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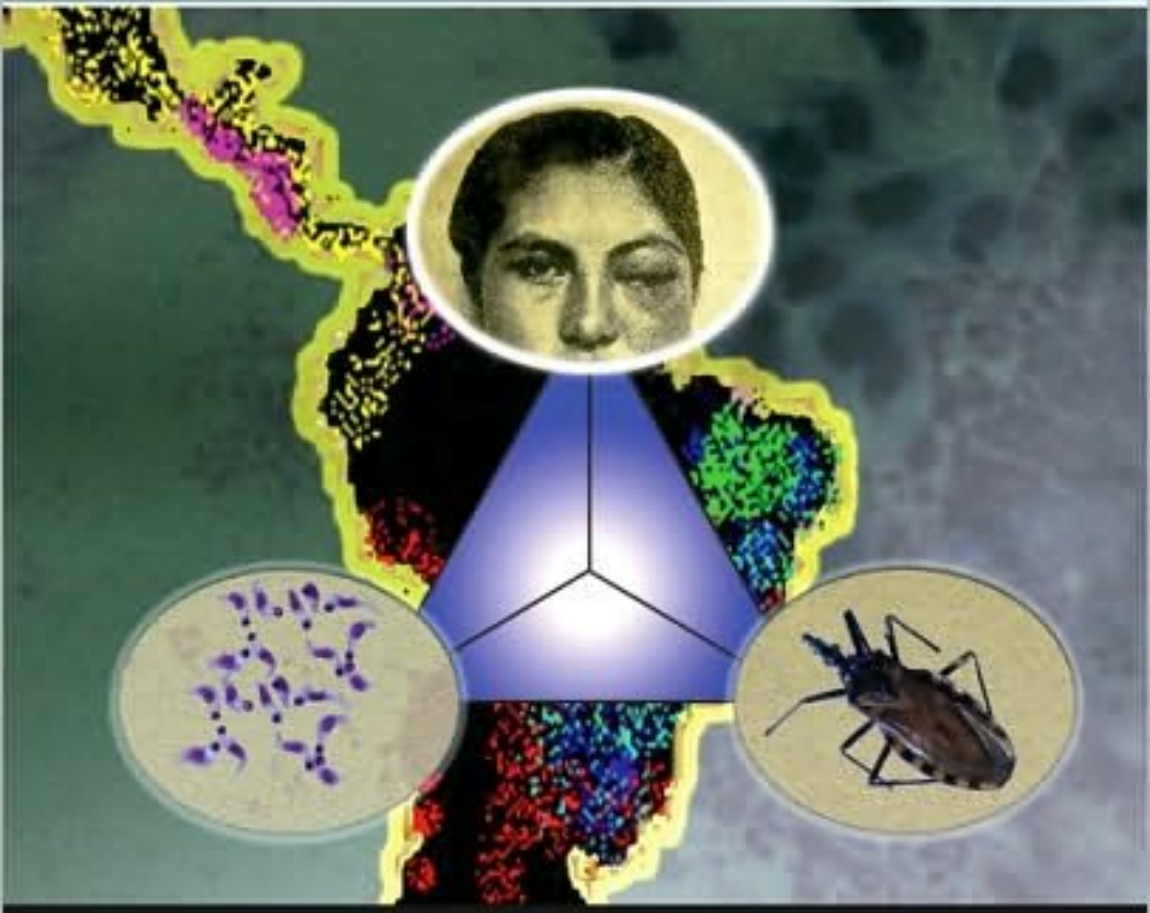

AMERICAN TRYPANOSOMIASIS CHAGAS DISEASE

ONE HUNDRED YEARS OF RESEARCH

Edited by JENNY TELLERIA • MICHEL TIBAYRENC

American Trypanosomiasis Chagas Disease
One Hundred Years of Research

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Preface

One century with Chagas disease is much too long.

In 1909, Carlos Chagas identified *Trypanosoma cruzi* as the agent of Chagas disease. In 1910, Chagas discovered that *Triatoma* bugs are vectors of the parasite and that various animals (first, the armadillo) are wild reservoirs for the parasite. In 1986, Michel Tibayrenc and collaborators discovered that the reproduction of the parasite is clonal, rather than sexual. The disease is, of course, much older than Chagas' discovery. It may have been associated with humans shortly after they arrived in the Americas some 15,000 years ago. *T. cruzi* has been found in mummies from northern Chile and southern Peru that are nearly 9000 years old.

Chagas disease is endemic in Central and South Americas, with significant prevalence of human infection in 22 Latin American countries, where it affects 10−12 million people and kills more than 15,000 humans each year. There are several hundred thousand people infected with *T. cruzi* in other parts of the world. Mostly, in the USA, Canada, Australia, Japan, Spain, and Portugal, where the carriers are typically Latin American immigrants, who are often unaware of their infection, but cause the infection of others through blood transfusions and otherwise.

In spite of counting among mankind's worst scourges Chagas disease has received relatively little attention from investigators and institutions. Pharmaceutical corporations typically have little or no interest in diseases that affect the world's poorest people. In the USA, Europe, and other industrialized countries, Chagas is largely perceived as a foreign disease, which does not motivate government agencies, foundations, and other institutions to invest substantial resources to discover curative drugs and medical treatments.

This neglect may be changing. In 1943, the Oswaldo Cruz Institute's Prophylaxis and Study Center for Chagas Disease was created in Bambuí, Brazil. In 1974, the World Health Organization set a special program for the study of Chagas disease. In 1991, Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay started the Southern Cone initiative for vector control. The Drugs for Neglected Diseases Initiative was established the same year to promote research against Chagas and other tropical diseases. The first complete genome sequence of *T. cruzi* was published in 2005, culminating an effort pioneered since 1998 by Björn Andersson and others. There are now very few treatment drugs in use, notably benznidazole and posaconazole, but research and testing of several other drugs are in the pipeline.

American Trypanosomiasis Chagas Disease: One Hundred Years of Research is a wonderful addition to current efforts. The coverage is broad, almost all-inclusive: from history and geography through vectors and nonhuman hosts, the biology and modes of transmission of the parasite, and the host−parasite immune interactions to the pathology, diagnosis, and treatment of the disease. I anticipate that this volume will be hailed as a landmark in the history and control of Chagas disease.

Francisco J. Ayala
University of California, Irvine, USA

List of Contributors

Tania C. de Araujo-Jorge
Laboratório de Inovações em Terapias, Ensino e Bioprodutos,
Instituto Oswaldo Cruz FIOCRUZ,
Rio de Janeiro,
Brazil

Arthur Aufderheide
Department of Pathology,
University of Minnesota,
Minnesota,
USA

Werner Apt
Laboratory of Parasitology,
Biological Science Institute,
University of Chile,
Independencia 1027 Santiago,
Chile

Christine Aznar
Université des Antilles et de la Guyane,
Cayenne,
French Guyana

Maria Dolores Bargues
Departamento de Parasitología,
Universidad de Valencia,
Valencia,
Spain

Christian Barnabé
Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS),
Montpellier,
France

Emile Santos Barrias
Instituto de Biofísica Carlos Chagas Filho,
Universidade Federal do Rio de Janeiro,
Rio de Janeiro,
Brazil

Daniella C. Bartholomeu
Department of Parasitology,
Federal University of Minas Gerais,
Minas Gerais,
Brazil

Miguel Angel Basombrío
Instituto de Patología Experimental,
Universidad Nacional de Salta,
Salta,
Argentina

Simone Frédérique Brenière
Institut de Recherche pour le Développement (IRD),
UR016 Caractérisation et Contrôle des Populations de Vecteurs,
Centre IRD, BP 64501,
34394 Montpellier, Cedux 5,
Montpellier,
France

Gregory A. Buck
Center for the Study of Biological Complexity,
Virginia Commonwealth University,
Virginia,
USA

Yves Carlier
Laboratoire de Parasitologie,
Faculté de Médecine,
Université Libre de Bruxelles (ULB),
Bruxelles,
Belgium

Tecia Maria Ulisses de Carvalho
Instituto de Biofísica Carlos Chagas Filho,
Universidade Federal do Rio de Janeiro,
Rio de Janeiro,
Brazil

Marcelo Corti
HIV/AIDS Division,
Infectious Diseases F.J. Muñiz Hospital,
Buenos Aires,
Argentina

Joffre Marcondes de Rezende
Faculty of Medicine,
Federal University of Goias,
Goias,
Brazil

Wanderley de Souza
Diretoria de Programas,
Instituto Nacional de Metrologia,
Normalização e Qualidade Industrial-Inmetro,
Rio de Janeiro,
Brazil

Marc Desquesnes
CIRAD-Bios,
UMR177-Trypanosomes,
Montpellier,
France; Kasetsart University,
Bangkok,
Thailand

Roberto Docampo
Department of Cellular Biology and Center for Tropical and Emerging Global Diseases,
University of Georgia,
350B Paul D. Coverdell Center,
500 D.W. Brooks Dr., Athens,
Georgia,
USA

Patricia Dorn
Department of Biological Sciences,
Loyola University New Orleans,
Louisiana,
USA

Jean-Pierre Dujardin
IRD (Institut de recherche pour le développement),
CNRS (Centre National de la recherche scientifique),
Av. Agropolis, F-34032,
Montpellier Cedex 5,
France

Najib M.A. El-Sayed
Department of Cell Biology and Molecular Genetics,
Center for Bioinformatics and Computational Biology,
Maryland Pathogen Research Institute,
University of Maryland,
College Park,
Maryland,
USA

David Gorla
Centro Regional de Investigaciones Científicas y Transferencia Tecnológica de La Rioja,
La Rioja,
Argentina

Felipe Guhl
Facultad de Ciencias,
Departamento de Ciencias Biológicas,
Centro de Investigaciones en Microbiología y Parasitología Tropical CIMPAT Universidad de Los Andes,
Bogotá,
Colombia

Patrick B. Hamilton
School of Biosciences,
University of Exeter;
Hatherly Laboratories,
Prince of Wales Road,
Exeter,
UK

Mireille Hontebeyrie
Institut Pasteur,
Paris,
France

Ana Maria Jansen
Laboratório de Biologia de Tripanosomatídeos,
Instituto Oswaldo Cruz FIOCRUZ,
Rio de Janeiro,
Brazil

Marta de Lana
Departamento de Análises Clínicas,
Escola de Farmácia,
Rua Costa Sena,
171, Centro,
Universidade Federal de Ouro Preto,
35300-000 Ouro Preto,
Minas Gerais,
Brazil

Michael D. Lewis
Department of Pathogen Molecular Biology,
Faculty of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine,
London,
UK

Alejandro O. Luquetti
Instituto de Patologia Tropical e Saúde Pública and Faculdade de Medicina,
Universidade Federal de Goias,
Goiânia,
Brazil

Martin S. Llewellyn
Department of Pathogen Molecular Biology,
Faculty of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine,
London,
UK

Andréa M. Macedo
Departamento de Bioquímica e Imunologia,
Universidade Federal de Minas Gerais and Researcher of Conselho Nacional de Pesquisas (CNPq) of Brazil,
Minas Gerais,
Brazil

Paula Marcet
Division of Parasitic Diseases,
Centers for Disease Control and Prevention,
Georgia,
USA

Evandro Marques de Menezes Machado
Departamento de Ciências Biológicas,
Instituto de Ciências Exatas e Biológicas
Universidade Federal do Ouro Preto,
Campus Universitário Morro do Cruzeiro,
CEP:35400-000 Ouro Preto,
Minas Gerais,
Brazil

Silvia N.J. Moreno
Center for Tropical and Emerging Global Diseases and Department of Cellular Biology,
University of Georgia,
Georgia,
USA

Álvaro Moncayo
Manager of the TDR Task Force on Intervention Research on Chagas disease from 1998 to 2001 at the World Health Organization,
Geneva,
Switzerland; Academia Nacional de Medicina,
Bogotá,
Colombia

Fernando Monteiro
Laboratório de Doenças Parasitárias,
Instituto Oswaldo Cruz FIOCRUZ,
Rio de Janeiro,
Brazil

Michael A. Miles
Department of Pathogen Molecular Biology,
Faculty of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine,
London,
UK

François Noireau
Institut de recherche pour le développement,
Centre National de la recherche scientifique,
Montpellier,
France

James S. Patterson
Facultad de Ciencias,
Departamento de Ciencias Biológicas,
Centro de Investigaciones en Microbiología y Parasitología Tropical CIMPAT Universidad de Los Andes,
Bogotá,
Colombia

Angel Marcelo Padilla
Instituto de Patología Experimental,
Universidad Nacional de Salta,
Salta, Argentina;
Center for Tropical and Emerging Global Diseases,
University of Georgia,
Georgia,
USA

Cecilia Perez Brandan
Instituto de Patología Experimental,
Universidad Nacional de Salta,
Salta,
Argentina

João Carlos Pinto Dias
Laboratório de Triatomíneos,
Centro de Pesquisa René Rachou FIOCRUZ,
Belo Horizonte,
Brazil

Carlos Ponce
Laboratorio Central de Referencia,
Tegucigalpa,
Honduras

Anis Rassi
Faculty of Medicine,
Federal University of Goias,
Goias,
Brazil

Anis Rassi Jr
Anis Rassi Hospital,
Goias,
Brazil

Jaime Rios Dalenz
Departamento de Patologia,
Facultad de Medicina,
Universidad Mayor de San Andrés,
Avenida Saavedra No. 2246,
La Paz,
Bolivia

André Luiz Rodrigues Roque
Laboratório de Biologia de Tripanosomatídeos,
Instituto Oswaldo Cruz FIOCURZ,
Rio de Janeiro,
Brazil

Gabriel A. Schmuñis
Former Officer of Communicable Disease Program,
Regional office of the World Health Organization for the Americas

C.J. Schofield
Department of Infectious and Tropical Diseases,
ECLAT network,
London School of Hygiene and Tropical Medicine,
Keppel Street,
London WCI EIHT
London,
UK

Marcela Segatto
Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais,
Minas Gerais,
Brazil

Antonio Carlos Silveira
Pan American Health Organization/ World Health Organization,
Brasilia,
Brazil

Jamie R. Stevens
School of Biosciences,
University of Exeter,
Exeter,
UK

Michal Svoboda
Laboratoire de Chime Biologique,
Faculté de Médecine,
Université Libre de Bruxelles (ULB),
Route de Lennik 808,
CP611, B-1070,
Bruxelles,
Belgique

Santuza M.R. Teixeira
Department of Biochemistry and Immunology,
Federal University of Minas Gerais,
Minas Gerais,
Brazil

Jenny Telleria
Unité Mixte de Recherche: Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS): “Génétique et Evolution des Maladies Infectieuses”,
Centre IRD, BP 64501,
34394 Montpellier Cedex 5,
France

Michel Tibayrenc
Unité Mixte de Recherche: Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS): "Génétique et Evolution des Maladies Infectieuses", Centre IRD, BP 64501,
34394 Montpellier Cedex 5,
France

Carine Truyens
Laboratoire de Parasitologie,
Faculté de Médecine,
Université Libre de Bruxelles (ULB),
Bruxelles,
Belgium

María F. Villafañe
HIV/AIDS Division,
Infectious Diseases F.J. Muñiz Hospital,
Buenos Aires,
Argentina

Matthew Yeo
Department of Pathogen Molecular Biology,
Faculty of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine,
London,
UK

1 History of the Discovery of American Trypanosomiasis (Chagas Disease)

Tania C. de Araujo-Jorge[1], *Jenny Telleria*[2] *and Jaime Rios-Dalenz*[3,*]

[1]Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, [2]Institut de Recherche pour le Développement/Centre National de la Recherche Scientifique, Montpellier, France, [3]Departamento de Patologia, Universidad Mayor de San Andrés, La Paz, Bolivia

1.1 A Beautiful History of Life and Work

American trypanosomiasis was named Chagas disease in honor of its discoverer, Carlos Ribeiro Justiniano Chagas (Chagas, 1909a), who was born on a coffee farm at Oliveira (Figure 1.1), state of Minas Gerais, on July 9, 1878 (Chagas Filho, 1979, 1993; Kropf and Sá, 2009).

Chagas' father was a tradesman named José Justiniano Chagas; his mother was Maria Ribeiro de Castro, born from a traditional family of coffee producers (Figure 1.2).

At an early age, Carlos Chagas lost three close family members: his father and his two brothers. Soon, he assumed the responsibility as the head of the family, helping his mother and sister. He spent his childhood (Figure 1.3) on the farm and his youth in a Catholic school in São João del Rey, where the priest João Sacramento exerted an enormous influence on his education (Chagas Filho, 1979, 1993).

Chagas' mother wanted him to be an engineer, but he did not pass the entrance exams, which depressed him. Chagas then decided to break from his mother's expectations and settled in Rio de Janeiro to study medicine. Two uncles from his mother's family profoundly influenced him in finding his medical vocation.

During his childhood and youth, Chagas' curiosity about medicine grew; moreover, Sacramento introduced him to the delights of the discovery of the natural world and the art world. Chagas learned the important mental tools for educating his imagination, as summarized by Robert and Michelle Root-Bernstein (1999): to

*E mail: jriosdal@gmail.com

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Figure 1.1 The house in Oliveira, state of Minas Gerais, Brazil, where Carlos Ribeiro Justiniano Chagas was born (July 9, 1878).
Source: Archives of the Instituto Oswaldo Cruz.

Figure 1.2 The parents of Carlos Ribeiro Justiniano Chagas: José Justiniano Chagas and Maria Ribeiro de Castro.
Source: Archives of the Instituto Oswaldo Cruz.

observe, to evoke images, to think in the abstract, to recognize and to form patterns, to think with the body, to empathize, to think in a dimensional way, to establish analogies, to create models, to play, to transform, and to synthesize. These are some of the tools that explain how creative thinking emerges, which are clear in Chagas' scientific work.

Figure 1.3 Carlos at the age of about 5 on the farm where he was born.
Source: Archives of the Instituto Oswaldo Cruz.

Carlos Chagas graduated in 1903 with a degree in medicine, concluding his clinical training under the influence of Professor Miguel Couto and with a well-grounded laboratory experience in the Manguinhos Institute, where he studied malaria. He accepted the invitation of Oswaldo Cruz and obtained a contract to work as a doctor at the Hygiene and Public Health Office/Ministry because of his expertise in malaria, and also because he needed to take a job with a fixed salary to marry the woman he had fallen in love with, Iris Lobo (Figure 1.4). Lobo, who came from a rich family in Rio de Janeiro, eventually became the mother of his two sons, Evandro and Carlos (Figures 1.4 and 1.5).

Chagas was then commissioned by Oswaldo Cruz to lead a campaign against malaria in Itatinga, a state of São Paulo. In this and other situations, he advocated a strategy of prevention based on the intrahousehold combat of the mosquito and succeeded in his goal of controlling malaria. After this success, Chagas became a member of the Brazilian National Academy of Medicine, which was a place especially created for him. He also received numerous awards and titles, including Doctor Honoris Causa (from institutions that included Harvard, Paris, Lima, and Belgium Universities), was nominated twice for the Nobel Prize (in 1913 and 1921), directed the Oswaldo Cruz Institute for 17 years, and coordinated the campaign against the epidemic of Spanish influenza in Brazil. His son, Carlos Chagas Filho (1993), wrote two books that illuminated the human and emotional aspects of the scientist. His son reported that the elder Chagas loved to hunt (Figure 1.6) and enjoyed football, supporting the Botafogo football club in Rio de Janeiro.

Figure 1.4 The family founded by Carlos Ribeiro Justiniano Chagas (his wife, Iris Lobo, and his two sons: Evandro and Carlos, Rio de Janeiro).
Source: Archives of the Instituto Oswaldo Cruz.

Figure 1.5 Carlos Ribeiro Justiniano Chagas' sons: Evandro and Carlos.
Source: Archives of the Instituto Oswaldo Cruz.

Figure 1.6 Carlos Ribeiro Justiniano Chagas (on right) with a group of friends after a hunting expedition, on the property at Rio Pardo, Avaré, Sao Paolo.
Source: Archives of the Instituto Oswaldo Cruz.

The political, scientific, and cultural context in which Carlos Chagas was immersed and made his discovery was a very rich one. Politically, the era of the Brazilian empire had ended with the abolition of slavery and the proclamation of the Republic of Brazil.

The American trypanosomiasis discovery also was determined by a peculiar health context in Brazil. In the Institute of Manguinhos, Oswaldo Cruz implanted a triad of assistance, research, and education. This public health model remains at Fiocruz, which continues today as an institution of science, technology, and innovation in health linked to the Ministry of Health.

After the campaign against the Spanish flu in 1918, Chagas created the National Department of Public Health, giving rise to the future Ministry of Health and leading to the Sanitary Code. The Code expanding assistance for other diseases, such as tuberculosis, venereal diseases, and workers' health diseases, and led to the creation of the School of Nursing and a program for prophylaxis in endemic areas with "endemic guards." A strong cooperation with the Rockefeller Foundation was initiated. Formal science education started in 1911 with the first "Course of Application" at the Institute, and in 1925 Carlos Chagas began the first Special Course in Hygiene and Public Health. He became professor of Tropical Medicine at the Faculty of Medicine in 1928 (Chagas Filho, 1993).

1.2 The History of a Significant Discovery

The great sociologist of science, Bruno Latour (1987), said that "The more controversy we articulate, the broader becomes the world." Carlos Chagas and his colleagues precisely articulated controversies and discovered a new world of research and of political action in public health.

The disease had already existed in the Americas for over 9000 years (Araújo et al., 2009). Mummies were found in Peru with physical evidence of clinical signs of Chagas disease from which samples of *Trypanosoma cruzi* DNA were recovered. In Brazil, paleoparasitology studies conducted by Adauto Araujo, Luiz Fernando Ferreira, and others have confirmed *T. cruzi* DNA in mummies dating back 7000 years. These findings changed the assumptions about the emergence of Chagas disease in the Americas, dating it back to the contact of hunters and gatherers with mammalian reservoirs and insect vectors, much earlier than the period when Andean men started home breeding small animals, as was previously thought. The disease had already been in Latin America for 9000 years, and no one had seen or detected it before Chagas did in 1909. Even Charles Darwin registered the presence of triatomines during his stay in South America in 1835 (Darwin 1899, cited in Neiva and Lent, 1943), but this was not associated with any specific disease: "The night I experienced an attack (for it deserves no less a name) of the Benchuca (a species of *Reduvius*) the great black bug of the Pampas. It is most disgusting to feel soft wingless insects, about an inch long, crawling over one's body. Before sucking, they are quite thin, but afterwards became round and bloated with blood, and in this state they are easily crushed. One which I caught at Iquique was very empty. When placed on a table, and though surrounded by people, if a finger was presented, the bold insect would immediately draw its sucker, make a charge, and if allowed, draw blood. No pain was caused by the wound. It was curious to watch its body during the act of sucking, as it changed in less than ten minutes, from being as flat as a wafer to a globular form." Darwin's biographers suspect that he also contracted Chagas disease on the Beagle voyage (Bernstein, 1984).

What enabled Chagas to make the triple discovery: the parasite, the vector, and the disease? All other parasitic diseases took years or decades to get their cycle completely elucidated, from the etiological agent, to the vector(s), the reservoir(s), and the clinical manifestations. How did Carlos Chagas do all that in less than 5 months?

First, Carlos Chagas was educated as a scientist, with a mind trained in the tools that educate the imagination; he received solid experience in the scientific method and reasoning. Second, he had excellent clinical training as a physician and in the laboratory, being a skilful examiner of blood smears for the diagnosis of malaria that was the subject of his MD thesis. Third, the scientific environment of the Manguinhos Institute allowed him to follow the development of medical entomology and the development of literature on tropical medicine and microbiology. And finally, at the Institute, he had laboratories to conduct experimental animal testing under controlled conditions. That is why, while on a mission for malaria control in the region of Itatinga, São Paulo, then in Xerém, Rio de Janeiro, and later in

Figure 1.7 Carlos Chagas with his work team at the railway station in Lassance Minas Gerais, Brazil (1908).
Source: Archives of the Instituto Oswaldo Cruz.

Figure 1.8 View of the railway station in Lassance Minas Gerais (1908).
Source: Archives of the Instituto Oswaldo Cruz.

Lassance, Minas Gerais (Figures 1.7−1.8), he articulated the daily work of the prophylaxis of malaria with the work of an investigator.

Chagas worked in two ways, first as a health professional and second as a health researcher. He was trained to embrace the idea of Patrick Manson's School of Hygiene and Tropical Medicine in London (Riley, 2000) that a tropical disease is "a more convenient than accurate concept" because bacteriology is cosmopolitan,

with variations in climate and geography, and protozoa and helminths are prominent in tropical climates where many vectors and hosts live. The concept of tropical medicine in Brazil reconciles the microbiology laboratory with the entomological collection, based on the classification of naturalists. All this gave him a more holistic environmental approach.

With these ideas in mind, when Chagas studied the real health situation in the interior of Brazil, he transformed science and public health into a tool for building a nation (Kropf et al., 2003; Kropf and Sá, 2009). He rethought the nation among its "hinterlands" and its people. Chagas took advantage of the modernization of Rio de Janeiro, then the capital of the New Republic, to call for the integration of the entire country.

His discovery took place in the context of expanding Brazilian economic frontiers, following the need for installation of railways for transportation of agricultural production, along with the intense collection of biological material that Chagas promoted during his missions (Sá, 2005; Kropf and Sá, 2009). In Lassance, the engineer of the train company showed him an insect. Carlos Chagas had already discovered a *Trypanosoma* when he examined the blood of monkeys in the region (it was not *T. cruzi*, but *T. minasense*; Chagas, 1908). He had sent the infected insects to Manguinhos, asking Oswaldo Cruz to perform experimental infections that confirmed that the parasite could cause disease in monkeys. After a train journey lasting more than 70 hours, Chagas went to the laboratory in the middle of the night. He had realized that the protozoon was not *T. minasense* but a new species, which he called *T. cruzi* in honor of Oswaldo Cruz.

This work described the stages of the evolution of *T. cruzi* in the insect, in cultures, and in organs of infected animals (Chagas, 1909a). Chagas had a certainty in his mind: a new parasite, a vector, wild reservoirs: only the human cases were missing. He then returned to Lassance and began to look carefully at every febrile child, always examining blood smears. In his work (Chagas, 1909a), he described finding a trypanosome in the blood of a cat, defining a domestic reservoir, and in the sequence, he found *T. cruzi* in the blood of Berenice, who would become case number 1 (Figure 1.9).

In an article published in 1909 (Chagas, 1909b) Chagas wrote: "In a febrile patient, deeply anaemic and with oedema, engorged with multiple ganglia, we found trypanosomes, whose morphology is identical to that of *T. cruzi*. In the absence of any other aetiology for the morbid symptoms observed and in accordance with previous experiments in animals, we believe this is a human trypanosomiasis, the disease caused by *T. cruzi*, transmitted by *Conorrhinus sanguissuga*." Chagas examined many other children because he did not find circulating trypanosomes in adults.

Berenice, his first patient, was found (Figure 1.10) at age 55 by (Salgado et al., 1962) and the strains are characterized by Lana et al. (1996). She still had circulating trypanosomes and had remained asymptomatic, living with an indeterminate chronic form of Chagas disease just as 75% of other patients with the disease have done. Berenice did not die from *T. cruzi* infection but from other causes at the age of 73.

This was the natural path traced by Carlos Chagas, in which the scientific fact was the result of a social construction caused by a public health problem. It begins

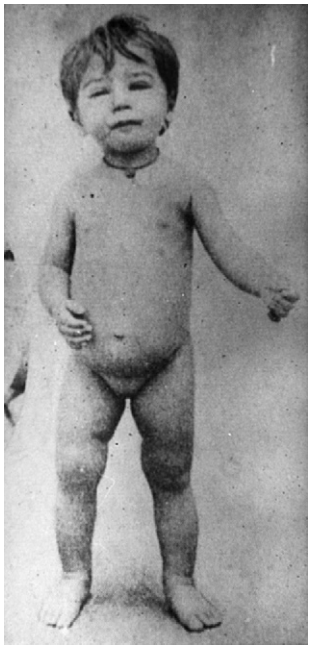

Figure 1.9 Berenice, Carlos Chagas' first patient.
Source: Archives of the Instituto Oswaldo Cruz.

Figure 1.10 Berenice, found again at the age of 55 and reassessed at age of 55 and 71 for further clinical studies.
Source: Archives of the Instituto Oswaldo Cruz.

with the identification of a vector, then the causative agent, and the epidemiological characteristics, in the search for an associated disease without previous clinical evidence.

The disease was described in the initial period, without definitive evidence of etiological and epidemiological studies. Nearly 10 years after the description, only 40 cases had been confirmed by laboratory procedures. Chagas claimed that the disease was a major public health problem and a major obstacle to the country's progress, and the eventual acceptance of the existence of a new disease led to a social and cognitive process that spanned three decades. In publications (Chagas, 1911), photos, and films, Chagas showed the whole country that this "new trypanosomiasis reached vast areas of Minas Gerais and other Brazilian states, giving rise to a degenerate population seriously jeopardizing the vitality and productivity of rural persons in the country." He made an error, associating trypanosomiasis with the goiter, which was endemic in this area of iodine deficiency. That would later be revised and corrected by several researchers.

From 1916 to 1920, Carlos Chagas led a large national campaign for rural sanitation, which was not an easy struggle. In 1917, after the death of Oswaldo Cruz, Chagas at the age of 39 became director of the Oswaldo Cruz Institute.

From 1909 to 1913, Chagas prospered. The Brazilian science was recognized internationally for its excellence, the Oswaldo Cruz Institute was consolidated, the "Movement of Rural Hygiene" was created, public health in Brazil was founded, and a nation began to be built and prepared to face the health challenges for economic and social development. Because of this success, Chagas was twice nominated for the Nobel Prize.

There were also struggles. The medical and political community doubted clinical aspects of his research, the epidemiological data and on the scale of the disease, claiming that it was a "rare disease entity" and not a "national evil." There was a political dimension concerning a possible negative image of Brazil abroad, questioning the project of tropical medicine.

During this period, Chagas and his assistants worked hard to achieve a double translation of parasitic thyroiditis (Kropf et al., 2003), with multinodule goiter and cretinism, for chronic heart and nervous disease (Chagas and Villela, 1922). The acute and chronic diseases were also determined to be two different entities. The silent indeterminate clinical form, "the potential cardiac patients," was also described. This changed the disease from a "Brazilian disease" into "American trypanosomiasis." To overcome these ordeals, it was essential to develop clinical simplified diagnostic features, such as Romaña's sign, which describes the swelling around the eyes and face (Romaña, 1935); the reports of cases found by Mazza in Argentina (Mazza, 1949); and the electrocardiogram (Laranja et al., 1956). In the 1940s, after the identification of cases in Argentina, after several scientific expeditions to the interior of Brazil, after the creation of the Centre for the Study and Prevention of Chagas disease in 1943 in Bambuí, the prevalence and relevance of Chagas disease was finally proved and the strategic method to control transmission using insecticides and house improvement was implemented (Dias and Peregrino, 1948; Dias, 2009).

Chagas made many important discoveries, such as the theory on the intradomicile transmission of malaria (Chagas, 1906), which was fundamental for its prophylaxis, and the description of *Pneumocysti carinii*, which was confused with a cystic form of *T. cruzi* (Chagas, 1909a; Chagas Filho, 1993). But, history will acknowledge him for this unique achievement in biomedical science: his 1909 description of a new etiological agent, the hemoflagellate *T. cruzi*, its life cycle, vectors, domestic and sylvatic reservoirs, and corresponding human disease (Chagas, 1909a). This brilliant scientist was more than a member of the "microbe hunter" generation, but decades were required for international recognition of the dramatic epidemiological picture of Chagas' disease. He died at the age of 56, after 32 years of a hard and fruitful scientific career that included 17 years of active direction of the Oswaldo Cruz Institute.

1.3 Salvador Mazza: The History of His Knowledge of the Disease

In 1915 and 1916, Maggio and Rosenbusch with Kraus noticed the absence of a link between infected bloodsucking insects and goiter and cretinism, which constituted the basis of a set of doubts relating to the pathogenic conceptions proposed by Chagas. This decade saw the beginning of the work by Salvador Mazza (1886−1946) on Chagas disease, which profoundly marked the history of knowledge of the disease.

Mazza, born in Rauch, Buenos Aires, Argentina, earned his medical degree in 1910 and was a specialist in chemical and bacteriological pathology. During his career, he was a member of the National Department of Hygiene, served as minister of health, was appointed professor of bacteriology at the University of Buenos Aires, and served as director of the Central Laboratory of the Clinics Hospital in Buenos Aires. In 1926, he founded the Jujuy Scientific Society. Between 1926 and 1927, subsidiaries of the society were created in Salta, Tucumán, Catamarca, Santiago del Estero, La Rioja, and Corrientes. The year 1928 saw the creation, with the support of José Arce, of an official extension of the universities dependent on the Institute of Clinical Surgery of the Faculty of Medicine of the University of Buenos Aires, called Mission de Estudios de Patologia Régional Argentina (MEPRA). During this time, Mazza met Carlos Chagas in Germany, and had been impressed by the clarity and strength of his arguments concerning the disease. Consequently, he initiated many studies that confirmed the existence and importance of this pathology. In 1926, Mazza found a dog infected with *T. cruzi* and, in 1927, he diagnosed clinically the first acute case in Argentina (Mazza, 1949).

During the 1930s, as the head of MEPRA, Mazza guided studies of this affliction, confirming its multiple aspects, the insect vectors, the mammal hosts, the epidemiology, and the pathogenesis. This work, carried out with tenacity, enabled the diagnosis of several hundred cases, both clinical and parasitological. Furthermore, the verification of Chagas disease in the zones where goiter is endemic enabled him to overcome the obstacles that Chagas had confronted. Following this work,

American trypanosomiasis was the main theme of the Sixth National Congress of Medicine in 1939. In 1940, Mazza and Jörg defined the three anatomo-clinical periods of the disease, which remain valid to this day. In 1946, Mazza died from a heart attack. MEPRA, which had become a multidisciplinary team carrying out treatment, teaching, and research, was temporarily directed by Jörg. However, he was unable to overcome the institutional and political vicissitudes that finally led to its closure in 1958.

1.4 Cecilio Romaña: His Contribution to the Identification of the Disease

Research carried out at MEPRA during the 17 years that Mazza worked for the mission enabled him to observe people and animals in the zone infected by *T. cruzi*, as well as the infestation of the bloodsucking bugs in homes. Among the descriptions of the clinical manifestations of the disease, a crucial point in its identification was the observation of an ocular edema (known as Romaña's sign in honor of the doctor a disciple of Mazza who suggested it).

Romaña's sign is a pathognomonic early sign of Chagas disease: unilateral severe conjunctivitis and swelling of the eyelid, inflammation of the tear gland, and swelling of regional lymph glands caused by the entry of *T. cruzi*. The sign has proved of great value in the identification of the infection in its acute phase. It constituted a fundamental element to establish a rapid diagnosis and a clinical characterization of the disease, and it enabled the confirmation of many cases, finally putting an end to doubts concerning its spread.

In 1946, the year of the transfer of MEPRA to Buenos Aires, Mazza and his collaborators registered 1400 cases of Chagas disease of which 1,100 revealed a direct presence of parasites in the blood (Sierra-Iglesia, 1990). During the same period, the Regional Medicine Institute was founded at the National University of Tucuman, directed by Cecilio Romaña. The institute dealt with the more precise characterization of the clinical symptoms of the disease, as well as trials on the efficiency of hexachlorocyclohexane, an insecticide capable of killing the bloodsucking bug.

During the first Pan-American meeting of Chagas disease, organized by Romaña in Tucuman in 1949, a decision was taken to create, in 1950, a management committee for research and prophylaxis on Chagas disease at the Ministry of Health, and the coordination was entrusted to Cecilio Romaña. This important step constituted the first institutional organization concerning Chagas disease outside of university research institutes already mentioned (Kreimer and Zabala, 2008).

1.5 First Evidence of Trypanosomiasis Americana (Chagas Disease) in Various Countries of Latin America

The discovery of Chagas disease slowly spread across various countries of Latin America. In Central America, the first report of tripanosomiasis humana americana was made by

Segovia in El Salvador in 1913 (Segovia, 1913); subsequently, other Salvadorian researchers reported on new cases: Reina Guerra (1939), Castro, Fasquelle, Garcia Montenegro.

In Costa Rica, the disease was originally described in 1922 by Picado (doctoral thesis) and was later studied by Von Bullon, Cespedes, Chen, and Zeledon. In Guatemala, the disease was first encountered by Reichnow in 1933. Subsequently, the work of De Leon in 1935 highlighted the importance of this disease in this country (León Gómez et al., 1960). However, it is important to emphasize the considerable contributions to the understanding of this disease realized by Montenegro, Esteres, Blanco, and Peñalver.

In Panama in 1931, the presence of the disease was proven with the report of 19 human cases in the area of the Panama Canal. Later on, other research was carried out by Calero, Johnson, and Rivas.

In Honduras, the disease was not officially reported until 1960, when A. Leon Gomez, A. Flores Fiallo, E. Poujeol, and M. Barilla reported a case in the Seventh Day of Honduran Medicine that took place in San Pedro Sula (León Gómez et al., 1960). In 1961, the same authors communicated seven cases of Chagas chronic myocarditis (León Gómez et al., 1961); Durón (1965), in a post-mortem study, encountered numerous pseudocysts of *Leishmania* in the myocardium of a girl suffering from acute Chagasic myocarditis who died suddenly. Nevertheless, in 1950, Zepada observed a vector in various zones of the country and in 1939 a possum infected with *T. cruzi* was discovered (Robertson, 1931). In 1968, Fernandez and Lainez reported the first two cases of the acute form of the disease, with the discovery of *T. cruzi* in peripheral blood, whose clinical characteristics presented a unilateral conjunctive reaction, temperature above 39°C, palpebral edema, hemifacial edema, and cervical adenitis.

In 1949, the first native case of the disease was described in Nicaragua, and as recently as 1969 Fray Bernadini de Schagen reported that homes were infested by vectors described as "poisonous bloodsucking insects like cockroaches."

In Mexico in 1928, Hoffman described the great abundance and domiciliation of *Triatoma dimidiata* in Choapas, Veracruz. In 1938, the same author spoke of a case in the same region described by Luis Mazzotti, who identified the first two officially recognized cases originating from Oaxaca as being abundantly infected with triatomine bugs (Mazzotti, 1940). In 1938, Bernal Flandes published on transmitter insects and trypanosomatides in Veracruz, and in 1940, Palomo Eroso described two other new cases in Yucatan. It was only in 1972 that the first formal identification of the disease was carried out with reports by Eugenio Palomo and Luis Mazzotti (Symposium: National Academy of Medicine, 1975).

In South America, Enrique Tejera reported the first case of Chagas disease in Venezuela (Tejera, 1919), 10 years after Carlos Chagas' discovery. Pioneer studies were carried out in Venezuela, directed by José Francisco Torrealba, who initiated studies in the state of Guàrico (Torrealba, 1935) and introduced the xenodiagnosis invented by Emile Brumpt, while Pifano (1941) studied the epidemiology of the disease in the state of Yaracuy. Both authors highlighted its importance as a public health problem and a social one with the maintenance of this zooanthroponosis. At the

Institute of Tropical Medicine of the Central University of Venezuela, Maekelt developed a protocol in 1960 for the preparation of antigen that is still used in the country, and Pifano published the first figures of national prevalence. As recently as 1961, the Ministry of Health and Social Welfare allocated budgets, effort, and expertise to fight Chagas disease and initiated the campaign to control its spread (Feliciangeli, 2009).

In Colombia in 1939, Ignacio Moreno Perez observed for the first time the pathogenic parasite provoking the disease, according to a report by Hernando Ucros in 1971. In 1947, Caicedo and Hernandez wrote a report describing the first proven chronic case of the disease in Colombia originating from Fusagasuga in Cundinamarca. In 1961, Marcos Duque began studies on Chagas' cardiopathy. Almost 10 years later, Hernando Rocha communicated his discoveries on the mega-esophagus, which is a consequence of the disease (Serpa Florez, 2000).

The first research on the epidemiology of Chagas disease in Chile is attributed to Juan Noe who, in 1921, observed the presence of *T. cruzi* in many samples of *Triatoma infestans* coming from the outskirts of the town of Santiago. Later, in 1931, Miguel Massa, under the authority and direction of Noe, demonstrated the specificity of the parasite in cardiac fibers in animals used for experimentation. With the creation of the Department of Parasitology of the State Health Office in 1937, systematic investigations began that led to the demonstration of the first human cases of this disease (Gasic, 1940).

In Peru in 1919, Escomel described the first human case demonstrating the presence of *Trypanosoma* in human blood, found in a border zone between Brazil and Bolivia where species of the flora and fauna are very abundant. Escomel said predecessors described *T. infestans* in the Vitor and Majes valleys, which were suspected as the cause of certain patients' clinical symptoms that turned out to be trypanosomiasis, but the confirmation of the parasite could not be made (Ayulo and Herrer, 1944). Roughly 25 years after Escomel's cited publication, the second human case of Chagas disease was verified in Peru, and at the same time the first epidemiological research was realized.

In Paraguay in 1924, the first discoveries were made from studies by Lutz, Sousa, Araujo, de Fonseca, and Migone, which showed the first infected bloodsucking insects (the Chagas Space Group). In this way, Gamiro in Uruguay carried out studies on the infection of these insects in his country.

In nineteenth-century Bolivia, when the country was part of the Spanish Viceroyalty of Peru, a priest, Fray Reginaldo de Lizarraga, made an interesting discovery while traveling to inspect the convents of this region. He noticed that in the valleys of Cochabamba, bloodsucking insects like cockroaches, called "vinchucas" by natives, during the night fell from the ceilings of the poor houses and bit people who were sleeping, particularly in the face and other exposed parts of the body. This was a description of the Triatominae as discovered later (De Lizarraga, 1928).

In 1916, Brazilian researcher Artur Neiva, from the Manghinhos Institute of Rio de Janeiro, found these Triatominae in Sococha, a small town of the Department of Potosi near the Argentinian border. These insects were similar to ones called "barbeiros" in Brazil, which were infected with the parasite *T. cruzi* (Dias and Torrico, 1939).

In 1929, Bolivian researcher Felix Veintenillas claimed that he found Triatominae with *T. cruzi* in the area of Yungas of La Paz (Veintenillas, 1931).

The Argentinean Salvador Mazza, who studied Chagas disease in the northern region of Argentina, did expeditions to several regions of Bolivia from 1937 to 1943. During these endeavors, he found infected triatomine bugs of the species *T. infestans* in 22% of the cases studied in Vitichi and Potosi and in 50% of the cases in the small village of Mollegrande. The researcher found a 2-year-old girl, who died a few days later, with *T. cruzi* in smears of her blood (Mazza, 1942; Mazza and Chacon, 1943).

Rafael Torrico (Figure 1.11) returned to Bolivia in 1943 after postgraduate studies at the Oswaldo Cruz Institute of Brazil and became professor of parasitology at the medical school in Cochabamba. He was named director of the Central Laboratory of Interamerican Cooperative Public Health Service of the Ministry of Health, where he did several studies in Chagas disease. Torrico and Dias published research in 1943 relating to infected triatomine bugs of the species *T. infestans* in several towns of Cochabamba (Dias and Torrico, 1943).

In 1946, Torrico described a 14-year-old girl from Capinota (Figure 1.12), a rural area of the Department of Cochabamba, as the first case in Bolivia of an acute form of Chagas disease. The girl was found to have edema involving the eyelid, the site of the bite by triatomine bugs identified as *T. infestans*, and a preauricular lymphadenopathy as described by Romaña in Argentina (Torrico, 1946; Romaña, 1961).

In the area where the patient lived, Torrico found that 84.9% of 427 insects of the species *T. infestans* were infected with *T. cruzi*. However, a Bolivian student in Chile, C.L. Ponce Caballero, published a study of seven cases of Chagas disease from the town of Colcapirhua, Cochabamba, reportedly confirmed by xenodiagnosis (Ponce, 1946). According to one of the participants, the town did not have any clinical manifestations of the disease. In 1947, Romaña, with a group of students of the University of Chile that included Ponce Caballero, reported 122 cases confirmed by xenodiagnosis in several areas of Bolivia (Romaña, 1947).

Figure 1.11 Rafael Torrico, 1943, the father of Chagas disease in Bolivia.

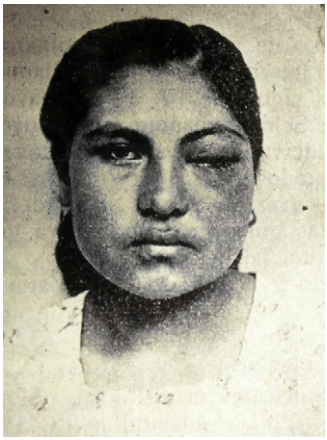

Figure 1.12 First case of Chagas disease in Bolivia, reported in a girl aged 14 in Capinota, Department of Cochabamba, Bolivia.

At the first Pan-American meeting of Chagas disease held in Argentina in 1949, Torrico presented a paper about the knowledge of Chagas disease in Bolivia. He stated that *T. infestans* was the principal and the most important vector because of its high infection index, its prevalence, and wide distribution, predominantly in the valleys, where it is an obliged host in most houses. He also stated that guinea pigs (*Cavia cobaya*), dogs, and cats were the only parasite reservoirs known of *T. cruzi* in Bolivia guinea pigs in particular were seen in houses inhabited by many peasants. Regarding human morbidity, he presented 211 cases collected (most of which had acute onset) from many areas of the country that were either studied by him or published by others; most cases were diagnosed by blood examination and mostly by xenodiagnosis (Torrico, 1949).

Finally, Torrico presented an up-to-date study of the situation of Chagas disease in Bolivia at the International Congress of Chagas disease in 1959. Here he confirmed his observations of the vectors, reservoirs, and 342 human cases (acute or chronic), including cases of Chagasic myocardiopathy with its classic electrocardiography, studied by J. Rodriguez Rivas (Rodriguez Rivas, 1957; Torrico, 1959).

1.6 Chagas Disease 100 Years after the Discovery

What is Chagas disease now, 100 years after its discovery? It remains a neglected and silent disease of poverty (Hotez et al., 2008). Millions of people are infected in Latin America and thousands of others in Europe, Asia, and North America.

The centenary of the discovery of Chagas disease (Lannes-Vieira et al., 2009) leads us to reflect on the evolution of ideas and concepts about it (Morel, 1999). The first phase from 1909 to 1934, mainly through the work of Chagas and the team of scientists from Manguinhos, ended immersed in controversies; the second phase, from 1935 to 1960, after the death of Chagas, when Mazza and Romaña confirmed acute Chagas disease in Argentina and when Evandro Chagas (Chagas' elder son) and his student Emmanuel Dias deepened studies and confirmed the concept of endemic chronic disease; and finally a third phase, in which political awareness

about Chagas disease, associated with the need for control and to the implementation of national and international policies of vector control and corresponding challenges, paved the way for more work to be done in the twenty-first century.

The control program brought many advances; about 200,000 new cases were reported in the year 2000 compared with 700,000 in 1983 (Moncayo and Silveira, 2009). Today, fewer than 100,000 new cases are reported each year. These numbers are possibly underestimated due to lack of attention to Chagas disease in health information systems that report mainly injuries.

The effectiveness of the two main drugs, benznidazole and nifurtmox, for the treatment of the chronic phase remains under investigation (Marin-Neto et al., 2009). There is no vaccine as of now. A new profile of oral transmission predominates and the disease still needs a lot of attention. With the Worldmapper tool (Dorling, 2007), it is possible to display the country areas' proportions comparing the relative burden of various diseases and the existing epidemiological data. The reality of cases of Chagas disease in the Americas can be seen at http://www.worldmapper.org/display extra.php?selected=392.

All Latin America has to honor Carlos Chagas' legacy and face such challenges. His old institution and many others on the continent are actively engaged in research on several topics concerning Chagas disease. At the clinical trials Web site (http://www.clinicaltrials.gov), very few studies are devoted to infectious diseases clinical trials: 390 on malaria, 336 on tuberculosis, but only 10 on Chagas disease: 5 on diagnosis and 5 on treatment. This expresses the real definition of a neglected disease. It is neglected by the pharmaceutical industry, which does not invest in research and technological development of drugs for the treatment of Chagas' disease because it does not provide a rich market for buying future products.

Chagas disease is still the most neglected among the kinetoplastid infectious diseases (leishmaniasis and African trypanosomiasis). From the total amount of resources invested in 2007 (over US$125 million) only 8% were for Chagas disease. This leads, of course, to a lack of research on interventions and therapeutic innovations in Chagas disease and emphasizes the need for public policies to help the affected countries.

Chagas disease has poverty as its major social determinant, with the vector and the susceptibility of human host as its major biological determinants. The affected countries need policies for studies and development of drugs and vaccines, but also for the reduction of poverty and social disparities that expose the poor to risks of contamination. So here we highlight the words from Peter Hotez (Morel et al., 2005): "We have the technology available to develop new drugs, vaccines and diagnostics to combat poverty. However, what is needed is more innovation to enable financial institutions to be able to lead the intensification of the process of developing manufacturing and clinical testing, in order to ensure global access to these new products." It is worth remembering the legacy of Carlos Chagas: his son Evandro Chagas (1935) wrote that "for Chagas, science was valid only if it was directed toward the welfare of humanity."

Carlos Chagas began in 1909 with the goal of broad social commitment. The words of Chagas fill us with hope and renew our commitment to the struggle for

control of the disease and care for people: “It won’t take long for us to pass on a beautiful and strong science which creates art in the support of life.”

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2 Chagas Disease in Pre-Colombian Civilizations

Felipe Guhl[1,*] *and Arthur Aufderheide*[2]

[1]Centro de Investigaciones en Microbiología y Parasitología Tropical—CIMPAT—, Facultad de Ciencias, Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia, [2]Department of Pathology, University of Minnesota, Minnesota, USA

2.1 Introduction

Reconstruction of the behavior of a modern disease during antiquity is a formidable challenge. However, success in such an endeavor would allow for the creation of a new database, and this new information could then spawn new hypotheses. Their results could then be blended with our present knowledge to produce an unbroken history of infectious diseases from deep antiquity to the present. Paleoecological integration of such data could help explain chronological changes whose causes could be exploited for novel modern therapeutic or preventive control of the condition. However, there are currently only three methodological tools that can be used in such searches: genetic variation, archaeology, and biochemistry.

2.1.1 Genetic Variation

More than 100 feral animal species in Central and South America can be septicemic with the trypanosome but demonstrate few or no clinical effects of the parasite in their blood. Although this phenomenon could have occurred via several mechanisms, the most likely is host or vector genetic changes produced by a process of coevolution. For example, new antigens could have been added to the surface of the parasite; alternatively, or in addition, the parasite could have developed the ability to cross cell membranes and complete the remainder of its life cycle in an intracellular location where antibodies could not gain access to it (evasion). Such changes commonly occur over an extremely long time period but result in an increased tolerance of both host and vector for each other (Anderson and May, 1991; Schmunis, 1994).

* E mail: fguhl@uniandes.edu.co

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2.1.2 Archaeology

The modern domestic cycle is the common environment through which humans are exposed to reduviid bugs, usually *T. infestans* and *Rhodnius prolixus*. These insects hide in the cracks and defects of a house's roof and wall during the day, emerging at night to obtain a blood meal from their prey. That prey includes not only humans but also domestic animals. Thatched roofs are especially attractive for these insects (Edgcomb and Johnson, 1976). Even today, many families supplement their meals by raising guinea pigs in cages in the home as well as such wild animals as armadillos and small mammals, a high fraction of which can become infected by *Trypanosoma cruzi*.

One of the best-studied populations dealing with Chagas disease is from the coastal area of northern Chile at the foot of the western Andean slopes, between about 19 and 23 degrees of south latitude. The extremely arid climate here generates rapid, spontaneous desiccation of buried bodies, arresting the decay process. The absence of rainfall then preserves these dried bodies (mummies) for millennia.

2.1.3 Biochemistry (Bioarchaeology)

Our attempt to reconstruct the behavior of Chagas disease in antiquity was based on the assumption that the soft tissues of naturally desiccated human remains (mummies) would also preserve trypanosomes that were present at the time of death. We had already demonstrated the survival of the tubercle bacillus in one of these mummies. We had dry soft tissue samples from several hundred mummies in our files; these spanned an interval of 9000 years until the nineteenth century. We felt that we could make a major contribution to our understanding of Chagas disease if we could identify the presence of *T. cruzi* in those individuals who were infected at the time of their death.

While the chemical methods of dealing with ancient DNA (aDNA) were still evolving at the time of this study (and are evolving still), we approached it by targeting a segment of mitochondrial DNA that was unique to *T. cruzi* (i.e., found in no other plant, animal, or human), (Guhl et al., 1997, 1999). We pulverized 400 mg of each sample of dried mummy. We then extracted the sample's aDNA content and amplified this minute amount by subjecting it to "nested" polymerase chain reaction (PCR). The sequence of the amplified aDNA was then determined (Madden et al., 2000).

The results indicated that an average of 40% of these mostly prehistoric people were infected with *T. cruzi* at the time of their death (Aufderheide et al., 2004). That frequency was common to all cultural groups studied and did not differ between time periods. We wondered whether it was really possible that nearly half of ancient South Americans were suffering from Chagas disease. To answer this question, we examined the available literature to compare modern frequencies with our results.

In the 1970s and 1980s, surveys of poorly constructed houses were carried out in endemic areas. In northern Chile, houses were inspected to determine whether they

contained living vectors of Chagas disease. About one-third housed the vector, and a fifth of these insects were infected with *T. cruzi*. All of the houses inspected in some endemic areas of Argentina contained the vector. Between one-fourth and one-half of domestic animals (such as guinea pigs, cats, and dogs) were found to be infected (Bastein, 1998). Not unexpectedly, humans living in these areas were also commonly infected. About 40−80% of rural Bolivians were shown to be infected; infection was also detected in northern Chile (20%), southern Peru (12−20%), and endemic areas of Venezuela (54%) (Briceño-Leon, 1987; WHO, 2002).

2.2 The Parasite Transmission Cycle

T. cruzi was originally transmitted directly between marsupials but was subsequently vectored to other mammals through the advent of the bloodsucking Hemiptera (Triatominae) that are now considered the major vectors. This transfer from marsupials to other mammals may have been the main factor promoting adaptation of the parasite from the original widespread form (*T. cruzi* I) to a range of other lineages now grouped as *T. cruzi* II, *T. cruzi* III, and *T. cruzi* VI.

Current estimates suggest that the first divergence from *T. cruzi* I to *T. cruzi* II (mainly human) occurred about 10 million years ago (Zingales et al., 2009). The epidemiological pattern of *T. cruzi* reveals that primitive transmission was restricted to established cycles in tropical forest environments. Triatomine insects fed on small mammals in broad areas of the South American continent, with no human involvement in the natural cycle. The same situation persists today in the wild, where the disease maintains enzootic epidemiological character. The presence of *T. cruzi* does not seem to affect triatomines significantly, nor does it impact the mammals that have been naturally infected, suggesting that a balance exists between species as a result of long periods of adaptation and coevolution (Guhl et al., 1999).

In general, the Hemiptera represent an ancient order, with fossilized remains dating from the Permian period nearly 232−280 million years ago; however, the triatomines may have evolved later, starting at different times and from diverse ancestral forms. The Hemiptera comprise a large order with over 80,000 species widely distributed in all tropical and temperate areas. Ancestral predatory habits among the triatomines can be inferred from the fact that some species occupy a relatively wide spectrum of ecotopes and are able to exploit different species of hosts while others occupy restricted habitats and hosts (Schofield and Matthews, 1985). The vectorial transmission of *T. cruzi* is restricted to the New World.

2.3 Insect Vectors Associated with the Human Habitats

T. infestans is the *T. cruzi* vector with the widest distribution in South America, occupying the geographic regions that today belong to Argentina, Bolivia, Brazil,

Chile, Paraguay, Uruguay, and southern Peru, usually found in rural dwellings inside the cracks of walls and roofs.

This domiciliated insect adapts well to harsh external conditions and human habitations and grows to high capacities and domiciliation indexes. As a result, it has been targeted for vector control activities in the past decade and has been eliminated from Chile, Uruguay, and Brazil as well as large areas of Argentina and Paraguay.

T. infestans reaches high population densities in human dwellings, maintaining similarly sized populations from year to year. Its generation time is about 6 months, so it is possible to have two generations per year. Unlike other medically important insects, such as mosquitoes, triatomines tend to adapt to efficiently exploit a stable environment like the nest of a mammal or a human habitation.

These insects were undoubtedly present in the human environment in pre-Colombian times and effectively transmitted *T. cruzi*, as we will see. The dispersion of the vectors is a high-risk factor for human settlements. Historical reconstructions suggest that the dispersal of *T. infestans* from its supposed origin in central Bolivia was associated with documented human migration (Schofield, 1994).

This also is true for *R. prolixus*, the primary domiciliated vector in regions of Colombia, Venezuela, and the vast majority of Central American countries. The development of *R. prolixus* from egg to adult takes 3−4 months, compared with 1−2 years for such species as *T. dimidiata*, a peridomiciliary/domiciliary species.

Triatomines show a high degree of dispersion, which involves two different mechanisms: one passive, by the vertebrate host, and the other active by walking or, in the case of adult insects, flying. Several authors have reported the passive transport of triatomines in clothing and baggage, and even the transport of eggs and nymphs in the feathers of birds (Gamboa, 1962; Foratini et al., 1971).

The geographic distribution of triatomine species extends from the Neotropics to Neoarctic regions and is closely related to environmental and ecological factors (Figure 2.1).

Historical data indicate that the disease was transmitted in South America and seriously affected the inhabitants of endemic regions, who referred the insects with vernacular names. The many indigenous names for the insect vectors, such as *vinchuca*, *hita*, and *chirimacha*, demonstrate the frequency with which pre-Colombian civilizations encountered these insects.

The Quechua word *vinchuca*, for example, means "bug that lets itself fall," which describes the behavior of the domiciliated insect after feeding on blood. *Hita* is also a Quechua word that means "bedbug," and the *chirimacha* meaning is "which fears the cold." These Quechua words clearly evoke the domiciliated behavior of triatomines.

Quechua ("qheshwa") is an indigenous language of the Andean region, spoken today by approximately 13 million people in Bolivia, Peru, Ecuador, northern Chile, Argentina, and Southern Colombia. It was the official language of *Tawantinsuyu*, the Inca Empire, which was the largest empire in pre-Colombian America. The administrative, political, and military center of the empire was located in Cusco in modern-day Peru. The Inca civilization arose in the highlands of Peru sometime in the early thirteenth century (Table 2.1).

Figure 2.1 Distribution of different triatomine species restricted to particular geoecoepidemiological factors.

2.4 Historical Overview

Historical data allow us to infer that when the Europeans arrived in the New World, there was already a local knowledge of triatomine insects, including their habits and some biological characteristics directly related to man; however, these insects were not known to be associated with the disease first described by Carlos Chagas in 1909.

Table 2.1 List of the Vernacular Names for Triatomine Insects in South American Geographical Areas and Cultures

Region	Names	Meaning
Argentina, Chile, Uruguay	*vinchuca*[a]	Bug that lets itself fall
Belize	*bush chinche*[h]	(Implies absence of domestic Triatominae in Belize)
Bolivia	*vinchuca*[a]	Bug that lets itself fall
	uluchi[a]	Bug without wings; refers to nymphal stages
	timbucú	
Brazil	*barbeiro*[b]	Barber, shaver
	furão[b]	Big piercing bug
	chupão[b]	Big sucking bug
	bicudo	Beaked bug
	fncão[b]	Big piercing bug
	cascudo[b]	Thick skinned bug, used mainly for nymphs
	chupança[b]	Sucking bug
	procotó[b]	Bug that hides in cracks
	gigolô[b]	Exploiter of women
	percevejo[b]	Wall bedbug
	gaudério[b]	Indigent thief
	rondão[b]	Big bug that observes from hiding
	percevejão[b]	Big bedbug
	percevejo do sertão[b]	Bedbug from the sertão (interior of Brazil)
	percevejo das pedras[b]	Bedbug among the stones
	piolho de piassava[b]	Louse from the piassaba palm, refers to *R. brethesi* in Amazon region
	vunvum[b]	Probably onomatopoeic for the sound of bug flight
	vosipak	Matacos Indians, Roraima
	îipi	Macuxi Indians, Roraima, and Venezuela, refers specifically to *T. maculate*
Colombia	*pito*[c]	Whistle or horn
	chupasangre[c]	Bloodsucker
	kajta in kággaba[d]	Kogi Indians, refers to the spirit of the insect
	kajta chiguibu[d]	The eggs of the triatomines
	kajta bulo[d]	First nymph star
	kajta yagua[d]	Second nymph star
	kajta tema[d]	Other star and adults
	kajta ungaga[d]	The place for payment where the spiritual Leader (Mamo), after consultation with the spirit of the triatomines, pays with offerings that vary greatly depending on the query to restore the natural balance
Cuba	*sangrejuela*[c]	Bloodstealer

(Continued)

Table 2.1 (Continued)

Region	Names	Meaning
Ecuador	*chinchorro*[c]	Large bug
Central America	*chinche besucona*[c]	Kissing bug
	talaje[c]	Cutting bug
	chuluyu	Needle
	polvoso[c]	Dusty
	chinche bebe sangre[c]	Blood drinking bug
	chinche picuda[c]	Biting bug
Mexico	*chinche besucona*[c]	Kissing bug
	chinche hosicona[c]	Trunked bug
	chinche picuda[c]	Biting bug
	chinchona[c]	Big bug
	pech[e]	Onomatopoeic for the sound of bug flight
	texcan[f]	
Paraguay	*chichá guazú*[g]	Big bug
	tchajuponja[g]	Bug sucker
	sham bui tá[g]	Insect that does harm by its dejections
	timbucú[g]	Long beak
Peru	*chirimacha*[a]	Bug that dislikes the cold
	yta[a]	
USA	*kissing bug*[h]	Cone nose bug, big bedbug
	china bug[h]	Refers to *T. protracta* on Pacific coast, once assumed to come from the Orient
	red banded	Cone nose (refers to *T. rubrofasciata* or *T. sanguisuga*)
Venezuela	*chipo*[c]	Little bug
	îipi	Macuxi Indians; refers specifically to *T. maculata*

[a]Quechua, a Native American language family spoken primarily in the Andes of South America, derived from an original common ancestor language, Proto-Quechua. It is the most widely spoken language family of the indigenous peoples of the Americas.
[b]Portuguese.
[c]Spanish.
[d]Kogui language, Colombia.
[e]Maya language.
[f]Azteca (Nahuatl) language.
[g]Guaraní.
[h]English.
Source: Adapted, modified, and expanded from Schofield and Galvao (2009).

Geronimo de Bibar, Chilean author of a chronicle from the early 1500s, wrote, "for six years now there is a type of bugs that sting very badly and give little itching, they are as big as cockroaches and their time is in summer" (Bibar, 1966).

Various chroniclers, such as Antonio de Ciudad Real, also make references to bugs with wings. There are other references to bugs with wings that correspond to Triatominae (i.e., *kissing bugs, pitos hyphae, hocicones*), (*texcan* in Nahuatl, *pec* in

Maya, and *yta* in Quechua). Lizárraga also refers to this group of insects, saying the following about Bolivia and Argentina:

> *Here called hyphae, cockroaches (black), as large as the insects found in the ships of the North Sea, and that colour, with wings: but with the difference that they are almost invisible, they sting and bite so delicately at night that it is not felt after the fire is finished, however after two days a welt rises as a bean, so itchy it is insuf ferable (...) The insects are afraid of fire, and when the fire goes out they fall down the walls or from the ceiling and bite the sleeper in the legs, in the head and face (...) They have clumsy feet, and when they have filled their bellies with the blood they have sucked, cannot walk.*

Felix de Azara also describes these bugs "like flat beetles," adding the important observation that "...when defecating on the wound, they leave an indelible stain."

When describing the existence of *vinchucas* in Venezuela, the Jesuit José Gumilla reported that the bite was painless; however, once the arthropod detaches, unbearable itching pain occurs. In Peru, especially in the area of Charcas, Bernabé Cobo commented that they are harmful, locate their victims by scent, and are known by the natives as hyphae (Gumilla, 1963).

Archaeological excavations have been carried out along the Aleutian Islands and the west coasts of Canada and the United States, as well as on the west coasts of Central and South America. The origin and spread of humans coincided with the latter part of the Pleistocene ice ages, and the geographical distribution of early humans was influenced by these ice ages in a number of ways. All of the excavations have yielded evidence of the presence of human activity since nearly 15,000 years ago. However, the precise date of the first human presence on the continent may be even earlier, as far back as 20,000−25,000 years, although this is still a matter of debate among archaeologists. For the purpose of this chapter, it is safe to conclude that humans were spreading south and east in North America and that these small bands of hunter-gatherers had reached the northern tip of South America around 10,000−12,000 years ago.

This amount of time was enough for the adaptation of various parasitic diseases to their new hosts. Cultural developments, such as agriculture and permanent or semipermanent settlement patterns, created an ideal environment for the spread of infectious diseases, including tuberculosis and syphilis. Human cultural adaptation to warm and humid environments allowed Chagas disease to spread widely.

The epidemiological pattern of *T. cruzi* reveals that primitive transmission was restricted to established cycles in tropical forest environments. The parasite was spread via anal gland secretions and urine from opossums, and later via triatomine insects that fed on small mammals in broad areas of the South American continent, with no human intervention in this natural cycle. This would suggest a balance between species.

Because of long periods of adaptation, the parasite has a wide range of wild mammal reservoirs, including *Didelphis marsupialis*, *Philander opossum*, *Dasypus novencinctus*, *Tamandua tetradactyla*, *Saimiri sciurius*, *Chiropotes satanas*, and

bats of the genus *Phylostomus*, as a result of long periods of adaptation. The same situation persists today in the wild, where the disease maintains an enzootic epidemiological character. The presence of *T. cruzi* does not seem to significantly affect triatomines, nor does it impact mammals that have been naturally infected. Humans might then have become infected as a single addition to the already-extensive host range of *T. cruzi*, which also includes other primates (Guhl, 2000).

Human Chagas disease is a purely coincidental occurrence. As humans came into contact with the natural foci of infection and caused different degrees of ecological transformations, infected triatomine insects were forced to occupy their dwellings. Thus began a process of adaptation and domiciliation to human habitations through which the vectors had direct access to abundant food as well as protection from climatic changes and predators. A good example of this adaptation is *T. infestans*, the main vector of *T. cruzi* in the Southern Cone countries of South America. *T. infestans* is considered to be an almost exclusively domiciliary species, and the same is true for *R. prolixus* in the northern region of South and Central America.

Estimates have been made about the age of the order Xenarthra (which contains armadillos, anteaters, and sloths), one of the four major clades of placental mammals reported to be hosts for *T. cruzi*. All four of these clades were isolated in South America following its separation from the other continental land masses. Xenarthrans diverged over a period of about 65 million years, leaving more than 200 extinct genera and only 31 living species.

The next placental mammal (*T. cruzi*) reservoirs to emerge in South America were the caviomorph rodents and platyrrhine primates during the Eocene epoch (Poux et al., 2006). These clades appear following colonizations by rafting or island-hopping across the Atlantic Ocean from Africa by their respective most recent ancestor. The continental isolation ended when the Isthmus of Panama land connection between South and North America emerged approximately 3.5 million years ago in the Pliocene, marking the beginning of the Great American Biotic Interchange (Stehli and Webb, 1985). With regard to mammals, the northern invaders of South America included carnivores, insectivores, noncaviomorph rodents, lagomorphs, artiodactyls, and perisodactyls. However, bats are conspicuously absent in this scenario despite of their contribution to 20% of the mammalian species diversity.

Based on molecular dating, interordinal diversification occurred in Laurasia during the Cretaceous Era, including the appearance of bats an estimated 85 million years ago (Springer et al., 2003) (Figure 2.2).

2.5 Pre-Hispanic Settlements in Areas of Transmission of *T. cruzi*

The process of triatomine domiciliation occurred simultaneously with the process of human settlement near zoonotic transmission cycles of *T. cruzi*. The process was gradual, as has been demonstrated with other species of triatominae today (Guhl, 2005).

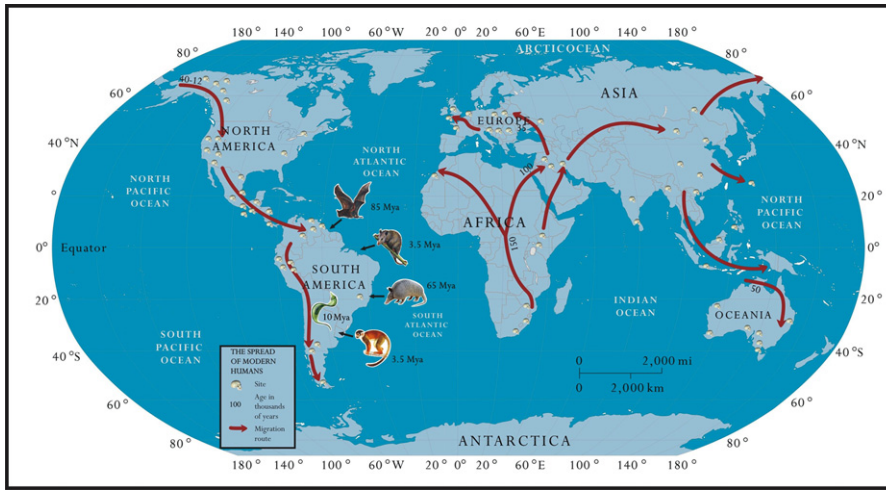

Figure 2.2 Migration routes and spread of modern humans. Hypothesized evolutionary sylvatic cycle of *T. cruzi* in America. Human contact with *T. cruzi* occurred as a simple addition to the already extensive host range 8000−10,000 years ago.

The insects found enough food sources, feeding on men and domestic animals, such as dogs, chickens, and guinea pigs. There is evidence that, in modern times, people living with pets in their homes in endemic areas enhanced the transmission of the parasite. By comparing the migration patterns of pre-Colombian cultures in different areas of Latin America with the distribution of triatomine species, it is possible to infer the degree of passive dispersion between the parasite/insect vector and pre-Hispanic settlements (León, 1962).

One of the most extensive and detailed studies of this type was performed by Viana and Carpintero in 1977 and 1979, from which one can draw the following information relating to the major pre-Hispanic cultures in the Americas.

2.5.1 Argentine-Bolivian Altiplano, Northwest Argentina

This area received direct and indirect influences from the high Andean cultures and had a society based on an intensive agricultural economy, with a variety of vegetables, livestock, and advanced bronze metallurgy. These cultures survived for a period of just over 1700 years, which were grouped into three periods: early, middle, and late.

The Early period ranges from the appearance of the earliest civilizations to the year 650 AD and encompasses the Tafi, Cienaga, Candelaria, and Condorhuasi cultures. Remains of *T. infestans* (which can be easily recognized by an expert eye) were found in the detritus deposited in urns of Tafi, Santamaría, and Aguada cultures. The sealing and subsequent burial of the urns would have prevented any

triatomine penetration *a posteriori*. Clearly, the insects were buried at the same time, probably hidden in the clothes of the corpse, which obviously suggests a close contact between the vector and pre-Columbian aboriginal communities.

The Middle period spans 650−850 AD and revolves around the La Aguada culture. The Late period ranges from 850 until about 1480 AD, beginning with the arrival of the Incas and ending with the first entry of the Spanish conquerors.

In the course of human settlements, natural pathways were formed, by which they conducted a lively exchange that enabled the economic complementarity of the different cultures. The most important archaeological sites (Quebrada de Humahuaca) show that the houses were important community nuclei and were built of stone walls with roofs of branches and mud, very good niches for triatomines. The economy was based on agricultural farming supplemented by the raising of llamas (*Lama glama*).

2.5.2 Sierras Centrales

This region, comprising the central region of Argentina-Sierras de Córdoba, San Luis, and Santiago del Estero, has been inhabited since the year 6000 BC. Interestingly, the region of Santiago del Estero continues to have one of the highest prevalence of Chagas disease in Argentina and is one of most triatomine-infested areas.

2.5.3 Sur del Perú

For the purposes of this chapter, we will highlight the Chilca culture (3800 BC) and Nasca culture (2500 BC). In Huanuco, Peru's Eastern Sierra, buildings were found that confirm the domestication of "cui" (*Cavia* sp.) dating back to 1200 BC.

In the Formative period (1200 BC–100 AD), and continuing through the Regional Development period (100−800 AD), the Wari Old Empire (800−1200 AD), and the Empire Tawantinsuyu (1430−1532 AD), the human migration of these populations increased in Northwest Argentina. The construction of large cities that still surprise us, agriculture, and the breeding of llamas and other mammals were all good environments for domiciliated populations of *T. infestans.*

New settlements and the displacement of entire villages as a result of wars of conquest facilitated the dispersion of triatomines into new areas. The mechanism of *T. infestans*' adaptation to human habitats was probably facilitated by the custom of pet storage near or even inside houses. This custom still persists in many parts of Bolivia, Perú, and Ecuador, where guinea pigs are bred in homes for human consumption.

These mechanisms of domiciliation helped *T. infestans* become one of the first insects to adapt to human habitats in South America, and it remains probably the most widespread and numerous. Although its settlement of Uruguay and Brazil occurred more recently (probably through Argentina), its dispersion increased, spreading northeast into Bahia (Brazil). *T. infestans* was one of the main vectors in the country (with *Panstrongylus megistus*) until a decade ago when efficient vector

Figure 2.3 Origin and dispersal of *T. infestans* in South America.

control was established. Paraguay could be settled from the provinces of Northwest Argentina and across the Gran Chaco (Figure 2.3).

2.5.4 Mesoamerica Mayan Culture

The great Mayan culture in the Peten region (northern Guatemala) lasted from 300 to 900 AD. However, the different communities that made up the amazing Mayan Empire probably originated from what is now northern Honduras (Santa Rosa de Copan). In the Early Formative Period (1200 BC−300 AD), the society was agricultural and built ceremonial buildings of stone. Constructions of this type also housed chiefs, priests, and dignitaries, although most of the townspeople occupied dwellings made of boughs and straw-reinforced adobe and poles, an environment conducive to the establishment of triatomines.

After an intermediate period (900−1000 AD), the New Empire or Mexican Period began, which took place primarily in Yucatan, Quintana Roo, Campeche, Tabasco, and Chiapas (1000−1200 AD). The disintegration of the Empire began due to internal wars and was completed by the arrival of the Spanish (1450−1550 AD). The economy,

mainly agricultural, reached a high level, and trade with the cultures of central Mexico was frequent and important. There is also sufficient evidence of an active relationship, mostly commercial, with the northern cultures of South America.

The keeping of poultry among the Toltecas, a Mayan village dominated by the Aztecs in the fourteenth century, certainly led to the adaptation of *T. dimidiata* and subspecies to human habitats by a mechanism similar to that for *T. infestans*.

Although its geographical distribution is wide, *T. dimidiata* is less efficient in transmitting *T. cruzi*. Its range extends north into Mexico and south to Colombia and Ecuador (Bargues et al., 2008).

Interestingly, a culture akin to the Maya flourished in the area occupied by the current state of Oaxaca: the Zapotec culture. This area is inhabited by *T. barberi*, the Protracta complex species that is best adapted to human habitats (Figure 2.4).

The species and subspecies of the Phyllosoma complex, which is found around dwellings and in domiciliary environments in southern and central Mexico, seem to have adapted more recently and by a mechanism similar to that of the species mentioned so far.

2.5.5 Andean Region, Northern South America

The Chibcha tribes stretched from Colombia to Ecuador and Nicaragua. Their houses were built of posts or poles, with or without added adobe, and the roofs were built with palm leaves. The domiciliation process of *R. prolixus* from its wild habitat occurred as it does today. Genetic studies of these insects have shown identity of genotypes among domiciled insects and insects found in the wild, especially those nesting in palm trees of the genus *Attalea* (Pinto et al., 2005).

One of the most prominent Chibcha groups inhabiting the highlands of Bogota and its nearby valleys were the Muiscas (men), who built several large communities (100 AD). They grew corn, potatoes, sweet potatoes, cotton, and cassava in the lower valleys, and their extensive housing groups formed stable communities for the creation of a solid economy based on trade with neighboring villages, primarily those to the north and west. Domiciliated *R. prolixus* penetrated into Colombia and Venezuela and then expanded northward reaching Guatemala (Figure 2.5).

2.6 Oral Infection by *T. cruzi*

In addition to insect transmission, Chagas disease may be acquired by ingestion. *T. cruzi* oral transmission is possible through food contamination by the feces of the vector or by the ingestion of raw meat from infected sylvatic reservoirs. Many aboriginal cultures and rural populations in South America still eat raw or semiraw meat from wild animals, which are reservoirs of the parasite (Coura, 1990), (Lainson et al., 1979).

In the last 10 years, for example, many outbreaks of orally acquired acute Chagas disease have been reported in different geographical areas in South America. This includes five deaths and 121 acute cases in Brazilian Amazonia, 34 cases including four deaths in Colombia, and a large urban outbreak of orally

Figure 2.4 Origin and dispersal of *T. dimidiata* in Mesoamerica and northern South America region.

acquired acute Chagas disease at a school in Caracas, Venezuela, that affected 103 children (Alarcón de Noya et al., 2010).

In general, these outbreaks lead to death in a percentage of infected individuals, which indicates a high pathogenicity of the parasites and demonstrates its capacity to penetrate through the gastric mucosa, despite the presence of gastric acid (Marsden, 1967; Maguire et al., 1986; Hoft et al., 1996). Additionally, the presence of metacyclic

Figure 2.5 The dispersion of *R. prolixus* in the Americas.

forms of *T. cruzi* in anal gland secretions of the possum (*D. marsupialis*), an animal with wild and peri-urban habits, cannot be overlooked as a source of oral transmission in the outbreaks (Deane, 1964, 1984). The traditional mechanism of transmission of Chagas disease, which involves contact with metacyclic forms in the feces of wild triatomines, may not be the most common type of transmission in wild ecotopes like the Amazon where several other types of transmission appear to be occurring.

2.7 Evidence of Human *T. cruzi* Infection in Pre-Colombian Civilizations

For more than a century, examination of skeletal tissue from ancient human remains has demonstrated information useful for the understanding of some diseases in antiquity (Aufderheide, 2003). Unfortunately, only a minority of human diseases leave a detectable impact on bone. Hence, during the past few decades, efforts have been made to evaluate whether other diseases could be detected by examination of the soft (i.e., nonskeletal) tissues in mummified human remains.

One of the first reports related to Chagas disease in human remains from South America was from Rothhammer et al. in 1984 and 1985, which described cardiac lesions compatible with the chronic clinical picture of the disease. The evidence was obtained from 35 bodies dating from 470 BC to 600 AD that were mummified in the desert of Atacama in Chile.

A Peruvian Inca mummy studied by Fornaciari et al. in 1992 showed evidence of Chagas disease in the lesions described; additionally, they demonstrated the presence of amastigote nests in the heart muscle of the mummy.

In 1997 and 1999, Guhl et al. initiated molecular studies of mummies from the Atacama desert. For the first time, they isolated *T. cruzi* DNA from 4000-year-old mummified tissue. In 2000, Ferreira et al. published an article on mummies from San Pedro de Atacama that confirmed chagasic infection in specimens up to 2000 years old.

All of these reports confirm the hypothesis discussed in this chapter on human migration and Chagas disease. Most of these studies have taken the form of individual case reports. These are valuable and will remain so for a long time; however, the study reported herein represents an effort to determine whether examination of such mummified human soft tissues can reconstruct the behavior of a disease in entire ancient populations.

We selected American trypanosomiasis, more popularly known as Chagas disease, as an appropriate candidate because of its high prevalence in the area of our study.

Initially, we attempted to detect the presence of *T. cruzi* using molecular biology methods that targeted a segment of the parasite's DNA in excess of 300 base pairs. Although we succeeded in that effort (Guhl et al., 1999), the target segment proved to be too long for the sensitivity needed for a study involving a large number of specimens. A shorter segment involving a probe had the necessary sensitivity but required considerable manipulation of the amplified product (Madden et al., 2000). Our final effort, described in detail in this report, used a short segment of kinetoplast DNA with less handling of our amplicon. This technique proved to have the sensitivity we needed with minimal manipulation to bring about the hybridization reaction. This was then applied to extracts of tissue specimens from 283 mummified human remains from a South Andean coastal zone. The results enabled us to construct the paleoepidemiology of Chagas disease in that area over a period of nine millennia, from the appearance of the first humans in that region to the near present (Aufderheide et al., 2004).

Members of the Chinchorro cultural group were the first to settle this coastal segment. The oldest body from this group was radiocarbon-dated to about 9000 years ago. Stable isotope reconstruction of their diet indicates that approximately 90% was of marine origin, consistent with some of their grave artifacts. We have only a few samples of their housing because of lack of rain, which meant they did not need a waterproof shelter. Reconstruction of several of these shelters indicates that a series of slender wood poles were arranged in a circular pattern, and the top ends were gathered together in the form of a wooden tepee. The cover of the pole skeleton did not survive, but was most probably composed of intertwined reeds harvested from the brackish water at the river mouth. Such a dwelling would be ideal for nesting by the Chagas insect vector.

After more than 5000 years of residence with this type of a marine-based strategy, the Chinchorros were replaced by highland migrants, that we call Alto Ramirez. Arriving at the coast in about 1000 BC, they introduced agriculture. Their shelters were slightly larger than those of the Chinchorros, with walls of cane and reeds; however, because they still employed plant products in parts of their structures, these remained attractive for insect nesting.

By 4000 BC, the rapidly expanding highland population called Tihuanaco extended their territory to the sea, where it remained the dominant force until their empire crumbled about in 1000 AD. Emerging from the resulting politico-cultural chaos of small, fragmented groups were the people now known as Maitas Chiribaya. By this time, they had drifted from a mixture of marine and agricultural strategies more toward the latter, and this remained the case until the Incas arrived, preceding the Spanish.

Beginning at the end of the Chinchorro period, the trend in these societies was one of progressive technology development, particularly in textile production. Their shelters also slowly increased in sturdiness. However, local resources were limited, so opportunities remained available for insect vectors to move in permanently. The arrival of the Spanish initiated the historic period. The following cultural and societal chaos rarely offered the native populations improvements with respect to living quarters.

Thus, the picture painted by archaeological findings of events in the past nine millennia begins with primitive dwelling structures that were altered by the succession of cultural groups until historic times, but not to a degree that would prevent infestation by the Chagas disease's insect vector.

Another interesting study of *T. cruzi* in human remains dating back 4500−7000 years were obtained from a Brazilian archeological site. From these remains the recovery of an ancient DNA (aDNA) sequence corresponding to the parasite lineage I (*T. cruzi* I) was recovered. The mummy was a woman approximately 35 years of age from a found-gatherer population. She was found in the Abrigo Malhador archeological site, Peruaçu Valley, Minas Gerais State. In this region, the semiarid ecosystem is predominant, thus it has a dry climate, and soil with a basic pH. These conditions have contributed to preservation of specimens (Araújo and Ferreira, 2000).

This report (Lima et al., 2008) showed that *T. cruzi* human infection in Brazil is ancient, dating back at least 4500 years and therefore occurring in hunter-gatherer

populations largely preceding *T. infestans* domiciliation. The presence of the *T. cruzi* I in humans 4500−7000 years ago in Minas Gerais State, where this genotype is currently absent, suggests that the distribution pattern of *T. cruzi* genotypes in humans has changed over time. Moreover, the recovery of an aDNA sequence and the possibility of genotyping parasites from human remains make it possible to reconstruct the early dispersion patterns of *T. cruzi* subpopulations. On the basis of these results, one may speculate that the current outbreaks of human *T. cruzi* infection, independent of triatomine domiciliation, are re-emergences of the ancient epidemiologic scenario of Chagas disease in Brazil.

Paleoparasitological studies of Chagas disease may clarify the antiquity of this disease in the Americas through extraction and amplification of *T. cruzi* aDNA from human remains, other animal hosts, and vector fragments found in archaeological findings.

Phylogenetic analysis of this material would also shed light on different aspects of host−parasite coevolution and parasite transmission cycles.

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3 Social and Medical Aspects: Morbidity and Mortality in General Population

João Carlos Pinto Dias[1,*] *and C.J. Schofield*[2]

[1]Oswaldo Cruz Foundation and Brazilian National Research Council, Minas Gerais, Brazil, [2]ECLAT network, London School of Hygiene and Tropical Medicine, London WC1 E7HT, UK

3.1 Introduction

In their recent publication about human Chagas disease (HCD), the Médecins Sans Frontières (MSF) declared that

> *when we say that Chagas disease is a silent disease, we are simply stating a fact: in most cases it is a disease that presents no suspicious signs or symptoms for sev eral years. Patients who suffer from it are not often aware that they are infected until heart or digestive dysfunction develops in its chronic stage. However, when we say that Chagas is a silenced disease we want to stress that there are those who wish to silence it.*
>
> *(MSF, 2005)*

In the reality, both aspects remain associated in the social context of HCD. Poverty, huts, economic instability, a lack of favorable production relations, and the usual absence of political and social priorities by the Latin American governments are some of the general historical factors influencing the expansion and control of the disease (Dias and Borges Dias, 1979; Dias, 1988; Briceño-León, 1990). In spite of evidence of its existence among very ancient indigenous settlements in South America, for example, by the detection of *Trypanosoma cruzi* DNA in Chilean and Peruvian mummies aged up to eight centuries before Christ, dispersion of Chagas disease occurred mainly after the time of Columbus. Its epidemiological apex occurred during the twentieth century as a result of extremely deep social and economic changes throughout the whole region (Dias et al., 1994; Carlier et al., 2002).

* E mail: jcpdias@cpqrr.fiocruz.br

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The appearance of the disease as endemic depended basically on the economic and social scenery of Latin American colonization involving complex and different anthropic factors, such as migration, inequity in the productive/extractive process, and very unstable and precarious ways of living. However, by the nineteenth century, other factors were involved, such as the complete absence of official planning for human settlement, mainly in terms of public health, public education, and environmental policy (Dias and Borges Dias, 1979; Bucher and Schofield, 1981; Candiotti, 1989).

The classical pattern of HCD has expanded throughout the poorest Latin American rural areas over many decades, particularly from the start of the twentieth century until 1990, when insecticide campaigns were launched in several endemic countries and the classical rural−urban migration process began to reverse in all regions (Dias and Schofield, 1999). Since then, from its original framework, the disease began to modify its general epidemiology. Important demographic changes, such as globalization and rural−urban migration, were involved in this process by which "chagasic" individuals have been detected in several Latin American urban centers, as well as in Europe and other nonendemic regions. Another new epidemiological fact concerns the crescent detection of acute cases due to oral *T. cruzi* transmission in the Brazilian Amazon region (Carlier et al., 2002; Dias, 2005). Looking to the future, the main challenges regarding HCD as a public health problem may be to:

1. consolidate vector and blood banks control in Latin American countries still without regu lar control programs;
2. implement a regular and sustainable epidemiological surveillance in those countries with advanced control programs;
3. take medical and social attention to the 12−14 million infected people living both in endemic and in nonendemic countries (Dias, 2004; Schofield et al., 2006).

A very crucial point in the history of controlling the disease has been the political priority of the official control programs and their sustainability. In most situations, the population under transmission risk remains very poor and dependent on continuous governmental public actions for disease prevention and medical attention. Disease transmission has been controlled in many areas of the endemic region by intensively controlling triatomines and monitoring blood banks (Dias and Schofield, 1999; WHO, 2002). As a consequence, present and future scenarios involve a progressive reduction of disease visibility and the need for permanent surveillance regarding savage triatomines and new possible epidemiological situations. Such risks cannot be ignored and will require adequate approaches, in a foreseeable context of reduced political interest and consequent lower operational budgets (Schofield et al., 2006).

In this context, the political activity of many scientists involved in the fight against HCD has been decisive to create and maintain the priorities for control of the disease. The basic strategy in this task was to research and publish epidemiological data, pointing out the medical and social frame of the disease in terms of its

geographical distribution, incidence, prevalence, morbidity, mortality, and social costs (Dias and Schofield, 1999).

In the last three decades, it has been recognized that the social and economic aspects of Chagas disease were extremely important in the production and dispersion of the disease. Moreover, such a "contextual" frame showed to be clearly linked with the clinical aspects, also being determinant of the possibilities of disease controlling and management (Dias and Borges Dias, 1979; Dias et al., 1994; WHO, 2002; Briceño-León, 2007). The consensus in the field is that the definitive fight against Chagas disease cannot be focused simply in the traditional biological angle; instead, action must involve all those aspects and determinants of its occurrence, "*understanding the 'chagasic' patient in his bio-psycho-social and cultural reality, in a political and economic context in which the common denominator is poverty*" (Dias et al., 1994, p. 527). We intend to reinforce some yet traditional ideas of such a context, focusing the main discussion in the related medical dimension, including some disease prevention aspects.

3.2 General Frame and Costs of HCD

Three major parameters define the social context of HCD in endemic areas: poverty, literacy, and politics. First, from the standpoint of disease spreading, the general picture of poverty can be represented by the typical Latin American rural huts, which are easily invaded and colonized by triatomine bugs. Furthermore, the weak local and regional public health systems are unable to provide adequate access, expertise, and action sustainability, including blood bank control. Overall, there is general inequity involving the production relationship along the whole region.

Secondly, from a cultural and sociologic standpoint, Chagas disease is substantially more prevalent in illiterate individuals. Generally, those who are infected or are at high risk for the disease are without a positive political expression and tend to offer very low self-appraisals.

Thirdly, from a political and economic standpoint, the disease does not always have the necessary governmental priority. Endemic countries chronically lack the human and financial resources needed for disease control and frequently discontinue their control programs.

Another important aspect concerns the very poor involvement of regular national education systems in the campaign against HCD along the endemic area. At the same time, the disease does not represent a good market, regarding either the political or the common capitalistic interests.

The costs of the disease have been estimated in several studies. In general, we agree that the costs of chronic disease are higher than acute ones, mainly in terms of mortality and morbidity. Most studies have shown that the principal element of cost remains in severe chronic Chagas heart disease, leading the affected people to premature death while they incur high medical and hospital expenditures and lose time from work. We also agree that the costs of prevention (triatomine and blood bank controls) are relatively low compared with the financial loss due to Chagas

disease in endemic areas (Schofield and Dias, 1991; Antunes, 1999; Akhavan, 2000; Salvatella, 2007; WHO, 2007).

3.3 The Medical Burden of HCD in Endemic and Nonendemic Areas

According to recent Pan American Health Organization (PAHO) data, 21 countries must be considered endemic for HCD in the Americas, with a general prevalence rate of 1.448 for every 100,000 inhabitants meaning at least 7,694,500 individuals are infected. Between 60 and 80 million individuals remain at risk of *T. cruzi* transmission in endemic countries.

The annual incidence rate was calculated to be 0.008 cases out of 100,000 inhabitants, with 41,200 cases of vectorial transmission and 14,385 cases of congenital transmission yearly (OPS, 2006). Since morbidity associated with Chagas disease varied by geographical regions and age groups, general data have been restricted to estimate the impact of the disease in terms of cardiac involvement and premature deaths, both in acute and in chronic phase of the infection. For the acute disease, it is accepted that the mortality of nontreated individuals will depend on the intensity and the virulence of the infection. Acute myocardiopathy is common and may or may not be associated with meningoencephalic compromise. Deaths in acute Chagas disease occur mostly in children up to 3 years age, meaning there is a high rate of transmission, chiefly of "domiciliated" infected triatomines.

The general mortality rate in nontreated individuals ranges from 2% to 12% in the acute phase, with rates being higher in younger age groups. A correlated consequence of severe acute disease has been demonstrated in longitudinal studies, in which the worst evolution of Chagas disease at long-term (50 years) follow-up corresponds exactly to those cases with severe acute onset detected in those younger than age 3 (Dias, 2006). On the other hand, acute Chagas disease has shown to be curable with the currently available drugs (nifurtimox and benznidazole).

Adequate treatment improves the clinical picture in most cases. Nevertheless, there are three major constraints limiting acute treatment: lack of medical expertise in endemic areas, making difficult and rare the correct diagnosis; the unclear clinical picture related to laboratory difficulties and symptoms that are many times unapparent or similar to other several febrile diseases; and limited medical access for the poor and rural population of endemic countries (Dias, 2005). An additional problem remains in the progressive loss of interest and visibility in this disease as it disappears from endemic areas after the control implementation (Schofield et al., 2006).

The chronic phase now constitutes the major problem in all endemic and nonendemic countries. Among the millions of infected individuals, at least 20% will develop chronic heart or digestive disease. All of these people are potential transmitters of the parasite by means of blood and organ transplantation, and those patients suffering Chagas heart disease certainly will have severe working limitations, high costs concerning medical

attention, and reduced life expectancy, according to several studies (Schofield and Dias, 1991; Dias et al., 1994; Brasil, 2005; MSF, 2005; WHO, 2007).

Another issue is the superposition of coinfections or chronic degenerative diseases in individuals primo-infected with *T. cruzi*. Both these situations are related with social and demographic factors involving the migration for urban centers and the survival of "chagasic" people (Dias, 2005, 2006). At present, the possibilities for chronically infected individuals are much better than 30 years ago. Not only the specific treatment but also the supportive management for chronic cardiopathy and digestive "megas" have been considerably improved in the last decades (Brasil, 2005; Dias, 2007). Following the evolution of disease management, the quality and quantity of life of the infected individuals can be improved, chiefly by preventing severe arrhythmias, the progression of cardiac failure, and the occurrence of sudden death. The same is valid for the "mega" syndromes, since a precocious intervention at the beginning of the symptoms can delay or even prevent later severe complications.

The correct management of HCD presupposes continuity for several years, with regular medical supervision included for indeterminate chronic patients. In this context, three main elements must be considered for an effective sector management policy: access, expertise, and drug availability. On the other hand, social security is deeply involved with the evolution of chronic disease, mainly for those patients who demonstrate initial and severe degrees of cardiac failure and complex arrhythmias. When adequately detected and managed, these classic heart disturbances can be effectively controlled through modern drugs, medical interventions (e.g., pacemakers and defibrillators), and rest and physical adequacy (Dias, 2000).

In Brazil, with about 2.5 million chronic infected individuals, 20% will develop a cardiac disease (500,000 individuals) and, among these, 5−10% (25,000−50,000) will develop severe degrees of "chagasic" cardiopathy (Salvatella, 2007). All of them will require not only permanent medical but also social attention. In such a context, probably half of Brazilian individuals who do not receive the care they need, which clearly affects their chances of survival. In our framework, the social consequences of Chagas disease are mainly due to chronic cardiopathy, involving work limitation and quality and quantity of life, thus generating severe social consequences at the individual and group levels. Besides the *T. cruzi* infection and some biological characteristics, some social factors actually may contribute to Chagas heart disease, such as compromised physical effort, undernourishment, alcoholism, and lack of medical attention.

The more severe "chagasic" cardiopathy often kills men aged 35−50 years, leaving a significant population of orphans, widows, and children who must leave school prematurely to help the family survive (Dias et al., 1979; Dias et al., 1994; Carlier et al., 2002). On the other hand, the majority of infected individuals remain several years in the chronic indeterminate clinical form. Most of them will never be diagnosed since neither symptoms nor physical signs are present. Some of them will be diagnosed by means of blood bank serological screening, other by population serology, presurgical procedures, or special public health programs.

Anyone whose illness is not discovered cannot be treated early and will miss out on current promising treatments that can delay chronic forms of the disease (Brasil, 2005; MSF, 2005). In the case of nonendemic areas, the social and macro-political aspects of globalization, underemployment, and immigration of people from endemic countries have been the main causes for the detection of thousands of infected individuals in Europe, Oceania, Asia, and North America (mainly USA). This relatively new situation involves serial problems concerning medical attention, labor affairs, and concrete possibilities of disease transmission, chiefly by blood transfusion, congenital, and organ transplantation mechanisms. Two correlated and crucial factors compounding the problem are medical professionals lacking the expertise to diagnose and treat the disease, and the clandestine situation of thousands of individuals who are socially unprotected (Dias et al., 2002; MSF, 2005; WHO, 2007).

3.4 The Particular Question of Specific Treatment of Chagas Disease

In recent years, the scientific community who deals with Chagas disease has been more and more involved with the theme of specific treatment: the classical drugs (nifurtimox and benznidazole) were employed during many years, and several experimental, clinical, and epidemiological evidences of concrete benefits for acute cases were established. Moreover, recent data show that parasitological cure is possible in young chronic patients and in a minor proportion (20%) of chronic older individuals (Dias, 2007; WHO, 2007).

The social aspects related to specific treatment correspond mainly to drug availability, medical expertise, and the political and administrative problems concerning the case detection and the organization of a public system for treatment provision. Drug production also has been a problem, since the market is very weak, considering the poor chagasic population and the lack of political priority in endemic regions. The solution has been drug fabrication.

The possibility of new and more effective drugs has also been a considerable constraint, since the research for new molecules is very expensive and Chagas disease does not interest the pharmaceutical industry. A strategy to take advantage of already existing products that would be effective against *T. cruzi* has been attempted and shows promise; such is the case for using third-generation antimycotic drugs able to inhibit the sterol metabolism of the parasite. The problem with this strategy, once more, has been social and economical, since these drugs are very expensive and because the industry has no interest in making them "social" (philanthropic) products (Dias, 2007; MSF, 2005).

3.5 Some Social Remarks Concerning the Control of HCD

Many years ago, a PAHO scientific group in Caracas organized by Jorge Rabinovich and Robert Tonn entered thousands of pieces of information into an

ancient computer concerning the available strategies for Chagas disease control in a typical Latin America rural environment. Considering a projection of 20 years, the results showed that adequate and sustainable triatomine chemical control would provide the best short-term impact, reaching the interruption of vector transmission in the first 4 or 5 years and sustaining this situation for the rest of the time. House betterment came in second place, reaching considerable reduction of transmission at medium term. An ideal vaccine and patient-specific treatment did not work.

The only alternative would be social development, which provided similar results to insecticide over the long term (TDR, 1981). Regarding this theoretical approach, we can now confirm the projection made in 1979. In several areas of endemic countries in which regular chemical programs were employed, not only was the incidence of HCD stopped but the degrees of prevalence, morbidity, and mortality also dropped (Dias et al., 2002; Brasil, 2005; Schofield et al., 2006; Dias, 2007). Naturally, in the scope of such results, there is a clear association between the chemical strategy and social and economic development.

Regular insecticide spraying and the sustained entomological surveillance produced naturally the first and more bruising impact, putting down dramatically the indoor population of *Triatoma infestans* and *Rhodnius prolixus*. This impact can be particularly certified in very poor and underdeveloped communities of Argentina, Bolivia, Brazil, Honduras, Paraguay, and even Venezuela, where social development did not arrive until recent years (Dias et al., 2002). On the other hand, it is unquestionable that the social improvement of several Latin American communities, the result of regional economical development, is also contributing with house improvement, better local health services, reduction of rural density, and so on. Some examples can be easily observed in São Paulo, Brazil, where industry and extensive crops of sugarcane and soybeans rapidly improved the social status of the whole population. Thus, the challenge for the future will necessarily involve social and political affairs, side by side with insecticides and medical attention. In transmission control and medical attention, the problems concerning sustainability, access, expertise, and public health systems will be crucial to overcome Chagas disease in Latin America. All of these goals are absolutely attainable, according to the current observation, all of them depending on political will (Dias et al., 2002; Schofield et al., 2006).

3.6 Final Remarks

It was not our purpose to have a deep discussion of the social aspects related to Chagas disease but to summarize, in few words, how much they are present in the production, impact, and management and control of this so-important (and neglected) disease. It can be expected for the endemic countries that, in the next 20 years, sustained control actions and strategies will control transmission in most parts of the region.

Other consequences concerning the reduction of morbidity and mortality will also depend on the improvement of the public health system. Undoubtedly, we

need more research to face new epidemiological and clinical situations, as well as to improve specific treatment and cure assessment. By the control side, the keys will be sustainable epidemiological surveillance, effectiveness of actions in peri-domestic ecotopes and, particularly, a better approach to congenital disease prevention (Schofield et al., 2006; Dias, 2007). All these questions involve naturally the regional capacity of research because Chagas disease is not an exciting market and does not affect the so-called First World (MSF, 2005). Finally, considering the next future of Chagas disease in the Americas, some risks and challenges must be highlighted:

(a) The disease will be definitely controlled in endemic areas according to political sustain ability, which means (Briceño León, 2007):
- Economic development
- Social development
- Environmental protection
- Cultural diversity.

(b) Due to political and administrative inconstancy in the region, and at the price of success fully limiting Chagas disease and thereby reducing the disease's visibility, some risks remain (Dias, 2007):
- Losing the regular structure of programs and of surveillance
- The progressive loss of human resources
- The loss of epidemiological information
- The absence or weakness of the educative component
- Several difficulties for the counter reference of cases
- The absence of a "basic basket" for "chagasic" people
- The loss of research priority
- The loss of consistence in the university curricula.

(c) Some very concrete constraints can be detected both at control and clinical programs at the present moment, such as (Schofield et al., 2006):
- The punishment of success
- *External enemies*: they do not provide more support, they withdraw resources
- *Internal enemies*: the programs are not changed to the new phase, they do not adapt to the risks of success, the same continues to be done
- Risks are based on believing that success is permanent and that it is unnecessary to adapt.

Finally, we have evidence that all these constraints and risks can be overcome by means of the technical and political association of the more authentic protagonists. The war against HCD did not start with the politicians, the health authorities, or the "chagasic" people. Since Carlos Chagas, the main efforts for recognition and control of the disease have resulted from the Latin American scientific community, also incorporating valorous partners from Europe and the USA. In the last two decades, such efforts have been directed by other very important partners and strategies, with a special distinction for the so-called Intergovernmental Initiatives for Chagas Disease Control. Such a frame represents a new logic for a sustainable and more effective control approach (Salvatella, 2007). The association among endemic countries can be considered the most visible political advance since Carlos Chagas stated, in 1911, that the disease might be considered a problem for the state (Chagas, 1911).

In particular, the role of WHO, PAHO, and some NGOs like the MSF has been more and more fundamental for the sustainability of Chagas disease control and management. It will be a real human and social tragedy if these organizations withdraw Chagas disease from their agenda in the next 20 years (Dias, 2004; Briceño-León, 2007). In conclusion, we must admit that the overcoming of Chagas disease clearly involves not only a technical logistic and a political affair, but also several aspects of social ethics.

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4 Current Trends and Future Prospects for Control of Chagas Disease ☆

Álvaro Moncayo[1,*] *and Antonio Carlos Silveira*[2]

[1]Academia Nacional de Medicina, Bogotá, Colombia, [2]Pan American Health Organization/World Health Organization, Brasilia, Brazil

4.1 Introduction

Chagas disease is named after Carlos Chagas, the pioneer researcher who first described it in 1909 (Chagas, 1909). Existing only on the American continent, the disease is caused by a flagellate parasite, *Trypanosoma cruzi*, and is primarily transmitted to humans by blood-sucking triatomine bugs and by blood transfusion.

Chagas disease has two successive phases, acute and chronic. The acute phase lasts 6–8 weeks. Once the acute phase subsides, most of the infected patients recover an apparent healthy status, where no organ damage can be demonstrated by the current standard methods of clinical diagnosis. The infection can only be verified by serological or parasitological tests. This form of the chronic phase of Chagas disease is called indeterminate form. Most patients remain in this form of the disease.

However, after several years of the chronic phase, 20–35% of the infected individuals, depending on the geographical area, will develop irreversible lesions of the autonomous nervous system in the heart, esophagus, colon, and peripheral nervous system. The chronic phase lasts the rest of the life of the infected individual. Chagas disease represents the first cause of cardiac lesions in young, economically productive adults in the endemic countries in Latin America.

Thanks to a coordinated multicountry program in the Southern Cone countries, the transmission of Chagas disease by vectors and by blood transfusion was interrupted in Uruguay in 1997, in Chile in 1999, and in Brazil in 2006.

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* E mail: amoncayo@uniandes.edu.co

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4.2 Modes of Transmission

4.2.1 Transmission Through Vectors

Chagas disease is a zoonosis transmitted in natural foci or ecological units within a well-defined geographical environment. The ecological unit is composed of sylvatic or domestic mammals and of sylvatic triatoma bugs, both infected with *T. cruzi*. Continuous transmission is assured with or without the involvement of human beings. These conditions of transmission are present from latitude 42°N to latitude 40°S, so *T. cruzi* infection occurs from the south of the USA to the south of Argentina.

There are two stages of the human disease: the acute stage, which appears shortly after the infection; and the chronic stage, which may last several years. After several years of a silent asymptomatic period, 25% of those infected develop cardiac symptoms that may lead to chronic heart failure and sudden death, 6% develop digestive damage, mainly mega-colon and mega-esophagus, and 3% will suffer peripheral nervous involvement (Coura et al., 1983, 1985; Pereira et al., 1985).

4.2.2 Transmission via Blood Transfusion

The rural-to-urban migration movements that occurred in Latin America in the decades of the 1970s and 1980s changed the traditional epidemiological pattern of Chagas disease as a rural condition and transformed it into an urban infection that can be transmitted by blood transfusion.

In most countries in Latin America it is now compulsory to screen for infected blood in blood banks and systems have been established to do so.

4.3 Methods and Measurement of Epidemiological Trends in the Continent from 1980 to 2006

Data on the prevalence and distribution of Chagas disease improved in quality during the 1980s as a result of the demographically representative cross-sectional studies carried out in countries where accurate information was not available. A group of experts met in Brasilia in 1979 and devised standard protocols to carry out countrywide prevalence studies on human *T. cruzi* infection and triatomine house infestation.

It should be noted that the prevalence and incidence of the disease as well as the mortality are constantly changing as a consequence of the impact of control programs, people migration, and changes in socioeconomic conditions of the population. The decrease in the frequency of new cases of infection by *T. cruzi* in the last decades as a result of vector control is presented later in this chapter in the sections on the subregional initiatives for the interruption of transmission of Chagas disease.

The estimation of the decrease in the incidence rates of infection by *T. cruzi* in the period under study was made by comparing the age-specific prevalence rates of

Table 4.1 Prevalence of Human *T. cruzi* Infection in Latin America, 1975–1985

Country	Population at Risk (thousands)	Percentage of Total Population	Number of Infected Persons (thousands)
Argentina	6900	23	2640
Brazil	41,054	32	6180
Bolivia	1800	32	1300
Chile	11,600	63	1460
Paraguay	1475	31	397
Uruguay	975	33	37
Colombia	3000	11	900
Ecuador	3822	41	30
Perú	6766	39	621
Venezuela	12,500	72	1200
Costa Rica	1112	45	130
El Salvador	2146	45	900
Guatemala	4022	54	1100
Nicaragua	ND	ND	
Honduras	1824	47	300
Panamá	898	47	200
México	ND	ND	ND
Total	99,895	25	17,395

ND: No data.

infection in a given age group in the period from 1980 to 1985 (at the time when the cross-sectional studies were carried out in the different countries) and the age-specific prevalence rates in the same age group in the period from 1997 to 2006 (i.e., 20 years later) (Figure 4.1; Table 4.1).

4.3.1 Transmission Through Vectors

Data on the prevalence and distribution of Chagas disease improved in quality during the 1980s as a result of the demographically representative cross-sectional studies carried out in countries where accurate information was not available. A group of experts met in Brasilia in 1979 and devised standard protocols to carry out countrywide prevalence studies on human *T. cruzi* infection and triatomine house infestation.

These studies were carried out during the 1980s in collaboration with the Ministries of Health of Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Panama, Paraguay, Peru, Uruguay,

and Venezuela. The accurate information obtained has made it easier for individual countries to plan and to evaluate the effectiveness of national control programs (Pan American Health Organization, 1974; Cedillos, 1975; Marinkelle, 1976; Zeledón et al., 1976; Cordova et al., 1980; Camargo et al., 1984; Ponce, 1984; Reyes Lituma, 1984; López, 1985; Matta et al., 1985; Schenone et al., 1985; Sousa, 1985; Franca, 1986; Acquatella et al., 1987; Salvatella et al., 1989; Valencia, 1990) (see Table 4.1).

On the basis of these individual countrywide cross-sectional surveys, it was estimated that the overall prevalence of human *T. cruzi* infection in the 18 endemic countries has reached 17 million cases. Some 100 million people (25% of all the inhabitants of Latin America) were at risk of contracting *T. cruzi* infection (see Table 4.1). The incidence was estimated at 700,000−800,000 new cases per year; the annual deaths due to the cardiac form of Chagas disease were estimated at 45,000 (UNDP/WORLD BANK/WHO, 1991).

The originally endemic area with vectorial transmission in the human domicile comprised 18 countries with higher *T. cruzi* infection rates in the regions infested by *Triatoma infestans* (Southern Cone countries) and *Rhodnius prolixus* (Andean countries and Central America), which were the triatomine species best adapted to the human domicile.

The epidemiological quantification was one of the reasons to prioritize the control of the disease, but the final political decision came from the demonstration of the high cost−benefit ratio of the control programs versus the costs of the medical care and the social security of the infected patients (Akhavan, 1997).

4.3.2 Transmission Through Blood Transfusion

The figures in Table 4.2 show the extent of the problem of transmission via blood transfusion in some selected cities of the continent between 1980 and 1989 (Schmunis, 1991). While the prevalence rates of *T. cruzi* infection in blood varied between 1.3% and 51.0%, these rates were much higher than those of hepatitis or HIV infection.

The transmission of Chagas disease via blood transfusion is a real threat even for countries where the disease is not transmitted by vector, such as the USA and Canada, where cases of acute Chagas disease have been documented (Grant et al., 1989; Kirchhoff et al., 1987; Nickerson et al., 1989).

The prevalence of infected blood samples in the Southern Cone countries has decreased, as shown by the consistently decreasing trend in all six countries of this subregion since 1994 (Table 4.3).

4.4 Feasibility of Interruption of Transmission

The tools for interrupting the domestic cycle of *T. cruzi* transmission, such as chemical control, housing improvement, and health education, are available. In

Table 4.2 Prevalence of *T. cruzi* Infected Blood in Blood Banks of Selected Countries, 1980–1989

Country	Number of Samples Tested	Percentage Positive	Reference
Argentina			
Buenos Aires (1987)	58,284	4.9	Pérez and Segura (1989)
Santiago Estero (1987)	2003	17.6	Pérez and Segura (1989)
Córdoba (1982)	2441	8.4	Pérez and Segura (1989)
Bolivia			
Santa Cruz (1990)	205	51.0	Carrasco (1990)
Brazil			
Brasilia (1984)	2413	14.6	Pereira (1985)
Paraná (1987)	3000	4.8	Marzochi (1981)
Sao Paulo (1982)	56,902	2.9	Dias and Brener (1984)
Chile			
Santiago (1983)	214	3.7	Liendo (1985)
Vicuña (1983)	62	14.5	Liendo (1985)
Colombia			
Bogotá (1990)	1128	2.5	Guhl et al. (1987)
Cúcuta (1987)	491	7.5	Guhl et al. (1987)
Costa Rica			
San José (1985)	602	1.6	Urbina (1991)
Ecuador			
Guayaquil (1971)	1054	3.2	Reports Ministry of Health
Honduras			
Tegucigalpa (1987)	1225	11.6	Ponce and Ponce, 1987
México			
Puebla (1986)	200	17.5	Velasco Castrejón and Guzmán Bracho (1986)
Peru			
Tacna (1972)	329	12.9	Reports Ministry of Health
Paraguay			
Asunción (1972)	562	11.3	Reports Ministry of Health
Uruguay			
Paysandú (1983–1984)	445	4.7	Franca (1986)
Salto (1983–1984)	71	4.2	Franca (1986)
Tacuarembó (1983–1984)	699	7.7	Franca (1986)
Venezuela			
Various cities	195,476	1.3	Schmunis (1991)

Source: Schmunis (1991).

fact, the prevalence of the infection has decreased in countries that have consistently applied control measures. For example, after 20 years of control programs in Argentina, positive serology in 18-year-old men has significantly decreased since 1980, and the number of reported new acute cases has decreased since the 1970s

Table 4.3 Estimated Number of Infections by *T. cruzi* and Annual Incidence in Latin America, 1975–2005

Country or Region	Total Number of Infections			New Cases		
	1975–1985	1995	2005	1990	1995	2005
Central America and Mexico	1,935,000[a]	...	1,906,600	209,187	72,677	16,200
Argentina	2,333,000[b]	2,100,000	1,600,000	...	...	1300
Brazil	4,500,000	1,900,000	1,900,000	...	...	0
Bolivia	1,134,000	...	620,000	86,676	...	10,300
Chile	1,239,000	157,000	160,200	...	...	0
Colombia	900,000	...	436,000	39,162	31,330	5250
Ecuador	300,000	450,000	230,000	7488	13,365	2350
Paraguay	397,000	...	150,000	14,680	...	900
Peru	643,000	...	192,000	24,320	19,072	3100
Uruguay	37,000	...	21,700	...	...	0
Venezuela	1,200,000	...	310,000	179,703	22,960	1400

(...) No data.
[a]Except Mexico 1995.
[b]1990.

(Segura et al., 1985). In Brazil, transmission by vector has been interrupted in the whole state of Sao Paulo since the mid-1970s. Decreasing rates of seropositive schoolchildren have paralleled the above control efficacy: in 1976, the incidence rate was 60%; in 1983, it dropped to 0% (Souza et al., 1984).

Transmission through transfusion could be prevented if blood is screened by serology and positive units are discarded. In most countries of the region, serology for *T. cruzi* is mandatory for blood donors.

Available knowledge therefore indicates that the most common ways of transmission of human *T. cruzi* infection could be interrupted by implementing vector control activities in houses to first reduce and then eliminate the vector-borne transmission of *T. cruzi,* and then by enabling blood banks to prevent transmission of Chagas disease by blood transfusion through the development and implementation of a policy for screening blood for human use.

4.5 Current Control Programs

The traditional vertical control programs in the Latin American countries have focused on the spraying of insecticides on houses and household annexes and buildings. National control programs aimed at the interruption of the domestic and peridomestic cycles of transmission involving vectors, animal reservoirs, and human beings are feasible and have proven to be very effective. Reaching the goal of eliminating vector-borne transmission is more feasible in areas where the vector is domiciliated, like *T. infestans* and *R. prolixus.*

Twelve countries of the Americas have active control programs that combine insecticide spraying with health education. The common pattern of the vertical, centralized control programs follows several operational steps or phases, namely:

- Preparatory phase for mapping and general programming of activities and estimation of resources.
- Attack phase, during which a first massive insecticide spraying of houses takes place and is followed by a second spraying 6–12 months later, with further evaluations for selective respraying of reinfested houses.
- Surveillance phase for the detection of residual foci of triatomines after the objective of the attack phase has been reached. In this last phase, the involvement of the community and the decentralization of residual control activities are essential elements.

A prime example is the program that was operating in Brazil since 1975 when 711 Brazilian municipalities had triatomine-infested dwellings that were the objective of control. Ten years later, in 1986, only 186 municipalities remained infested. This represents a successful accomplishment of the program objectives in 74% of the originally infested municipalities. In 1993, only 83 municipalities were infested, which represents a reduction of 86%. In 1983, 84,334 *T. infestans* bugs were captured by field workers, but in 1998 only 485 insects were found in the whole country.

In large parts of the Southern Cone countries, programs that have entered the surveillance phase are characterized by monitoring of house infestation and, where necessary, focal spraying.

4.6 Economic Impact

4.6.1 Program Costs and Cost-Effectiveness of Control Interventions

The countries of the Southern Cone Initiative spent in the period 1991–2000 more than US$345 million from their national budgets to finance the vector control activities in their territories.

The Ministry of Health of Brazil carried out a study aimed at the analysis of the cost-effectiveness and cost–benefit of the country's national Chagas Disease Control Program. Due to the chronic nature of the disease and the protracted period of evolution, a period of 21 years was chosen for the analysis. The time interval from 1975 to 1995 includes data from different sources of information that were used to carry out this evaluation (Akhavan, 1997).

Effectiveness was defined using various parameters, but the main one was the measurement of the burden of disease prevented in disability-adjusted life-years (DALYs). From 1975 to 1995, the program (excluding blood banks) prevented an estimated 89% of potential disease transmission, avoiding some 2,339,000 new infections and 337,000 deaths. This translated into the prevented loss of 11,486,000 DALYs, 31% from averted deaths, and 69% from averted disability, showing the large role of disability in the overall burden of disease caused by Chagas disease.

The estimated benefits (expenditures prevented) of the program (excluding blood banks) were US$7.5 billion, 63% of the savings being health care expenditures and 37% social security expenditures (disability insurance and retirements).

The cost-effectiveness analysis demonstrated that for each US$39 spent on the program, 1 DALY was gained. This places the Program and its activities in the category of interventions with a very high cost-effectiveness. The results of the cost−benefit analysis indicated savings of US$17 for each dollar spent on prevention, also indicating that the program is a health investment with good return. The analysis of other diseases with socioeconomic causes demonstrated that the decline in Chagas disease infection rates is due to the preventive activities, and not due to general improvement in life conditions.

The economic impact of the disease during the chronic stage is very high as shown by data from Brazil. If we consider that about 30% of the infected persons will develop severe cardiac and digestive lesions, such as cardiac arrhythmia (75,000 cases), mega-esophagus (45,000 cases), and mega-colon (30,000 cases) each year, the estimated costs for pacemaker implants and corrective surgery (average US $5000) would amount to approximately US$750 million per year. This expenditure would be enough for the improvement or construction of more than 700,000 rural dwellings at a minimum estimated cost of US$1000 each in Brazil in 2000.

Between 1979 and 1981, there were 14.022 deaths due to Chagas disease in Brazil, which represented approximately 259,152 years of potential life lost (YPLL) before the age of retirement. Assuming that all the patients were unqualified rural workers only and that the minimum daily wage was at the time US$2.50, the total economic loss due to premature deaths would amount to US$237 million.

4.7 Epidemiological Impact in the Region

The average reduction of incidence in the Southern Cone countries is 94%, as shown in Table 4.4. By cutting the transmission of the disease in the countries of

Table 4.4 Changes in Epidemiological Parameters Due to Interruption of Transmission and Decrease of Incidence, 1990−2006

Epidemiological Parameters	1990	2000	2006
Annual deaths	>45,000	21,000	12,500
Annual new cases	700,000	200,000	41,200
Prevalence (million)	30	18	15
Population at risk (million)	100	40	28
Distribution	18 countries	16 countries	15 countries (transmission interrupted in Uruguay in 1997, Chile in 1999, and in Brazil in 2006)

Source: World Health Organization, TDR (2006), p. 7.

the subregion in this proportion, the incidence of Chagas disease in the whole of Latin America has been reduced by 70%. The number of incident cases was reduced from an estimated 700,000 new cases per year in the whole region in 1983 to fewer than 200,000 new cases per year in 2000 and to 41,200 in 2006. Also, the annual number of deaths dropped from more than 45,000 to 12,500. The number of endemic countries was 18 in 1983; in 2006, this number was reduced to 15, as shown in Table 4.4 (World Health Organization [WHO]/TDR, 2006).

The intradomiciliary infestation by *T. infestans* has been eliminated in Brazil in 2006, in Chile in 1999, and in Uruguay in 1997.

At present, the major challenge is to ensure the sustainability of this program in an epidemiological context with very low *T. cruzi* infection rates and a political–institutional context of health sector reforms in which the decentralization of operations may result in the risk of the activities losing priority. The new institutional order requires that Chagas disease control be integrated into other services and programs and become part of a broader scheme for meeting the health needs of the population. In these circumstances, the integrated activities must sustain the significant progress so far achieved in the way of elimination of Chagas disease.

4.8 Initiative of the Southern Cone Countries: Epidemiological Trends

Accepting that the epidemiological and entomological spaces did not overlap with political divisions in Brasilia in June 1991, the Ministers of Health of Argentina, Brazil, Bolivia, Chile, Paraguay, and Uruguay launched the "Initiative for the elimination of transmission of Chagas Disease" (MERCOSUR, 1991). Since the vector of *T. cruzi* in these countries, *T. infestans*, is intradomiciliary, sustained implementation of control measures have successfully interrupted transmission of Chagas disease, as indicated here.

At the time of implementation in these countries, there were 11 million infected persons and 50 million were at risk. This represented 62% of the prevalence of infected individuals of the whole continent.

Technical representatives of each ministry were designated to constitute an intergovernmental commission in charge of implementation and evaluation of the control programs. The Pan American Health Organization (PAHO) was appointed as the secretariat of this commission and has played a leading role in promotion and coordination.

A program guide was designed by the commission to incorporate revisions submitted by the professional staff of the control programs and was used for the development of the country programs. The proposed plans for Argentina, Brazil, Bolivia, Chile, Paraguay, and Uruguay are approved on a yearly basis by their respective governments.

The objectives of the "Southern Cone Initiative" were clearly established at its inception and comprised the interruption of vectorial and transfusional

transmission. The cooperation among countries was ensured by the formal commitments of the countries, which introduced the agreed-upon activities in their national control programs. Later, other objectives of this Initiative were introduced, such as etiological treatment and medical care of the infected patients as an ethical imperative.

Current data, which include disinfestations of houses, blood bank screening, and incidence of infection in children younger than 5 years, indicate that the vector-borne and transfusional transmission of Chagas disease were interrupted in Uruguay in 1997, in Chile in 1999, and in Brazil in 2006 (WHO, 1994, 1995, 1996, 1997, 1998a, 1999a, 2000a,b; WHO/TDR, 2006; PAHO, 2007). Chagas disease has been targeted for elimination by the World Health Assembly in Resolution WHA51.14 approved in May 1998 (WHO, 1998b) and recently reviewed by the WHO Executive Board in January 2009 (WHO, 2009).

The model implemented in the Southern Cone was adapted to the Initiatives of the Andean Countries in 1996 (IPA) and Central America in 1997 (IPCA), (WHO, 2002), and more recently the Amazon Initiative in 2004 (AMCHA).

The advances in control of Chagas disease accomplished from 1991 to 2006 changed the epidemiological model of the disease. From a general point of view, the most important changes obtained by the Southern Cone Initiative are:

- Interruption of transmission of *T. cruzi* by *T. infestans* certified in Brazil, Chile, and Uruguay, eastern Paraguay, and in five of the endemic provinces of Argentina.
- Important reduction of vectorial transmission in Bolivia due to the coverage of house spraying with insecticides in the endemic area and regular activities of vector control in southern Peru, where the house infestation is also caused by *T. infestans*.
- Reduction of the transmission by secondary species in Brazil.
- Coverage of blood screening in close to 100% in all the countries, as shown in Table 4.2.

Progress in control in each country is reported as follows.

4.8.1 Argentina

The area of transmission covered 60% of the country north of parallel 44°. The main vector is *T. infestans*. In 1980, the average house infestation rate for the country as a whole was 30%; in 1998 it was 1.2%, and in 2002 it dropped to 1.0%, which is equivalent to 98% reduction in house infestation by the main vector.

The seroprevalence rate for the whole country for the age group 0−4 years is 0.9%, which confirms the very low number of acute cases among children in this age group. In the age group 0−14 years the rate is 1.9%. In the age group of 18-year-old men, the seroprevalence rates have dropped from 5.8% in 1981 to 1.0% in 1993 and 0.5% in 2002. The interruption of vectorial transmission has been achieved in 10 of the 13 endemic provinces of the country (Ministerio de Salud, 2002).

Finally, there is 100% coverage of the blood donations screened against Chagas disease in the blood banks of the public sector and 80% coverage in the private ones (WHO, 1996).

4.8.2 Bolivia

The endemic area covers 80% of the extension of the country, which corresponds to seven of the nine departments. *T. infestans* is the main vector. In 1982, an estimated 1,300,000 persons were infected, 26% of whom had electrocardiograph alterations. The house infestation rate for the whole country was 41.2% in that year, and the infection rate in the vectors was 30.0%. Infection rates of more than 50% have been reported in blood donors in Santa Cruz (Carrasco, 1990).

Data on serological prevalence shows a rate of 28.8% in the general population. In the age group of 0−4 years, prevalence is 22.0% in Cochabamba but 0.0% in Potosi where there is an active vector control program. In Tupiza, another department where there is an active control program, the house infestation rate is 0.8% (PAHO, 1998b).

4.8.3 Brazil

The main vector was *T. infestans.* Other two common species, *Triatoma brasiliensis* and *Panstrongilus megistus*, are less important in disease transmission. In 1975, the endemic area comprised 36% of the total extension of the country, and the most extensive endemic area on the continent. This area included 2493 municipalities in the States of Alagoas, Bahía, Ceará, Espirito Santo, Goiás, Piauí, Mato Grosso, Mato Grosso do Sul, Maranhao, Paraiba, Paraná, Pernambuco, Río de Janeiro, Río Grande do Norte, Río Grande do Sul, Sergipe, Tocantins, and the Federal District of Brasilia. At present, only the states of Bahía and Rio Grande do Sul are still considered infested by the main vector in residual foci with low density.

House infestation due to *T. infestans* has been reduced from 166,000 insects captured in the endemic areas by the control program in 1975 to 611 insects captured in 1999 in the same areas, which corresponds to a reduction of 99.7% of the infestation by this vector. This represents an average of 1 insect per 10,000 houses surveyed (i.e., an infestation rate far below the minimum required for effective transmission of the parasite into new patients).

The prevalence of human *T. cruzi* infection in the 7- to 14-year-old group in 1999 was 0.04%, compared with 18.5% in 1980. This represents a 99.8% reduction of incidence of infection in this age group. Results of 94,000 serological tests carried out in a population sample in the population of the 0- to 5-year-old group in 2007 indicate that the seroprevalence in this age group is 0.0%, which can be interpreted as a proof of the interruption of vectorial transmission of Chagas disease in Brazil (Luquetti, 2006).

The aforementioned data confirm the interruption of transmission of Chagas disease by *T. infestans* vectors in Brazil. Based on this epidemiological and entomological data, an international commission in charge of evaluating the interruption of vectorial transmission in this country issued a certification to declare the country as free of transmission in 2006 (WHO, 1997, 2000b; PAHO, 1998a,b, 2007).

4.8.4 Chile

The vector responsible for disease transmission was *T. infestans*, which has been eliminated from human dwellings hence, the transmission has been interrupted. The overall infestation rate for the country has been reduced from 3.2% in 1994 to 0.14% in 1999, a reduction of 95.6%. In 1999, only 26 *T. infestans* insects were captured in the interior of dwellings of the endemic areas in the whole country, which represents 2.5 insects in every 1000 houses, an infestation rate far below the threshold required for effective transmission of the parasite to new persons. The infection rate in the age group 0–4 years in 1999 was 0.016%, which represents a reduction of 98.5% compared with 1.12% found in the same age group in 1995.

The screening in blood banks in the endemic areas is mandatory since 1996, and the prevalence of infected samples has been reduced to 0.5%. An independent commission visited the endemic areas of the country in November 1999 and, based on the aforementioned data, certified the interruption of vectorial transmission (WHO, 1999a, 2000a).

4.8.5 Paraguay

The main vector is *T. infestans*. Chagas disease is endemic in all rural areas of the country, and the house infestation rate in 1982 varied from 10% in the Department of Misiones to 20% in Cordillera. In a serological survey carried out in 1997 in a representative sample (940 individuals) of children less than 13 years old in marginal areas of the capital city of Asuncion, a significant decrease of prevalence rates was observed in all age groups when compared with data of 1972. Rural/urban migration to these marginal areas of Asuncion comes mainly from Paraguari, Cordillera, and Central, which have the highest domiciliary infestation rates by triatomines. However, the zero prevalence rate in the age group of less than 4 years old indicates interruption of transmission by triatomines in the urban areas of the capital (PAHO, 1998a,b).

4.8.6 Uruguay

T. infestans was the only intradomicile vector. Since 1997, this species has been eliminated from the intradomiciles in the whole country. In 1975, the endemic area comprised the Departments of Artigas, Cerro Largo, Colonia, Durazno, Flores, Florida, Paysandú, Rio Negro, Rivera, Salto, San José, Soriano, and Tacuarembó. The house infestation rate dropped from 5.65% in 1983 to 0.30% in 1997. The interruption of vectorial and transfusional transmission was certified in 1997, and the whole country is under surveillance. There is 100% coverage of blood screening in blood banks. The incidence of infection in the age group of 0–12 years was 0%, which confirms the interruption of vectorial and transfusional transmission of Chagas disease in Uruguay since 1997 (WHO, 1998a).

4.9 Initiative of the Andean Countries: Epidemiological Trends

In these countries there are 5 million infected individuals and 25 million are at risk of contracting the infection. This represents 27% of the prevalence of infected individuals of the whole continent.

As the vectors of Chagas disease in these countries are not strictly domiciliated, it is necessary to adapt and test the vector control strategies to the local entomological conditions.

In the Andean Countries of Colombia, Ecuador, Peru, and Venezuela, the elimination of the vectorial transmission was launched at an intergovernmental meeting held in Bogotá in February 1997 where detailed country-by-country plans of action including annual goals, budgetary needs, evaluation mechanisms, and research needs were prepared (OPS/OMS, 1997).

The advances made in these countries, both in the development of new methods for evaluation and in control activities, include:

- Development of methodologies for risk stratification;
- Stratification of vectorial transmission risks and limited control programs in Colombia;
- Establishment of a national control program in Ecuador and implementation of activities following risk stratification criteria;
- Implementation of regular control activities in the MACROSUR region of Peru;
- Re establishment of activities of vector control and entomological surveillance in Venezuela;
- Screening of blood for transfusion close to 100% as shown in Table 4.1.

Progress in control in each country is reported as follows.

4.9.1 Colombia

The main vector is *R. prolixus* but *Triatoma dimidiata* has also been described as an effective *T. cruzi* vector.

It has been estimated that 5% of the population living in the endemic areas is infected, or approximately 700,000 persons. The departments with higher infection rates are Arauca (21.1%), Casanare (10.0), Santander (6.3), Norte de Santander (5.2), Boyacá (3.7), Cundinamarca (1.9), and Meta (1.7).

The screening in blood banks is mandatory since 1995 and there is 100% coverage. In 2001, the prevalence in blood donors was 0.65% as compared with 2.1% in 1998.

The preparatory phase of the national Chagas disease control program has been advanced and a map of the country featuring the risk municipalities has been prepared. The vector control activities with insecticide sprayings have been decentralized to the departments but there are no data available to monitor the impact of the control programs.

4.9.2 Ecuador

The main vector is *T. dimidiata.* The transmission occurs in the provinces of the Pacific coast including El Oro, Manabí, and Guayas. It is estimated that between 30,000 and 50,000 persons are infected. However, there are no data on prevalence of infection by age groups or on house infestation rates by province.

The preparatory phase of the national Chagas disease control program has been advanced. The law reorganizing the control program was issued in December 1998 and places the program under the Secretary of Tropical Medicine with specific functions and budget. The law for compulsory blood screening against *T. cruzi* was issued in August 1998. The seroprevalence of infected blood in blood banks for the whole country is 0.2%.

4.9.3 Peru

The highest prevalence of human infection is found in the Departments of Arequipa, Moquegua, Ica, and Tacna, which comprise 8% of the total population of the country. The main vector is *T. infestans* and it is estimated that there are some 394,000 houses infested with 24,000 persons infected with the parasite. Acute cases are regularly reported from this endemic area, which indicates active transmission. There are no screening programs in blood banks in spite of a prevalence of infected donations estimated at 2.4% in 1993.

4.9.4 Venezuela

The main vector is *R. prolixus.* The endemic area comprised in 1987 is 591 municipalities in an area of 700,000 km^2 with a population of 12 million.

The control program was officially established in 1966. The objective of the program was to interrupt intradomestic transmission through vector control by insecticide spraying. The program for improvement of rural housing, originally initiated in the 1960s, assists the rural inhabitants to substitute palm roofs, plaster adobe walls, and cement earthen floors. In addition, routine screening for *T. cruzi* in blood banks is mandatory since 1988.

In children under 10 years, the figures of seroprevalence rates for *T. cruzi* infection have decreased steadily in the last four decades from 20.5% in 1958−1968 to 3.9% in 1969−1979, and further to 1.1% in 1980−1989 and to 0.8% in 1990−1999.

The incidence of infection in the age group 0−4 years old has been reduced by 90% to less than 1.0% from 1990 to 1999. The geographical distribution of *T. cruzi* transmission is restricted to the states of Portuguesa, Barinas, and Lara (WHO, 1999b).

The prevalence of infected blood in blood banks has been reduced from 1.16% in 1993 to 0.78% in 1998 (Aquatella, 1987; Aché and Matos, 2001).

4.10 Initiative of the Central American Countries: Epidemiological Trends

In these countries there are 2 million infected individuals and 26 million are at risk of contracting the infection. This represents 11% of the prevalence of infected individuals of the whole continent.

As the vectors of Chagas disease in these countries are not strictly domiciliated, it is necessary to adapt and test the vector control strategies to the local entomological conditions.

In the Central American countries, Belize, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, and Panama, progress in blood banks control is also proceeding well and all of them except one have issued legislation for compulsory blood screening against blood infected by *T. cruzi*.

Similarly, the elimination of the vectorial transmission was launched at an intergovernmental meeting held in Tegucigalpa in October 1997 where detailed country-by-country plans of action including annual goals, budgetary needs, evaluation mechanisms, and research needs were prepared (OPS/OMS, 1997).

The advances made in vector control and in control of blood transfusions in these countries include:

- Interruption of transmission of *T. cruzi* by *R. prolixus* certified in Guatemala;
- Interruption of transmission of *T. cruzi* by *R. prolixus* in Honduras and Nicaragua in the process of certification;
- Reduction of transmission by *T. dimidiata* in several countries;
- Testing of alternative methodologies for control of *Rhodnius pallescens*;
- Screening of blood for transfusion close to 100%, including Costa Rica where it was compulsory, as shown in Table 4.1;
- The elimination of *R. prolixus* was certified in El Salvador in 2010.

Progress in control in each country is reported as follows.

4.10.1 Belize

The only vector species of epidemiological importance is *T. dimidiata*, but is restricted to the wild environment. There are sporadic reports of insect adults attracted by light that are frequently found in the periphery of the cities and villages. The seroprevalence in the general population is very low and the seropositives found are migrants from neighboring countries. The screening of blood banks has 100% coverage and the prevalence among blood donors in 2000 was 0.5%.

4.10.2 Costa Rica

The main vector is *T. dimidiata*. The vectors are found in the central plain, extending primarily to the northwest and southwest regions of the country. Seroprevalence of 1.94% was found in some blood banks of the country that participated in one study in 2000. A recent survey carried out in the Heredia Province in 2001 in schoolchildren, 7−12 year old, showed an infection rate of 0.2%. Chagas disease is not considered a public health problem (OPS/OMS, 2001).

4.10.3 El Salvador

T. dimidiata is the main vector. *R. prolixus* was detected in the country in the 1980s, but this species disappeared from El Salvador in the last decade. *T. dimidiata* is the only vector currently detected in all departments with a house infestation rate of 21% in the dwellings in rural areas and in the small or medium townships. In 2000, the prevalence of the infection in schoolchildren of 7−14 years was 0.3% and 2.1% in the population older than 14 years.

The blood screening coverage was 100% in 2000 when the prevalence of infected blood was 2.48%. The vector control program treated in that year 67.3% of infested dwellings in areas where there is coexistence of infestation by anophelins and triatominos.

4.10.4 Guatemala

T. dimidiata is found in 18 of the 22 departments and *R. prolixus* in 5 departments. The infestation rate varies from 12% to 35%. The infection rate in schoolchildren in the five most endemic departments, namely, Zacapa, Chiquimula, Jalapa, Jutiapa, and Santa Rosa, was 4.9% in a survey carried out in 2000. There is a poor blood bank control system and prevalence of seropositive blood donations in 2000 was 0.84%.

Vector control activities are carried out by the control program of the Ministry of Health in the aforementioned departments with highest house infestation rates (OPS/OMS, 2001). Interruption of transmission of *T. cruzi* by *R. prolixus* was being certified by an international commission in 2005.

4.10.5 Honduras

The main vector *R. prolixus* is present in 11 departments of the country and the second vector *T. dimidiata* is present in 16 departments. Vectors are present in the Departments of Choluteca, Comayagua, Copan, Francisco Morazan, Intibuca, Lempira, Ocotepeque, Olancho, El Paraiso, La Paz, Santa Barbara, and Yoro. In 1983, the highest infection rates were found in the western and eastern departments and in the southern region. About half of the population is estimated to be at risk. Infection rates of 32% or more in the vectors have been reported. The most frequent clinical manifestation is cardiopathy.

Vector control activities are carried out in six of the nine Health Regions of the country. A recent seroepidemiological survey carried out in areas under chemical control in children aged less than 5 years was 0.36% and in schoolchildren 7−14 years was 3.3%. The coverage of the control of transfusional transmission is 100% and the national seroprevalence in blood donors in 2000 was 1.53% as compared to 11% in 1985 (OPS/OMS, 2001). Interruption of transmission of *T. cruzi* by *R. prolixus* is in the process of certification.

4.10.6 Mexico

Vectors and infected mammals are found in the states of Chiapas, Guanajuato, Guerrero, Hidalgo, Jalisco, Mexico, Michoacan, Morelos, Nayarit, Oaxaca, Puebla, Sonora, Yucatan, and Zacatecas. The prevalence of the disease is highest in the Pacific coast states from Chiapas to Nayarit, in the Yucatan peninsula, and in some areas of the central part of the country. Although most of the human infections and clinical forms in Mexico are considered to be mild, there have been recent reports of some cases of mega-viscera. Mexico has not introduced routine screening for *T. cruzi* in blood banks where 850,000 donations are made every year and where around 12,760 units of blood could be infected.

There is no vector control program, although there is a renewed interest of the health authorities to organize national and state vector control activities.

4.10.7 Nicaragua

T. dimidiata is present in 14 of 17 departments of the country and *R. prolixus* in 5 departments. The control of transfusional transmission is made in 70% of the blood banks with a prevalence of infected blood of 0.33%. A seroepidemiological survey carried out in 2000 in schoolchildren showed a prevalence rate of 3.3% in this age group (OPS/OMS, 2001). Interruption of transmission of *T. cruzi* by *R. prolixus* is in the process of certification.

4.10.8 Panama

The main vector is *R. pallescens* found inside the dwellings of the Chorrera district. This vector is present also in palms in the wild environment. *T. dimidiata* is also an important vector. There is no compulsory screening in blood banks or vector control programs.

4.11 Amazon Initiative

The "Initiative for Surveillance of Chagas disease in the Amazon Region" (AMCHA) was launched in 2004.

The epidemiological data to assess the magnitude of the problem are scanty. In the report of the first meeting held in Bogota there is no mention of the burden of disease except for a mention of the presence of 279 autochthon cases (252 acute cases and 27 chronic cases) in the whole of the 5,000,000 km^2 extensive territory of the Brazilian Amazon region. In addition, it is mentioned that in the national serological survey (1975−1981) the rate of positivity by state oscillated between 0.0% in Amapá and 1.9% in Amazonas. There are no data for tendencies or data of annual incidence (Guhl, 2004, pp. 17, 103).

In the same report it is only mentioned that in the Colombian Amazon region, there is positive serology in nine communities of the Department of Guainía but

there are no indications as to the methodology of selection of the population samples (Guhl, 2004, pp. 27, 102). On the other hand, for Guyana, Surinam, and French Guyana it is concluded that Chagas disease "is not a public health problem for these three countries" (Guhl, 2004, p. 36). Ecuador, Peru, and Bolivia do not have information on the morbidity by Chagas disease in the departments or provinces of their respective Amazon regions.

In the report of the second meeting held in Manaus in September 2004, the risk of the establishment of endemic Chagas disease is discussed and it is mentioned that up to 1998, 17 episodes with 85 cases have occurred of possible oral transmission of *T. cruzi* due to the ingestion of açai juice (*Euterpa catinga*) in the States of Pará, Acre, Amapá, and Amazonas. Apart from the above information, there are no further epidemiological data on the trends in Brazil or in any of the countries (http://cdiaecuniandes.edu.co/AMCHA.htm).

The advances made in vector control and in control of blood transfusions in the countries of the AMCHA include:

- Development and standardization of a surveillance model based on the detection of anti bodies against *T. cruzi* through serological analysis of samples collected for the diagnosis of malaria.
- This surveillance model has been evaluated in Brazil and partially in Ecuador.

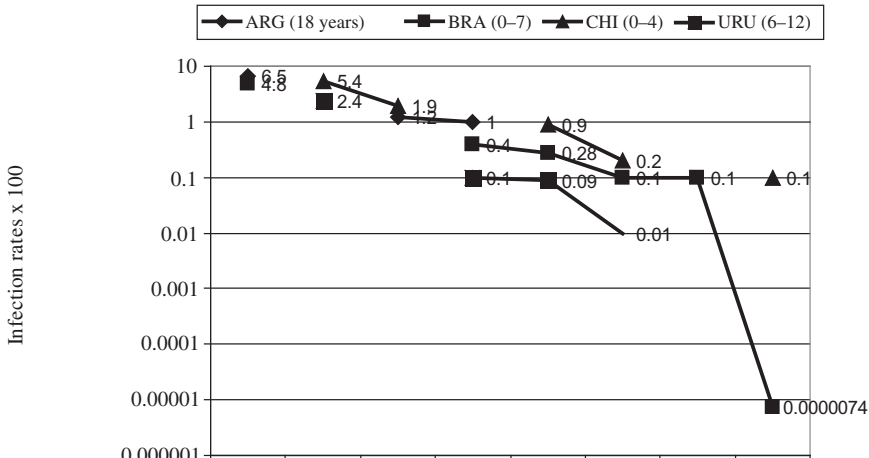
4.12 Epidemiological Impact

The most significant results in the interruption of transmission have been achieved in the Southern Cone and the Central American countries. The national programs of Argentina, Brazil, Chile, and Uruguay had shown important results even before the launching of the Initiative. Figure 4.1 shows the current trends of the incidence of infections in the Southern Cone countries as a consequence of elimination of vectorial transmission in Uruguay, Chile, and Brazil (Moncayo, 1997, 2003).

In Central America, after the creation of the IPCA, the response of the government was very effective and complemented by the participation of international cooperation agencies in financing and execution of control activities. In the Andean countries the difficulties encountered by the IPA were twofold: on the one hand the variety of epidemiological and entomological situations and on the other side the different degrees of political commitment of the governments. The concept of risk stratification was developed in the context of this Initiative and was an important contribution to the other ones (Guhl, 2000; OPS/OMS, 2004; Silveira, 2004). In addition, it was felt the need to better define objectives, such as the elimination of *T. dimidiata* in Ecuador, *R. ecuadoriensis* in Peru, and *R. Prolixus* in Colombia.

With respect to the AMCHA it is recognized that it is still in an initial phase of generation of knowledge on the magnitude of the problem and development of control methodologies, in particular in Brazil and to a lesser extent in Ecuador.

The impact of vector control programs and the possible influence of other variables such as the general socioeconomic development of the populations at risk can be



	82	90	92	94	98	2	4	6
ARG (18 years)	6.5		1.2	1				
BRA (0–7)	4.8			0.4	0.28	0.1	0.1	7.4×10^{-6}
CHI (0–4)		5.4	1.9		0.9	0.2		0.1
URU (6–12)		2.4		0.1	0.09	0.01		

Figure 4.1 Interruption of transmission of Chagas disease, infection rates × 100 Southern Cone countries, 1982−2006.
Source: WHO, 2002; INCOSUR, 1993−2006.

better evaluated by the trends of the number of infected cases in different moments. Table 4.1 shows those data in three different moments: 1975−1985, 1995, and 2005 to estimate the prevalence and 1990, 1995, and 2005 to estimate the incidence.

As the information compiled in this table has different sources (WHO/OPS, 2006; Schmunis, 2007a), the interpretation should be cautious and should be considered as the best estimation of the real epidemiological situation.

The impact on the decrease of the burden of disease measured in DALYs due to Chagas disease has been the most important in the period 1990−2001 for Latin America and the Caribbean. The number of DALYs dropped from 2.8 million in 1990 to 0.8 million in 2001, a reduction of 78%, the highest among the top eight communicable diseases in the region (Schmunis, 2007a,b).

The impact on mortality due to Chagas disease is evidenced by the reduction in mortality from 45,000 annual deaths in 1980 (Moncayo, 2003) to 14,000 in 2001, a net decrease of 70%. Table 4.3 summarizes the reduction in incidence, prevalence, mortality, and distribution of Chagas disease on the continent (WHO/TDR, 2006).

In Table 4.5 the data on coverage of blood banks screening and the percentage of positive samples for 2005 are shown. The sources of this information are the official reports of the governments to the meetings of the Intergovernmental

Table 4.5 Coverage of Screening of Blood for Transfusion for *T. cruzi* (%) and Proportion of Positive Samples in Endemic Countries, 2005

Country	Coverage of Screening for *T. cruzi* (%)	Positive Samples (%)
Southern Cone		
Argentina	100	2.47
Bolivia	80	8.0
Brazil	100	0.21
Chile	87	0.6
Paraguay	99	3.2
Uruguay	100	0.47
Andean countries		
Colombia	100	0.44
Ecuador	100	0.15
Peru	99	0.57
Venezuela	100	0.6
Central America and Mexico		
Belize	ND	0.4
Costa Rica	100	0.09
El Salvador	100	2.4
Guatemala	100	0.79
Honduras	100	1.4
Nicaragua	100	0.9
Panama	97.6	1.4
Mexico	100	0.51

ND: No data.
Source: Document OPS/HDM/CD/425-06, Reunión Iniciativa de los Países de Centroamérica 2006, Reunión Iniciativa de los Países Andinos 2006.

Commissions of the subregional Initiatives. High coverage and low percentage of seroreactivity can be observed in these different countries.

4.13 Future Challenges

In spite of the progress achieved, there are a number of future challenges to pursue the way ahead. They can be divided in three categories: epidemiological, technical, and political.

4.13.1 Epidemiological

4.13.1.1 Oral Transmission

This route of transmission is well established and documented (Shikanai-Yasuda et al., 1991; Valente et al., 1999; Camandaroba et al., 2002; Rodríguez-Morales, 2008). The

most salient characteristic of the oral transmission is the fact that several persons are affected simultaneously pointing to the occurrence of a common source outbreak through contaminated food.

The challenge here is the prevention of the oral transmission via the ingestion of beverages such as açai juice (*Euterpe oleracea, E. catinga*) in the Brazilian Amazon region.

In other documented cases of outbreaks of oral transmission the contaminated food was served in family celebrations and in circumstances that were unpredictable. This was the case in the outbreaks of Teotonia, Catolé do Rocha, and Santa Catarina in Brazil (Shikanai-Yasuda et al., 1991) and in Caracas, Venezuela (Rodríguez-Morales, 2008).

The approach for prevention of these outbreaks is based on surveillance, prevention, and management similar to the control of diseases transmitted by food as recommended by a group of experts convened by the PAHO in Brasilia in 2006 (OPS/ DPC/CD/CHA, 2006).

4.13.1.2 Transmission in the Amazon Region

The patterns of vectorial transmission in this region are unusual and different from those that were recognized as necessary to maintain the endemic levels such as the presence and colonization of the household by the vectors. So, the few known cases of infection by *T. cruzi* are being transmitted (i) by the oral route, (ii) by vectors that enter the dwelling but do not develop intradomicile colonies, and (iii) by infection of persons that enter the jungle and have contact with sylvatic triatomines such as *Rhodnius brethesi* in the extraction of "piaçaba," a vegetal fiber (*Leopoldinia piassaba*) (Silveira and Passos, 1986; Coura et al., 1994).

Most of the documented cases are due to the ingestion of açai juice and are concentrated in the States of Pará and Amapá, where the production of this fruit is intensive.

The special characteristics of the transmission of the disease in this region required a new model of epidemiological surveillance adapted from the traditional used in the endemic areas that is based on the detection of the intradomicile vector, the screening of blood for transfusion, and the identification and treatment of the congenital form. In addition, it was necessary to take into account the enormous extension of the territory of this region, which implied very serious operational difficulties.

However, there were other favorable factors to be considered such as the financial resources allocated by the Brazilian government for malaria surveillance and the feasibility of the parasitological diagnosis of *T. cruzi* to be carried out simultaneously in the same slide as for *Plasmodium falciparum*. However, it is recognized that the sensitivity of this examination for the detection of *T. cruzi* is low.

The following surveillance activities were proposed at the meeting held in Cayenne in 2005 that were accepted by the PAHO (OPS, 2005):

- Detection of cases using the infrastructure of malaria surveillance.
- Identification and mapping of environmental markers for the identification of current or potential vector species.
- Research and monitoring of entomological situations when vector colonization is suspected by some species such as *Triatoma maculata, Panstrongylus geniculatus, Panstrongylus herreri, Rhodnius neglectus,* and *Rhodnius stali.*

4.13.1.3 Globalization of Transmission

The increasing mobility of populations and the migration toward nonendemic countries have extended the infection to these countries through blood transfusion, organ transplantation, and the congenital form among migrants. The risk is related with the country of origin of the migrants and the rate of prevalence in that given country.

However, the advances observed in control of the transmission indicate that this potential extension to Europe, the USA, and Canada might be transitory or decreasing.

The WHO recently launched the Global Network for Chagas disease elimination (WHO Global Network for Chagas Elimination GNChE) to meet this situation.

4.13.1.4 Local Epidemiological Situations

There are a number of situations that depend on localized circumstances that have implications for vector control operations in the following areas.

The Chaco Region

In spite of the sustained regular activities of vector control in this territory that is shared by Argentina, Bolivia, and Paraguay, the rates of infestation by *T. infestans* remain high. The reasons are associated to the complex peridomiciles of the rural houses, the emergence of resistance of the vector to the pyrethroid insecticides in the Provinces of La Rioja and Salta in Argentina and the Departments of Tarija and Cochabamba in Bolivia, and the presence of sylvatic foci of *T. infestans* in Bolivia.

An integrated approach is being proposed that includes the improvement of peridomiciles and the use of higher doses of insecticide in these peridomiciles (Gürtler et al., 2007).

Areas with Vectorial Transmission Without Intradomicile Colonization

In these areas the transmission occurs because of the "visit" of the vector to the houses from the natural ecotopes to feed on the inhabitants after which the insect comes out. This form of transmission has been documented for *R. pallescens* in Panama and Colombia where the natural ecotopes of this species are several types of palm trees which are planted around the rural dwellings. The infection rates of this insect with the parasite are very high.

The surveillance and control in these particular situations are based on the analysis of variables that influence transmission such as the ratio houses/ecotopes, the insect infection rates by *T. cruzi*, the vulnerability of the dwelling to the insect "visit," and the frequency of these "visits" (Zeledón, 2003).

Several alternatives of control have been proposed including physical or chemical barriers and the use of light sources that are not attractive to the insects.

4.13.2 Technical

4.13.2.1 Chemical Vector Control in the Peridomicile

The peridomicile comprises the annexes, fences, corrals, and poultry yards that are built around the rural dwellings. The efficacy of the insecticides sprayed in this part of the house is low due to the degradation of the active principle by the rains and the continued exposure to sunlight. Other reasons of this low efficacy include the operational difficulties for spraying and the lack of order and cleanliness in these spaces. So, residual infestations are invariably situated in the peridomicile.

It seems that the only alternative is the physical management of these spaces, including reordering of the structures and the objects found there.

4.13.2.2 Resistance of Vector Populations to Insecticides

"Control failures" that evidence the cross-resistance of vectors to pyrethroids have been documented in areas of Tarija and Cochabamba in Bolivia and in Salta and La Rioja in Argentina. Some field tests have been carried out with other insecticides as carbamates and phosforates with poor results. It is necessary to develop and test new molecules to overcome the emerging resistance.

4.13.2.3 Low Sensitivity of Entomological Methods

The available methods for direct detection of domiciled triatomines have a low sensitivity especially in circumstances where the insect densities are low, as it is the case in advanced control phases. The direct reporting by the inhabitants of the presence of domiciled vectors is more effective, and there are studies to evaluate the efficacy of traps with and without attractants such as pheromones.

4.13.3 Political and Structural Factors

These factors refer to the ranking of disease control priorities within the national health policies.

4.13.3.1 Maintaining the Political Priority

At present, the major challenge is to ensure the sustainability of the national control programs in an epidemiological context with very low *T. cruzi* infection rates in

the younger age groups of the populations of several countries and negligible house infestation rates.

The national programs should be adapted to the new epidemiological circumstances but should be maintained with emphasis in entomological surveillance to avoid that the government efforts, financial and otherwise, to attain the interruption of transmission, be lost. The successes accomplished in this respect should not be "punished" but maintained.

It is proposed the stratification of the risk be the central criterion to shape the required surveillance and control activities (OPS/OMS, 2004; Silveira, 2004).

4.13.3.2 Decentralization of Control Programs

Since the 1980s, the traditional vertical programs for disease prevention and control are being dismantled in accordance with the current political and institutional context of health sector reforms, where the decentralization of operations may result in the risk of the activities losing priority.

The vertical programs were characterized by high specificity of activities, strict planning, and clear definition of measurable goals and investment of important financial resources.

The decentralization of programs has resulted in the lack of recognition by the local authorities of the priority given to Chagas disease control in view of more pressing needs for immediate attention.

The new institutional order requires that Chagas disease control be integrated into other services and programs and become part of a broader scheme to meet the health needs of the population. In these circumstances the integrated activities must sustain and expand the significant progress so far achieved in the interruption of transmission of Chagas disease in several countries of Latin America.

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5 Geographical Distribution of Chagas Disease

*James S. Patterson and Felipe Guhl**

Centro de Investigaciones en Microbiología y Parasitología Tropical—CIMPAT—, Facultad de Ciencias, Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia

5.1 Introduction

Just over a century ago, the Brazilian physician Carlos Chagas was the first to describe the disease that was to subsequently take his name (Chagas, 1909). He went much further than recognizing the clinical aspects of the disease; he also isolated and described the etiological agent (*Trypanosoma cruzi*) and identified the insect vector, triatomine bugs (blood-feeding true bugs of the family Reduviidae).

A full understanding of the etiology and epidemiology of Chagas disease across its distribution was to prove elusive and complex, and remains under intense investigation to the present day. The difficulty in completely defining the epidemiology of Chagas disease is attributable to several factors. Firstly, Chagas disease is a zoonosis, and a variety of widely distributed mammals serve as reservoirs for *T. cruzi*. Moreover, all mammals are susceptible to infection. A further factor that contributes to the complexity of Chagas disease as a zoonosis is the variety of vectors involved, being not simply represented by a range of related species or genera, as is the case for all other insect vectors, associated with any given disease. Triatomine bugs are a subfamily of insects and across this relatively broad taxonomic range there are members from all groups that can harbor *T. cruzi*. Most transmission, however, is attributable to three main genera: *Rhodnius*, *Panstrongylus*, and *Triatoma*, but this diversity still represents two different tribes of the subfamily (Rhodniini and Triatomini). Furthermore, the insects vary in more than ancestry, having a diverse range of vertebrate host and ecological associations. The third factor that complicates Chagas disease epidemiology and accounts for variation in the clinical manifestation of the disease is the subspecific diversity of *T. cruzi* itself. Much work has been conducted over the past 40 years to elucidate the variation of *T. cruzi* across its geographical distribution and associations with hosts and vector species.

Even the basic epidemiology was elusive at first, as it was not until the mid-1920s was it resolved that transmission occurs via contamination with feces of

* E mail: fguhl@uniandes.edu.co

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infected triatomine bugs rather than by the bite, as is the usual route of transmission of protozoa transmitted by insects. This key epidemiological factor limits those at risk to vectorial transmission to people living in infested houses with prolonged exposure, essentially poor rural communities. However, this factor, that in most scenarios limits transmission to rural communities, historically caused the disease to have a limited political impact and low profile and priority as a public health issue. This hurdle was difficult to overcome, and appropriate recognition was not forthcoming until scientists began to demonstrate the economic impact of the disease.

In addition to vector transmission, a small percentage of cases are attributable to unscreened blood transfusions, congenital transmission, and incidences of oral transmission by contamination of food. In the 1990s, it was estimated that greater than 80% of transmission was due to vectors and approximately 16% were due to blood transfusion (Schofield, 1994). Most endemic countries have since implemented approaching 100% screens of donated blood.

In this chapter we relate factors of vector−host−parasite associations that highlight the evolutionary course of Chagas disease and how these interactions account for the observed distribution of the disease in domestic and sylvatic cycles in the Americas. We also present changes in prevalences and distribution of Chagas disease in Central and South America in recent times and particularly in response to control programs. We also report on the global dispersal of Chagas disease due to migrant South and Central American populations to Europe, North America, Japan, and Australia. Finally, we highlight the need for nonendemic countries to maintain and develop an awareness of Chagas disease as a consequence of globalization.

5.2 Vector Phylogeography and Ecology

The endemic transmission of Chagas disease in humans and wild hosts is restricted to the Americas and corresponds largely with the distribution of triatomine bugs approximately from latitudes 42°N to 46°S (i.e., from the mid-USA to Patagonia). The triatomines constitute a subfamily of an otherwise predatory group of bugs, and is relatively small compared to the thousands of predatory reduviids. The Triatominae comprises some 140 species. This diversity is classified into six tribes and 19 genera.

Importantly, the main factor that constrains Chagas disease to the Americas is the same reason that African trypanosomiasis is restricted to Africa the vectors are solely endemic to their respective continents. This is certainly true for tsetse flies, but strictly speaking not entirely the case for triatomine bugs. Seven endemic species of *Triatoma* are reported from southeast Asia (Ryckman and Archbold, 1981), and one genus (*Linshcosteus*) is endemic to India. None of these endemic Asian triatomine species have been reported to have close contact with human populations.

A further species, *Triatoma rubrofasciata*, has a global tropicopolitan distribution and is recorded from port areas throughout the tropics and subtropics.

T. rubrofasciata is frequently found in coastal cities of Brazil, especially the city of São Luis, Maranhão, where it causes public concern (Macario Rebelo et al., 1999). Natural infection of *T. rubrofasciata* with *T. cruzi* has been reported in Brazil (Schofield, 1994), although the feeding and defecation habits of this species make it a relatively inefficient vector (Braga and Lima, 1999). Also, in Brazil and parts of southern Asia, *T. rubrofasciata* is commonly infected with the rat trypanosome, *Trypanosoma conorhini*, and is considered the main vector of this parasite which, like *T. cruzi*, is transmitted by host contamination with infected bug feces (Hoare, 1972). *T. rubrofasciata* seems to have a close association with rats (*Rattus rattus*) and is assumed to have been transported on ships along early international trade routes during the sixteenth to nineteenth centuries (Schofield, 1988; Gorla et al., 1997; Patterson et al., 2001). Most reports of *T. rubrofasciata* in the Old World are old. Indeed, the oldest description of any triatomine refers to a specimen of *T. rubrofasciata* collected in the then Dutch East Indies (now Indonesia) (DeGeer, 1773). Ryckman and Archbold (1981) summarized its global distribution and to our knowledge the most recent report of *T. rubrofasciata* outside the Americas is of domestic infestations in coastal villages in southern India in 2000 (Patterson, unpublished data).

There are no records of any triatomine infected with *T. cruzi* beyond the Americas. Furthermore, the recent migratory trends of human populations that we will address later pose little or no threat to the establishment of transmission cycles beyond the American continent.

However, there has been a trend in recent years for immigrant-descended Latin American populations to return to their countries of ancestry (see Section 5.5.6). If this should ever become the case for the large ethnical Indian populations of the Guyanas to migrate back to southern India, it is possible that they could take infected *T. rubrofasciata* (or another species) back with them, and endemic Asian triatomine species could theoretically be involved in initiating sylvatic and domestic *T. cruzi* transmission cycles outside of the Americas.

There are some general trends in the geographical distribution of triatomine bugs. Fundamentally, it has been established that there is a relationship between latitude and species richness (Rodriguero and Gorla, 2004), with increasing diversity toward the equator and easterly in latitude, thus corresponding to the Amazon (Figure 5.1).

There are also some patterns in geographical distribution that can be related to the taxonomic groups. For example, *Rhodnius* species are mostly associated with the Amazon and periphery (Abad-Franch and Monteiro, 2007) extending westerly to the northern Andean countries and to parts of Central America.

There are notably high concentrations of closely related *Triatoma* species from southern and central Brazil and the southern USA. Several studies have demonstrated large phylogenetic distances between the *Triatoma* of South America and those from North and Central America (Bargues et al., 2000; Marcilla et al., 2001; Hypsa et al., 2002). *Panstrongylus* species are also thought to be divided into two phylogenetically defined groups (Lent and Wygodzinsky, 1979), also indicated by Marcilla et al. (2002), that correspond roughly to regions North and South of the Amazon (Patterson et al., 2009) and West and East of the Andes.

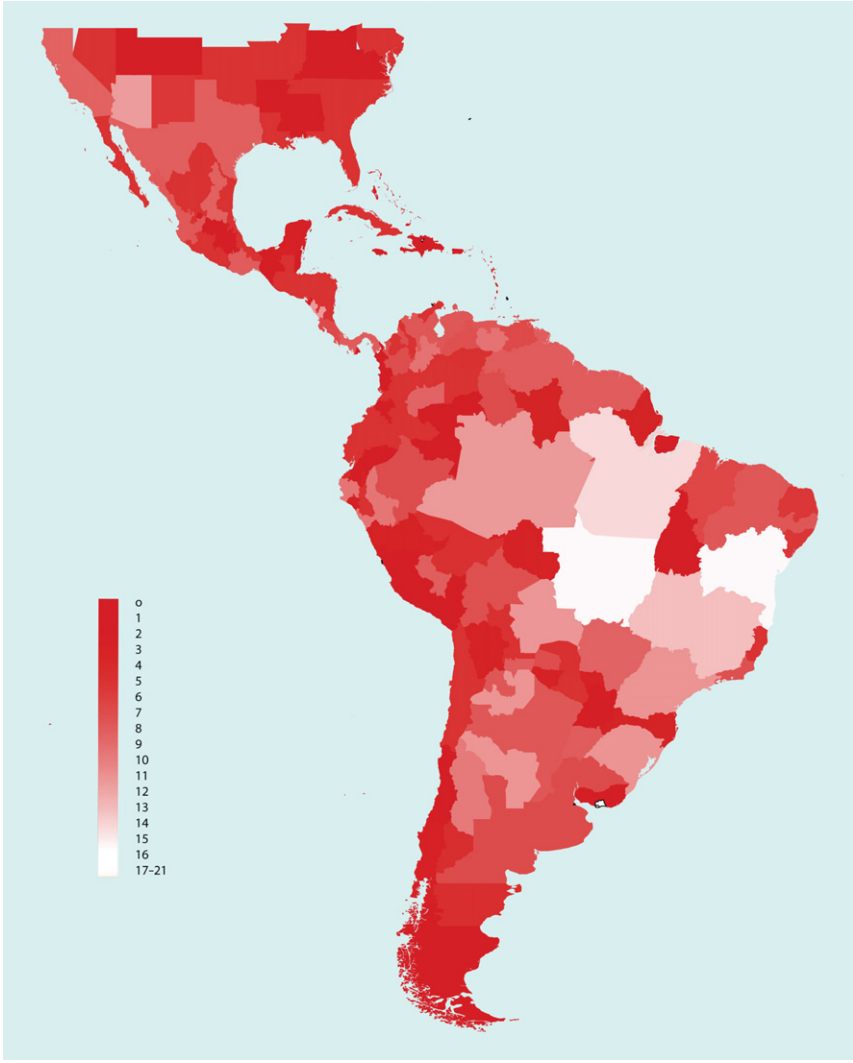

Figure 5.1 Map showing distribution of species richness for 118 species of triatomines. *Source*: Redrawn from Rodriguero and Gorla (2004).

In general, any given species of triatomine bugs has a relatively limited distribution. This is evident in some of the taxonomic nomenclature, where a number of species are named after countries or regions. One notable exception is *Panstrongylus geniculatus*, which has the most extensive continuous distribution of any triatomine species, from the Atlantic to Pacific coasts and from mid-Central America to northern Argentina. Its region of origin is thought to be the Amazon (Abad-Franch and Monteiro, 2007). *P. geniculatus* has a strong association with subterranean burrowing

mammals, such as armadillos. Subsequently, it has been speculated that after initial adaptation to environments of high humidity in the Amazon, association with the humid microclimate of armadillo burrows has facilitated its observed broad geographical distribution well beyond the limits of the Amazon (Abad-Franch and Monteiro, 2007).

A few other species also have broader distributions, possibly due to their potential to exploit a broader range of habitats, or because their ecological niche is widespread. The potential to exploit and adapt to a variety of environments is one of the factors that might facilitate some species to invade domestic environments and initiate disease transmission. Certainly three of the most important vector species, *Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiata*, have distributions across several countries (Figure 5.2).

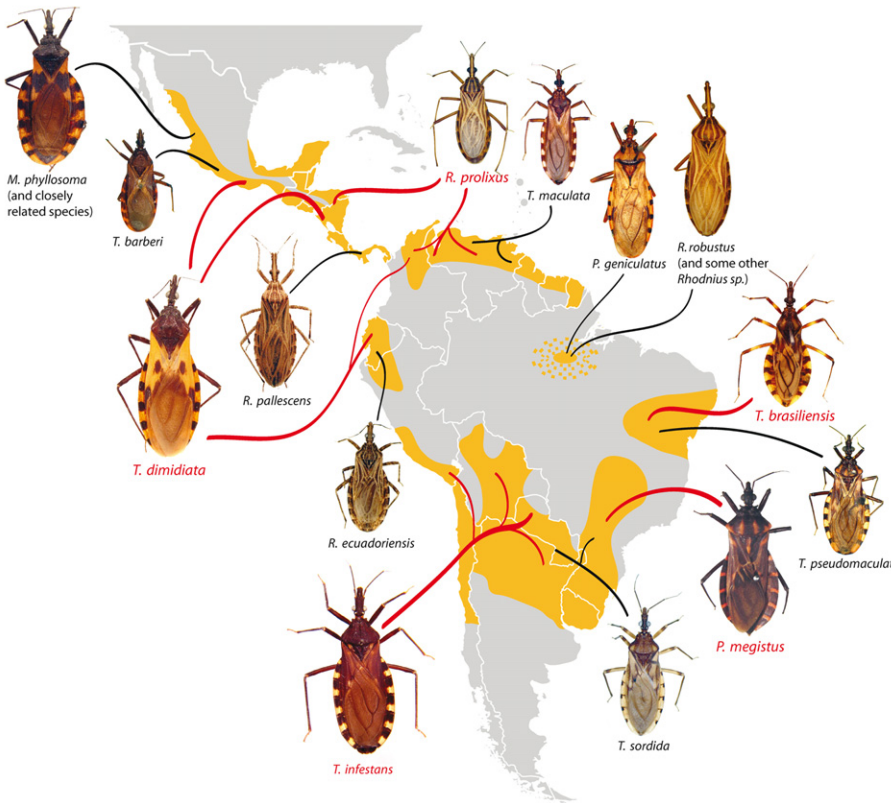

Figure 5.2 Map of Chagas endemic regions of Central and South America showing respective associations with the 14 most important vector species. The five most important vector species are *T. infestans, R. prolixus, T. dimidiata, P. megistus,* and *T. brasiliensis*. Genera are *Triatoma Rhodnius, Panstrongylus*, and *Meccus* (*Meccus* is synonymous with *Triatoma* in some literature).

Most of the 140 Triatominae species occupy sylvatic ecotopes in association with their respective vertebrate hosts. Examples include palm crowns, bird nests, possum lodges, rock piles, hollow trees, rodent nests, and bat caves. In most cases triatomine species are exquisitely adapted to their ecotopes with little propensity to invade human habitations. Therefore, fortunately or unfortunately, there are only some 10−15 species of triatomines that show anthropophilic tendencies and are regularly implicated in disease transmission (see Figure 5.2). Several factors can initiate the development of domestic tendencies in wild populations of triatomines, such as a successful control of one species in houses can offer an open niche for other species to invade. Another factor is habitat destruction, in particular deforestation, which can potentially drive species toward invading domestic environment.

Schofield et al. (1999) discuss the process of domestication, and highlight that when domesticated populations undergo a process of simplification through adaptation to the stable domestic environment, the resulting optimum genotypes would offer reduced fitness in sylvatic ecotopes. This has proven to be the case for *T. infestans*, which until recently had a huge distribution across Brazil and occurred in all Southern Cone countries. However, through most of its range it was solely domestic, having originally adapted to domestic environments from a relatively small sylvatic distribution in Bolivia. As a result of the Southern Cone program (see Chapter 4), it has largely been eliminated from much of its previous range.

There have been numerous studies to assess the relationships between domestic and sylvatic populations of triatomines in attempts to address the question of whether there is population differentiation due to domestication. Also, other studies have been concerned with establishing the extent of gene flow across geographical areas to help plan a strategy for control. Many different methods have been used to measure population differentiation, including antennal sensilla patterns, morphometrics of head and wing shape, isoenzyme profiles, and various other molecular techniques. Potentially the best approach for measuring gene flow and population structure of vectors is microsatellite markers. Markers have been developed for some of the main vectors, including *Rhodnius pallescens* (Harry et al., 1998), for *T. infestans* (Marcet et al., 2008), for *T. dimidiata* (Anderson et al., 2002), for *R. prolixus* (Fitzpatrick et al., 2009), and for *Triatoma pseudomaculata* (Harry et al., 2008).

With the eminent success of controlling predominantly domestic species, such as *T. infestans*, there is an increasing emphasis on defining the role that invasive species play in Chagas disease transmission (Guhl et al., 2009). Careful surveillance of potential vectors ecology and distribution (e.g., for Colombia [Guhl et al., 2007] and Mexico [Cruz-Reyes and Pickering-Lopez, 2006]) are important for the identification of trends leading to the domestication or potential proximity to human habitations and activity. A prominent example is that proximity of human settlements to sylvatic foci, palm trees in particular, pose a risk of invasion and colonization in the Andean and Amazon regions. For example, in the case of *R. prolixus* in Venezuela (Sanchez-Martin et al., 2006) and other palm-dwelling triatomines related to incidences of transmission by contamination in food production in the Amazon

(see Section 5.5.3 for more details). Such factors need to be considered long term in the context of surveillance and control (Guhl et al., 2009).

Risk mapping using distribution records, bioclimatic data, and geographical information systems has recently been developed to aid the design of intervention. Examples of risk mapping triatomine species' distributions include *T. dimidiata* in Mexico (Dumonteil and Gourbiere, 2004), *R. pallescens* in Colombia and Central America (Arboleda et al., 2009), and *T. infestans* in the Southern Cone countries (Gorla, 2002). Also, detailed ecological analysis and niche characterization can be used to estimate areas at risk, according to sylvatic ecology such as for *Rhodnius ecuadoriensis* (Abad-Franch et al., 2005) and other *Rhodnius* species (Abad-Franch et al., 2010), and domestic ecology (Cohen and Gurtler, 2001; Campbell-Lendrum et al., 2007). Finally, predictive ecological niche modelling algorithms have been applied to the Brazilian species *Rhodnius neglectus* (Gurgel-Goncalves and Cuba, 2009) and *Triatoma brasiliensis* (Costa et al., 2002), and to the Triatominae of North America (Ibarra-Cerdena et al., 2009) and Mexico (Cruz-Reyes and Pickering-Lopez, 2006; Sandoval-Ruiz et al., 2008).

Dispersal of triatomines can be either passive or active, and there are clear examples of both in the literature. The most striking example of passive dispersal is the earlier discussed global dispersal of *T. rubrofasciata*. Another long-distance dispersal associated with human movements is the introduction of *T. dimidiata* to Ecuador from Central America (this will be addressed in more detail in a later section). Passive dispersal can also be facilitated by sylvatic vertebrate hosts; small nymphs can be carried in fur or feathers, some species have been observed to attach their eggs to feathers (Forattini et al., 1971; Schofield, 1994). Active dispersal by flight is observed to be seasonal for some species, brought on by hot dry weather. Other related factors that may trigger dispersal are high densities (Schofield, 1994), and for several triatomine species it has been observed that starvation is the main factor that initiates dispersal by flight (Sjogren and Ryckman, 1966; Ekkens, 1981; Lehane and Schofield, 1981; McEwen and Lehane, 1993).

5.3 Parasite Phylogeography and Ecology

T. cruzi is genetically diverse and is classified into a series of strains or subtypes. This genetic diversity was initially discovered using a panel of isoenzyme markers to investigate differences between parasites involved in the domestic and sylvatic cycles in Bahia state of Brazil (Miles et al., 1977). This study was a breakthrough, revealing that in Bahia there were substantial genetic differences between the parasites in sympatric sylvatic and domestic transmission cycles. These described variants were designated zymodemes I and II (ZI and ZII). It was soon after revealed that the widespread strain associated with the sylvatic cycle in Brazil (ZI) was the predominant cause of human disease in Venezuela (Miles et al., 1981). These groundbreaking findings opened the door to investigating the etiology of Chagas disease, allowing host−vector−parasite associations and comparative geographical

Table 5.1 Nomenclature of *Trypanosoma cruzi* Strains

Zymodeme[a]	DTU[b]	Reference strains	New DTUs[c]
ZI	TcI	Sylvio X10/1	TcI
ZII	TcIIb	Esm cl3	TcII
ZIII/ZI ASAT	TcIIc	M5631 cl5	TcIII
ZIII	TcIIa	CanIII cl1	TcIV
Bolivian ZII	TcIId	Sc43 cl1	TcV
Paraguayan ZII	TcIIe	CL Brener	TcIV

[a]Based upon multilocus enzyme electrophoresis (MLEE) (Miles et al., 1977, 1978b, 1981; Tibayrenc and Miles, 1983; Chapman et al., 1984; Povoa et al., 1984).
[b]Based upon MLEE, random amplification of polymorphic DNA, and nuclear loci (Brisse et al., 2001).
[c]Most recent consensus discrete typing unit (DTU) designations (Zingales et al., 2009).
Source: Modified from Miles et al. (2009) to include a comparison with the most recent nomenclature.

distributions to be explored as reviewed by Miles et al. (2009). Subsequently, four further zymodemes were described from Brazil, Paraguay, and Bolivia (Table 5.1). In the following two decades, various authors proceeded to characterize strains, applying other molecular methods as they became available. As a result, further diversity within the original zymodemes was discovered. However, designations of subtypes in the literature started to become confusing. To standardize the nomenclature, it was decided by the scientific community to formally recognize two main groups that corresponded with the original zymodemes; these were called TcI and TcII (Anon, 1999). In light of further molecular studies, a system of five discrete typing units (DTUs) was establish to represent the diversity within TcII, these were called TcIIa−e (Brisse et al., 2001). Most recently, the scientifc community has redesignated the six strains as TcI−VI (Zingales et al., 2009) (see Table 5.1 for a summary of the nomenclature and synonyms). (From now on we will use the new nomenclature [TcI−TcVI].)

The ordering of the new nomenclature better indicates the perceived phylogenetic relationships between the strains: TcI and TcII are the most anciently divergent (Machado and Ayala, 2001). TcIII and TcIV show similarities. Some argue that they represent the result of ancient hybridization events between TcI and TcII (Westenberger et al., 2005), alternatively they could represent a separate lineage (de Freitas et al., 2006). Finally, TcV and TcIV are generally accepted to be hybrids derived from parental stocks of TcII and TcIII (Machado and Ayala, 2001).

TcI has remained a constant grouping in the nomenclature since first described. However, in recent years at least four subgroupings within TcI have been identified (Herrera et al., 2007, 2009; Falla et al., 2009) (Figure 5.3) with further characterization with microsatellite markers (Figure 5.4) (Llewellyn et al., 2009b). Both sequence variation (Herrera et al., 2009) and microsatellite markers demonstrate that genotypes/haplotypes involved in human infections in Venezuela and Colombia are distinct from sympatric sylvatic variants. This is intriguing as it was recently demonstrated that the main vector in the region, *R. prolixus*, demonstrates panmixia between domestic and sylvatic environments across the western endemic region in Venezuela (Fitzpatrick et al., 2008).

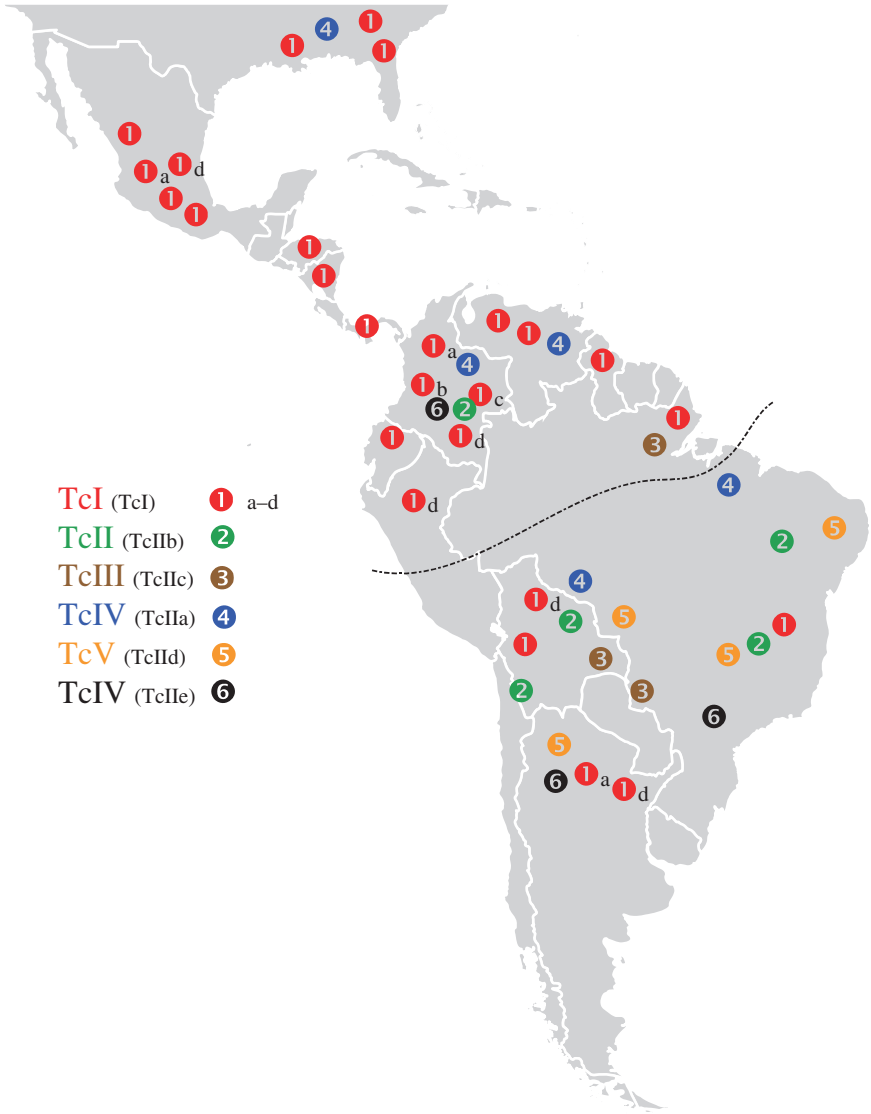

Figure 5.3 Geographical distributions of *Trypanosoma cruzi* strains using the latest nomenclature (Zingales et al., 2009). In parenthesis are the discrete typing units (DTUs) of Brisse et al. (2001). The distribution of TcI subtypes (haplotypes a−d) are also indicated according to Herrera et al. (2009). The dotted line indicates the division where TcI predominates in humans north of the Amazon and TcII−TcVI in the Southern Cone region. *Source*: Adapted from Vallejo et al. (2009).

5.3.1 Epidemiological Implications of Parasite Distributions

In a sense, the findings of Miles et al. (1977) were the tip of the iceberg, but at the same time they hit the nail on the head. The observed predominance of TcI in the human population in Venezuela and TcII mostly infecting human hosts in Brazil was to prove representative (i.e., it has since been demonstrated that TcI predominates in countries north of the Amazon and TcII–TcIV in the Southern Cone countries). This is particularly illuminating given that there are distinct clinical differences between patients presenting with Chagas disease in the two geographical regions.

Strains appear to differ in terms of pathogenicity and response to treatment. Both TcI and TcII–IV are associated with cardiac lesions in human infections, but it seems that only TcII, TcV, and TcIV are also associated with digestive tract lesions (Prata, 2001). In general, TcI is considered to be less pathogenic with lower parasitemias (Burgos et al., 2007) and more chronic cases being asymptomatic

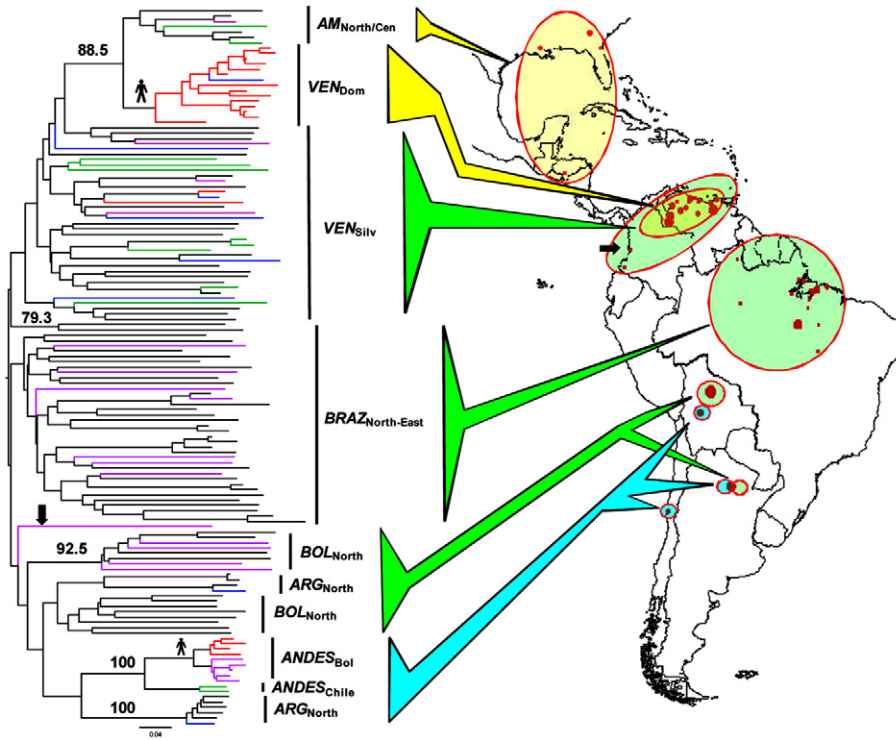

Figure 5.4 TcI phylogeography: a neighbor joining tree based on multilocus microsatellite profiles showing TcI population structure across South America. Branch colors indicate strain origin: black, *Didelphis* species; grey, non *Didelphis* mammalian reservoir, sylvatic triatomine, human or domestic triatomine. (The original figure is in colour and should be viewed from the source to facilitate a full interpretation).
Source: Reproduced with permission from Llewellyn et al. (2009b).

compared to Chagas caused by TcII, TcV, and TcVI in Argentina, Brazil, Chile, Paraguay, and Uruguay. TcI is almost the only form found in human infections north of the Amazon region. Moreover, there is an observed general partitioning of TcII subtypes between sylvatic and domestic transmission cycles; the human disease is associated with TcII, while TcV is rarely associated with sylvatic hosts (Yeo et al., 2005), and TcIII and TcIV are predominantly sylvatic.

5.4 Vector–Parasite–Host Interactions and Implications for Chagas Disease Distribution

There have been relatively few in-depth investigations to test coevolutionary relationships between triatomines, *T. cruzi*, and vertebrate hosts. There is a close association among TcI, *Rhodnius* species (and other arboreal triatomines), and arboreal marsupials, in particular *Didelphis marsupialis*. There is also a close association

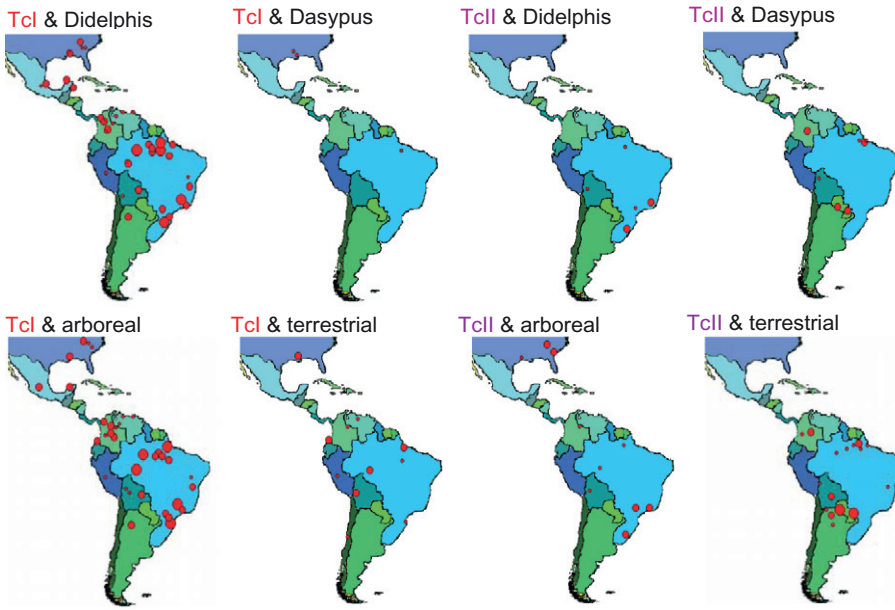

Figure 5.5 Maps indicating TcI/TcII host/habitat associations: in general, TcI is associated with *Didelphis* and the arboreal habitat, whereas TcII–TcVI is associated with armadillos and the terrestrial environment (samples from the strains TcI–TcVI were pooled in the analysis, referred to here as just TcII). The upper four maps show specific associations between strains and *Didelphis* and armadillo species, and the lower four show associations between all terrestrial and arboreal mammalian reservoirs.
Source: The data represents a literature survey collated by Yeo et al. (2005); this figure has been modified and reproduced with agreement of the authors.

between TcII−TcVI *Triatoma* (and other terrestrial triatomines) and armadillo species, particularly *Dasypus* (Gaunt and Miles, 2000; Yeo et al., 2005; Llewellyn et al., 2009a,b) (Figure 5.5).

However, the associations are not absolute, and in the case of TcI, there was no apparent clustering of particular TcI genotypes with *Didelphis* in comparison to isolates from other arboreal mammals (Llewellyn et al., 2009b) (see Figure 5.4). Also for phylogeographical analyses of TcIII, the results indicate that isolates cluster according to geography rather than host association (Llewellyn et al., 2009a; Marcili et al., 2009a).

Two interesting studies of host response to different strains have confirmed, by comparative artificial infection, that in the southern USA two species of opossum (*Monodelphis domestica* and *Didelphis virginiana*) seem to be resistant to TcIV (Roellig et al., 2009a,b). This highlights a mechanism for the association of a vertebrate host with one strain over others. The strong association between TcI and *Rhodnius* species can also be explained by a similar mechanism: comparative artificial infection studies of *R. prolixus* with various strains revealed a tendency for it to be resistant to infection by TcII (Mello et al., 1996).

For triatomines, susceptibility or resistance to trypanosome infections seems to be modulated by the intestinal symbionts, which are vital for development. *T. cruzi* is considered to be subpathogenic to triatomines, whereas *Trypanosoma rangeli* is another species that commonly infects *Rhodnius* species and causes pathogenicity based on a reduction of the number of symbionts (Vallejo et al., 2009). A study of four *Rhodnius* species artificially infected with different strains of *T. rangeli* showed a measurable difference in response and indicated adaptation of trypanosome strains to the local vector species (Machado et al., 2001). At least half of all species of triatomine bugs have been found naturally infected with *T. cruzi* (Lent and Wygodzinsky, 1979; Schofield, 1994). Unfortunately the vast majority of these records do not include specific strain associations. Clearly this is an area of potential research.

In the context of dispersal triggered by starvation, there is evidence that starvation decreases *T. cruzi* infection in triatomines (Kollien and Schaub, 1998) and in some species starvation may clear infection altogether (Phillips and Bertram, 1967; Vargas and Zeledon, 1985). This factor could go toward explaining paradigms such as in Venezuela where sylvatic and domestic bugs seem to be in panmixia but TcI shows discrete general clustering of sylvatic and domestic cycles (Fitzpatrick et al., 2008; Llewellyn et al., 2009b).

Triatomine bugs directly determine the etiology of the strains of *T. cruzi* involved in human transmission cycles. This is clear because despite TcI and *Didelphis* being widespread, it is the northern distribution of *Rhodnius* that corresponds with occurrence in human cycles.

Overall, the aspects of epidemiological relevance are that associations between terrestrial ecology, *T. infestans*, terrestrial mammals, and *T. cruzi* strains TcII−TcIV have lead to the prominence of TcII, TcV, and TcIV in human infections in the southern region of South America. In the northern regions human

infections stem from TcI associated with arboreal *Rhodnius* and arboreal mammals.

5.5 Assessment of Regions Affected by Chagas Disease

Chagas disease is a threat to almost a quarter of the population of Central and South America, associated with poverty in rural areas of 21 countries. In the early 1990s, approximately 16−18 million people were thought to be infected with 100 million at risk (WHO, 1991). In 1996, it was estimated that of those infected, 6 million would develop clinical symptoms and 45,000 would die per year (Rozendaal, 1997). As a result of efforts to control disease transmission, by 2003 an estimated 13 million people were infected with approximately 3 million symptomatic cases and a reduced annual incidence of 200,000 cases in 15 countries (WHO, 2003). The most recent estimates are as low as 7−8 million (Table 5.2).

Chagas disease is thought to be one of the principal reasons for the impoverishment of many areas of South America, and is believed to be responsible for the loss of 649,000 disability-adjusted life years (DALYs) (WHO, 2002).

We present here a summary of prevalence data for Latin and South America spanning the last 35 years and represent these data in graphical format (Figure 5.6) that allows easy comparative assessment of control program success by country and region. In particular, the map of incidence data from 2005 identifies current transmission hot spots, highlighting those countries where vector control is difficult to achieve or requires coordinated implementation.

We will now address the status of Chagas disease by region, paying special attention to nonendemic regions and the globalization of Chagas disease. For more detailed accounts of control programs and prevalence data, see Chapter 4.

5.5.1 Chagas Disease in North America

5.5.1.1 Vectors and Parasite Ecology

In the USA, Chagas disease exists almost exclusively as a zoonosis; only six autochthonous cases have been reported in humans, and the two most recent (Herwaldt et al., 2000; Dorn et al., 2007) were both attributable to *Triatoma sanguisuga*. *T. sanguisuga* has a broad distribution across the south from Texas to Maryland. *T. gerstaekeri* also shows anthropophilic tendencies and is found in Texas and New Mexico (Dorn et al., 2007).

The southern states of the USA have an active sylvatic transmission cycle involving numerous wild animal reservoirs. Recent serological surveys suggest that raccoons and opossums are the main hosts (Brown et al., 2009) with prevalences as high as 68%. Different *T. cruzi* strains have been associated with these two main hosts (Roellig et al., 2009a): TcI and TcIV with raccoons and TcI with opossums.

Table 5.2 Prevalence Rates by Region/Country and Recent Incidences with Attribution to Vector Transmission

Region/Country	Estimated Prevalence 1975/85	Population 2002 (000)	Estimated Prevalence Rate 2002 (%)	Estimated Prevalence Ranges 2002		Population 2005	Estimated Prevalence Rate 2005	Estimated Prevalence 2005	Incidence Rates 2005	Incidence 2005	Incidence due to Vector Transmission
				Conservative	High						
Southern Cone	**8,124,000**	**247,627**	**2.2–2.8**	**5,564,417**	**6,970,063**	**259,805,650**	**1.714**	**4,453,068.84**	**0.005**	**12990**	**12500**
Argentina	2,640,000	37,981	4.4–5.8	1,671,164	2,202,898	38,747,000	4.129	1,599,863.63	0.003	1162	1300
Bolivia	1,300,000	8,645	19–24	1,642,550	2,074,800	9,182,000	6.752	619,968.64	0.112	10284	10300
Brazil	3,600,000	176,257	1.1–1.3	1,961,000	2,291,341	186,405,000	1.019	1,899,466.95	0	0	0
Chile	150,000	15,613	0.4–0.6	62,452	93,678	16,267,300	0.985	160,232.91	0	0	0
Paraguay	397,000	5,740	3.9–5.0	223,860	287,000	5,898,650	2.543	150,002.67	0.015	885	900
Uruguay	37,000	3,391	0.1–0.6	3,391	20,346	3,305,700	0.656	21,685.39	0	0	0
Andean Initiative	**3,121,000**	**108,329**	**2.3–3.0**	**1,879,700**	**3,240,498**	**113,545,000**	**1.029**	**1,168,378.05**	**0.011**	**12490**	**12100**
Colombia	900,000	43,526	3	700,000	1,305,780	45,600,000	0.956	435,936.00	0.012	5472	5250
Ecuador	400,000	12,810	1.0–1.6	165,000	170,000	13,228,000	1.739	230,034.92	0.018	2381	2350
Peru	621,000	26,767	2.4	600,000	680,000	27,968,000	0.686	191,860.48	0.011	3076	3100
Venezuela	1,200,000	25,226	1.7–4.3	414,700	1,084,718	26,749,000	1.159	310,020.91	0.005	1337	1400
Central America	**2,697,600**	**37,976**	**2.5–4.5**	**785,966**	**1,881,651**	**39,656,200**	**2.034**	**806,607.11**	**0.021**	**8328**	**8500**
Belize	600	251	0.26	600	650	270,000	0.741	2,000.70	0.009	24	20
Costa Rica	130,000	4,094	1.9–3.2	77,786	130,000	4,327,000	0.532	23,019.64	0.001	43	30
El Salvador	900,000	6,415	2.5–5	192,000	320,750	6,881,000	3.372	232,027.32	0.036	2477	2500
Guatemala	1,100,000	12,036	2.8–6.1	337,008	734,196	12,599,000	1.984	249,964.16	0.017	2142	2200
Honduras	300,000	6,781	1.19–1.53	103,750	300,000	7,205,000	3.053	219,968.65	0.039	2810	2800
Nicaragua	67,000	5,335	1.29–3.3	68,822	176,055	5,142,200	1.140	58,621.08	0.015	771	750
Panama	200,000	3,064	<0.1–7.59	6,000	220,000	3,232,000	0.006	193.92	0.007	226	200
Mexico	**1,564,800**	**101,965**	**1.6–2.1**	**1,605,872**	**2,100,479**	**107,029,000**	**1.028**	**1,100,258.12**	**0.007**	**7492**	**7700**
Guyanas[a]	–	–	–	–	–	**1,397,000**	**1.288**	**17,993.36**	**0.029**	**405**	**400**
Total: 18 countries	**15,507,400**	**495,897**		**9,835,955**	**14,192,691**	**521,432,850**		**7,523,119.45**		**40989**	**41200**

[a]French Guyana, Guyana, and Suriname.

Source: 1975–1985 data was collated by Strosberg et al. (2007) and the 2005 data was sourced from PAHO (2006).

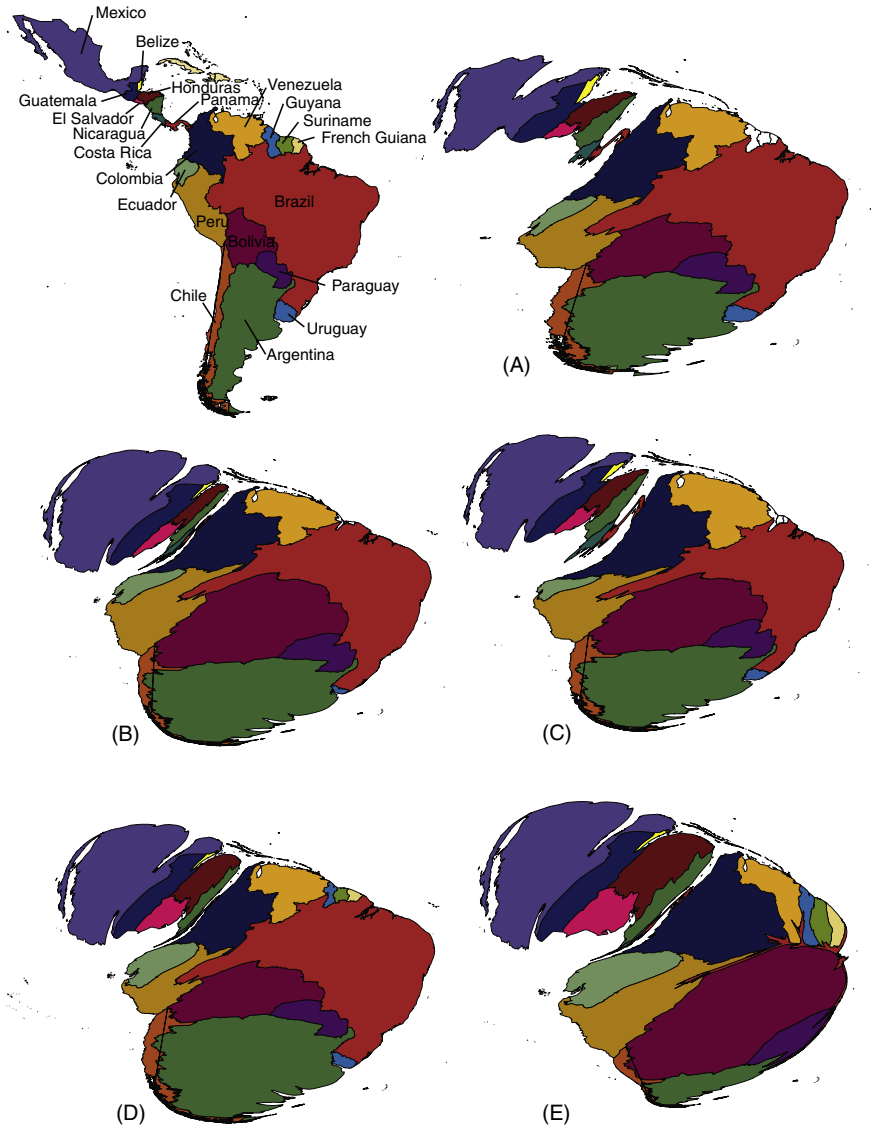

Figure 5.6 Maps showing the prevalence and incidence of Chagas disease in Central and South America. (A) Prevalence 1975−1985; (B) Prevalence 2002 (conservative estimates); (C) Prevalence 2002 (high estimates); (D) Prevalence 2005; (E) Incidence 2005. Countries shown are distorted in size and shape according to the data. Shadings of countries are kept constant to reference map. The maps were generated using the diffusion based algorithm for producing density equalizing maps (Gastner and Newman, 2004) with ScapeToad (Andrieu et al., 2010).
Sources: Data of 1975−1985 and 2002 from Strosberg et al. (2007); 2005 data from PAHO (2006).

Other reported hosts are armadillos (Dorn et al., 2007), and incidences of peridomestic transmission have been reported in domestic dogs (Beard et al., 2003). In Georgia, lemurs have been found with high rates of infection, and isolates have also been characterized as TcIV (Hall et al., 2007).

In reality, it is probable that there are more than six autochthonous cases in the USA, but due to lack of public awareness, serological data, and the asymptomatic nature of chronic Chagas disease, the true incidence remains occult. However, due to general higher standards of housing in the USA compared with Latin America, levels of autochthonous transmission are unlikely to become a serious problem.

5.5.1.2 Chagas Disease Status

Despite low risk of vector-borne transmission in the USA, the numbers of infected individuals living in the USA are estimated to be of the same magnitude as prevalence estimates of several South American countries (Figure 5.7).

This is due to enormous numbers of immigrants from Latin America to the USA in recent years. The most recent estimates from 2007 are that 2% of the 17 million Latin American immigrants in the USA could be infected (325,671) (Schmunis and Yadon, 2010); of these, an estimated 65,000 would need medical attention. Similar estimates come from other authors (Milei et al., 1992; Bern and Montgomery, 2009), and another recent report estimated that the number of infected individuals in the USA could be as high as 1 million (Simon and McKay, 2009).

Estimates for Canada from 2006 are that 3.5% of approximately 160,000 immigrants could be infected (approximately 5500) (Schmunis and Yadon, 2010) (see Figure 5.7). A recent serological survey of approximately 100 Latin American

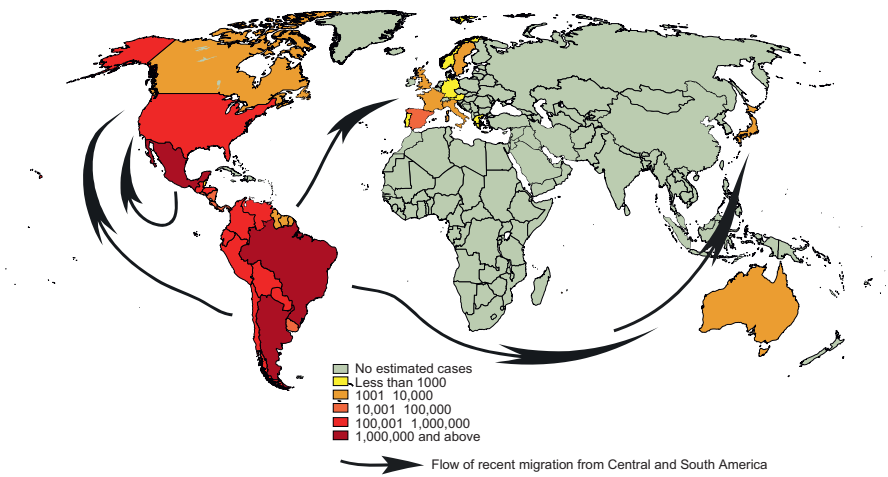

Figure 5.7 Current estimated global population infected by *Trypanosoma cruzi*.
Sources: PAHO (2006); Schmunis (2007); Guerri Guttenberg et al. (2008); Paricio Talayero et al. (2008); Perez de Ayala et al. (2009).

immigrants in Canada revealed a 1% prevalence (Steele et al., 2007). There is awareness of the risk to health professionals, and blood and organ donors are screened by questionnaire (Lennox et al., 2007).

The transmission of Chagas disease through infected blood transfusion is manifest in North America (Castro et al., 2009; Milei et al., 2009). Large concentrations of Latin American immigrant populations in Los Angeles and Miami have historically recruited many immigrant donors. Recently, the US Centers for Disease Control and Prevention (CDC) has implemented the screening of all donated blood for *T. cruzi* antibodies in the USA (CDC, 2007).

5.5.2 Chagas Disease in Mexico and Central America

5.5.2.1 Vectors and Parasite Ecology

R. prolixus appears to be exclusively associated with domestic environments in Central America and is thought to have been introduced from domestic South American populations, as has been demonstrated through morphometric and genetic comparisons of samples from Honduras and Colombia (Dujardin et al., 1998). The other two main species implicated in transmission in Central American countries are *T. dimidiata* and *R. pallescens. T. dimidiata* is endemic to the region and the most widespread vector species; it regularly invades domestic and peridomestic ecotypes from a variety of natural habitats including rock piles, palms, and caves. It has been linked to rural and recently to urban transmission in Merida City, Yucatan, Mexico (Guzman-Tapia et al., 2007). *R. pallescens* occurs in Panama, where it is associated with *Attelea* palm trees and domestic infestations; recent reports demonstrate that TcI circulates in both domestic and sylvatic *R. pallescens* (Sousa et al., 2006; Samudio et al., 2007).

Mexico has some 30 vector species (including *T. dimidiata*), with eight that pose a risk to transmission (Cruz-Reyes and Pickering-Lopez, 2006). They are members of the *Triatoma (Meccus) phyllosoma* complex and *Triatoma barberi* (Figure 5.2).

As in northern South America, TcI predominates in Central America and Mexico in both sylvatic and domestic cycles. However, recent reports from Guatemala found domestic *T. dimidiata* harboring TcI and "TcII" concurrently (Pennington et al., 2009). This surprising finding highlights a need for further molecular epidemiological studies in the region. The TcI phylogeographical analysis of Llewellyn et al. (2009b) shows that TcI samples from the sylvatic cycle in Central America form a separate clade (Figure 5.4), with the US strains similar to domestic strains from northern South America. Further studies including strains from humans are needed to help elucidate the molecular epidemiology of the region.

5.5.2.2 Chagas Disease Status

The Central American initiative for Chagas control (IPCA) was established in 1997 during the 13th meeting of the Central American Health Sector, which included representatives from Belize, Costa Rica, El Salvador, Guatemala, Honduras, Mexico,

Nicaragua, and Panama. The objectives of the initiative were to eliminate *R. prolixus* from Central America, reduce domiciliary *T. dimidiata*, and halt transfusion transmission (Ponce, 2007). The occurrence of *R. prolixus* is much reduced, and there are good prospects for eliminating it from Central America, as has been the case for *T. infestans* over large regions of the Southern Cone. However, the trends in prevalence for the region have risen since the 1980s (Figure 5.6). Our maps graphically

Figure 5.8 Screening of donated blood. Percentages of blood donations screened by country. Countries without data are in light gray.
Source: From Schmunis (2007).

demonstrate that the estimated prevalence in Central America and Mexico is approaching that of South America, and in terms of incidence (Figure 5.6E) exceeds half the level estimated for South America. The rise represents an increased awareness and surveillance throughout the region.

An aspect of control for the region that is clearly lacking is the relatively low proportion of blood transfusions currently screened in Panama and Mexico (Figure 5.8).

5.5.3 Chagas Disease in the Amazon Region

5.5.3.1 Vectors and Parasite Ecology

The Amazon region is vast, extending across much of northwestern Brazil, the Guyanas, northern Bolivia, eastern Peru and Ecuador, and southern Colombia and Venezuela. The region has a high number of sylvatic triatomine species (see Figure 5.1). However, only the following seven (mostly *Rhodnius* species) have been implicated in disease transmission: *R. prolixus, R. pictipes, R. robustus, R. stali, Panstrongylus geniculatus, P. herreri*, and *Triatoma maculata*. There are also a plethora of reservoir hosts (e.g., marsupials, bats, rodents, edentates, carnivores, and primates). In the Amazon, the observed tendency is that infestation by triatomine species is occasional and sporadic. Most Amazon species seem to be constrained from readily exploiting domestic environments by preadaptation to humid environments. The same factor makes such species notoriously difficult to culture in the laboratory, unless increased humidity is maintained (e.g., *P. geniculatus* [D. Feliciangeli, personal communication], *R. robustus*, and sylvatic *R. ecuadoriensis* [Abad-Franch and Miles, personal communication]).

Human activities that negatively impact the natural ecology of triatomines species, such as deforestation, are likely to drive adaptation to the domestic environment (Abad-Franch and Monteiro, 2007; Abad-Franch et al., 2009).

There is a diverse range of *T. cruzi* strains reported from the Amazon (Figure 5.3) from TcI to TcV. Predominantly TcI and TcIII circulate (Gaunt and Miles, 2000), and both have been associated with human infections along with TcIV (Marcili et al., 2009a,b).

5.5.3.2 Chagas Disease Status

There are many reports of acute cases of disease in humans (Coura et al., 2002), but chronic forms of the disease are considered to be relatively rare in the region (Aguilar et al., 2007). As described by Miles et al. (2003, 2009), currently the main type of transmission cycle in the Amazon region is enzootic (i.e., no/few domestic colonies of triatomines exist), but infrequent, sporadic cases of Chagas disease may occur due to bugs sporadically flying into houses and infecting people or contaminating food. There are numerous reports of small epidemics in the Brazilian Amazon, mainly attributed to oral transmission (Miles et al., 1978a; Pinto et al., 2001, 2008, 2009; Beltrao Hde et al., 2009; Valente et al., 2009). Often oral

contamination involves palm-dwelling species (most likely *Rhodnius* species) that are accidentally collected along with palm fruits, such as acai, and subsequently pressed to make juice. The most recent outbreak was reported the same month that this chapter was written (Sekkides, 2010). Another paradigm is communities are frequently attacked by *Rhodnius brethesi* when venturing into the forest along the Rio Negro (Brazil, Colombia, and Venezuela) to harvest piçava palm fibers (Miles et al., 2003; Aguilar et al., 2007). There are also reports of foci of transmission associated with vector infestation, such as *P. geniculatus* in the Amazon basin (Valente et al., 1998), *R. stali* in Bolivia, and *Panstrongylus herreri* in Peru.

An initiative for Chagas disease surveillance and prevention in the Amazon (AMCHA) was officially launched with the backing of Pan American Health Organization (PAHO)−WHO and ECLAT (European Community−Latin American Network for Research on the Biology and Control of Triatominae) in 2004 (Guhl and Schofield, 2004).

5.5.4 Chagas Disease in the Andean Region

5.5.4.1 Vectors and Parasite Ecology

The main vector in the Andean region is *R. prolixus*, principally in Venezuela and Colombia where it is mainly associated with the foothills of the Andes and adjacent plains (Llanos). *R. prolixus* is a close relative of the Amazon species *R. robustus* (Monteiro et al., 2003), and it seems probable that *R. prolixus* represents a lineage that adapted to exploit drier environments north of the Amazon, whereby predisposing it to be an efficient invader of domestic environments. However, unlike *T. infestans*, *R. prolixus* has widespread sylvatic foci that pose the threat of reinvasion of houses postintervention; this was demonstrated by a population genetics study comparing domestic and sylvatic samples in Venezuela (Fitzpatrick et al., 2008) and by the identification of palm trees as a risk factor to domestic infestations (Sanchez-Martin et al., 2006). However, previous studies have suggested that the risk of invasion by sylvatic *R. prolixus* is unlikely (Feliciangeli et al., 2003; Guhl and Schofield, 2004).

T. dimidiata is implicated in transmission in Colombia and Ecuador. However, there is strong evidence to suggest that *T. dimidiata* in Ecuador was introduced from mid-Central America in association with human migration patterns (Bargues et al., 2000, 2008). Therefore, it may represent a more domiciliated population with fewer sylvatic refuges and better prospects for control.

R. ecuadoriensis is an important vector implicated in transmission in Ecuador and northern Peru (Abad-Franch et al., 2002; Cuba et al., 2002). Like *R. prolixus*, it maintains sylvatic populations in palm trees that pose the risk of reinvasion after the control of domestic populations (Abad-Franch et al., 2005).

The main strain of *T. cruzi* associated with human disease and sylvatic transmission cycles in the region is TcI, and recent molecular epidemiology studies (Herrera et al., 2007, 2009) have demonstrated that there are subtypes associated

with different transmission cycles and geographical regions (see earlier) with a particularly high diversity within Colombia (see Figure 5.3).

5.5.4.2 Chagas Disease Status

The Andean initiative (Colombia, Ecuador, Peru, and Venezuela) was inaugurated in 1997. This program, like the Central American countries, has more complications than the Southern Cone initiative because there are more vector species involved which maintain sylvatic habitats and pose the threat of reinvasion postcontrol (Guhl, 2007).

In 1993, the preliminary phase of the Andean initiatives and Central American began with the emphasis on screening blood banks for parasites. The vector control phase was launched in 1997, triggering a surge in research on the biology and systematics of triatomine species in these regions, with the aim to provide governments with recommendations on how to best proceed with control. The incidence of Chagas disease remains relatively high in the region (Figure 5.6; Table 5.2), highlighting the need for the development and implementation of control and surveillance. Currently the level of blood donations screened is high for the region, with further implementation necessary in Peru.

5.5.5 Chagas Disease in the Southern Cone Countries

5.5.5.1 Vectors and Parasite Ecology

Historically the main vector for the region was *T. infestans*. However, with the eminent success of the Southern Cone program, having effectively eliminated *T. infestans* from Chile and Uruguay, much of Brazil and parts of Argentina its importance as a vector has been significantly reduced. It has been demonstrated that the center of radiation for the species is in Bolivia (Bargues et al., 2006), where it most likely continues to cause a problem due to ready reinvasion from sylvatic foci. Domestic infestations continue to persist in the periphery of its origin in Paraguay, northern Argentina, and southern Peru.

Several other species are implicated as secondary vectors, particularly since the control of *T. infestans*, and the presentation of the niche to other species. In northeastern Brazil, *T. brasiliensis* and *T. psuedomaculata* show strong tendencies to colonize domestic habitats along with *Panstrongylus megistus* in southern Brazil (Figure 5.2). Other species that have shown some potential to domiciliation are *R. neglectus*, *T. vitticeps*, and *T. rubrovaria* in Brazil. *Triatoma sordida* is an important secondary vector in Paraguay, Bolivia, northern Argentina, and southern Brazil. *T. sordida* and a related species, *T. guasayana*, demonstrate mass dispersal during the dry season, which can result in the invasion of domestic environments (Noireau and Dujardin, 2001).

T. cruzi subtypes TcII−TcVI predominate in both sylvatic and domestic cycles in this region (see earlier). Interestingly TcI is also present in many arboreal hosts, such as *Didelphis* species (see Figures 5.4 and 5.5). The absence of *Rhodnius* species in the southern part of this region and the lack of data for other triatomines

carrying TcI present the possibility that transmission of TcI, in *Didelphis* at least, commonly occurs without vectors by direct contamination from the anal scent glands (Carreira et al., 2001). However, *P. megistus* has been implicated with TcI transmission in southern central Brazil (Diotaiuti et al., 1995), and a species of *Rhodnius*, *R. neglectus* (Fernandes et al., 1991), which has a distribution extending from the Amazon to southern Brazil. Also, *Triatoma (Mepraia) spinolai* in Chile has been reported infected with TcI (Miles et al., 1984). Other vector species may yet to be implicated in TcI transmission because it is often difficult to sample from sylvatic habitats directly.

5.5.5.2 Chagas Disease Status

In Brasilia, in June 1991, Ministers of Health of Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay met with the PAHO and launched an initiative for the elimination of Chagas disease in these countries by the end of the twentieth century. The emphasis of the campaign was to use established triatomine control methods in a cooperative, international effort to eliminate domestic *T. infestans* populations from these six southernmost countries of South America. *T. infestans* was responsible for 80% of *T. cruzi* transmission in the Southern Cone countries (Schofield and Dias, 1999), with other species such as *T. brasiliensis* in northern Brazil and *P. megistus* in Atlantic coastal regions being responsible for 10% (the remaining 10% being attributable to unscreened blood transfusion and congenital routes). The most important achievements of the Southern Cone program include:

- The incidence of human infection in children and young adults was reduced by 72% in the countries of the initiative.
- In 1997, Uruguay was certified free of vectorial and transfusional transmission of Chagas disease.
- In 1999, Chile was certified free of vectorial (by *T. infestans*) and transfusional transmis sion of Chagas disease.
- In 2000, Brazil was certified free of vectorial (by *T. infestans*) and transfusional transmis sion of Chagas disease.

Recent incidence data (PAHO, 2006) report zero incidence of Chagas disease for Brazil, Chile, and Uruguay (Figure 5.6; Table 5.2). While this might be accurate for Uruguay with 100% of blood donations screened and few reports of threats from secondary vectors, it is unlikely to be true for Brazil where secondary vectors are reported to be involved in transmission (Costa et al., 2003; Herrera et al., 2003). Also, Chile is likely to have some incidence without comprehensive screening of donated blood (see Figure 5.8). Bolivia continues to have the worst Chagas disease situation (Figure 5.6) of the region with fewer than 100% of blood donations screened and continued problems with transmission by *T. infestans*.

5.5.6 Chagas Disease in Europe and Other Nonendemic Countries

During the 1960s through 1980s, political unrest and economic problems spurred migration from Latin America to developed countries. The USA was the most

popular destination, but Latin Americans also migrated to Australia and Canada. The 1990s saw an increasing flow of migration from Latin America to Europe constituting a reverse of the mass European migrations to Latin America of the late nineteenth century until the 1940s. This recent trend of migration to Europe from Latin America has been facilitated by economic downturns in Latin America and opportunities created by the formation of the European Union. By 2005, there were more than 2 million people born in Latin America living in Western European countries (Schmunis and Yadon, 2010). In the US, it was estimated that there were millions of Latin American immigrants according to the census of 2000 (Schmunis and Yadon, 2010).

Recent analyses of migration trends and numbers of migratory persons, factoring in the proportions from respective Latin American countries and the estimated prevalence of Chagas disease from each, have produced some rather alarming figures for the estimated numbers of infected people living in nonendemic countries (Schmunis, 2007; Guerri-Guttenberg et al., 2008; Schmunis and Yadon, 2010) (see Figure 5.7).

5.5.6.1 Spain

Spain has by far the most Latin American immigrants in Europe, with annual numbers increasing dramatically almost fivefold over the past decade from 446,000 in 2001 to more than 2 million in 2008 (Gascon et al., 2010). Another estimate for 2008 is slightly lower, stating that Spain has approximately 1.7 million Latin American immigrants. Of these, an estimated 5.2% were potentially infected with *T. cruzi*, approximately 17,000 of whom may be expected to eventually present with Chagas disease (Schmunis and Yadon, 2010). The same study suggested that there may have been as many as 92 congenital infections of newborns in Spain in 2007. A survey of recent literature (Castro et al., 2009) reveals that numerous cases have been reported from the immigrant population, mostly by congenital (vertical) transmission and by contaminated blood transfusion.

A case study (Gonzalez-Granado et al., 2009) assessed the prevalence of Chagas disease in Bolivian pregnant women in a Spanish hospital for 2 years and found a 17.7% rate (71/401), with a 1.4% vertical transmission rate (1/71). Screening for *T. cruzi* has been implemented in Spain since 2005. However, as yet no national screening protocol for pregnant women has been established.

5.5.6.2 Other European Countries

The survey of literature by Castro et al. (2009) reveals that Chagas disease has been reported from immigrants in Denmark, Germany, Italy, Netherlands, and Switzerland, and congenital cases have been documented in Romania and France. Estimates as of 2008 state that Italy has 230,000 Latin American immigrants from Chagas disease−endemic countries, while Portugal has 121,001 and Switzerland has 57,000 such immigrants (Gascon et al., 2010). The estimated number of Latin Americans living in France has risen from 27,400 in 1999 to 105,000 in 2005

(Lescure et al., 2008). Figure 5.7 summarizes the number of infected individuals for countries where immigration estimates are available.

The varying estimates and recent increases in immigration trends to Europe in general highlight the need for European Union–centered assessment of Chagas disease for the region, including comprehensive efforts to halt transfusional and congenital transmission.

5.5.6.3 Oceania and Asia

Australia has an estimated 80,579 Latin American immigrants with an estimated 1392 cases of *T. cruzi* infection (Gascon et al., 2010). Japan accommodates some 371,700 people from Chagas-endemic regions, 84% of whom are from Brazil with an estimated 3592 *T. cruzi*-infected residents (Gascon et al., 2010). These immigrants represent the descendants of people that migrated to Brazil in the early twentieth century to work on coffee plantations. The 1980s saw a reversal of this migration back to Japan. In Brazil, these immigrant Japanese were called *dekasseguis*, and now more than 300,000 *dekasseguis* Brazilians live in Japan (Castro et al., 2009). Several cases of Chagas cardiomyopathy have been reported in Brazilian immigrants in Japan (Ueno et al., 1995; Nishimura et al., 1997).

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6 Classification and Phylogeny of the Triatominae

Maria Dolores Bargues[1], *C.J. Schofield*[2] *and Jean-Pierre Dujardin*[3,*]

[1]Departamento de Parasitología, Universidad de Valencia, Valencia, Spain, [2]ECLAT network, London School of Hygiene and Tropical Medicine, London WC1 E7HT, UK, [3]Institut de recherche pour le développement, Centre National de la recherche scientifique, Montpellier, France

6.1 Introduction

In understanding biodiversity, one must understand that taxonomy (classification) and systematics (including phylogenetics) work together, although the two terms are often confused. The objective of systematics is to understand the natural mechanisms responsible for the biodiversity, whereas the task of taxonomy is to set up a useful classification of the organisms concerned. In a sense, systematics provides guidelines for taxonomy to classify organisms according to accepted rules of nomenclature (ICZN, 1999). But nevertheless, the two concepts face an inevitable conflict because classification and nomenclature are designed to be stable (ICZN, 1995) and of use to those considering other aspects of the organisms. Systematics, on the other hand, is essentially a dynamic approach suggesting changes and adjustments as new data become available. Also, systematics is an inherently dynamic system in which the units of study species or populations are liable to change with time and circumstance.

Divergence between the modern concepts of systematics starts at the definition given to the **taxa** they wish to analyze: single individuals (Vrana and Wheeler, 1992), reproductively isolated populations (Mayr, 2000; Meier and Willmann, 2000), populations (Mishler and Theriot, 2000), or agglomerations of populations (Wheeler and Platnick, 2000). In the case of the Triatominae, these problems are also evident from the epidemiological requirement (e.g., are these important vectors of Chagas disease or not?) and the increasing wealth of data offering new insights to their evolution. These problems are compounded by evidence that the Triatominae represent a polyphyletic group yet it would make no epidemiological sense to try to reclassify them in that light (Schofield and Galvão, 2009) and also by their evident capacity for **phenetic drift** that can give rise to morphological and

*E mail: dujardinbe@gmail.com

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morphometric variants (Dujardin et al., 1999b) that tempt yet more specific designations.

Other problems arise from the lack of clear consensus on taxonomic concepts. Not only is the subfamily itself poorly defined, such that some predatory Reduviidae have been erroneously described as Triatominae (Lent, 1982; Forero et al., 2004), but there is no consistent concept of features meriting tribal or generic rank, and there is considerable divergence on concepts of species, subspecies, and species complexes. In this chapter, we summarize the current classification and offer ways in which these concepts might be usefully reviewed.

6.2 Subfamily: Triatominae

The Triatominae are classified as a subfamily of the Reduviidae (Hemiptera, Heteroptera) and are believed to have derived from predatory forms of the Reduviidae. They are customarily defined by their bloodsucking habit and associated morphological adaptations particularly the straight three-segmented rostrum in which the final segment is capable of flexing upward when the rostrum is extended for feeding. This definition is not entirely satisfactory, because although most other Reduviidae are predators on other invertebrates, several will also suck vertebrate blood on occasion (Schofield and Dolling, 1993). Conversely, an increasing number of triatomine species have been shown to be at least facultative predators (Lorosa et al., 2000; Schofield and Galvão, 2009; Zeledón et al., 2010) and some particularly of the tribe Bolboderini appear to be almost entirely predaceous in habit (Sandoval et al., 2004, 2007). Over half of the 141 currently recognized species of Triatominae have been shown to be naturally or experimentally infected with *Trypanosoma cruzi* (causative agent of Chagas disease), and all are suspected to have this capacity. However, although of great epidemiological relevance (and a primary stimulus to research on the group), this characteristic is not used in the definition of the subfamily because of the capacity of *T. cruzi* to infect a wide range of other arthropods (Schofield et al., 2009), even though other arthropods appear to have no epidemiological significance as vectors.

For this review, we follow the classification of Triatominae of Lent and Wygodzinsky as updated by Dujardin et al. (2002) and Schofield and Galvão (2009). For this, the subfamily is classified into five tribes with 15 genera and 141 species (Table 6.1). The classification has a morphological basis and has been challenged by various other arrangements based on morphology and genetic **characters** (see Tables 6.2 and 6.3). Difficulties in reaching a stable classification are discussed more fully by Schofield and Galvão (2009).

6.2.1 Tribes and Genera

6.2.1.1 Tribe: Alberprosenini (Genus Alberprosenia*)*

The two similar species of *Alberprosenia* forming this tribe are unusually small Triatominae (adults up to 5 mm in length) with unusually short heads that have

Table 6.1 Classification of Triatominae

Tribes	Genera	Number of Species
Alberproseniini	*Alberprosenia*	2
Bolboderini	*Belminus*	8
	Bolbodera	1
	Microtriatoma	2
	Parabelminus	2
Cavernicolini	*Cavernicola*	2
Rhodniini	*Psammolestes*	3
	Rhodnius	17
Triatomini	*Dipetalogaster*	1
	Eratyrus	2
	Hermanlentia	1
	Linshcosteus	6
	Panstrongylus	13
	Paratriatoma	1
	Triatoma	80
Total	15	141

Source: From Schofield and Galvao (2009) with the addition of *Rhodnius zeledoni* (Jurberg et al., 2009).

Table 6.2 Comparison of Classification of Triatominae by Concept

Concepts	TYPO	BIOL	HENN	EVOL	PHG I	PHG II
References	(1)	(2,3)	(4,5)	(6,7)	(8)	(9)
RI	no	yes	yes	no	no	no
TD	no	no	yes	yes	yes	yes
ST	/	/	no	yes	yes	no
SC	/	/	yes	/	no	yes
Problems	S	A	A	S	M	H

TYPO, typologic concept; BIOL, biological concept; HENN, Hennigian concept; EVOL, evolutionary concept; PHG I, phylogenetic concept *sensu* Wheeler and Platnick; PHG II, phylogenetic concept *sensu* Mishler and Theriot; /, not relevant; (1) Carl von Linnaeus (2) Earl of Buffon (3) Mayr (2000) (4,5) Hennig (1965); Meier and Willmann (2000) (6,7) Wiley (1978); Wiley and Mayden (2000b) (8) Wheeler and Platnick (2000) (9) Mishler and Theriot (2000); RI, reproductive isolation; TD, temporal dimension; ST, stem species survival; SC, exclusive use of synapomorphic characters; A, allopatry; H, hybridism; M, exclusive use of morphologic characters; S, subjectivity.

been little studied beyond their original descriptions. They have no known epidemiological significance. The tribal, generic, and species concepts are entirely morphological (Lent and Wygodzinsky, 1979), although *Alberprosenia goyovargasi* has been reared in the laboratory by feeding on human blood (Martinez and Carcavallo, 1977), which supports their inclusion in the Triatominae classification. *Torrealbaia martinezi*, previously ascribed to this tribe (Carcavallo et al., 1998), has been shown to be a Harpactorinae (Forero et al., 2004).

Table 6.3 Techniques and Molecular Markers Most Commonly Used in Systematics

Techniques and Molecular Markers		Strengths	Weaknesses
1 MLEE	Multilocus enzyme electrophoresis	Provides information on several gene loci (and individuals) simultaneously; enzyme mobility differences can be related to different alleles at the gene locus for the enzyme in question; mobility variants are called electromorphs	Samples must be fresh or frozen
2 RAPD	Random amplification of polymorphic DNA	Provides information on many gene loci (and individuals) simultaneously; has been used for population genetic studies similar to MLEE	Dominance may hide heterozygous forms; problems of reproducibility; little guarantee of homology between comigrant bands
3 PCR RFLP	Polymerase chain reaction−restriction fragment length polymorphism	Widely used for epidemiological typing of many organisms; used for population genetic studies; established marker; relatively inexpensive; codominant marker	Might reveal little polymorphism; partial digestion can lead to problems; gives information on one locus at a time
4 Cytogenetics	Chromosome analyses	Necessary for studies of genome organization and its association with chromatin; can be informative on population expansion processes; used for intraspecific variability studies and to detect cryptic species	Gonads must be fixed from live specimens
5 Microsatellites	Short tandem repeats (period of 1−6 bp)	Provides information on several loci (and individuals) simultaneously; highly polymorphic; may be considered as neutral Mendelian markers; used for analysis of population genetic structure	Poor markers for phylogenetic inference; requires access to sequencer; development of primers can be time consuming

6 DNA sequencing		Highly informative; allows for studies of any taxonomic level and for comparisons between different laboratories through DNA data banks	Relatively expensive; access to sequencer required; labor intensive
7 Molecular markers PCR and sequencing			
7a Nuclear rDNA	Nuclear ribosomal DNA	Generally evolves more slowly than mtDNA; follows Mendelian inheritance; special interest in studies on bisexual species; useful at different taxonomic levels and for evolutionary inference; follows a concerted evolution	Occurs in lower copy number and often is more difficult to work with than mtDNA; sometimes more difficult to amplify by PCR; difficulties in alignments due to high number of insertions/deletions
28S	Gene 28S rRNA = large subunit of rRNA (LSUrRNA); total length approximately 4000 bp; region analyzed usually the D2 domain of 633 bp	Evolves faster than 18S; presents divergent domains (D1, D2, D3, etc.); can be used to distinguish between species, but mainly used for intermediate and higher taxonomic levels	Less used for phylogenetic analyses; usefulness of the domains varies depending on organisms analyzed
18S	Gene 18S = small subunit of rRNA (SSUrRNA); total length 1913−1918 bp; region analyzed is usually the complete gene	Very low evolutionary rate; useful for distant species, genera, and tribes; provides inferences about ancient to very ancient relationships of distantly related organisms (>100 Mya)	Restricted to comparisons of distant taxa
ITS 1	Internal transcribed spacer 1; total length 573−750 bp; region analyzed is usually the complete **spacer**	Evolves faster than ITS 2; useful for closely related taxa that have diverged recently (<50 Mya); good complement to the ITS 2 for systematic and taxonomic purposes from species to tribe levels	Presents microsatellites and minisatellites that seem specific to particular populations

(Continued)

Table 6.3 (Continued)

Techniques and Molecular Markers		Strengths	Weaknesses
ITS 2	Internal transcribed spacer 2; total length 470−722 bp; region analyzed is usually the complete spacer	Useful for closely related taxa that have diverged recently (<50 Mya); useful in the analysis of species, subspecies, hybrids, and populations as well as for problematic taxa such as cryptic or sibling species	Not appropriate for the comparison of different tribes as no clear alignment could be obtained; microsatellites are numerous and are only population markers
7b mtDNA	Mitochondrial DNA; complete genome is 17,015 bp	Evolves faster than nuclear genome; clonally inherited (maternal lineage); useful for studies of closely related taxa that have diverged recently; amplification and sequencing is easier than for nuclear rDNA genes	High substitution rate can be disadvantageous for resolving divergences of more than 5−10 Mya; saturation is a major cause of homoplasy and erases phylogenetic signal
12S	Gene 12S rRNA = mitochondrial small ribosomal subunit (mtsrRNA); total length 781 bp; region analyzed is usually 339−371 bp	Shows the lowest evolutionary rate within mtDNA markers; useful up to the level of species within the same genus; can reveal differences at population level	Tends to become saturated at higher taxonomic levels; its usefulness for comparison of species of different genera and tribes does not appear to be recommendable
16S	Gene 16S rRNA = mitochondrial large ribosomal subunit (mtlrRNA); total length 1270 bp; regions analyzed are usually fragments of 284 bp and from 501−510 bp	Appears to evolve in parallel with 12S, but is more variable, and so tends to reveal greater differences between species of different tribes	Differences between species of different genera can appear similar to those within the same genus; its usefulness for analyses at genus or higher levels does not appear to be recommendable

COI	Cytochrome *c* oxidase subunit 1; total length 1534 bp; regions analyzed are fragments of 1431−1447 bp and fragments of 636−661 bp	Evolves slightly faster than NAD1 but slower than *Cyt b* at low level comparisons; useful for studies of closely related species; used for "bar coding" of various organisms	Saturation becomes a problem in comparison of distant species and at higher taxonomic levels
NAD1	NADH dehydrogenase subunit 1; total length 912−933 bp; region analyzed is usually the complete gene	Evolves faster than ITS 2 at low level comparisons; useful for studies of closely related species	Saturation is already occurring when comparing distant species of the same genus
Cyt b	Cytochrome *b*; total length 1132 bp; several different fragments are analyzed from 313 to 682 bp	Fastest evolving mtDNA gene; useful for comparison of subspecies (intrapopulational, interpopulational, between morphs and subspecies)	Saturation becomes a problem in analysis of distant species and higher taxa

Techniques 1−6 adapted from Abad-Franch and Monteiro (2005); 7, including molecular markers used in Triatominae (7a to 7b), adapted from Mas-Coma and Bargues (2009).
Bp = base pairs; Mya = million years ago. Gene and fragment lengths refer to published studies on Triatominae.

6.2.1.2 *Tribe: Bolboderini (Genera:* Bolbodera, Belminus, Microtriatoma, Parabelminus*)*

The 13 species of Bolboderini are typically small Triatominae (adults up to 12 mm in length). *Bolbodera* is known only from Cuba, whereas the other genera are reported from isolated locations spanning the region from Mexico to southern Brazil. The tribal, generic, and species concepts are entirely morphological (Lent and Wygodzinsky, 1979), and it may be that intermediate forms may be found as more natural populations are sampled. Extensive study of the feeding habits of *Belminus* suggests that these species may be at a very early stage in adaptation to hematophagy (Sandoval et al., 2000, 2004, 2007). A morphological **cladogram** of the four genera (Lent and Wygodzinsky, 1979) placed *Bolbodera* as the most primitive, with *Microtriatoma* and *Parabelminus* as the most derived. However, this is difficult to reconcile with the known geographical distribution because *Bolbodera* is known only from Cuba. Comparison of 28S-D2 sequences placed *Microtriatoma trinidadensis* basal to the Rhodniini (Patterson, 2007), although no other Bolboderini have yet been studied by such means.

6.2.1.3 *Tribe: Cavernicolini (Genus:* Cavernicola*)*

The two species of *Cavernicola* are small Triatominae (adults up to 11 mm in length) with unusually shaped heads and necks reminiscent of some Apiomerinae. They seem invariably associated with bats and bat roosts but will feed from other vertebrates in the laboratory. The tribal, generic, and species concepts are entirely morphological (Lent and Wygodzinsky, 1979; Barrett and Arias, 1985).

6.2.1.4 *Tribe: Rhodniini (Genera:* Psammolestes, Rhodnius*)*

The three species of *Psammolestes* are small Triatominae (adults up to 15 mm in length) with truncated heads. They are invariably associated with nests of woven sticks as made by dendrocolaptid or furnariid birds (irrespective of the vertebrates currently occupying the nest). Although considered a **monophyletic** genus by morphological and behavioral similarity, this is difficult to reconcile with their distribution: *Psammolestes arthuri* occurs in the llanos of Colombia and Venezuela north of the Amazon region, whereas *Psammolestes tertius* and *Psammolestes coreodes* seem to form a cline down the caatinga-cerrado-chaco corridor of open vegetation south of the Amazon region.

By contrast, *Rhodnius* species are distributed throughout the Amazon region, into the llanos northward, and throughout the caatinga and cerrado to the south and east. They are primarily associated with palm tree crowns but also occur in bird nests and in domestic and peridomestic habitats. By morphological, behavioral, anatomical, and genetic features, they appear to form a monophyletic genus (Schofield and Dujardin, 1999) with two main lineages the "prolixus" group (*prolixus, robustus, milesi, neglectus, neivai, dalessandroi, domesticus, nasutus*) mainly east of the Andes, and the "pictipes" group comprising one subgroup east of the Andes (*pictipes, stali, brethesi, paraensis, amazonicus*) and one subgroup

west of the Andes (*pallescens, colombiensis, ecuadoriensis*) (Abad-Franch et al., 2009). *Rhodnius pictipes* may be closest to the ancestral form because it is the most widely distributed, is of more generalist habit, and shares genital characteristics with other Triatominae that are not shared with other Rhodniini except *Rhodnius stali* (Jurberg, 1996).

The Rhodniini appear to represent a monophyletic tribe with clear morphological, physiological, anatomical, and genetic characteristics that distinguish them from other Triatominae. Relationships between the two genera are less clear, largely because of the paucity of studies on *Ps. arthuri*. Various phylogenetic studies based on multiloci enzyme electrophoresis (MLEE) (Monteiro et al., 2002), mtDNA (Lyman et al., 1999; Monteiro et al., 2000), or ribosomal mtDNA (Hypsa et al., 2002) indicate **paraphyly** between *Rhodnius* and *Psammolestes*. The two genera have different morphologies and ecological habits but both are **arboricolous**. They seem to represent ecological adaptations to either the tree crown (*Rhodnius*) or the bird nests (*Psammolestes*).

6.2.1.5 *Tribe: Triatomini (Genera:* Dipetalogaster, Eratyrus, Hermanlentia, Linshcosteus, Panstrongylus, Paratriatoma, Triatoma*)*

The Triatomini is the most speciose of the tribes of Triatominae, with the widest geographical distribution covering a broad variety of ecotopes although most seem naturally associated with **rupicoline** habitats. The original tribal and generic concepts are morphological (Usinger, 1944; Lent and Wygodzinsky, 1979), although several phylogenetic studies now offer extensive support for these concepts (García and Powell, 1998; Bargues et al., 2000; Hypsa et al., 2002; Paula et al., 2005).

Phylogenetic analyses using nuclear or mitochondrial **gene** fragments generally indicate that the Triatomini forms three main **clades** that are broadly consistent with their geographical distribution: the *Triatoma* of Central and North America (and the Old World species) (i.e., north of the Amazon region) with which *Dipetalogaster*, *Eratyrus*, *Linshcosteus*, *Paratriatoma*, and *Panstrongylus* are usually clustered; the *Triatoma* of South America (i.e., south and east of the Amazon region, except for *Triatoma maculata*; *Hermanlentia* has not yet been included in such studies); and representatives of the dispar complex that occur mainly along the Andean cordillera from Venezuela to Bolivia (i.e., west of the Amazon region). Perhaps significantly, all species of all genera so far examined that form the "northern clade" (except for *Triatoma lecticularia*) show multiple X chromosomes, whereas those of the southern clade generally show single X chromosomes (Panzera et al., 2010). Of the exceptions, *Triatoma tibiamaculata* and *Triatoma vitticeps*, both Brazilian species, show two and three X chromosomes, respectively, and often cluster with the northern Triatomini in phylogenetic analyses. The other exceptions are species of the spinolai complex (*spinolai, gajardoi, eratyrusiformis, breyeri*) that show two or three X chromosomes (Ueshima, 1966; Panzera et al., 2010) and often cluster apart from others of the southern clade of Triatomini. The morphological similarities that placed these species as the spinolai complex (Lent and Wygodzinsky, 1979),

together with the recent karyotype studies, lend support to the concept of these species forming a separate genus: *Mepraia* (Lent et al., 1994b).

Within the northern Triatomini, several other concerns have been raised. The genus "*Meccus*" was originally proposed for some members of the phyllosoma complex, which were subsequently reduced to subspecific rank by Usinger; these subspecies were then divided and raised to specific rank by Lent and Wygodzinsky and grouped as the phyllosoma complex. The genus was re-proposed by Hypsa et al. (2002) but without including all species that had been assigned to the phyllosoma complex, nor including *Triatoma dimidiata*, which appears very closely related by phylogenetic analyses (Garcia et al., 2001; Marcilla et al., 2001; Dujardin et al., 2002; Pfeiler et al., 2006; Bargues et al., 2008). Moreover, the phylogenetic approach to sustain such a genus (Hypsa et al., 2002) is questionable because the terminal entities are not reproductively isolated. Their interfertility has been demonstrated many times and recently confirmed by molecular marker analysis on natural populations (Martinez-Hernandez et al., 2010). Thus, according to the Hennigian phylogenetics and to the biological type of species, the members of this genus constitute a single **polytypic species**. Even as evolutionary species, they are not convincing. Very small genetic divergences were detected between them by comparison of nuclear **DNA sequences** (Bargues et al., 2000, 2002, 2008; Marcilla et al., 2001). The relatively higher number of nucleotide differences found in the mtDNA genes analyzed (16S, COI, and Cyt b) (Hypsa et al., 2002; Martinez et al., 2006; Pfeiler et al., 2006; Bargues et al., 2008; Mas-Coma and Bargues, 2009) cannot be used to support species level, as argued by Pfeiler et al. (2006). Some concern is also valid for the idea of resurrecting the genus "*Nesotriatoma*" for the flavida complex of *Triatoma* (*flavida, bruneri, obscura*), which is a set of geographical populations assembled on the basis of morphological characters (Lent and Wygodzinsky, 1979). Phylogenetic confirmation as a monophyletic clade has not been obtained by using a sample that includes *Triatoma obscura* and the dimidiata/phyllosoma complex of species.

The Old World species of Triatomini have also raised concern, with the six species of *Linshcosteus* raised to tribal rank (as "Linshcosteusini") by Carcavallo et al. (2000) on the basis of their uniquely Indian distribution and morphological characters not shared with most other Triatomini especially the abbreviated rostrum that does not reach the prosternal sulcus. However, phylogenetic analyses tend to show *Linshcosteus* close to *Triatoma rubrofasciata* (Patterson et al., 2001; Hypsa et al., 2002), which is believed to have been spread from North America to port areas throughout the Old and New World tropics and subtropics by its association with rats on sailing ships (Gorla et al., 1997; Patterson et al., 2001).

6.3 Concept of Species

Perhaps the greatest challenge for the classification of Triatominae is the lack of a unifying concept of species. To discuss some of the conflicts that arise from applying modern concepts to traditional classification, and to highlight some recurrent

practices regarding the systematics of the subfamily, we have developed our discussion in parallel with the traditional and modern concepts of species. The most important of them are shortly described in the following sections.

6.3.1 Historical Concept

6.3.1.1 Morphological or Typological Concept of Species ("Morphological Species," "Morphospecies")

This is the historical concept of species, the one accepted by many generations of naturalists, zoologists, and even today, by many biologists.

Most, if not all the presently known species of Triatominae are morphospecies (Lent and Wygodzinsky, 1979). They have been described on a few individuals (Lent and Wygodzinsky, 1979), very few individuals (Beranger and Pluot-Sigwalt, 2002), or even a single individual only (Galvão and Palma, 1968; Martínez et al., 1994). This might be one of the most recurrent problem in morphospecies description: the lack of consideration for natural variation. When more sampling is available, it may become difficult to draw a clear demarcation between simple geographical variation and species difference (Dujardin et al., 2009). Although the idea is that morphological differences are the reflection of biological divergence, it may happen that morphology appears disconnected from other biological properties. *Triatoma platensis* and *Triatoma infestans* are two morphospecies, but they could appear as the same biological entities: not only they interbreed, but also they are genetically very similar (Pereira et al., 1996; Garcia et al., 2001; Bargues et al., 2006).

The most important objection to the typological concept is the existence of sibling species. Sibling (also "cryptic," or "isomorphic") species are morphologically identical or nearly identical entities recognized as different species according to other concept(s) of species. This objection to the typological concept is weakened by the modern possibilities of morphological comparisons, such as electron microscopy of the eggshell (Barata, 1995), biometric methods exploring the antennal phenotype (APH) (Catalá, 1995, 1996), or geometric morphometrics (Baylac et al., 2003; Dujardin, 2008). Sibling species do not seem to be frequent in Triatominae (Dujardin et al., 1999b). Known cases are *Triatoma sordida* (Panzera et al., 1997; Noireau et al., 1998) and *T. dimidiata* (Panzera et al., 2006; Bargues et al., 2008), which have been split into sibling species on the basis of cytogenetic and molecular characterization techniques. However, even accepting that sibling species are infrequent in Triatominae, many morphospecies are very similar. Triatominae do not tend to show discrete characters that would allow clear-cut species differentiation, and some morphospecies are difficult to recognize without dissection of the genitalia. For morphologically very close species, the typological concept can produce confusion. For instance, *Rhodnius prolixus* and *Rhodnius robustus* are so similar that their geographical distribution is not ascertained. In their revision of the Triatominae from French Guiana, the type locality of *R. robustus* (Larousse, 1927),

Chippaux et al. (1985) reported *R. prolixus*, not *R. robustus*, whereas the presence of *R. prolixus* in French Guiana is considered very doubtful (Bérenger et al., 2009). To help distinguish these two entities and some other closely related species of the so-called prolixus group, geometric (Matias et al., 2001; Villegas et al., 2002; Gurgel-Goncalves et al., 2008) and molecular tools (Pavan and Monteiro, 2007) have been suggested.

6.3.2 Modern Concepts

The modern concepts of species make use of other criteria than simple morphological comparison, with some of them even completely free of any character examination. Species may be subdivided according to whether or not they take into account the temporal dimension of the species (see row TD, Table 6.2). The biological concept of species (see column BIOL, Table 6.2), like the cohesion and recognition concepts, does not consider the birth and the death of the species.

The modern concepts of species could also be subdivided according to the reproductive strategy of the organisms: biological and Hennigian concepts (Table 6.2) only consider sexually reproducing organisms, whereas the other concepts apply to the entire range of living organisms.

6.3.2.1 The Biological Concept of Species ("Biospecies")

Probably the most often used concept in entomology, the biological concept, considers as species any natural interbreeding populations reproductively isolated from other such natural populations (Mayr, 1969, 2000). The concept has ancient roots in the texts from a contemporary of Carl von Linnaeus: the Earl of Buffon (1707−1788), may be the first naturalist to consider the criterion of interfertility in the definition of species. In this concept, a temporal dimension is not necessary. Indeed, the reproductive isolation criterion would be poorly contributive when considering fossils or extinct species. Other related or similar concepts insist on prezygotic barriers (Paterson, 1985) or on gene flow within species rather than reproductive isolation (Templeton, 1989, 1994).

The biological concept is not a character-related concept. Instead, it relies only on reproductive isolation. In addition, like the evolutionary concept but contrary to the phylogenetic concepts, the biological definition of a species does not include the operation to be performed to verify it. Nevertheless, reproductive isolation can be demonstrated in various ways, which makes the biological concept a refutable one. Thus, in spite of ignoring the time dimension of a species (i.e., ignoring evolutionary biology and phylogenetics), the concept is not an arbitrary one.

It is, however, difficult to reject the propensity to interbreed when natural populations are allopatric (Costa et al., 2003). It might also be confusing to observe partial reproductive isolation (see Section 6.4.2), more so between separated populations (insular regions, see Section 6.4.9). Such situations, not infrequent

in Triatominae, may be interpreted as incipient speciation and invite reconsideration of the use of the subspecies category (Mas-Coma and Bargues, 2009).

According to the biological concept of species, several well-recognized species of Triatominae could be considered as one single species. As an example, *T. platensis* and *T. infestans* are interfertile in the laboratory as well as in nature, although the latter is not frequently detected because of ecological separation. According to the biological concept, separation even ecological separation does not mean reproductive isolation. *T. platensis* and *T. infestans* are two morphospecies but one single biospecies. The same conclusion could be derived from the *R. prolixus*−*R. robustus* question: these morphologically close species are interfertile but ecologically and, to some extent, geographically separated. Again, separation does not mean reproductive isolation in the sense of the biological concept: *R. prolixus*−*R. robustus* are one single biospecies. Similar reasoning could be applied to the members of the phyllosoma complex, some members of the brasiliensis complex (see Section 6.4.6), the dimidiata complex (see Section 6.4.8), and others.

6.3.2.2 The Hennigian Concept of Species ("Hennigian Species")

The Hennigian concept (Hennig, 1965) also considers that species are reproductively isolated, but it gives them a beginning and an ending in time. The inclusion of the temporal dimension in the definition of species appears as an obvious reality, but this is exactly where discrepancies begin with other related concepts. The Hennigian concept considers that speciation is a splitting event (**cladogenesis**), which means the birth of two reproductively isolated **sister species** and the dissolution of the stem species (Meier and Willmann, 2000). Not only the survival of the stem species is not admitted, but, coherently, the idea of **phyletic speciation** (**anagenesis**) is rejected. The speciation event is the dissolution of the stem species into a reproductively isolated pair of sister species. Table 6.2 indicates which definitions agree or disagree with this point of view (see row ST, Table 6.2).

Reproductive isolation (from the sister species) is a very important component of the Hennigian concept because it avoids the idea of groups linked by netlike relationships. This restriction allows clean definitions of monophyletic groups.

The Hennigian concept has been applied to draw hypothetisized relationships within the genus *Panstrongylus*. Distinctions between *Panstrongylus lignarius* and *Panstrongylus herreri* could not be resolved (Lent and Wygodzinsky, 1979), suggesting one single species as supported later by their close genetic (Marcilla et al., 2002) and morphometric similarity (Gumiel et al., unpublished data). However, when applied to the Rhodniini excluding the *R. robustus* species since it is interfertile with *R. prolixus* the cladistic tree performed on the basis of isoenzyme characters could not reveal the paraphyly between *Psammolestes* and *Rhodnius* genera (Dujardin et al., 1999a). This paraphyly was revealed later by molecular phylogenetic approaches using more sophisticated algorithms (Lyman et al., 1999; Monteiro et al., 2000, 2002).

6.3.2.3 The Phylogenetic Concepts of Species

Two different positions are competing for the title of "phylogenetic" concept of species (Cracraft, 2000). One is defining species prior to cladistic analysis on the basis of an exclusive combination of character states (Wheeler and Platnick, 2000), while the other is defending the idea of using **synapomorphies** alone to define monophyletic groups of demes, hence species (Mishler and Theriot, 2000).

6.3.2.4 The Phylogenetic Concept Sensu Wheeler and Platnick

The phylogenetic concept of species *sensu* Wheeler and Platnick (PHG I in Table 6.2) defines hypothetic species, prior to any cladistic analysis, as being "the smallest aggregation of (sexual) populations or (asexual) lineages diagnosable by a unique combination of character states" (Wheeler and Platnick, 2000). The "unique combination of character states" is completely independent from the circumstances of the speciation event: either a splitting into sister species with dissolution of the stem species, a **vicariance** event, or an allopatric or a sympatric speciation (hence with survival of the stem species). Neither synapomorphy nor monophyly (both of critical importance to **cladistics**) nor reproductive isolation are prerequisites to define a phylogenetic species. A new species typically contains a fixed state of a previously polymorphic character in the ancestor species. The concept does not reject the idea of phyletic speciation (anagenesis) but rather accepts the idea of stem species survival, relying on morphological characters. The authors of this concept recognize that it could dramatically increase the number of species in certain groups, especially in groups where many subspecies were described, which has been the case for Triatominae (Schofield and Galvão, 2009). This approach does not seem to have been used in the systematics of Triatominae, probably because of the paucity of discrete morphological characters.

6.3.2.5 The Phylogenetic Concept Sensu Mishler and Theriot

This concept of species (PHG II in Table 6.2) provides a species definition quite distinct from the previous one, but it might be considered as very close to the Hennigian one. It rejects the idea of stem species survival and does not accept the hypothesis of phyletic speciation. Its first criterion for a species is its monophyletic nature. The important difference from the Hennigian concept relates to the entry groups. According to the Hennigian concept, the entry groups must be reproductively isolated, a characteristic that is not required by the PHG II concept. Terminal entities may be species, demes, or local populations. This concept makes species dependent on cladistic analysis: species are the result of phylogenetic analysis of infraspecific units, and they are defined by their monophyly. The resulting criticism is that monophyletic groups may be difficult to retrieve correctly when terminal entities are interbreeding units.

Furthermore, even the authors of the concept admit that to define a monophyletic group is not enough to raise it as a species: "any application of fixed names to

phylogenetic trees has to be arbitrary to some extent" (Mishler and Theriot, 2000). The definition includes additional steps to rank the monophyletic group as a species (e.g., bootstrap percentage, number of synapomorphies, decay index, biological criteria). Moreover, even if they are well supported, the following monophyletic groups should not be named: groups defined only by "selectively neutral apomorphies" and groups "marked only by molecular apomorphies and thus nearly impossible to distinguish for practical use" (Mishler and Theriot, 2000).

In Triatominae, the *R. prolixus* and *R. robustus* question offers a good example of this approach. *R. prolixus* and *R. robustus*, known as interfertile taxa, appeared as different monophyletic groups in molecular cladistic analyses, and, in a second step of the same analysis, were claimed to be different species because of relatively large mtDNA sequence divergence between groups (Monteiro et al., 2000). Two different steps were (i) to recognize distinct monophyletic groups and (ii) to consider them as distinct species. The first step might be considered as a rigorous application of phylogenetic rules, although even on that matter phylogeneticists may disagree because of **reticulation** (Wheeler and Meier, 2000). The second one is a subjective ranking (the amount of genetic **distance**), which is similar to a quantitative approach (also called the "distance approach" or the "phenetic concept of species").

Thus, the phylogenetic concept *sensu* Mishler and Theriot is not free of subjective appreciation, and its main problem remains the reticulate structure of terminal entities. Interspecific or interdeme natural hybridism might be the main obstacle to the blind application of synapomorphies to define monophyletic groups. The problem is worth mentioning for Triatominae for which interspecific hybridism is known to be frequent (Usinger et al., 1966; Barrett, 1991; Costa et al., 2008), and the so-called "prolixus" group is far from being an exception (Barrett, 1995). If the relationships among the terminal taxa are reticulate rather than hierarchical, as they could be in the present concept, apparent synapomorphies might not correspond to monophyletic groups. Reticulate evolution produces confusing patterns of synapomorphy (Funk, 1985; Smith, 1992).

6.3.2.6 The Evolutionary Concept of Species

An evolutionary species is "an entity composed of organisms that maintain its identity from other such entities through time and over space and that has its own independent evolutionary fate and historical tendencies" (Wiley, 1978; Wiley and Mayden, 2000b).

As for the biological concept, and contrary to the other modern concepts, the evolutionary concept does not specify a particular operation to verify its properties. Its strength, which is unique among the various concepts presented here, is that its acceptance does not mean rejection of the other (modern) concepts. Biological and Hennigian species are "evolutionary" species, as are phylogenetic species (to the extent that they retrieve correct ancestry patterns).

The evolutionary concept's weakness is some arbitrariness in its definition: "historical tendencies" and "evolutionary fate" are not easy to define or reject.

However, this objection also makes the concept potentially attractive. The definition of the evolutionary species does not dictate any rigorous system nor any kind of rigid algorithm to identify independent units. Their identification requires discussion and consensus; however, the phylogenetic approach does not have a preponderant place in the discussion, which is based more frequently on ecology, population genetics, geography, and morphology.

For instance, the *Rhodnius* genus has been discussed in terms of an assemblage of evolutionary units derived from each other in which *R. pictipes* is considered as the survival of the stem species of the genus, and where geography, vicariance, and ecological adaptation are the main forces explaining the groups observed within the genus by other approaches (Schofield and Dujardin, 1999). In this study, different criteria were considered and contrasted to evaluate the fate and destiny of each recognized member of the genus.

The example of the *T. infestans* and *T. platensis* pair is also a good model of evolutionary units where genetic and ecological criteria were applied together to reach a consensus. In spite of natural interbreeding and genetically similar units, their obvious ecological adaptation makes them two evolutionary species. This observation is true for the third species of the infestans subcomplex, *Triatoma delpontei*, which has partial reproductive compatibility with *T. platensis* but has apparently adapted to other bird hosts.

6.4 Examples of Specific Questions

At the species level, most of the conflicts between traditional and modern concepts of species were treated using the subjective argument of "large" or "low" genetic and phenetic distances. Thus, in the current literature, the molecular approach to species resolution is widely inspired by the evolutionary concept of species, as well as by the subjective step of the phylogenetic concept (PHG 2) asking whether two monophyletic clades are two different species.

6.4.1 R. prolixus *and* R. robustus

The *R. prolixus*—*R. robustus* pair received phylogenetic recognition as distinct monophyletic groups (Lyman et al., 1999; Monteiro et al., 2000), but the question has been treated mostly by considering the degree of genetic distances (see Sections 6.3.1 and 6.3.2). The two species present the particularity of no (Harry et al., 1992; Harry, 1993) or very low nuclear DNA divergence (Monteiro et al., 2000; Mas-Coma and Bargues, 2009) but relatively high mitochondrial genetic distances (Monteiro et al., 2000). Phenotypic distances have been found to be significant (Matias et al., 2001; Villegas et al., 2002). Thus, the recognition of monophyletic clades was not sufficient to consider them as separate species; instead, the distinction was made on the basis of the amount of sequence divergence of mitochondrial DNA and the known ecological adaptations.

6.4.2 T. infestans, T. platensis, T. delpontei

In the more abundant genus *Triatoma*, many species have been revisited using molecular tools, first by using isoenzymes (Pereira et al., 1996; Costa et al., 1997; Monteiro et al., 1998; Noireau et al., 1998, 2002), then by DNA analyses.

The *infestans*−*platensis*−*delpontei* question has been addressed again by considering genetic distances. Contrary to the *R. prolixus*−*R. robustus* pair, these do not show significant mtDNA divergence (García and Powell, 1998; Garcia et al., 2001) but do show a consistent, although low, genetic distance derived from MLEE (Pereira et al., 1996) or ITS-2 and ITS-1 (Bargues et al., 2006), as well as cytogenetic differences (Panzera et al., 1995). The genetic differences at the nuclear level were among the arguments to decide their species status (Dujardin et al., 2002), although the ecological specialization also provided a very strong argument (Pereira et al., 1996; Dujardin et al., 1999b).

6.4.3 T. infestans, T. melanosoma

The *infestans*−*melanosoma* pair has been explored by geometric morphometrics (Gumiel et al., 2003) and DNA analyses. *Triatoma melanosoma* was originally described as a subspecies of *T. infestans* and later raised to species rank (Lent et al., 1994a, 1995). Only the black color of its exoskeleton distinguishes it from *T. infestans*, with which it is interfertile. This melanic characteristic appears to be recessive in crossbreeds (Monteiro et al., 1999; Dujardin et al., 2002). Analyses of Cyt b (Monteiro et al., 1999) and ITS-2 and ITS-1 (Bargues et al., 2006) considered that genetic differences were not sufficient to justify two different taxa and thus supported the synonymy of *T. melanosoma* with *T. infestans* after morphometric comparison (Gumiel et al., 2003).

6.4.4 *The "Dark Morphs" of* T. infestans

The "dark morphs" of *T. infestans* (Noireau et al., 1997) have been examined by random amplified polymorphic DNA (RAPD) analysis (Noireau et al., 2000), by APH patterns (Catalá and Torres, 2001), and by morphometric characters (Gumiel et al., 2003). Low or lack of phenotypic and genetic distances **converged** to the conclusion of intraspecific variation. Recently, ITS sequences showed that the dark morph **haplotype** (T.inf-CH5A) was identical to the majority at ITS-1 and differed from the most dispersed *T. infestans* (H2) by only one transversion at ITS-2 level. In the phylogenetic reconstruction, the dark morphs appeared in the branch grouping all non-Andean *T. infestans* haplotypes. These results suggested that these melanic forms did not need any taxonomic distinction (Bargues et al., 2006).

6.4.5 T. sordida, T. garciabesi

T. sordida is morphologically close to *Triatoma guasayana* and *Triatoma patagonica*, but its variable morphology seems to cover different species. *Triatoma garciabesi* has

been revalidated mainly on basis of genetic differences with *T. sordida* (García and Powell, 1998; Jurberg et al., 1998). The species has been shown through cytogenetics (Panzera et al., 1997) and MLEE (Noireau et al., 1998) to contain two sibling species, but because of the apparent lack of morphological difference, no new name was created.

6.4.6 T. brasiliensis

Triatoma brasiliensis is composed of at least four geographic populations (*brasiliensis*, *melanica*, *macromelasoma*, and *juazeiro*) that have distinct chromatic, morphologic, biologic, and ecologic patterns and genetic composition. This group has been evaluated using different approaches, exactly in the spirit of the evolutionary concept of species. Reciprocal crosses revealed genetic incompatibility between *melanica* and *brasiliensis* samples (Costa et al., 2003). Thus, according to the biological and the Hennigian concepts of species, the group contains certainly two species. A large Cyt b study indicated that the four geographic populations of *T. brasiliensis* were genetically distinct (Monteiro et al., 2004). The conclusion was to consider the *brasiliensis*−*macromelasoma*, *melanica,* and *juazeiro* forms as three separate species (Monteiro et al., 2004). No independent taxonomic status was concluded for the *brasiliensis* and *macromelasoma* forms despite their representing distinct evolutionary lineages (Monteiro et al., 2004). Geometric morphometric analyses, morphological comparisons with laboratory hybrids, and ecological considerations led Costa et al. (2008) to suggest the hypothesis of *T. macromelasoma* as a historical hybrid.

6.4.7 T. eratyrusiformis

Hypsa et al. (2002) presented the Argentinian species *Triatoma eratyrusiformis* as belonging to the same monophyletic group as *Mepraia spinolai*, supporting its inclusion in the genus as "*Mepraia eratyrusiformis.*" Combined cytogenetic and nuclear rDNA and mtDNA markers indicate that populations within the *Mepraia* genus could be divided into two separate lineages, with specific status supported by the level of divergence between their nuclear and mitochondrial sequences that correspond to recently diverged species (Calleros et al., 2009), with similar nucleotide divergence to that found between the closely related species from the infestans subcomplex (Bargues et al., 2006). This phylogenetic approach supported the recently described *Mepraia gajardoi* (Frías et al., 1998); unfortunately, its validity is questioned since an **ingroup** species, *eratyrusiformis*, was used as an **outgroup** (Calleros et al., 2009).

6.4.8 T. dimidiata

Morphometric variation (Bustamante et al., 2004), chromosomal variation, and genome size (Panzera et al., 2006) suggested the existence of cryptic species of *T. dimidiata*. The *T. dimidiata* complex of species has also been suspected from a

phylogenetic (PHG 2) approach (Marcilla et al., 2001; Bargues et al., 2002, 2008). According to ITS-2, *T. dimidiata* populations from the Yucatan part of Guatemala and northern Honduras belong to a cryptic species (= *T.* spp. *affinis dimidiata*) different from *T. dimidiata* (Bargues et al., 2008). Cyt b and ITS-2 confirmed their specific status and extended their distribution to Belize (Dorn et al., 2009). This distinction agrees with results from multidisciplinary studies using RAPD-PCR, genital structures, morphometrics (Dujardin et al., 2009), APH (Catalá et al., 2005), cuticular hydrocarbons, and chromosomes (Calderón et al., 2005; Panzera et al., 2006) and has been confirmed by ND1 analyses and the absence of introgressed sequences in overlapping zones (Mas-Coma and Bargues, 2009).

6.4.9 T. hegneri

Triatoma hegneri is a species originally described from the Mexican island of Cozumel. Although chromatically distinguishable from most forms of *T. dimidiata* (Lent and Wygodzinsky, 1979), it is known to produce fertile hybrids when experimentally crossed with *T. dimidiata* (R.E. Ryckman, unpublished). The two ITS-2 haplotypes of *T. hegneri* differ by only three mutations from haplotypes of *T. dimidiata* from Mexico and Guatemala. This reduced number of nucleotide differences and the location of *T. hegneri* haplotypes within the phylogenetic clade of *T. dimidiata* do not support its taxonomic status. These data suggest that it is an intraspecific morphological variant of *T. dimidiata* (Bargues et al., 2008).

6.4.10 P. lignarius *and* P. herreri

P. lignarius is an exclusively sylvatic species of Brazil, Guyana, Suriname, Fr. Guiana, Venezuela, and perhaps Ecuador, and found naturally infected by *T. cruzi*. *P. herreri* from Peru and Ecuador adapted to other habitats through its trophic link to guinea pigs and is the main domestic vector in northern Peru (Dujardin et al., 2002; Abad-Franch et al., 2009). Both species are difficult to distinguish and they cross-fertilize to give rise to hybrids (Barrett, 1988). No nucleotide difference was detected between their ITS-2 sequences, suggesting that there is only one species (Marcilla et al., 2002). They have been synonymized by Galvao et al. (2003).

6.5 Conclusions

As can be seen from the aforementioned examples, the phylogenetic concepts (mainly PHG II) have been the most efficient techniques to retrieve ancestry and discuss the validity of supraspecific levels. They were much less useful (or much less used) to discuss the species status of various entities. For species status discussion, the biological and evolutionary definitions of the species were much more helpful (or much more used).

6.5.1 The Species Level and the Phylogenetic Concepts

The phylogenetic concepts put evolutionary meaning to the supraspecific organization, removing the arbitrariness of taxonomists. They are less suited to decide about the species status of natural populations. To rigidly name species according to the phylogenetic concepts (HENN, PHG I, and PHG II) leads to disagreements between concepts, but also presents the risk of unnecessarily modifying the number of species.

The Hennigian concept, because of its reliance on reproductively isolated terminal entities, has the effect of drastically reducing the apparent number of species, compelling biologists to create many subspecies as the only alternative. The PHG I concept presents the risk of dramatically amplifying the number of species (cf. Reinert et al., 2004; Savage, 2005). The PHG II concept, especially when relying on molecular phylogenetics, presents the risk of creating unstable taxa (subject to frequent changes as more characters and taxa are discovered), trivial species in evolutionary terms (*minor, selectively neutral apomorphies*), as well as cryptic species impossible to distinguish for practical use.

6.5.2 The Species Level and the Nonphylogenetic Concepts

Neither the biological nor evolutionary definitions of species are useful to understand the evolution from the past to the present state of biodiversity. However, both of them, especially the evolutionary concept, compel us to gather biological information specific to the populations under study. Their openness to various analytical approaches leads us to define species in a more informative way, in accordance with species definitions and known speciation mechanisms.

Taking into account known speciation processes to understand the species status of a population is where the evolutionary concept could be the most useful. Its receptiveness to discussion provides richer biological information on populations and their possible species status. Importantly, the evolutionary concept is also the only modern concept allowing discussion about infraspecific populations and decisions about the utility of giving them subspecific names. For instance, the partial reproductive isolation of *T. platensis* with *T. delpontei* would make them subspecies according to the biological or Hennigian concepts. Whereas the ecological adaptation of *T. delpontei* to particular bird nests makes it a true evolutionary species (see also Sections 6.4.6 and 6.4.8).

6.5.3 Speciation Event and Speciation Process

As previously demonstrated, biologists can strongly disagree about the species definition (Wheeler and Meier, 2000). However, with the exception of possible disagreement about the speciation event itself (survival, or not, of the stem species), there is generally much less discrepancy about the likely grand mechanisms of speciation (Schluter, 2001): **allopatry**, mutation, **genetic drift**, **disruptive**

selection (Dujardin et al., 2009), ecological adaptation (Schofield, 1988)[1], hybridization (Usinger et al., 1966; Barrett, 1991; Costa et al., 2008; Dujardin et al., 2009), and so on. Interestingly, most of these mechanisms, when considered for sexually reproducing insects, are processes tending to increase the reproductive isolation between "future" species, which is an indirect recognition to the importance of the biological concept of species.

6.5.4 The Quantitative Approach to the Species

The mechanisms supposed to be at work in producing new species justify the use of phenotypic and genetic differences as an argument for species rank recognition. Indeed, in the case of an allopatric speciation or genetic drift, we expect the species to show the accumulation of genetic and corresponding phenetic differences (Dujardin et al., 2002). In the case of disruptive selection, we expect two optimal phenotypes to coexist (Dujardin et al., 2009). In the case of ecological adaptation, we expect them to show morphological attributes related to the specific ecological niche (Schofield, 1988). In the case of hybridization, we expect the hybrids to occupy a different territory to the parental species, with intermediate morphological characters and possibly some genetic evidence of **introgression** (Costa et al., 2008). In the case of reproductive isolation, we expect to find biological mechanisms explaining the isolation (e.g., genital morphology, symbionts, pheromones).

This information is crucial to define evolutionary species, but it is not needed by the phylogenetic concepts of species nor even by the biological concept. Rates of molecular (and/or morphological) divergence or acquisition of niche specializations vary independently of the acquisition of reproductive isolation (Mayr, 2000). The reproductive isolation required by the biological and Hennigian concepts does not infer any amount of phenetic or genetic differences between species, nor do the phylogenetic concepts care about any biological differences between taxa. Only the evolutionary concept would benefit from such information, which is frequently used by entomologists to support their description of a new species. Thus, MLEE, cytogenetics, DNA sequences, nuclear or mitochondrial sequence divergence (Table 6.3), as well as the amount of morphometric and APH differences, have been used to support systematic studies in Triatominae. Since the results of such approach depend on possible speciation mechanisms, and since the latter are probably different according to species, very different situations have been obtained.

6.6 Recommendations

Triatominae display a relatively high degree of morphological plasticity (Dujardin et al., 1999b, 2009), which can also be referred to as "phenetic drift" (Schofield

[1] An ecological concept of species (Van Valen, 1976) was progressively abandoned because of two reasons: (i) many subspecies or even simple local populations of the same species may show different ecological adaptations, and (ii) the rate of speciation largely exceeds the rate of ecological changes (Wiley and Mayden, 2000a).

and Galvão, 2009). Morphological variation (e.g., in color, patterns, shape) should not lead by itself to the erection of a new species name. Modern approaches, and especially the quantitative approach (see Section 6.5.4), should be applied before reaching a consensus. Conversely, molecular species (i.e., species discovered by genetic techniques) should not be given a new name unless clear and usable morphological characters can be described.

Thus, possible changes should be limited to very particular circumstances, they should be applied to morphologically diagnosable entities and should provide a benefit to the biological or to the epidemiological knowledge of the group.

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ECLAT http://eclat.fcien.edu.uy/.

Glossary

The following terms were not defined in the text. Sources of the glossary: Wikipedia, UCMP Phylogenetics at www.ucmp.berkeley.edu/glossary/gloss1phylo.html. See also Table 6.3 for the definition of most molecular tools and techniques.

Allopatry as opposed to "*sympatry*." *Allopatry* refers to the spatial distribution of two populations, either separate (allopatric) or not (sympatric).

Anagenesis evolutionary change along an unbranching lineage; see **Phyletism**.

Apomorphy derived character. See also **Synapomorphy** for derived characters shared by different species. Note that *apomorphy* is a relative concept depending on its position in the phylogeny.

Arboricolous ecologically associated with trees.

Character heritable trait possessed by an organism; characters are usually described in terms of their states, e.g.: "OCELLI present" versus "OCELLI absent," where "OCELLI" is the character, and "present" and "absent" are its states.

Clade a monophyletic taxon; a group of organisms that includes the most recent common ancestor of all of its members and all of the descendants of that most recent common ancestor. From the Greek word "klados," meaning branch or twig.

Cladistics cladistics (from ancient Greek *klados*, meaning "branch") is a form of biological systematics that classifies organisms into hierarchical monophyletic groups. It can be distinguished from other taxonomic systems, such as phenetics, by its focus on shared derived characters (synapomorphies).

Cladogenesis the division of a single species into two or more genetically distinct ones. Cladogenesis is often contrasted with anagenesis (see also **Phyletism, Phyletic speciation**), in which gradual changes in an ancestral species lead to its eventual "replacement" by a novel form.

Cladogram a diagram, resulting from a cladistic analysis, which depicts a hypothetical branching sequence of lineages leading to the taxa under consideration. The points of branching within a cladogram are called *nodes*. All taxa occur at the endpoints of the cladogram.

Convergence similarities that have arisen independently in two or more organisms that are not closely related. Similar in meaning to the words parallelism, homoplasy.

Disruptive selection also called *diversifying selection*. Disruptive selection is a descriptive term used to account for changes when selection favors extreme over intermediate phenotypes.

Distance (genetic distance, phenetic distance) a variety of parameters used for phenotypic or genetic characterization of two taxa are considered to compute a single index represen tative of the differences between taxa.

Gene a DNA sequence coding for a given polypeptide. More broadly, any given DNA sequence.

Gene sequence (genetic sequence, DNA sequence) can be compared with a series of let ters corresponding to the primary structure of a real or hypothetical DNA molecule or strand. The possible letters are A, C, G, and T, which correspond to the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine, and thymine). This coded sequence corresponds to the basic genetic information. A DNA sequence may code for proteins. In this case, it directly monitors the succession of amino acids that constitute the primary structure of the protein. Many DNA sequences have no known coding function.

Genetic drift the standard deviation of allele frequency. Genetic drift is due to random sam pling and chance.

Haplotype a group of alleles of different genes on a single chromosome that are closely linked enough to be inherited usually as a unit. In medicine, the term is more commonly understood as a contraction of the term "haploid genotype."

Ingroup in a cladistic analysis, the set of taxa that are hypothesized to be more closely related to each other than any are to the outgroup.

Introgression spread of genes from one population or species into another as a result of hybridization.

Monophyly, monophyletic a group of species descending from a single, common ancestor (stem species). A monophyletic group forms a clade, meaning that it consists of an ances tor and all its descendants.

Outgroup a group of organisms that serves as an external reference group for determination of the polarity of the characters describing the ingroup (i.e., the group that is of immedi ate interest). External means that the outgroup does not belong to the ingroup, it is phylo genetically outside the ingroup.

Paraphyly, paraphyletic a group of taxa containing its last common ancestor but not all the descendants of that ancestor.

Phenetic drift the progressive morphological change observed among separate populations, probably due to underlying genetic drift (also known as morphological plasticity).

Phyletism, phyletic speciation the transformation of one species into another one. Thus, it is the replacement of one species by another, without any increase in the number of species. Another term describing the same event is "anagenesis."

Polytypic species composed of genetically isolated populations needing a separate morpho logical description a species composed of various subspecies.

Reticulation joining of separate lineages on a phylogenetic tree, generally through hybrid ization or through lateral gene transfer.

Rupicoline rock inhabiting. Also described as "rupicolous," or "saxicolous."

Sister group, sister species the two clades (or species) resulting from the splitting of a sin gle lineage (or species).

Spacer in eukaryotic and some viral genomes, untranscribed DNA segments that flank func tional genetic regions or cistrons.

Synapomorphy the possession of derived features by two or more taxa in common. Only synapomorphic character states can be used as evidence that taxa are related and used to build phylogenetic trees.

Taxon any named group of organisms, not necessarily a clade; a taxon may be designated by a Latin name or by a letter, number, or any other symbol.

Vicariance speciation that occurs as a result of the separation and subsequent isolation of portions of an original population.

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7 Biology of Triatominae

*François Noireau and Jean-Pierre Dujardin**

Institut de recherche pour le développement, Centre National de la recherche scientifique, Montpellier, France

7.1 Introduction

Of the 140 species of Triatominae currently recognized (Schofield and Galvão, 2009), research has traditionally concentrated on those of greatest epidemiological significance as domestic vectors of *Trypanosoma cruzi*, the agent of Chagas disease. The main concentration has been on *Triatoma infestans*, *T. brasiliensis*, and *Panstrongylus megistus* from the Southern Cone countries, and *Rhodnius prolixus* and *Triatoma dimidiata* from the Andean Pact countries and parts of Central America. These five species, the main vectors of Chagas disease, represent three genera in two tribes, while the Triatominae are admittedly composed of 15 genera and 5 tribes. Our knowledge on the biology of Triatominae is thus obviously fragmentary.

Most of the Triatominae are found in the New World, with a very few others in the Old World. They are hematophagous bugs living in close association with their sylvatic hosts in such habitats as palm tree crowns, bird nests, rodent burrows, opossum lodges, and rock piles. For some genera, the classification of Triatominae reflects these associations, with for instance the *Rhodnius* adapted to the palm trees, the *Psammolestes* living in bird nests, and the *Panstrongylus* and *Paratriatoma* associated with burrows. However, with a very few exceptions, these species are opportunistic and feed on other hosts too, including the human host.

Since one of their commonly observed behaviors is to enter domestic and peridomestic structures ("intrusion," see Section 7.3.3.1), with some of them trying to colonize the human habitat ("domiciliation," see Section 7.3.3.2), the sylvatic species of Triatominae represent a possible source of infection by *T. cruzi*, thus they deserve much more interest. Due to their generally nocturnal habits and hidden refuges, they may be hard to collect in the field. In this regard, the design of a new trapping device was a welcome initiative (Noireau et al., 2002).

7.2 General Biology of Vectors

The domestic vector species are generally easy to rear in the laboratory and have provided excellent models for fundamental studies of insect physiology, as well as

*E mail: dujardinbe@gamil.com

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studies related to their control. In recent years, they have also been used for studies of population genetics and basic evolutionary trends, largely linked to their process of adaptation to human dwellings, which is seen as a future risk for transmission of *T. cruzi* by less-known triatomine species.

Thus, a few species have adapted to the domestic structures ("domestication," see Section 7.3.3.3) and represent the main providers of human Chagas disease. The biology of these species received most of the attention of the biologists, and what will follow is mainly related to them. They belong to two tribes: the Triatominii and the Rhodniini. Be the Triatominae a **monophyletic** group or not, these two tribes have already been proved to be very different in many aspects. As a consequence, what is known in one tribe should not be generalized and applied to the other tribe. However, domestic populations of Triatominae, whatever their tribal origin, tend to show a similar behavior. This chapter deals with these populations, and mentions otherwise the reference to sylvatic species.

7.2.1 Development

Triatominae are **exopterygote** insects. There is no pupal stage and metamorphosis is described as incomplete (hemimetabolous insects). The five successive immature stages increasingly resemble the adult, and rudimentary wings are apparent only in later stages. The cycle is composed of eggs, five nymph stages, and the male and female imagos. At temperatures between 20°C and 30°C, the development takes approximately 5−6 months, but this can vary according to species. Nymphs differ from the adults primarily by their lack of fully developed wings or genitalia, although they generally occupy the same habitat and feed on the same hosts as the adults. For Triatominae, this means that all five nymphal stages and both sexes of adults feed on vertebrate blood and are capable of becoming infected and transmitting *T. cruzi*.

Eggs have an opercule. They are ovoid, and their envelope present a texture with some aspects of taxonomic importance (Barata, 1981). They are white at the oviposition, then pink, becoming darker with hardening of chitin, and finally dark when arrived at maturity. The hatching occurs generally after 10−40 days since oviposition.

After a few days, the hatched nymphs are able to have their first blood meal. One nymph is able to take a quantity of blood equivalent to 8−9 times its own weight (Figure 7.1). Blood taking may last 20 min for a fifth nymph (and an adult). If the quantity of blood is sufficient, the wall distention may produce a nervous stimulus to initiate molt to the next stage. A triatomine becomes infected with *T. cruzi* by feeding on the blood of an infected person or animal. At the end of the blood intake, the triatomine generally deposits urine and dejection on the host's skin. If they contain the parasite, it can be transmitted to the host. If blood taking is interrupted by the movements of the host or under other circumstances, there will be no defecation and the molt is not initiated.

Adult Triatominae can generally be distinguished from other reduviids by the straight, slender, three-segmented **proboscis** adpressed to the underside of the

Figure 7.1 Blood fed fifth stage nymph of *Triatoma infestans*. The otherwise flat abdominal shape has converted into a rounded ball distended by blood. Hematophagy is the key feature to understand the biology of kissing bugs.
Source: From "Cyberatlas of Triatominae" at http://www.mpl.ird.frmorphometrics/cat/index.html.

cone-shaped head, which reaches the prosternal stridulatory sulcus[1] in all genera except *Cavernicola* and *Linshcosteus*.

By contrast, the proboscis of predatory reduviids is often curved, and usually more heavily chitinized. However, many predatory Reduviidae, especially of the subfamily Reduviinae (>900 species), can appear very similar to Triatominae.

Thus, blood intake must be complete to trigger molt. Domestic bugs generally feed on sleeping hosts to reduce the likelihood of blood meal interruption. When there are many bugs feeding on the same host, domestic animal or human, the host's skin will present local allergic reaction. The skin reaction has been shown to be repulsive for bugs, interrupting blood-feeding or discouraging tentatives from blood taking. In cases of high population density, which is relatively common for domestic bugs, many bugs will understandably have delay in their development, with particular consequences on population dynamics (see Section 7.2.6.2). Interrupted blood-feeding also means failure to transmit the parasite if present in the intestine.

On average, the time length of a development cycle goes from a few months (*R. prolixus*, *T. infestans*) to more than 1 year (*T. dimidiata*, *P. megistus*, *Dipetalogaster maximus*). This depends on population density, as previously explained, but also on external parameters like temperature in domestic (Gorla and Schofield, 1985, 1989) and sylvatic (Jimenez and Palacios, 2002) environment.

7.2.2 Reproduction

Copulation lasts for 5−15 min, male in dorsolateral position on female. Eggs are deposited 10−30 days after successful copulation. They can be laid individually one after the other during the whole life of the female (e.g., *T. infestans*), mainly in the burrow and saxicolous species. The eggs do not glue to the substrate, except in some species and in the Rhodniini, where they also can be deposited in sets (*Psammolestes arthuri*).

[1] The stridulatory sulcus is a vibratory communication organ of the head playing a role in the sexual behavior of the bugs (Manrique and Lazzari, 1994).

A single female of *T. infestans* oviposits around 100 eggs during its life, but depending on density conditions, it can oviposit a few times more. Under similar conditions of feeding, one female will oviposit a lot more of eggs if population's density is low. Fecundity, as well as developmental cycle, is densito-dependent (see Section 7.2.6.2). The number of eggs is also nicely correlated to the quantity of blood which has been ingested: blood availability and fecundity are tightly linked. Females can be fecundated by more than one male (Lima et al., 1987). The use of a genetic marker allowed Diotaiuti et al. (2002) to show that although a single mating can provide sufficient sperm for the whole reproductive life of the female *T. infestans*, multiple matings can result in balanced assortative sperm usage from the spermatheca.

7.2.3 Hematophagy

As a general rule, all Triatominae are hematophagous. This character is actually the one defining the subfamily, with likely related morphological characters of the head like a narrow and straight line rostrum with an articular membrane between the second and third segments (Lent and Wygodzinsky, 1979; Schaefer and Coscaron, 2001).

In the hypothesis of polyphyly (Schofield, 1988), other Hemiptera should show some hematophagic habits, like observed in *Clerada apicicornis* (Hemiptera: Lygaeidae) (Torres et al., 2000) or *Cryptophysoderes* (Physoderinae), and the characters defining the subfamily Triatominae would then be considered as an evolutionary convergence. In the alternative hypothesis of monophyly, the ancestor should already have these characters: Lent and Wygodzinsky (1979) suggested the Physoderinae as a possible candidate.

The hematophagy is not restricted to one sex as in mosquitoes or sandflies, for instance: it is obligatory for both males and females, as well as for each of the five nymphal stages. Exceptions to an exclusive hematophagy are situations where nymphs, and sometimes adults, still can feed or try to feed on other invertebrates or arthropods, suggesting their remote ancestry as predators (Schofield, 1988). Nymphs of the sylvatic *Eratyrus mucronatus* (Triatomini) or adult *T. circummaculata* (Salvatella, personal communication) may feed on other insects. In domestic Triatominae, at temperatures between 20°C and 30°C, the frequency of blood meal is around one meal every 4–9 days (Catalá, 1991). The bugs may starve for more than 1 month: in our experience, some specimens of *T. infestans* could survive up to 9 months without a blood meal.

Adults typically take 3–5 times their body weight of blood at each meal if allowed to engorge. Thus, during adult life, a female *T. infestans* will ingest about 10 g of blood, while larger species, such as *P. megistus* may take twice this amount. In the laboratory, a female *D. maximus* has been recorded ingesting 4.5 g of blood in a single feed. From the amount of blood ingested, it is deduced that domestic infestations of Triatominae can make a significant contribution to chronic iron-deficiency **anemia** (Schofield, 1981). Calculations based on studies of *R. prolixus* in Venezuela, and *T. infestans* in Brazil, have suggested that each person living in a

typically infested house was losing an average of about 2.5 g of blood per day due to the feeding bugs (Rabinovich, 1972).

In most Triatominae, including strictly sylvatic ones, it is worth noting a relative lack of host specificity in the feeding habits. Almost any vertebrate blood appears to be suitable, and different animals can be used in laboratory to feed Triatominae. First, instar nymphs may even feed on older congeneric insects when the latter are fully alimented (kleptohematophagy) (Sandoval et al., 2000). Exceptions to this lack of specificity are observed in the genus *Psammolestes* (sylvatic species of Rhodniini associated with some birds) or in other sylvatic bugs like *T. delpontei* (associated with the parrot *Monacha* species) or the **tropicopolitan** *T. rubrofasciata* (found in domestic structures but feeding preferentially on rodents). Rodents are also the preferred host of the North American protracta group of bugs and *Paratriatoma hirsuta*. In the Cavernicoli tribe, *Cavernicola pilosa* also has restricted host preferences.

7.2.4 Habitat

In sylvatic as well as in domestic species, the behavior is generally similar: the insect seeks body contact with the elements of the habitat (e.g., walls), a feature called thygmotropism. The insect remains hidden inside its refuge (e.g., cracks and crevices) without movement during hours (ataxia) so that it is not visible during the day (Figueiras and Lazzari, 2000). When obscurity is coming, then it may move (walking) to look for blood.

As a general rule, and not only for domestic insects, the habitat of a Triatominae offers shelter conditions, easy access to blood, and some stability of hosts. For arboreal species, it is bird nests or the crowns of palm trees; for other species with terrestrial habits, the habitat is a rodent burrow or marsupial lodge. The association is not a strict one; some arboreal species may sometimes be found in other places. What seems more important than the habitat structure is the host availability.

The human habitat (and peridomestic dependencies) offers the best features to Triatominae, such as stability, shelter, and blood abundance and availability. In rural areas, the domestic animals are frequently protected with wood pens and other natural elements, which could attract some sylvatic species because of some similarities with their own sylvatic habitats. However, domestic species do not seem to depend on the habitat structure or composition: again, what seems important is the host availability. Human bodies provide a large amount of blood and occupy their habitat for a longer time.

7.2.5 Dispersion

It is important to distinguish in Triatominae two dispersion modes: active and passive. Passive dispersion is transportation of generally immature stages by the animal host (eggs gluing on the feathers for instance) or with the familiar objects carried or worn by the human host (even a hat). Active transportation is ensured by both walking (nymphs, but also adults) and flying.

7.2.5.1 Active Dispersion

Here is maybe one aspect of the biology of Triatominae, together with population density, where we can find clear-cut differences between triatomines in domestic or sylvatic conditions. Active dispersion is performed not only by flying (Schofield et al., 1991; Noireau and Dujardin, 2001), but also and perhaps more frequently in domestic colonies by walking.

Domestic adult bugs when discovered in their hiding place do not fly; instead, they try to escape by walking. In sylvatic conditions, the same bug discovered under a stone might decide to walk away or might remain absolutely immobile, simulating a dead body. This behavior was observed by us in sylvatic *T. infestans*, probably because in sylvatic situation (and not in domestic places) the bug is surrounded by predators and cannot beat them at running. Some strictly sylvatic species are able, however, to immediately fly if disturbed, like *Parabelminus yurupucu* and *Microtriatoma trinidadensis* (Barrett, 1991).

The distance of flight of *T. infestans* is around 1 or 2 km, however, there are exceptions and some observations give *T. infestans* a much wider range (Schweigmann et al., 1988). The flight activity generally needs a physiological preparation (Lehane and Schofield, 1976). It requires a previous heating during which the bug shakes its wings for a few minutes, and it is more frequent in starved specimens (Lehane and Schofield, 1982). The flight of *T. infestans* seems to occur more often during the hot season, and at night (Lehane and Schofield, 1981).

The factors inducing flight are many, among which the nutritional status of the bug, external temperature and relative humidity, population density, and their respective roles are not easy to define (Barrett, 1991). The presence of light sources does not affect the spontaneous takeoff (Minoli and Lazzari, 2006). Flight orientation is apparently random, but it seems that during its flight, the bug can be attracted by light, as proven by the many observations of bugs either caught inside light traps set up outside villages to catch sandflies (F. Lepont, personal communication) or, in a village, observed landing on brightened window sills during night (La Fuente, personal communication).

Laboratory experiments have confirmed that a true attraction by white light rather than arrival by chance does exist (Minoli and Lazzari, 2006). Walking of nymphs toward a light source was reported during light trapping. It is more mysterious how a sylvatic habitat is left for another one. Possible but still not confirmed factors helping orientation in sylvatic conditions could be a specific odor attractant (Pires et al., 2002; Vitta et al., 2002) or the warmth (e.g., infrared) emitted by animal bodies, all signals probably collected by the receptors located on antennae (Carbajal de la Fuente and Catalá, 2002).

7.2.5.2 Passive Dispersion

This mode of dispersion is the most important one to explain the territorial expansion of the main vectors (Schofield, 1988). Triatominae migrate with their hosts, especially when highly adapted to their host. Dujardin (1998) hypothesized that the main

domestic populations of Triatominae (e.g., *T. infestans*, *R. prolixus*, *T. dimidiata*, and *T. rubrofasciata*) realized a passive migration with humans because of their high adaptation to the human habitat and the hosts living there or around. *T. infestans* has migrated from its sylvatic foci, mainly in Bolivia, to seven other countries in South America; *T. dimidiata* is found, like *R. prolixus*, in Central and South America; and *T. rubrofasciata* is a pantropicopolitan species.

The large territories of these species may present discontinuities (*R. prolixus* is absent from Panama and Nicaragua, for instance) suggesting passive transportation by man, and appear as recent conquests, as suggested by their genetic structure and by historical records when available. The hypothesis is that they have been transported by their hosts (human hosts, but domestic rodents for *T. rubrofasciata*) far outside their natural ecotope, losing their contact with original sylvatic foci, and increasing their dependency to human hosts.

The restricted habitat, the high dependency to humans and domestic animals, the loss of genetic resources from sylvatic original populations, and the genetic material reduction as observed by cytogenetic techniques (Panzera et al., 2004) have produced a vulnerable insect with a likely homogeneous response to control measures throughout its territory (Dujardin, 1998). Some Triatominae increase their possibility to follow the host by producing gluing eggs. For arboreal species that may feed on birds, one can imagine the important and fast migrations possible for a small population of eggs, leading to founder effects if peripheral colonization is successful. This mechanism has been suggested to explain the colonization of Central America by *R. prolixus* (Gamboa, 1962), but the theory was challenged by the historical revision made by Zeledon (1996), who suggested that Central America was colonized by human activities. The latter thesis was supported by genetic studies (Dujardin et al., 1998b).

7.2.6 Population Dynamics

Since populations are not fixed entities, the characters defining them include an information about change with time, about their dynamics, and refer mainly to reproduction, density, and demography.

7.2.6.1 Reproduction

What is meant by "reproduction" is "how many individuals will exist in a population after a given lapse of time." The lapse of time is often the generation time, and the question becomes: "What is the change in density from one generation to another?" The numeric answer to that question receives the symbol *r*. Birth (*b*) and death (*d*) are affecting the reproduction of any population. If *r* is the growth rate of the population, then:

$$r = b \quad d$$

But in a finite model, the population cannot grow indefinitely. There are some limitations, like space available, and a maximum of individuals are considered

under the new variable K (maximum capacity of growth). The rN (N being the number of individuals) is modified as long as it approaches K. The new equation becomes:

$$dN/dt = rN[((K \quad N)/K)]$$

Growth depends on the population density relative to its maximum capacity. The growth of a population is a density-dependent concept. Thus, insecticides reducing population density are also modifying the growth rate.

7.2.6.2 Density

In this aspect, striking differences are observed between domestic and strictly sylvatic species, or between the domestic and sylvatic habitats of the same species. Most sylvatic populations of Triatominae tend to be relatively small, composed of a few adults and nymphs. Most domestic populations show very high densities, with hundreds or thousands of adults and nymphs occupying one single house.

Field Definition

How many individuals are there in a given unit of space? The question looks simple, but the complete counting of all the individuals is generally impossible to perform in field situations, so an estimation is done from samples. Sampling natural populations has been suggested through various techniques (Schofield, 1978). The most often-used approach is called "capture by effort unit": the bugs are collected during a limited amount of time and its number formulated as, for instance, in "man/hour" unit (the number of insects captured in 1 h by one man). In the frame of control interventions, only one specimen found may be enough to decide insecticide application in the house or in the village. In some cases, the following strategy may be preferred: houses are inspected during 1 h but inspection stops whenever one specimen only is found.

Density-dependence

Studies of houses infested by *T. infestans* in Brazil between 1976 and 1978 showed that there was no change in density from 1 year to another (Schofield, 1980). The question was why these populations remained at the same density level. Why did they not increase their density, since no active control intervention was in development? Why, since it had been shown that in laboratory the bug was able to grow at a rate of 25 from one generation to the next (Rabinovich, 1972; Feliciangeli and Rabinovich, 1985)?

The search for a limiting factor considered many possibilities, among which availability, external temperature, the presence of predators, and the availability of blood. Space had been considered as a limiting factor by Gomez-Nunez (1965). Some houses indeed do not offer a lot of hidden places where a large population could freely grow. When no hidden places are available any more, the bug become vulnerable because of predators (e.g., hens and dogs) and population cannot grow further. This hypothesis was examined in a longitudinal comparative study performed in the field (Brazil) where untreated houses were compared with

semitreated ones. In the latter, cracks and crevices in the walls were filled in half the space of the house. After treatment, the density decreased as expected. After 1 year, in spite of the experimentally reduced availability of space in semitreated houses, the density increased back to the values of the untreated houses.

Mortality tables have been examined to identify the possible factor able to reduce the growth rate from 25 (the laboratory observed growth rate) to 1 (the field one). It was shown that a slight increase in the time from one stage to another could considerably reduce the growth rate, and that such increases could be the consequence of a reduction in blood availability (Schofield, 1980). The same reduction in blood supply had other effects, among which was the reduction in the number of eggs. The hypothesis became that denso-regulation would be the effect of competition for blood access, less blood meaning less fecundity as well as a longer time from one stage to another (Schofield, 1980).

Field observations were congruent with that hypothesis. Density of *T. infestans* in a house was apparently correlated with the number of people and domestic animals living there (Piesman et al., 1983). Host availability was also demonstrated as a critical factor by laboratory (Schofield, 1982) and experimental field populations protocols (Gorla and Schofield, 1985, 1989). An additional effect was observed: flight probability had an apparent negative correlation with blood availability. Thus, another factor modifying density, the dispersal of specimens, was dependent on nutritional factors.

However, some aspects were still obscure. For instance, there is more blood in one human than is necessary for feeding a complete population of *T. infestans*. Why then exactly was there a correlation between bug density and the number of humans? Laboratory experiments provided the answer. They showed indeed that the host irritability was increasing with the number of bugs feeding (Wier Lopez, 1982; Schofield et al., 1986); the probability for each insect to reach complete repletion (and subsequent molt) was a function of host irritability.

One obvious cause of host reaction is the saliva of the insect. It is released soon after the bite, and salivation occurs during the entire feeding process. In the probing phase, as was observed in *R. prolixus*, saliva is pumped continuously in the host skin, including around the blood vessels (Soares et al., 2006). Adopting a finalist point of view, in order for the insect to feed properly, it should produce the smallest possible irritation to the host. Which could mean (i) to have small and thin mouthparts entering the skin and (ii) to have nonirritating saliva. Mouthparts entering the skin are indeed very thin, with a small 10-μ diameter canal allowing just one red blood cell to move. This means that anticoagulant factors of the saliva must be strong to avoid obstruction of the canal. Other mechanisms controlling the contact with saliva seem related to the cibarial pump activity. It can regulate the quantity of saliva deposited in the microcirculation as necessary, and consequently minimize the host's immune response to salivary antigens (Soares et al., 2006).

7.2.6.3 Demography

MacArthur and Wilson (1967) distinguished "*r*" and "*K*" strategies as defining populations occupying unstable or stable habitats, respectively. The first demographic

strategy is typically the one of mosquitoes. They are generally relatively small insects producing a very abundant progeny, having a short developmental cycle (less than 1 month), high dispersal capacities, and an aggressive behavior to exploit at its maximum the available resources of their environment. The "*K*" strategy is the opposite one, congruent with domestic species of Triatominae. They are relatively larger insects producing a much smaller quantity of descendants (1000 times less than what a mosquito can produce), they have extended developmental cycles (various months), poor active dispersal capacities, and a timorous feeding behavior. They do not try to exhaust the resources of their environment, but seem to opt for an optimum use of it. Whereas the "*r*" strategists can recover quickly after a catastrophic mortality, or they can move and disperse to other more wealthy environments, the "*K*" strategists in the same situation would probably be unable to recover or to escape (Rabinovich, 1974).

Thus, extinction would be the fate of "*K*" strategist when confronted with an adverse environment, as observed for the domestic species of Triatominae that have been targeted by international programs of vector control (Schofield and Dias, 1999). However, such "*K*" populations have the possibility to significantly increase their development rate and recover their previous effectiveness relatively quickly if a few of them can survive. Again, the explanation of this recovering capacity is obtained through what we know about density regulation. In lower densities, there is no more competition to feed, and each insect would take complete blood meals, which in turn would shorten the developmental cycle and increase both fecundity and fertility of females. A good control program must avoid the survival of a few insects even a very few of them.

7.2.7 Insight into the Biology and Ecology of Triatominae in the Sylvatic Environment

In many areas of Latin America, the domestic intrusion of species that were, until now, considered strictly sylvatic has led to take more interest in their study. The observation of a species in its natural environment supplies a "basic pattern" that may help to understand its process of adaptation to a new environment (for instance, when populations are displaying **synanthropic** behavior). However, the studies carried out in the sylvatic environment are often fragmentary, principally because the field observations and collections of specimens are laborious and time-consuming.

7.2.7.1 Interest of a Trapping Device

The detection and collecting of triatomines in their natural environment, necessary precondition to biological and ecological observations, has benefited from the use of a simple trapping device (Noireau et al., 2002). In many cases, the use of a trap provides the only way to detect and collect triatomines in little accessible ecotopes, such as rock piles, hollow trees, or palm trees, and avoids ecological damage caused, for example, by tree dissection or logging. Because starved bugs are preferentially attracted, the

device does not allow accurate estimating of the density of insects and population structure. On the other hand, it has allowed some interesting observations: thus, for instance, wild populations of *T. infestans*, *T. brasiliensis*, or *T. pseudomaculata* may exhibit high motivation for food search during the day hours (Noireau, unpublished data) whereas they are supposed to leave their refuges and make for food source during the night (Guerenstein and Lazzari, 2009).

7.2.7.2 Habitat

According to Schofield (1988), each of the three most epidemiological important genera of Triatominae is virtually associated with a type of habitat. So, species of the genus *Rhodnius* are primarily associated with palms, the genus *Panstrongylus* has predominantly evolved in burrows and tree cavities, and the genus *Triatoma* in terrestrial rocky habitats or rodent burrows. This assumption is generally true for the genera *Rhodnius* and *Panstrongylus* even though some species were found in other sylvatic habitats. Thus, in the genus *Rhodnius*, *R. domesticus* has been reported in bromeliads and hollow trees in Amazonia, and *R. neglectus* in *Cereus jamacaru* (mandacaru), a cactus species characteristic of the Caatinga in northeastern Brazil (Lent and Wygodzinsky, 1979; Dias-Lima et al., 2003). Although some species of the genus *Panstrongylus* can be found in palm tree crowns (e.g., *P. megistus*), all species are associated with terrestrial burrows, tree-root cavities or hollow trees (Gaunt and Miles, 2000). On the other hand, the preference of the genus *Triatoma* for terrestrial habitats is more questionable. Species can be found in arboreal as well as rocky habitat (e.g., *T. infestans*, *T. sordida*, and *T. guasayana)*, of which Andean populations live in rock piles and those of the lowlands live in trees. Others are exclusively arboreal, found in hollow trees and bird nests (e.g., *T. ryckmani*, *T. pseudomaculata*, *T. platensis*, and *T. delpontei*).

Triatomine species may exhibit a great range of ecotopes (*P. megistus*, *T. dimidiata*) when others display a close relationship with one ecotope. *Psammolestes* species are only associated with nests of Furnariidae. Some *Rhodnius* species may be associated with a particular type of palms (*R. brethesi* with *Leopoldinia piassaba*, *R. ecuadoriensis* with *Phytelephas* species), whereas others do not exhibit a palm preference (*R. pictipes* and *R. robustus*). A theory suggests the occurrence of rapid morphological divergence in response to different ecological factors (Dujardin et al., 1999). Indeed, we observe obvious chromatic differences between arboreal *T. infestans* (*melanosoma* and dark morph populations; Martinez et al., 1987; Noireau et al., 1997a) and terrestrial specimens collected in rocky habitat in Bolivia (clearer morphs). Nevertheless, this model cannot be generalized when considering chromatic variation only. So, arboreal and terrestrial wild *T. sordida* do not display chromatic differences. Similarly, the genetically closely related species *T. pseudomaculata* and *T. wygodzinkyi* are probably issued from a common ancestor and have undergone an ecological divergence (the first species is arboreal whereas the second is terrestrial) without detectable morphological differences.

Many triatomine species exhibit a behavioral plasticity as related to habitat selection in different environments. In a sylvatic environment, the habitat of *T. pseudomaculata*

and *T. juazeirensis* is never shared. The first species is found in trees and bird nests whereas the second is exclusively **rupicolous**. When they invade the peridomestic area, they are highly adaptable to different habitats and can occupy substrates that they do not colonize in a sylvatic environment. So, *T. juazeirensis* leaves a rupicolous habitat for colonizing, in the peridomicile, wood material in more than 80% of cases (Carbajal de la Fuente et al., 2008).

7.2.7.3 Access to Host

Host-seeking classically results in the feeding. Such behavior, considered as basic in Triatominae, applies mostly to domestic/peridomestic colonies living in the closeness with synanthropic animals or humans. It also applies to adult wild triatomines, which can fly to find their feeding. This last assumption is strengthened by the uncommon capture of adult forms in traps placed in hollow trees, habitats considered as unfavorable for the permanence of feeding host. However, the starved nymphs, which are profusely collected by trapping in such unfavorable ecotopes, enjoy another type of access to host that may be called "host waiting."

This passive behavior is justified by the reduced locomotor activity of nymphal instars that have to wait for the intrusion of a host in order to feed. Another example of host waiting was noticed with relation to sylvatic colonies of *T. guasayana* occurring in bromeliads in Bolivia. Bromeliad beds covered no burrows, and the detection of blood sources indicated the cattle as the main feeding host (Baune, personal communication).

7.2.7.4 Survival Strategy

The environmental disturbance caused by man and succeeding damage of triatomine biotopes often results in a condition of chronic distress in wild populations of insects. This was demonstrated in *T. pseudomaculata* in northeastern Brazil, where the sylvatic insects exhibit a great weight deficit in relation to peridomiciliary ones (Carbajal de la Fuente, personal communication). This adverse condition leads the insect to apply a survival strategy related to the election of habitat, breeding behavior, and host preference, and is certainly the main cause of flight dispersal and possible subsequent settlement in artificial structures.

T. pseudomaculata is an autochtonous species of the Caatinga. In its survival strategy, this arboricolous species does not exhibit preference for any tree species. On the contrary, it can be captured in all the predominant trees of the area (more than 10 species), in hollows or nests of Furnariidae (Dias-Lima et al., 2003; Carbajal de la Fuente et al., 2008). The high percentage of positive trees (>50%) and the presence of small colonies of insects, rarely exceeding 10 individuals, suggest that females lay a small quantity of eggs in a large number of ecotopes to increase their chance of survival (Noireau et al., 2005).

With relation to the hematophagy, some works have pointed out the occurrence of alternative feeding behavior in natural populations of Triatominae. So, wild specimens of *T. circummaculata* (Salvatella, personal communication), *T. pseudomaculata*,

Figure 7.2 *Triatoma brasiliensis* nymphal instar feeding on the hemolymph of an immobilized *Scolopendra*.

T. sordida, *T. brasiliensis*, and *Psammolestes tertius* may feed on hemolymph of invertebrates (Carbajal de la Fuente, unpublished data). The importance and consequences of this biological trait, influenced by unfavorable environmental conditions, are unknown. Relationships between hemolymphagy and population dynamics, and vector−*T. cruzi* interaction, might be contemplated. This feeding habit is derived from the predaceous behavior in other Reduviidae. Nevertheless, when the assassin bugs prey upon arthropods, predigest their tissues, and kill their prey, the Triatominae only immobilize temporarily their prey and suck their fluids (Figure 7.2).

7.3 Vectorial Capacity and Domesticity

The vectorial capacity of a mosquito or a fly, be it an insect transmitting a virus or a protozoan, is often a matter of "host−parasite" specificity: the parasite is transmitted by one mosquito species, or a very few of them. The situation is very different for Chagas disease vectors, and this might be related to the mechanism of parasite transmission. The **stercorarian** parasite adapts to the lumen and epithelium of the bug intestine; it is evacuated with the feces dropped on the host's skin, and penetrates actively through a local wound (the one produced by the bite, for instance), or directly through the eye mucosa (Romaña's sign).

7.3.1 Adaptation to T. cruzi

Adaptation to the protozoan *T. cruzi* does not seem to be a critical issue in the vectorial capacity of Triatominae. Different tribes, different genera, different species are actively transmitting the diverse genetic entities assembled under the name of *T. cruzi*. Moreover, other orders of insect seem to be able to ensure the complete cycle of *T. cruzi* inside the intestine: the Diptera *Musca domestica* (Diaz-Ungria,

1966), the *Cimex lecticularius*, and even Arachnidae were found infected by the *T. cruzi* (Dujardin et al., 2000).

7.3.2 Blood-Feeding Habits

Hematophagy, an almost exclusive and obligatory habit in Triatominae at all their development stages and both sexes, appears as the key feature of their biology, by which much of their behavior and their microevolution is explained. Even if the transmission of the parasite is due to the contact of insect dejection on the host skin, hematophagy is a crucial feature of vectorial capacity. It allows the infection of the vector, and it ensures the regular and prolonged contact of the vector with the vertebrate host.

Since the parasite is transmitted by the feces, the presence of the feeding insect on the host must last long enough for the defecation to occur. The blood meal lasts approximately 20 min. The main vectors need less than this amount of time to deposit their urine and dejection, but some species (like the northern group of *Triatoma*) may defecate up to an hour after having left their host and so do not represent a high peril of transmission.

For each species, defecation also seems to be a density-dependent process. In high-density populations, each insect will have less blood due to competition with others, and an incomplete engorgement can delay or cancel defecation, hence transmission. In low-density conditions, however, each insect can feed without the stress of competition, and defecation will certainly occur (Kirk and Schofield, 1987; Trumper and Gorla, 1991). The transmission of *T. cruzi* is more likely in vector low-density conditions.

7.3.3 Domesticity

In the same way hematophagy is the dominant character to consider for understanding the biology of the bugs, domesticity is the key factor to evaluate their vectorial capacity. Only the species adapted to human dwellings are actively contributing to the transmission. They are called "domestic species" in the sense of species associated with human (synanthropic species), not in the sense of species domesticated by man. These species are less than 5% of the total number of Triatominae, which means that such adaptation is not easy to obtain in spite of continuous contacts reported between man and sylvatic vectors.

As observed in the literature, the frontiers between domestic, peridomestic, and domiciliary populations are not clear. The following distinction between intrusion, domiciliation, and domestication might help in defining the epidemiological importance of some populations or species of Triatominae.

7.3.3.1 Intrusion

Many adult specimens of sylvatic species are reported from inside human dwellings, probably attracted there by light or introduced by passive carriage (marsupials, for

instance). Some of them were unknown and described from the human habitat without knowing more about their biology (*T. jurbergi*, for instance). In this situation, there is no evidence of colonization (eggs, nymphs, and exuviae).

7.3.3.2 Domiciliation

What is different here is the presence inside the house of an adult and of nymphs, eggs, and exuviae, which means the complete cycle of the insect was occurring inside the house. The resulting colonies are not very abundant and represent merely a tentative adaptation to the house. This situation has been described for *R. pallescens* in the North of Colombia (Moreno, personal communication); *E. mucronatus* in Bolivia (Noireau et al., 1995), and in Venezuela (Viva et al., 2001); *T. sordida* (Noireau et al., 1997), *R. stali* (Matias et al., 2002), *M. trinidadensis* (De la Riva et al., 2001), and *P. rufotuberculatus* (Noireau et al., 1994; Dujardin et al., 1998a) in Bolivia. It is not necessarily a permanent situation. For instance, the domiciliary *R. pallescens* in the northern Colombia progressively disappeared from the houses without any control intervention (Moreno, personal communication).

7.3.3.3 Domestication

The definition includes the aforementioned observations for domiciliation, with an additional criterion related to the type of geographic extension. It is no more a local, geographically restricted observation but rather concerns a more widely extended territory with obvious arguments supporting migration by passive carriage. It is, for instance, a discontinuous geographic extension, with gaps apparently unexplained unless the human intervention is admitted. More research is needed to understand the factors allowing a species to reach a high level of adaptation to the domestic habitat, an adaptation which systematically reduces the size of the insect (Dujardin, 1998; Caro-Riaño et al., 2009).

It is important to recall that the existence of "domesticated" species does not exclude the existence of sylvatic foci. Wild populations of *T. infestans* and *R. prolixus* were recorded in Bolivia and Argentina (for *T. infestans*) and Venezuela (for *R. prolixus*).

7.3.3.4 About an Ecoepidemiological Classification of Triatominae

To provide a first operational evaluation of each species, an ecoepidemiological classification could be suggested that includes the aforementioned definitions. The ecological classification of Triatominae could be based on the nature of the sylvatic habitat (rupicolous or arboreal, for instance). As vectors of disease, the Triatominae could also be classified according to their epidemiological importance (species documented or not as responsible for the occurrence of Chagas disease cases). However, such a classification would only recognize the species documented as proven vectors of the disease and would discard all the putative vectors able to infest peridomestic/domestic areas near to humans. Consequently, it would be useful to adopt a classification system based both on the synanthropic process (restriction to the sylvatic environment, infestation of peridomestic structures, intrusion

into houses, domiciliation, and domestication) and epidemiological importance (proved or putative vector versus species without documented role in the transmission of *T. cruzi* to humans).

7.4 Vector Control Strategy

7.4.1 Entomological Surveillance

Domestic species have been severely hit by international programs (Schofield and Dujardin, 1997; WHO, 1997; Schofield and Dias, 1999), but not all of them have been controlled in every part of their territory (Dias et al., 2002). What we learned from their biology is that no country should allow the persistence or the development of domestic populations. The entomological surveillance of Chagas disease vectors is the surveillance of the human dwellings. A serious alert should be addressed to the local health authorities in case of domiciliation (see Section 7.3.3.2), a regional alert should be raised in case of domestication (see Section 7.3.3.3), be it observed for another species than the one already known as "domestic species."

7.4.2 Eradication, Elimination, and Reduction

The densito-dependent character of the delay between blood intake and defecation allows us to understand why it is crucial to completely eliminate bugs from the domestic and peridomestic structures. The most dangerous bug is the infected bug allowed to feed toward complete engorgement because it will certainly deposit its infected feces on the host's skin during the blood meal. This situation is obtained when the population density is low and no strong competition for food exists. It could be produced by poorly executed or partial vector control interventions. Because they lower the density of remaining insects, the control measures producing only the reduction of vector populations can be more dangerous than no control at all.

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Glossary

Most of the very specialized words are explained in the text; a few others are defined hereunder.

Anemia a deficiency of red blood cells.

Exopterygotes insects with incomplete metamorphosis, changing form only gradually from the immature stages to the adult; the young resemble adults but have externally develop ing wings (e.g., triatomine nymph to adult).

Monophyletic descending from a single, common ancestor; the alternative is "polyphyletic."

Proboscis long mouthpart or feeding tube of the kissing bug, appearing as an elongated appendage from the head; in kissing bugs, it is an articulated appendage.

Rupicolous thriving among or inhabiting rocks ("saxicolous" has similar meaning).

Synanthropic ecologically associated with humans.

Stercorarian of fecal origin; said of trypanosomes passed to the recipient in the feces (an alternative mode of transmission is "salivarian").

Tropicopolitan inhabiting all tropical climate countries.

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8 Population Genetics of Triatomines

Fernando Monteiro[1], *Paula Marcet*[2] *and Patricia Dorn*[3]

[1]Laboratório de Doenças Parasitárias, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, [2]Division of Parasitic Diseases, Centers for Disease Control and Prevention, Georgia, USA, [3]Department of Biological Sciences, Loyola University New Orleans, Louisiana, USA

8.1 Introduction

8.1.1 Purpose of the Chapter and How it is Structured

This chapter intends to introduce the reader to the world of triatomine **population genetics**. To achieve this goal, we start with an introductory section highlighting the importance of population genetics in epidemiology and vector control. We then explain basic concepts, such as the Hardy–Weinberg equilibrium (HWE) and genetic variation. These are important for understanding the next section on population structure and gene flow.

We proceed with a description of the first molecular markers used in population genetics of Triatominae, the **allozymes** (as they will serve as examples), and then describe the two most reliable molecular techniques used today (**microsatellites** and DNA sequencing).

With all the theoretical aspects understood, we present a discussion on how to elaborate a study design that will allow you to answer a specific scientific question (or to test your null hypothesis). Finally, we give a thorough description of the relevant population genetics inferences on the three most important Chagas disease vector species: *Rhodnius prolixus*, *Triatoma dimidiata*, and *T. infestans*.

8.1.2 Introduction

The field of systematics can be subdivided into three major areas of investigation known as *alpha*, *beta*, and *gamma* systematics (Mayr, 1969). *Alpha* systematics deals with species identification and description. *Beta* systematics studies the relationships among species (i.e., phylogeny). Lastly, *gamma* systematics investigates

E mail: fam@ioc.fiocruz.br, pvm3@cdc.gov and dorn@loyno.edu

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within-species phenomena (i.e., population variability and genetic structure). The latter is the focus of this chapter.

In biology, a population is defined as a collection of interbreeding organisms of a particular species. But in practical terms, a population can be defined according to different (usually arbitrary) criteria (e.g., by geographic proximity, by sample availability, or even by geopolitical limits). Population genetics studies can reveal the precise dimensions of real, biologically meaningful (i.e., **panmictic**) populations. We will address the importance of being clear and consistent in the definition of the population that is being studied in order to reduce biases in the interpretation and comparison of results obtained.

8.1.3 Important Epidemiological Questions and How They May Be Answered

How much migration and interbreeding occur among nearby populations? What are the barriers that prevent gene flow (e.g., mountain ranges, biogeographical limits)? When insects reappear in houses following insecticide treatment, do they represent recrudescent **domestic** populations or are they **sylvatic** colonizers? Do genetically different subpopulations differ in terms of vectorial competence or capacity? How fast would an insecticide-resistance allele spread throughout the population? The answers to these questions are of fundamental importance regarding the epidemiology and control of vector-borne diseases.

Population genetics studies the distribution of allele frequency and change in order to understand the influence evolutionary processes (e.g., mutation, **genetic drift**, natural selection, and migration) might have on the genetic variability and structure of natural populations. It thus provides the theoretical framework and tools to address the "real-life" questions presented here. This approach helps us to better understand disease epidemiology, and thus design novel and rational control-surveillance strategies to interrupt transmission.

8.1.4 The Importance of Population Genetics in Chagas Disease Epidemiology and Control

Human Chagas infection is endemic throughout Latin America, where its associated burden is larger than the combined burden of malaria, leprosy, leishmaniasis, filariasis, schistosomiasis, dengue, and the major intestinal nematode infections (Mathers et al., 2006). It is worth mentioning that Chagas disease recently became a concern in nonendemic areas, including the USA and Europe. This is a consequence of the immigration of infected individuals who act as blood or organ donors.

There is no vaccine against the etiological agent, *Trypanosoma cruzi*, and, in spite of recent advances, large-scale disease treatment remains impractical. As a consequence, disease control relies heavily on the elimination of domestic vector populations by the spraying of infested houses with residual insecticides, and

serological screening of blood donations. As the most prevalent form of transmission is vector mediated, there is a constant need for the development and optimization of vector control strategies to keep pace with the ever-changing epidemiological scenarios (Abad-Franch et al., 2009).

This is why the furthering of knowledge on triatomine population structure and gene flow, as well as the detection of cryptic taxa (which may exhibit different vectorial capabilities) within the main vector species, is so important (Abad-Franch and Monteiro, 2005).

8.1.5 Historical Account of Control Efforts

Launched in 1991, the "Southern Cone Initiative" was the first intergovernmental control program designed to eliminate domestic populations of *T. infestans*, the main vector in the region. It was followed by the Andean and Central American initiatives in 1997. Although implementation of control activities in the Andean subregion has been slow and coverage incomplete (Guhl, 2007), these three initiatives have been highly successful in eliminating domestic vector populations, thus interrupting disease transmission throughout Latin America (Dias et al., 2002; Feliciangeli et al., 2003; Schofield et al., 2006). However, less success has been realized in the Gran Chaco region (northern Argentina, Bolivia, and Paraguay), mainly due to high levels of bug reinfestation after spraying (Gürtler, 2007). A key question to be answered is whether recurrent infestations are due to residual domestic populations that survive insecticide spraying, or to reinvasion of bugs from external sources (either from unsprayed communities or from sylvatic foci).

In the Southern Cone, for example, 10 years of coordinated control actions reduced disease incidence by 94% (WHO, 2002). Nonetheless, recent estimates show that there is still work to be done, as infection prevalence remains at 12 million, with 200,000 new cases per year in 15 countries (Morel and Lazdins, 2003). But what is the cause of these new cases?

With the elimination of domestic vector populations, the problem now involves autochthonous (native) vectors. Differently from introduced *T. infestans* and *R. prolixus* domestic populations in several countries, native species are distributed across extensive sylvatic ecotopes, making the recolonization of treated villages a recurrent phenomenon. We must not forget that even *T. infestans* and *R. prolixus* have their own respective "home" areas in the Bolivian highlands and Venezuelan llanos. Such situations will require continued community-based vector surveillance and the respraying of any new domestic focus identified (Ramsey and Schofield, 2003).

New epidemiological scenarios are also a matter of concern. In Amazonia, where disease is hypoendemic, foci of relatively intense transmission, related to large-scale harvesting or consumption of forest products (such as *açaí* fruits or *piaçava* fibers) are overlaid onto a background of low-intensity, widespread, continuous vector-borne transmission (Aguilar et al., 2007).

8.2 Hardy–Weinberg Equilibrium

Godfrey H. Hardy and Wilhelm Weinberg defined a population in "equilibrium" as that in which allele (gene) and genotype frequencies remain stable from one generation to the next. This artificial state of equilibrium only occurs when the evolutionary forces of mutation, selection, migration, and drift are not operating (and applies only to sexually reproducing organisms that belong to panmictic (randomly breeding) populations).

So the rationale is to calculate allele frequency data from the observed genotype frequencies of our real samples, and then use the Hardy–Weinberg formula to estimate the genotype frequencies in the next generation assuming the population was in equilibrium. This can be done using a Punnett square (Table 8.1). The frequency of one allele is given the designation "p" (e.g., the F allele (for "Fast")), and the frequency of the alternative allele "q" (e.g., the S allele (for "Slow")), and we can see that $p + q = 1$, or 100% of the alleles.

In a two-allele system in a diploid organism, the expected frequency of the genotypes in the next generation would be:

$$\text{F/F} = p^2$$
$$\text{S/F} = 2pq$$
$$\text{S/S} = q^2$$

And of course,

$p^2 + 2pq + q^2 = 1$, or 100% of the genotypes.

To illustrate, we will use data from the phosphogluconate dehydrogenase allozyme locus (*Pgd*) of a *Triatoma sordida* subpopulation ($n = 68$) from the locality of Espinosa, Minas Gerais, Brazil, as an example (from Monteiro et al., 2009). More details on the sampling strategy used and how populations were defined are given in the genetic structure section.

$$\text{Freq F allele } (p) = \left(2 \times \text{homozygotes F/F} + \text{heterozygotes S/F}\right)/2N$$

N = number of individuals

And to calculate q, it is simply $q = 1 \quad p$

Table 8.1 Expected Genotypic Frequencies for a Population in HWE

			Alleles in female gametes (and their frequencies)	
	Alleles		F	S
Alleles in male gametes		Frequency	p (0.22)	q (0.78)
(and their frequencies)	F	p (0.22)	p^2 (0.05)	pq (0.17)
	S	q (0.78)	pq (0.17)	q^2 (0.61)

So, for our example, $p = [(2 \times 6 \text{ F/F individuals}) + 18 \text{ S/F}]/2 \times 68 = 0.22$, $q = 1 \quad p = 0.78$.

Now we can calculate what would be the expected numbers of each genotype in the next generation, if the population is in HWE:

F/F = $p^2 = (0.22)^2 = 0.05 \times 68$ individuals = 3.3 individuals
S/F = $2pq = 2 \times (0.22)(0.78) = 0.34 \times 68$ individuals = 23.4 individuals
S/S = $q^2 = (0.78)^2 = 0.61 \times 68$ individuals = 41.3 individuals

At this point, an important explanation is required. In certain situations (other than when using laboratory-reared populations), it might be impossible to go back to the field and find the offspring (or next generation) of the sample we collected and analyzed. So the "trick" usually employed consists of comparing the expected genotype frequencies obtained with the initial observed genotypic frequencies by using a Chi-squared analysis (Table 8.2). If a nonsignificant P value is obtained (i.e., $P \geq 0.05$), we accept (or better, are unable to reject) the null hypothesis that the population is in HWE. On the other hand, if a significant P value is obtained ($P < 0.05$), we reject the HWE null hypothesis and start looking for possible biological and evolutionary phenomena (discussed later) that might have caused the disequilibrium.

As we obtained a P value of 0.07 (>0.05), we are unable to reject our null hypothesis and thus conclude that the Espinosa *T. sordida* subpopulation is in HWE.

Note that the Hardy−Weinberg formula also allows us to calculate the expected heterozygosity:

$$H_e = 2pq$$

as shown above, S/F = $2pq = 2 \times (0.22)(0.78) = 0.34$. Thus 34% of the individuals are expected to be heterozygotes for that particular locus. The expected heterozygosity (H_e) is the measure most frequently reported when describing population variability based on codominant markers in which both alleles are expressed in a heterozygote.

Table 8.2 Chi squared (χ^2) Calculations to Test if the Population is in HWE

Genotype	Observed (O)	Expected (E)	|O E|	(O E)^2	(O E)^2/E
F/F	6	3.3	2.7	7.3	2.2
F/S	18	23.4	5.4	29.2	1.2
S/S	44	41.3	2.7	7.3	0.2
					$\chi^2 = 3.6$
With 1 degree of freedom					$P = 0.07$

8.3 Evolutionary Forces

8.3.1 How Does Genetic Variation Arise?

Although evolution has equipped our cells with an extremely efficient (and complex) DNA replicating machinery, the system is not perfect and, occasionally, mistakes do occur. Such mistakes are called mutations. When a nucleotide in a gene mutates into another nucleotide, a new allelic variant for that sequence is created. Therefore, mutations increase the amount of genetic variation in a population. In population genetics, we focus on germ-line mutations (as opposed to somatic) since these are the ones that will be transmitted to the next generation.

8.3.2 What Are the Forces That Reduce Genetic Variation?

There are several types of natural selection. Purifying selection is the most prevalent form of selection as it constantly sweeps away deleterious mutations that are produced in each generation. It is what keeps us fit. However, once in a while a mutation may arise that increases the fitness of the individual. These selectively advantageous alleles can replace other alleles and become "fixed" in the population (i.e., reach the frequency of 1) through directional selection. Strong directional selection, such as frequent pesticide application, may result in recurrent **bottlenecks** so that the population contains only the variation present in a small, surviving subpopulation. Therefore directional selection, in theory, has the effect of reducing the diversity of alleles and therefore the genetic variation in a population.

But genetic variation can also be decreased because of chance alone, through a process known as genetic drift. Genetic drift is the change in allele frequencies from generation to generation that occurs in finite-sized populations due to the random sampling of gametes (containing particular alleles) that will constitute the zygotes of the next generation. Its effect is more pronounced in smaller populations and inevitably leads to the fixation of a particular allele (and the loss of others). Inbreeding also increases as populations get smaller, further decreasing population variability. Genetic drift can be distinguished from selection because the whole genome is affected, not just a particular locus. Migration is a counteracting force to genetic drift. By mixing alleles among populations, migration distributes and homogenizes genetic variation among populations.

8.4 Genetic Variation and Population Structure

8.4.1 Genetic Variation

8.4.1.1 DNA Sequence and Nucleotide Diversity

DNA sequencing is the most efficient and versatile technique used today in molecular systematics (discussed in the next section). Technologic advances have greatly reduced sequencing costs, making the method more accessible. Consequently,

many sequences are available on publicly accessible databases, such as GenBank, providing a vast and cumulative source of data. A review of nuclear and mitochondrial markers used in triatomine studies, including a discussion on the strengths and weaknesses of individual markers, was recently published by Mas-Coma and Bargues (2009).

DNA sequence diversity in a population can be expressed as the nucleotide diversity (π), which describes the average proportion of nucleotide differences between all possible pairs of sequences obtained for that population (Nei, 1987). We will use nine published *T. infestans* 412 bp *cyt b* **haplotypes** (Table 8.3; Monteiro et al., 1999) to illustrate how to calculate nucleotide diversity. We start by finding the average pairwise difference among all possible haplotypes (i.e., 1 compared with 2, then with 3, and 4, etc.) for each variable site, and then replace the values in the following formula:

$$\begin{aligned}\pi &= \frac{\text{number of pairwise differences}}{\text{possible pairwise combinations}} \\ &= \frac{\text{number of pairwise differences}}{\text{number of nucleotides analyzed} \times (n(n-1))/2} \quad n = \text{number of sequences} \\ &= \frac{17+8+20+8+20+8+8+20}{412 \times (9 \times 8/2)} = \frac{109}{14832} \\ \pi &= 0.0074\end{aligned}$$

Table 8.3 Variable Sites among *T. infestans* Haplotypes Detected in Natural Populations from Bolivia (BOL), Argentina (ARG), and Brazil (BRA)

	Variable Sites (Nucleotide Position)							
	0 6 3	**1 8 3**	**1 8 6**	**2 4 3**	**2 8 8**	**2 9 7**	**3 5 4**	**3 8 4**
T. infestans (BRA BA)	A	T	T	T	A	A	T	C
T. infestans (BRA RS)	A	T	T	T	A	A	T	C
T. infestans (ARG)	A	T	T	T	A	A	T	C
T. infestans (BRA PR)	A	T	T	C	A	A	T	C
T. infestans (BOL Syl)	G	T	C	T	C	A	T	T
T. infestans (BOL Dom)	G	T	C	T	C	A	T	T
T. infestans (BOL SA)	G	T	C	T	C	A	T	T
T. infestans (BOL CO)	G	T	C	T	C	A	T	T
T. infestans (BOL DM)	A	C	C	T	C	G	C	T
Number of pairwise differences	17	8	20	8	20	8	8	20

Dom, domestic; Syl, sylvatic; DM, dark morph; BA, Bahia; PR, Parana; CO, Cochabamba; RS, Rio Grande do Sul.
Source: Monteiro et al. (1999).

Population diversity can also be described by the number of haplotypes present. The term haplotype refers to a set of linked single nucleotide polymorphisms (SNPs) that are typically inherited together (in this case, a unique haploid DNA sequence). Haplotype diversity is a measure of the relative frequency of each individual sequence variant (haplotype) in a population.

8.4.2 Population Structure

The movement of individuals between populations is known as migration. When there is migration, and subsequent reproduction, it is referred to as effective migration. Effective migration is a synonym for gene flow between populations. When gene flow among populations is restricted, they are likely to become genetically structured due to localized effects of drift and selection.

A randomly breeding population is described as panmictic (Figure 8.1A), implying that all individuals have an equal chance to mate with any other member of that group. However, several factors can serve as barriers and subdivide a population into several smaller subpopulations with varying degrees of effective migration (Figure 8.1B).

Ancient events, such as continental drift and the uplift of mountain ranges, or more recent climatic changes, such as the Pleistocene glaciations and its ecological consequences, all have important roles in separating and isolating natural populations. In addition, present circumstances like environmental factors (the amount of rainfall or vegetation type), or human impacts (e.g., road construction, deforestation, development), or insect behavioral attributes, such as habitat or food source preference, all contribute to population subdivision.

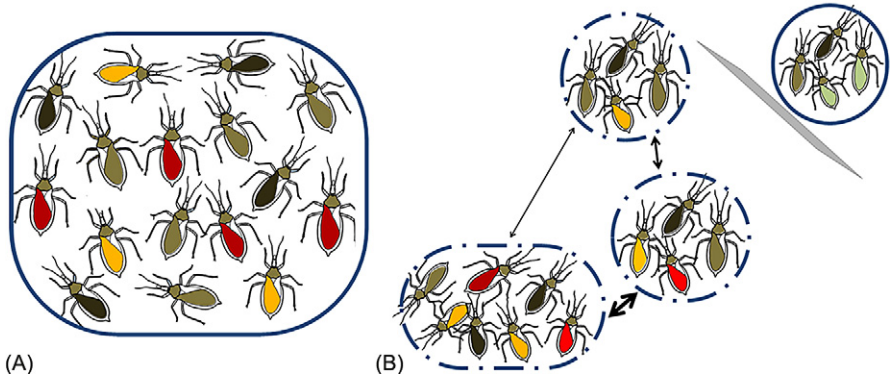

Figure 8.1 Varying degrees of population genetic structure. (A) panmixia (or lack of structure); (B) discrete subpopulations displaying different amounts of gene flow among them.

8.4.2.1 How Can We Quantify Population Subdivision?

Wright quantified the degree of population subdivision (genetic structure) in a hierarchical fashion, based on the reduction in heterozygosity that results when a population is separated into subpopulations. This measurement is relative to the hypothetical scenario of panmixia of the total population and is known as the "fixation index," F_{ST} (Wright, 1978).

$$F_{ST} = (H_T \quad H_S)/H_T$$

H_S = average heterozygosity among all subpopulations
H_T = hypothetical heterozygosity in the total population

F_{ST} values range from 0 (no structure) to 1 (complete differentiation among subpopulations), and are calculated per locus between all subpopulations studied. When there is no population structure (equal allele frequencies in all subpopulations) the mean $H_S = H_T$ (there is no loss of heterozygosity), and $F_{ST} = 0$. When there is structure (subpopulations with different allele frequencies), the mean $H_S < H_T$ (some variability would have been lost due to structure), and $F_{ST} > 0$.

This statistic can be estimated using allozyme or DNA sequence data based on the infinite alleles model (Tajima, 1996). This model assumes that a mutation can generate any new allele in the population. A modified version of the F_{ST} statistic, called R_{ST}, was developed by Slatkin (1995) for microsatellite data. This statistic is based on the stepwise mutation model that assumes that alleles of similar size are more closely related.

Wright (1978) suggests the following ranges as guidelines for the interpretation of F_{ST} values:

0 0.05 indicates little genetic differentiation
0.05 0.15 indicates moderate genetic differentiation
0.15 0.25 indicates great genetic differentiation
>0.25 indicates very great genetic differentiation

To illustrate how F_{ST} is calculated, we will again use data from the *Pgd* allozyme locus of Brazilian *T. sordida*, but now including all four subpopulations (Table 8.4;

Table 8.4 Allele Frequencies and Sample Sizes per Subpopulation

Pgd	**Espinosa**	**Mamonas**	**Januária**	**Corinto**
Allele F	0.22	0.33	0.23	0.76
Allele S	0.78	0.67	0.77	0.24
	($n = 68$)	($n = 32$)	($n = 37$)	($n = 35$)

Monteiro et al., 2009). Subpopulations were defined at the locality level and each was composed of insects collected from the peridomicile of 10 houses.

As a reminder,

$$F_{ST} = (H_T - H_S)/H_T$$

H_S = average heterozygosity among all subpopulation
H_T = hypothetical heterozygosity in the total population

So, let us first calculate H_e for each of the four subpopulations (H_S)

H_S = average heterozygosity among all subpopulation

$$\begin{aligned} H_S \text{ Espinosa} &= 2pq = 2 \times (0.22)(0.78) = 0.34 \\ H_S \text{ Mamonas} &= 2pq = 2 \times (0.33)(0.67) = 0.44 \\ H_S \text{ Januária} &= 2pq = 2 \times (0.23)(0.77) = 0.35 \\ H_S \text{ Corinto} &= 2pq = 2 \times (0.76)(0.24) = 0.33 \end{aligned}$$

Average $H_S = (0.34 + 0.44 + 0.35 + 0.33)/4 = 0.37$

Now, let us calculate H_e for all four subpopulations combined as if they belonged to a single large population ($\boldsymbol{H_T}$):

H_T = hypothetical heterozygosity in the total population ($n = 172$)

Freq F allele (p) = $\left(2 \times \text{homozygotes F/F} + \text{heterozygotes S/F}\right)/2N$
$N = 172$

And to calculate q, it is simply $q = 1 - p$.

So, for our example, $p = [(2 \times 87$ F/F individuals) + 50 S/F]/2×172 total individuals = 0.65, $q = 1 \quad p = 0.35$.

$$H_T = 2pq = 2 \times (0.65)(0.35) = 0.46$$

(Note that H_T is larger than average H_S, indicating that population substructure is contributing to a loss in total heterozygosity.)

Now, if we substitute the values in the F_{ST} formula

$$\begin{aligned} F_{ST} &= (H_T \quad H_S)/H_T \\ F_{ST} &= (0.46 \quad 0.37)/0.46 \end{aligned}$$

We find that

$$F_{ST} = 0.20$$

This indicates that there is "great genetic differentiation" among the four *T. sordida* subpopulations studied from Brazil, according to Wright's guidelines (1978), and that evidently, they do not belong to the same panmictic unit. It also indicates that population substructure is causing a 20% loss in total heterozygosity.

However, regardless of the magnitude of the F_{ST} value obtained, its statistical significance needs to be tested. Only F_{ST} values that are significantly different from zero are amenable to be biologically interpreted. Moreover, care must be taken when comparing F_{ST} values of different organisms, especially when they differ in vagility.

F_{ST} can be used to estimate the number of mating migrants per generation between subpopulations (assuming that all subpopulations satisfy the conditions of the infinite island model of population structure, and that they are in migration-drift equilibrium):

$$F_{ST} = 1/(1 + 4Nm)$$

which, when rearranged, becomes:

$$Nm = (1 - F_{ST})/4F_{ST}$$

where

N = effective population size (i.e., breeding adults)
m = migration rate between populations (proportion of alleles that are replaced by alleles from migrant organisms each generation)

If the F_{ST} values suggest that there is subpopulation heterogeneity, it might be important to investigate whether this heterogeneity is part of a clinal variation. Analysis of individuals in the geographic region between subpopulations may reveal: (a) a mixture of the two subpopulations, (b) yet a third subpopulation, or (c) intermediate forms between the two subpopulations, in which case the **isolation by distance model** should be tested.

The F_{ST} index can be considered a good measure of the overall picture of population substructure. It has also been used to estimate rates of gene flow among subpopulations, as presented earlier. However, there are conditions under which F_{ST} is inappropriate for gene flow estimation and can lead to incorrect conclusions (Neigel, 2002). In such cases, more recent programs based on the coalescent theory such as Migrate-n (Beerli, 2006), or Isolation with Migration, (Hey and Nielsen, 2004), should give better results.

Population genetic structure can be studied using many different statistical analyses and programs (Excoffier and Heckel, 2006). One of the most frequently used programs is Arlequin (Schneider et al., 2005). It handles both genotype and sequence data, does hierarchical analysis of molecular variance (AMOVA), and assesses degrees of population subdivision (F_{ST} and R_{ST}).

The Mantel test (Mantel, 1967), as implemented in Arlequin, can be used to evaluate the statistical significance of the correlation between genetic distance (for diploid/**codominant** data sets) and geographic distance (isolation by distance

model). To test for isolation by distance with DNA sequence data, a good option is to use the Isolation by Distance Web Service (http://www.bio.sdsu.edu/pub/andy/IBD.html). A Bayesian clustering analysis can reveal genetic differentiation in the population and is available in the software Structure (Pritchard et al., 2000). Relatedness among individuals can be determined using the Relatedness software (Queller and Goodknight, 1989).

8.5 Molecular Markers

8.5.1 Allozymes

Allozymes are enzymes with identical function but distinct electrophoretic migration patterns that are encoded by different alleles of the same locus. Allozyme electrophoresis provided the earliest assessment of the genetic variability of natural triatomine populations. Not more than a decade ago, allozymes were the markers of choice in triatomine molecular systematics, accounting for nearly 65% of the published work (Monteiro et al., 2001). Allozyme markers have, however, been gradually substituted by the more variable (and also codominant) PCR-based microsatellites (described below).

To perform allozyme analysis, protein extracts are isolated from each individual being studied, applied to a support medium (traditionally starch gels) and submitted to electrophoresis. This separates proteins based on charge and size. Gels are then histochemically stained for a particular enzyme. Discrete bands are produced for different allozymes encoded by their respective alleles for that locus. The next step is to genetically interpret the pattern obtained for each individual assayed. This technique enables the genotyping of several specimens and loci simultaneously. As allozymes are codominant markers (i.e., both alleles are expressed in a given locus, which permits the identification of all alleles present in the sample), populations can be tested directly for deviations from the HWE.

The drawbacks include the need for fresh or frozen samples and for considerable practice with the methodology and gel scoring. There is also a particular weakness with respect to triatomines; while there is substantial variation in other insect groups such as mosquitoes and sandflies, the variability is very low in triatomines.

8.5.2 Microsatellites

Microsatellites are neutral (i.e., not under positive selection), noncoding, fast-evolving, codominant markers. They consist of two to five nucleotide motifs repeated *in tandem* (e.g., ACACAC. . .) (Figure 8.2). The markers' high variability results from polymerase slippage during DNA replication, leading to the insertion (or deletion) of a motif in a given individual. Recombination further increases microsatellite variability. A microsatellite locus is PCR-amplified with the use of primers that anneal to the more conserved 5′ and 3′ regions that flank the repeat units. Individuals presenting both alleles with the same number of repeats are

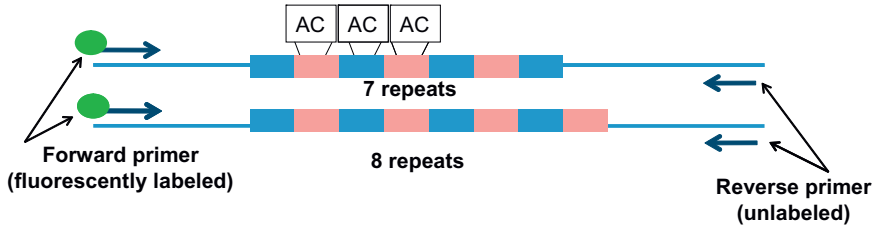

Figure 8.2 Schematic representation of the amplification of a microsatellite locus (ACACAC, etc., in this example). Alleles differ in number of repeat units and are identified as different sized bands after amplification using the primers that flank such units.

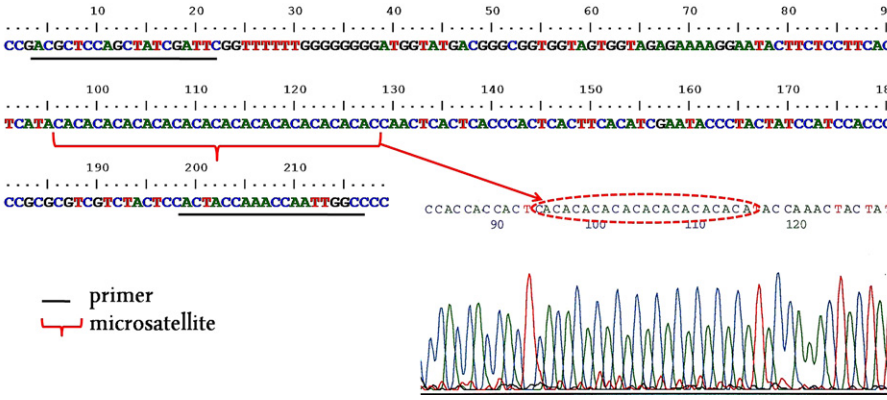

Figure 8.3 A microsatellite locus seen at the nucleotide level. Note that the primer binding sites (underlined in black) that flank the AC repeat region (or the microsatellite itself; an $(AC)_{17}$ allele) do not need to be contiguous to it. A chromatogram of a microsatellite locus is shown in the lower right portion of the figure.

homozygous, and individuals with different-sized alleles are heterozygous for that locus (Figures 8.2 and 8.3).

The presence of "null alleles" can be a concern. "Null alleles" are caused by mutations in the primer-binding site, which prevent annealing. Thus, no amplification will result for that particular allele. This may lead to underestimates of heterozygosity levels (Lehmann et al., 2003).

Therefore, an evaluation of each locus should be conducted before its use in population genetics studies, including the testing for linkage disequilibrium to determine if loci are independent, evaluation of polymorphism in the sample, and PCR failure rate. The analysis of several loci (>10) is recommended in order to avoid biases.

Microsatellites are currently the most appropriate markers for population genetics studies. A weakness of these markers is that the development of the primers that will amplify the loci can be costly and time-consuming. These markers have

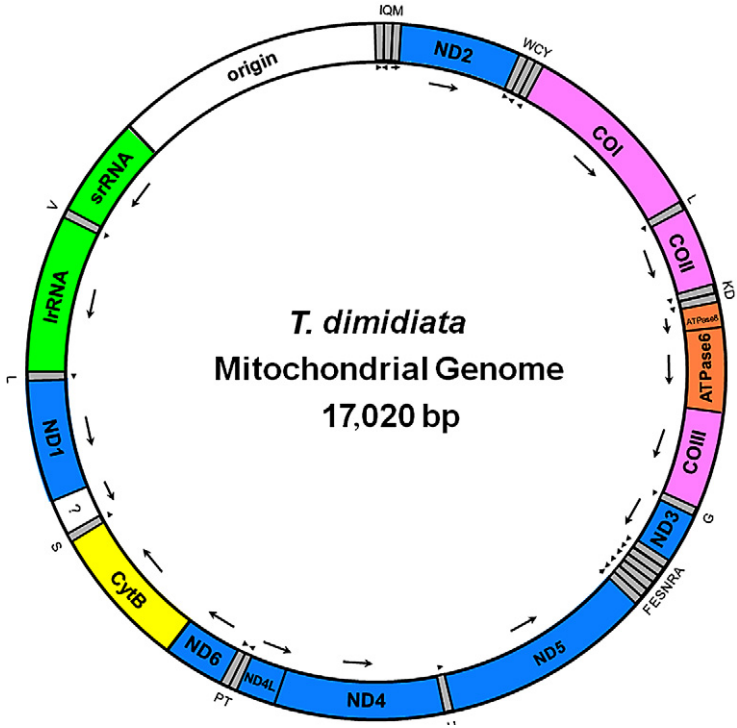

Figure 8.4 Schematic representation of the mitochondrial genome of *T. dimidiata*. (from Dotson and Beard 2001).

already been developed for several triatomine species: *R. pallescens* (Harry et al., 1998), *R. prolixus* (Harry et al., 2008b), *T. dimidiata* (Anderson et al., 2002), *T. infestans* (García et al., 2004; Marcet et al., 2006), and *T. pseudomaculata* (Harry et al., 2008a).

8.5.3 DNA Sequencing

In terms of objectivity and resolution, DNA sequencing is the ultimate molecular technique because it provides information on every single nucleotide of a given genomic fragment. In addition, the availability of different genomes (e.g., mitochondrial and nuclear, in the case of insects), and of genes with a wide range of degrees of conservation, means that any problem in systematics can be addressed.

Mitochondrial DNA (Figure 8.4) has been very important in the understanding of Triatominae taxonomy and phylogeny (Garcia and Powell, 1998; Lyman et al., 1999; Monteiro et al., 1999), phylogeography (Monteiro et al., 2004; Piccinali et al., 2009), and population structure (Giordano et al., 2005).

The mitochondrial genome evolves approximately 10 times faster than the nuclear genome, making it a very good source of markers, particularly for the

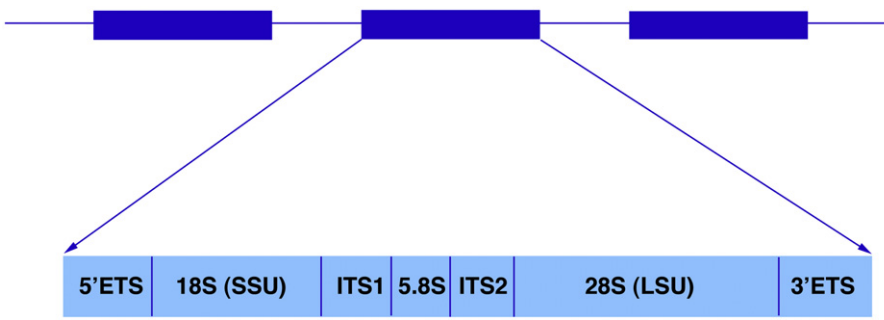

Figure 8.5 Schematic representation of the nuclear rDNA array (above) and its primary transcript (below).

comparison of closely related species, and for phylogeographical and population genetics studies. Its haploid nature is also helpful as complications in the analyses due to crossing-over are not a concern. However, as mitochondria are maternally inherited, it should be kept in mind that when measuring and discussing gene flow, we are making inferences on the movement of females only.

Ribosomal DNA (rDNA) sequences have also been useful in triatomine systematics. The rDNA cistron encodes three structural parts of the ribosome itself (18S, 5.8S, and 28S, in that order), which are interspersed by two internal transcribed spacers (ITS1 and 2, Figure 8.5).

Each of these complete units is separated by an external transcribed spacer (ETS). Units might be present in high copy number (over 100 copies per nuclear genome), making the rDNA a good PCR target. Independent mutations that arise in different copies tend to be homogenized through a process known as concerted evolution. However, this process apparently does not work effectively for some taxa, which might lead to intraindividual variation. When this occurs, an extra step of cloning will be required in the DNA sequencing methodology.

The variable ITS2 has been the rDNA region most often used in phylogeography and population level studies in triatomines. Its use has led to the discovery of **cryptic species** (Dorn et al., 2007; Bargues et al., 2008), **hybrids** (Herrera-Aguilar et al., 2009), and **introgression** events (Mas-Coma and Bargues, 2009). In triatomine molecular systematics studies in general, mitochondrial DNA markers have been applied more than twice as much as nuclear rDNA (16 papers used mtDNA, 7 used rDNA, and 5 used both).

8.6 Study Design and Marker Choice

8.6.1 Sampling Strategy

The first step of a population genetics study is the definition of the question(s) to be addressed, like the ones mentioned in the introduction of the chapter.

The next step is to delineate an appropriate sampling strategy. The geographic scale of the sample collection, the number of study sites, and number of specimens to be collected per site are, of course, dependent upon the question asked. For example, the large-scale question "Are there any cryptic species within the nominal species I am studying?" requires collections over a large geographic area, perhaps the entire range of the species. If the question is, "Are insects from sylvatic sites reinfesting treated houses?" insects may be collected in the surrounding forest and houses in a village or several villages (smaller, regional scale). If the question is, "Are animal corrals and other **peridomestic** structures the source of reinfesting bugs?" then insects may be collected from houses and the peridomestic areas that surround them within a village (even smaller, locality scale).

Since population genetics studies measure the differences in allele frequencies among subpopulations, a pilot study might be needed to assess the amount of variation present. For population genetics studies in which small differences in allele frequencies are important, a large enough sample size is needed to accurately represent the diversity in the subpopulation.

8.6.2 Choice of Genetic Marker

Once the research question and adequate sampling strategy have been determined, the next step is to decide which genetic marker(s) use. The resolution of a given marker depends on its degree of functional constraint. Protein coding genes that play vital biological roles are usually more conserved due to the strong purifying selection to which they are subjected. Genetic markers subjected to less constraint will evolve faster and therefore be more variable.

There is no "perfect" marker. However, microsatellite loci and DNA sequences (both mitochondrial and nuclear) are currently the most commonly used markers in triatomine population genetics.

It is important when comparing results of triatomine population studies to keep in mind the geographic scale, the sampling strategy used, and the resolution of the marker. For example, it is not valid to compare the amount of genetic variation between different studies when in one the bugs from a single village represent a population, while in the other the population is represented by bugs from several villages in multiple countries. Likewise, results of studies based on allozyme markers should not be compared with those from microsatellites.

The existence of hidden species-level variation within nominal species can further confound results. Recent studies have revealed that several Chagas vector species actually comprise cryptic **species complexes** which, if unrecognized, can result in an overestimation of the diversity present (Marcilla et al., 2001; Monteiro et al., 2003, 2004; Dorn et al., 2007; Bargues et al., 2008; Herrera-Aguilar et al., 2009; Martinez-Hernandez et al., 2010).

8.7 Genetic Variation and Population Structure of *R. prolixus*, *T. dimidiata*, and *T. infestans*

8.7.1 R. prolixus

8.7.1.1 A Historical Introduction

R. prolixus is the primary Chagas disease vector in Venezuela, Colombia, and certain areas of Central America (Figure 8.6).

The main reason for its epidemiological relevance is its high degree of adaptability for living in human habitations, where it can build up very large colonies (Sandoval et al., 2000). Control efforts against *R. prolixus*, although quite successful in Central America, have been hampered in South America due to taxonomic uncertainty. It was initially unclear if *R. prolixus* was in fact the same species as the morphologically indistinguishable *R. robustus*.

The validity of the species *R. prolixus* and *R. robustus* has long been a matter of controversy, especially in Venezuela, where they occur in high numbers in both houses and palms (Gamboa, 1963, 1973; Gomez-Nunez, 1963; Lent and Valderrama,

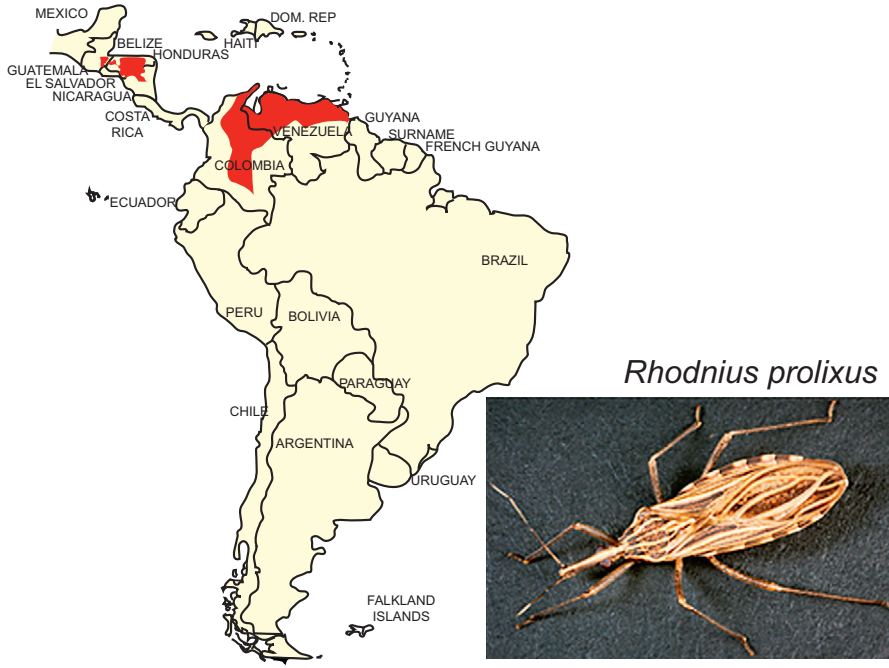

Figure 8.6 Approximate current distribution of *Rhodnius prolixus*.
Data from WHO (1989). Artist, Linda Waller; Photographer, James Gathany (Centers for Disease Control and Prevention).

1973). Lent and Wygodzinsky (1979) proposed slight dissimilarities in the male genitalia, and chromatic differences on the hind tibia of fourth- and fifth-stage nymphs as diagnostic characters for the separation of the two species (now known to present limitations; Monteiro et al., 2001). However, the observation that the smaller *R. prolixus* often forms large domestic colonies, whereas *R. robustus* seems to be exclusively sylvatic, led also to the use of "smaller size and domesticity" versus "larger size and non-domesticity" as a practical means to separate *R. robustus* from *R. prolixus* (Pavan and Monteiro, 2007). Nonetheless, the latter was often referred to as a domestic derivative of *R. robustus* (Schofield et al., 1999). Most importantly, it was believed that this domestication event occurred only once and that all *R. prolixus* populations became reproductively isolated from their sylvatic counterparts (Dujardin et al., 1998a). This taxonomic interpretation (*R. prolixus* as a recent domestic derivative of the sylvatic *R. robustus*) was advanced and accepted by the scientific community, even though genetic (allozymes) and cross-mating evidence showed otherwise (Harry, 1993; Barrett, 1995).

Epidemiologically, the possibility of *R. prolixus* and *R. robustus* being the same taxon was the worst imaginable, as it would imply in a scenario of potential free gene flow between insect populations from palms and houses, greatly complicating the logistics required to achieve the elimination of domestic vector populations. The realization of the importance of this taxonomic controversy stimulated researchers to search for alternative techniques to address the issue. DNA-based molecular markers came as a natural choice due to their efficacy in the guidance of control strategies (Miles et al., 2003).

The first markers used were RAPD-PCR (Garcia et al., 1998) and single-stranded conformational polymorphism (SSCP) (Stothard et al., 1998). The main limitation of the former, besides lack of reproducibility, was how to biologically interpret the genetic distance values obtained. The latter, although properly used to screen for variable samples to be sequenced (at a time when DNA sequencing was not as affordable as it is today), was not informative. Only with the use of mitochondrial DNA sequence analyses this issue was finally resolved. It was demonstrated that *R. prolixus* and *R. robustus* are separate species, and that *R. robustus* comprises a species complex (Lyman et al., 1999; Monteiro et al., 2000). Moreover, preliminary evidence indicated that, in Venezuela, *R. prolixus* was present not only in the domestic environment, but in adjacent sylvatic ecotopes (palm tree crowns) as well, thus potentially representing an important challenge to local insecticide-based vector control efforts (Monteiro et al., 2003).

Although these findings were of great taxonomic relevance, and broadened the understanding of vectorial disease transmission, they still did not conclusively prove that sylvatic and adjacent domestic *R. prolixus* specimens belonged to the same panmictic population (or gene pool), thus freely interchanging genes. The assessment of this kind of matter requires study designs that are based on population genetics concepts, which are presented later. The four members of the *R. robustus* species complex, on the other hand, are restricted to palm tree crowns. And although they are occasionally attracted to light in human habitations (Feliciangeli et al., 2002) they do not reproduce and form colonies inside houses.

8.7.1.2 *Genetic Variation in* R. prolixus *Populations*

Most of the available information on the levels of genetic variation of *R. prolixus* populations comes from allozyme studies of laboratory colony specimens, and thus should be interpreted with caution. The first studies were performed by Harry et al. (1992), based on 19−22 loci, on specimens collected from the Venezuelan states of Lara, Trujillo, Merida, Cojedes, and Guárico. Variability was low, with heterozygosity values ranging from $H_e = 0.053-0.227$ ($n = 22-43$). *R. prolixus* colony populations from Tolima, Colombia, also showed low variability ($H_e = 0.091$; $n = 41$, number of loci undetermined; Lopez et al., 1995). Another study by Dujardin et al. (1998a) on specimens from Honduras and Colombia revealed lack of variation, although sample sizes were small ($H_e = 0.0$; $n = 8-13$, 17 loci). The allozyme-based results of Monteiro et al. (2002) for samples from Cojedes, Venezuela, show the same trend ($H_e = 0.080$; $n = 12$, 12 loci).

In 2003, Monteiro and collaborators reported the first values of genetic variability for natural *R. prolixus* populations (Monteiro et al., 2003). Besides the novelty of using field-collected specimens, this study also represented a change in the way genetic variability was measured. First, because the samples used were collected according to a previously planned design; and second, because it was based on a more variable and objective marker a fragment of the mitochondrial gene cytochrome *b*. Eighteen insect samples were obtained from the Guatemalan villages of Tituque, Tuticopote, and Las Palmas, and also from Orica, Honduras. Tituque is separated from Tuticopote by a valley of 1 km in breadth, but these two villages are 4 km apart from Las Palmas. The three villages are approximately 250 km from Orica. Three houses were sampled from each Tituque and Tuticopote, whereas six houses were sampled from Las Palmas. Sampled houses were separated by at least 200 m. Two haplotypes were found, one in Guatemalan samples and one in Honduran samples. These differed from one another by a single substitution, leading to remarkably low levels of nucleotide diversity: $\pi = 0$ for populations of each country, and $\pi = 0.0006$ when combined. The addition of colony samples derived from six populations in Venezuela and two populations in Colombia only slightly increased nucleotide diversity ($\pi = 0.0008$).

Much higher levels of variability were reported in the recent comprehensive investigation conducted by Fitzpatrick et al. (2008) in Venezuela, where 551 specimens from 34 populations from six states were analyzed for the same *cyt b* fragment used in the previous study. Values of nucleotide diversity as high as $\pi = 0.002$ were obtained from samples from single palms, in contrast with the lower variability detected in samples from single houses. In contrast to the data from Mesoamerica, there was a high variability (six different haplotypes in total) when samples from more than one house per village were combined. This seems to indicate that house colonization by insects from nearby palms is not a frequent event.

8.7.1.3 *Genetic Structure of* R. prolixus

Data on the genetic structure of *R. prolixus* populations are scarce. The first study to estimate F_{ST} values for *R. prolixus* populations (Harry et al., 1992) was

conducted based on allozyme markers. However, as the specimens used originated from laboratory colonies, the biological interpretation of the genetic structure values obtained is compromised. Another study carried out by Lopez and Moreno (Lopez et al., 1995) estimated very high levels of genetic structure between domestic and sylvatic *R. prolixus* populations from Tolima, Colombia, also based on allozymes ($F_{ST} = 0.598$), indicating lack of gene flow. However, it was later shown that the alleged sylvatic *R. prolixus* population used (then referred to as "Tolima form," due to its unusual characteristics) was actually a new *Rhodnius* species, later described as *Rhodnius colombiensis* (Mejia et al., 1999).

The sole available study to date to evaluate the genetic structure of natural *R. prolixus* populations is the same mentioned in the earlier section, by Fitzpatrick et al. (2008), carried out in Venezuela. The comparison of all 34 populations analyzed revealed that populations are highly structured throughout the country ($F_{ST} = 0.44$). But most importantly, the authors not only showed that in the six Venezuelan states studied, *R. prolixus* is present in both rural houses and adjacent palms, but also demonstrated the existence of gene flow between populations found in palms and nearby houses (in five adjacent ecotopes in Barinas and Portuguesa). F_{ST} and AMOVA analyses of data for 10 microsatellite loci corroborated these results. The relationship between genetic isolation and increasing geographic distances was also investigated. Although the tests between populations, between localities, and within states were significant, distance was ruled out as a possible causative agent as correlations were weak. These findings indicate that sylvatic *R. prolixus* populations constitute a real challenge for vector control actions, as they will serve as a perennial source of migrants to recolonize insecticide-treated areas.

8.7.1.4 Was R. prolixus *Introduced into Central America from South America?*

If few individuals of a population disperse and colonize a new geographic area, this new subpopulation is likely to have less genetic variability than the original population. This phenomenon is called founder effect.

It was proposed that *R. prolixus* was introduced in Central America from northern South America because it showed a reduced genetic diversity compared with samples from South America (Dujardin et al., 1998a). However, this proposal rests on the RAPD-PCR analysis of 13 specimens from Honduras, and 8 specimens from Colombia (each sample set possibly coming from a single house), not a large enough sample size to adequately allow for the assessment of the levels of genetic diversity of *R. prolixus* in the referred region. The results of the analyses of 17 allozyme loci were uninformative because no variability was detected, and to further complicate the issue, morphometrics results actually show a greater variability for the Honduran samples (Dujardin et al., 1998a).

The migration of storks (*Mycteria americana*) carrying *R. prolixus* eggs and nymphs from South America to El Salvador, Honduras, and Guatemala, is tentatively presented as an explanation for the vectors' absence in Panama and Costa Rica (Gamboa, 1962, 1963). The problem here is that the storks nest in palm trees, but no sylvatic *R. prolixus* population was ever found in Central America. Another hypothesis suggests that the insects could have been disseminated throughout Central America as a consequence of an accidental escape from laboratory colonies (Schofield and Dujardin, 1997).

This particular issue exemplifies one of many fascinating unresolved questions in triatomine biogeography that are waiting to be properly addressed with appropriate population genetics markers, sampling strategy, and analytical tools.

Knowledge on the genetic structure of natural *R. prolixus* populations is likely to advance quickly, now that primers for 20 microsatellite *loci* have been described (Fitzpatrick et al., 2009; Harry et al., 2008b).

Another important scientific accomplishment that will certainly revolutionize the field is the release of the *R. prolixus* genome (http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/RevisedRhodniusSeq.pdf), which will allow for the selection of many new nuclear markers.

8.7.2 T. dimidiata

8.7.2.1 Introduction

T. dimidiata is the most important Chagas vector in Mesoamerica, and its distribution ranges from southern Mexico, across all of Central America, and extending into northern South America (Figure 8.7).

Unlike the mostly domestic nature of *T. infestans*, *T. dimidiata* occupies domestic, peridomestic, and sylvatic ecotopes; interestingly, different populations show quite distinct behaviors. *T. dimidiata* is nearly exclusively domestic in southeastern Guatemala, sylvatic with occasional entry into houses in the Peten region in northern Guatemala (Monroy et al., 2003), and it seasonally enters houses in the Yucatan, Mexico (Dumonteil et al., 2002). In fact, this species varies enormously in morphologic, behavioral, phenotypic, and genetic characteristics across its range (reviewed in Dorn et al., 2007). Many attributes have significant implications for the epidemiological importance of particular populations (e.g., domestic habitat or preference for human blood). Its diversity across its range and presence in multiple ecotopes make it quite difficult to control. Recently, *T. dimidiata* has been proposed to be a species complex (a group of morphologically similar species) (Panzera et al., 2006). At least one cryptic species has been identified (Dorn et al., 2007; Bargues et al., 2008), which seems to extensively hybridize with *T. dimidiata sensu stricto* (Herrera-Aguilar et al., 2009).

8.7.2.2 Genetic Variation in T. dimidiata *Populations*

As far as we are aware, there is only one study on *T. dimidiata* using allozymes. In three populations ($n = 18$, 1−13 per community), from two states in Mexico, *T. dimidiata*

Figure 8.7 Approximate current distribution of *T. dimidiata*.
Data from WHO (1989). Artist, Linda Waller; Photographer, James Gathany (Centers for Disease Control and Prevention).

showed a high number of **polymorphic loci**, P (0.95) = 0.50 (17 loci, insects collected in domestic and peridomestic habitats) (Flores et al., 2001). This is similar to other triatomine species in Mexico; most showed a polymorphism of P (0.95) = 0.29 0.5 (n = 28–39, 17 loci) (Flores et al., 2001). This is reported to be higher than levels found in *T. infestans* and many other South American *Triatoma* species (Pereira et al., 1996; Costa et al., 1997; Noireau et al., 1998); however, these other studies used different sample sizes and sampling strategies, so comparisons must be done with caution. In this *T. dimidiata* study, no heterozygotes were observed, and the mean expected heterozygosity was H_e = 0.187 (Flores et al., 2001). Genetic diversity within houses or villages was higher by RAPD-PCR (H_e = 0.299–0.326, P (0.95) = 0.97–1; H_e = 0.273–0.301, P (0.95) = 0.79–0.97, respectively), as might be expected by a higher resolution marker, in two villages in southeastern Guatemala (n = 35–53 per house and n = 33 or 66 per village).

8.7.2.3 Genetic Structure of T. dimidiata *Populations*

T. dimidiata is quite diverse across its geographic range, resulting in splitting and merging of the species several times since its discovery (reviewed in Dorn et al.,

2007). A cryptic species of *T. dimidiata* was suggested by many phenotypic and genetic markers (reviewed in Dorn et al., 2007) and confirmed by an $F_{ST} = 0.802$ (nearly 1!) using ITS2 DNA sequence comparisons among *T. dimidiata* "groups" (Bargues et al., 2008). This cryptic species has been identified in Peten, Guatemala; Yucatan, Mexico; and Belize (Dorn et al., 2007, 2009; Bargues et al., 2008). Therefore, to understand genetic differentiation among *T. dimidiata* subpopulations, one must take this taxonomic discovery into account and determine the identity of the samples before they are used.

Among nearby domestic populations in Guatemala (where the cryptic species has not been found), there appears to be high migration among houses within villages and among nearby villages. Populations of *T. dimidiata* from three houses within one Guatemalan village showed low genetic differentiation, Nei's (1978) genetic distance (D) = 0.018 and $F_{ST} = 0.025$, by RAPD-PCR markers ($n = 133$, distance separated <0.2 km, 29 loci) (Dorn et al., 2003). This panmictic unit appears to extend at least to a nearby village (distance 27 km) as a low genetic distance, $D = 0.02$ and $F_{ST} = 0.019$ were observed between two villages ($n = 99$) (Dorn et al., 2003). These results translate into very high average number of mating migrants per generation, $Nm = 9.7$ and 12, among houses and villages, respectively. Another study aimed at testing the relatedness of individuals within a house in a Guatemalan village, based on 33 RAPD-PCR loci, revealed that nearly half of the individuals were unrelated. The average family size was 2.17 individuals ($n = 89$, 41 families), again suggesting substantial migration (Melgar et al., 2007). More recently, two *T. dimidiata* populations from Costa Rica were shown to be genetically differentiated based on *cyt b* sequences, likely due to an approximate 200 km separation ($n = 19$ and 39) (Blandon-Naranjo et al., 2010). Only 1/15 haplotypes were shared between the two locations.

A positive correlation between genetic distances among *T. dimidiata* populations and their respective geographic distances is in agreement with the Isolation by Distance model (Wright, 1943). Moderate genetic differentiation was observed among either domestic (mean $D = 0.072$; $F_{ST} = 0.097$) or sylvatic (mean $D = 0.161$; $F_{ST} = 0.135$) populations collected within different provinces in Guatemala. Great genetic differentiation was observed among subpopulations sampled across the country and from distinct habitats (mean $D = 0.141$; $F_{ST} = 0.175$) (Calderón et al., 2004). The sylvatic samples showed greater genetic diversity than did the domestic; however, they were also collected over a larger geographic area (Calderón et al., 2004). Nearby populations from distinct habitats (domestic, peridomestic, and caves, 10−200 m apart) showed low to moderate genetic differentiation among habitats (overall $F_{ST} = 0.07$) and movement of at least three mating migrants per generation, suggesting that peridomestic and sylvatic populations could pose a risk for transmission (Ramirez et al., 2005).

In the Yucatan Peninsula in Mexico, *T. dimidiata* enters houses seasonally but does establish domestic colonies. By microsatellite analysis of *T. dimidiata* in and around 14 villages ($n = 11-36$ per site), high migration among nearby

houses ($F_{ST} = 0.037$), more distant houses ($F_{ST} = 0.055$, out to 250 km) and between the forest and houses ($F_{ST} = 0.01-0.03$, 1−280 km) was observed (Dumonteil et al., 2007). (Migration among the longer distances is likely due to passive transport.) In addition, 10−22% of the bugs found inside houses came from peridomestic or sylvatic habitats. Conformity to the Isolation by Distance model was not observed. However, since not only *T. dimidiata* but also the recently described cryptic species occur in this area (along with their putative hybrids) (Herrera-Aguilar et al., 2009), these results must be interpreted with caution.

8.7.2.4 *Implications for Control and Future Studies for* T. dimidiata

Therefore, from the limited data we have available, *T. dimidiata* appears to be a species with generally a higher diversity and migration rate than *T. infestans*. In localities where it is exclusively domestic and peridomestic, *T. dimidiata* moves readily among houses within villages and among nearby villages. Where sylvatic populations exist, insects can seasonally enter homes and thus pose a risk for transmission. For long-distance dispersal, the passive transportation of insects (or eggs) in human belongings or agricultural products is important. Increasing genetic differentiation correlates with geographic distance consistent with the isolation by distance model.

Because of the extensive peridomestic and sylvatic populations and the high migration rate, simply spraying insecticide in houses is unlikely to be effective. House improvements, community participation, and control strategies designed for the local situation will be more effective and sustainable. Population genetics studies are needed to determine the geographic range of differentiated subpopulations and their epidemiological importance. In addition, it will be important to determine the factors (e.g., geographic, environmental, anthropogenic) that are keeping subpopulations apart.

8.7.3 T. infestans

8.7.3.1 *The Hypothesized Origin of* T. infestans *Populations*

T. infestans (Figure 8.8; Schofield et al., 2006) is highly adapted to human dwellings.

Until recently, sylvatic *T. infestans* foci were only known in the Andean valleys of Cochabamba, Bolivia, leading to the assumption that this region was the center of origin and dispersion for the species (Schofield, 1988). At the time, two hypotheses were advanced to explain the origin of the wild insect populations: (a) they were a relic of the ancestral population from which domestic *T. infestans* derived; or (b) they represented feral populations (Dujardin et al., 1987). Interestingly, studies based on various molecular markers did not detect significant differences between domestic and sylvatic populations from the area (Dujardin et al., 1987; Lyman et al., 1999; Panzera et al., 2004; Giordano et al., 2005). However, microsatellite loci had shown a significant differentiation between one domestic population and sylvatic bugs from three

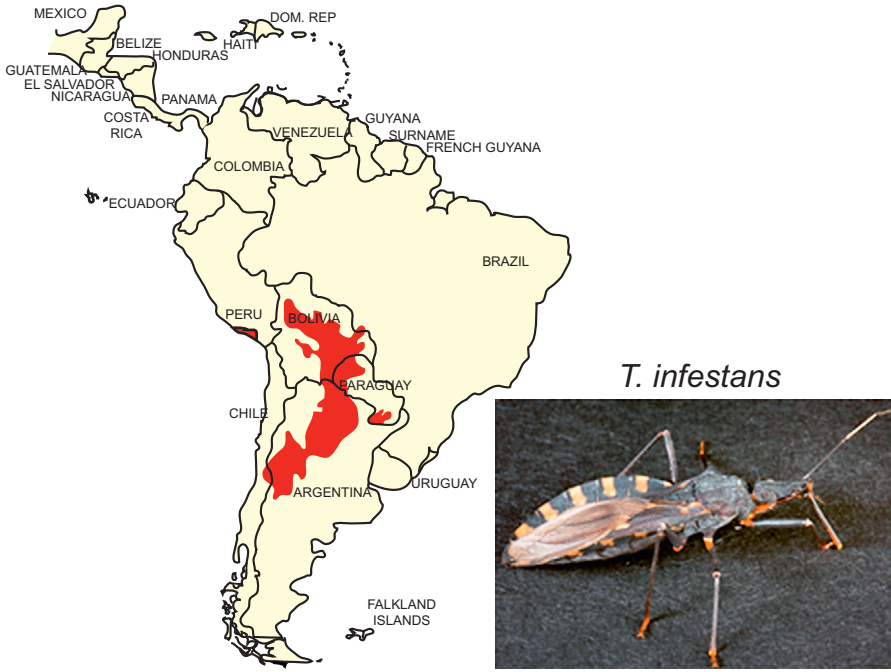

Figure 8.8 Approximate current distribution of *T. infestans*.
Data from Schofield et al. (2006). Artist, Linda Waller; Photographer, James Gathany (Centers for Disease Control and Prevention).

capture sites from the surrounding areas of the Cotapachi locality, Bolivia (Richer et al., 2007). This last observation should be interpreted with caution because the domestic population analyzed was a pool of 10 individuals captured in two houses 1 year after spraying.

Two cytogenetically distinct forms of *T. infestans* have been described: one from the Andean highlands (Bolivia and Peru), and another "non-Andean" from the lowlands further east (in Argentina, Paraguay, Brazil, Uruguay, and the Bolivian Chaco). Andean populations have, on average, 30% more haploid DNA and 50% more heterochromatin than the non-Andean populations (Panzera et al., 2004). The authors hypothesize that during the species dispersion from the Andean region toward the lowlands a reduction of the genome size occurred.

ITS2 results support this separation. Andean individuals shared a single haplotype, different from the four haplotypes found in non-Andean populations (with one exception). The authors also found a correlation between the presence/absence of minisatellites of the ITS1 and 2 and larger/smaller genome size (Bargues et al., 2006). Moreover, sequencing of the mitochondrial genes *cyt b* (Monteiro et al., 1999), and cytochrome oxidase I (COI) (Piccinali et al., 2009), have shown

significant levels of nucleotide differentiation between the two forms, suggesting a long-established genetic isolation.

However, the recent finding of genetically intermediate forms in the Chaco area (Panzera et al., 2007; Noireau, 2009) further complicates the clear-cut division and underscores the need for a thorough genetic analysis with independent molecular markers in populations across *T. infestans* distribution. The hypotheses to be tested now are: (a) these intermediate forms are natural occurrences within a genome-reduction gradient (isolation by distance model), or (b) they are the product of **hybridization** by secondary contact of Andean with non-Andean insects (Panzera et al., 2007).

8.7.3.2 Other Wild Populations of T. infestans

The first "wild" melanic form of *T. infestans* was discovered in peridomestic trees in the humid forest of Misiones, Argentina. It was initially considered to represent a subspecies of *T. infestans* (*T. infestans melanosoma*; Martinez et al., 1987), to be later raised to the species level as *T. melanosoma* (Lent et al., 1994). However, a comprehensive analysis has recently placed it as a chromatic variant of *T. infestans* (Gumiel et al., 2003).

Wild populations of *T. infestans* were later discovered in bromeliads, parrot nests, and hollow trees in the Bolivian Chaco (Noireau et al., 1997, 2000b). These bugs were much darker and had smaller yellowish spots on the connexivum than the typical *T. infestans*. They occurred in isolated dry forests, very far from the closest human settlements. The finding of these sylvatic "dark morphs" (Carcavallo, 1998) in the dry subtropical Chaco region challenged the traditional view of the Andean valleys in Bolivia as the center of origin of *T. infestans* (Carcavallo et al., 2000; Noireau et al., 2005) suggesting that these dark morph populations may be the most ancient ones.

Evidence from different molecular markers (ITS2, COI, *cyt b*) repeatedly provided evidence for the rejection of this new hypothesis (Monteiro et al., 1999; Bargues et al., 2006; Piccinali et al., 2009). However, there is yet no final agreement on whether these darker bug populations from the dry Chaco are a result of the recolonization of sylvatic habitats by domestic bug populations from the same region (Dujardin et al., 1998b; Noireau et al., 2000a; Panzera et al., 2004; Bargues et al., 2006) or if they are consequence of a direct expansion from the Andean populations (Monteiro et al., 1999; Piccinali et al., 2009). Several unique mtDNA COI mutations in the dark morph lineage (Piccinali et al., 2009) indicate a strong isolation between the dark morph from the Chaco and all other domestic and sylvatic Andean *T. infestans* populations (and also from all non-Andean domestic populations).

Outside Bolivia, *T. infestans* was collected in sylvatic habitats in Brazil (Barretto et al., 1963), Chile (Bacigalupo et al., 2006), and Argentina (Ceballos et al., 2009). *T. infestans* nymphs were also recently found in the interface between peridomestic and sylvatic habitats in the Paraguayan Chaco (Yeo et al., 2005). Further genetic assessment on these populations is needed in order to determine

how they relate to domestic populations. Nevertheless, these reports indicate that sylvatic *T. infestans* populations may be much more widespread than previously assumed, and call for further research on their role in the recolonization of insecticide-treated villages (Noireau et al., 2005; Bacigalupo et al., 2006).

8.7.3.3 Genetic Variation and Genetic Structure in T. infestans *Populations*

Diversity

The varying levels of genetic diversity estimated for *T. infestans* populations in several studies could result from either differences in the resolution of the markers used, or natural variation in diversity across the distribution range of the species. Allozyme analyses have revealed somewhat contradictory results. Low levels of heterozygosity and polymorphism were found in four field (wild and domestic) natural populations from Cochabamba, Bolivia ($H_e = 0.024-0.047$; $P = 0.1$) (Dujardin et al., 1987) or 22 Andean populations from Peru and Bolivia ($H_e = 0.020-0.047$; $P = 0.16$) (Dujardin et al., 1998b). In contrast, Garcia and colleagues (Garcia et al., 1995a,b) using 17 allozyme loci, detected higher levels of variability in bugs from colonies from different provinces of Argentina and Brazil, with values of heterozygosity and polymorphism that fell within or above values previously reported for other insects ($P = 0.41-0.71$, mean 0.59; $H_e = 0.08-0.12$, mean 0.095). Authors attributed the higher levels of variability observed to differences in the method used to process the samples, and to the fact that the colonies were founded with individuals from different subpopulations, thus artificially increasing variability (Garcia et al., 1995b).

Results from later studies using markers with higher resolution showed greater genetic variability in natural *T. infestans* populations and heterogeneity in the diversity estimations across the species range. Monteiro et al. (1999) were the first to analyze nucleotide variation of the mitochondrial *cyt b* gene. Though analyzing only one to six bugs per population, they detected two main clusters: one with haplotypes from Bolivia, and another with haplotypes specific to Argentina and Brazil. Subsequent studies using *cyt b* on four populations (defined at the province level and containing bugs from 30 localities) from Chuquisaca, Bolivia, showed substantial haplotype diversity per region (province) ranging from 0.29 to 0.67 (Giordano et al., 2005). Another comprehensive study including several locations across the *T. infestans* distribution, based on a different mtDNA marker, the COI gene, showed 48 variable sites distributed in 37 haplotypes, and great heterogeneity among sampled locations, with haplotype diversity ranging from 0.23 to 0.84, and π from 0.0052 to 0.0012 (Piccinali et al., 2009). Moreover, the DNA sequence comparisons of the 12S and 16S (878 bp) mtDNA genes of 40 specimens from 4 provinces of Argentina (Garcia et al., 2003) revealed 13 haplotypes determined by 17 variable sites. The percentage of within-populations nucleotide variation ranged between 5.6% and 94.4%. Later, the same authors, applying the same marker, evaluated more specimens from 11 more populations (defined at locality level) and detected a total of 18 haplotypes constituted by 21 variables sites, whereas nucleotide and

haplotype variation ranged from 0% to 0.84% and from 0% to 0.29%, respectively (Segura et al., 2009).

On the other hand, the estimations of total intraspecific variability (absolute nucleotide differences) based on ITS1 for *T. infestans* was 2.70% (13/482), which is the highest hitherto known among Triatominii species (Bargues et al., 2006). This marker also revealed heterogeneity in the diversity estimations among populations (defined at the province or location level) with π ranging between 0.0 and 0.8 and H_e from 0.0 to 3.2. The populations from Bolivia and Argentina presented the highest genetic diversity.

Microsatellite markers (applied at macro- and micro-scales of analysis) also revealed high variability. The variation was unevenly distributed throughout the samples studied. Between 80% and 90% corresponded to the "among individuals" level, 7.8−10% were among houses within a region (i.e., group of villages), and only 3% were found among regions (Marcet et al., 2008; Pizarro et al., 2008).

Genetic Structure

Allozymes results showed the same three polymorphic loci throughout Bolivia, Peru (Dujardin et al., 1998b), and Uruguay (Pereira et al., 1996).

Results from mtDNA loci of *T. infestans* populations from several locations of Argentina and Bolivia, including Andean and non-Andean samples, revealed the existence of few (two or three) high-frequency haplotypes (Monteiro et al., 1999; Garcia et al., 2003; Giordano et al., 2005; Piccinali et al., 2009; Segura et al., 2009). From these, several low-frequency similar haplotypes (differing in one or two nucleotide substitutions) would stem, forming star-like haplotype networks.

A similar pattern was observed in a study based on rDNA ITS markers. There was one widely distributed composite ITS1/2 haplotype (CH2A), with a few slightly different low-frequency composite haplotypes in some of the studied locations in Argentina (Bargues et al., 2006). This general pattern, observed in various studies with different markers, suggests a recent population expansion (Dujardin and Tibayrenc, 1985), and a shallow population substructure due to local differentiation processes. Within Argentina, COI results from populations (defined at the province level) presented levels of polymorphism that departed from neutrality expectations, thus supporting the "recent expansion" hypothesis (Piccinali et al., 2009).

As mentioned, different definitions of *T. infestans* population have been used in different studies (i.e., country, province, locality, habitat, household), and there is yet no agreement on what would be the "real" size of the panmictic unit for this species. Employing allozymes in *T. infestans* populations from Argentina and Bolivia, results suggested that the panmictic unit is defined at the locality level or above (neighboring localities; Dujardin et al., 1987, 1998b); or is represented by individual houses or capture sites (Brenière et al., 1998). A microgeographical scale of analysis based on microsatellite markers revealed high levels of population substructure within a locality, including differentiation among neighboring households

as well as between domestic and peridomestic sites (Pérez de Rosas et al., 2007, 2008; Richer et al., 2007; Marcet et al., 2008; Pizarro et al., 2008).

Regional analyses encompass arbitrarily defined populations based on bug availability and do not necessarily represent a true panmictic unit. The conclusions and interpretations of these studies are therefore limited to the context of speciation, phylogenetics, and relatively old genetic processes. In contrast, microgeographic (local scale) studies are conducted with populations defined at the capture site level (houses or individual structures a corral), using markers with higher resolution. In this scale of analysis, in which the entire sample with all populations evaluated may fall within the dispersion range of the species, other factors (such as host availability or microclimatic conditions) may have more impact on the gene flow among populations than geographic distance alone.

Adjustment to the isolation by distance model of genetic differentiation was found at different geographic scales (houses, villages, provinces, or countries) using allozymes in Bolivia and Peru (Brenière et al., 1998; Dujardin et al., 1998b), mtDNA, and microsatellites in Argentina (Pérez de Rosas, 2007; Piccinali et al., 2009). However, lack of fit to the isolation by distance model was observed in *T. infestans* populations at the microgeographic scale (populations defined at houses or capture site level), based on microsatellite markers (Richer et al., 2007; Marcet et al., 2008; Pizarro et al., 2008). The contradiction between these results is due to the variation in the geographic scales involved, differences in the definition of a population, and resolution of the markers used.

These results emphasize the need to study *T. infestans* population structure at a microgeographical level (within a locality), and determine the panmictic unit within the sample set *a priori*, before establishing the typical genetic parameters to characterize population variability, structure, and gene flow. This can be done through a hierarchical AMOVA (Excoffier et al., 1992) to determine the contribution each level of analysis (alternative population level definitions; i.e., house, village) has on the global variability of the samples studied. The number of distinguishable populations (or genetic clusters) present in the sample can also be determined in advance by using a Bayesian approach (Pritchard et al., 2000; Falush et al., 2003, 2007).

Genetic Structure and Vector Control Actions Domestic *T. infestans* populations subjected to vector control actions repeatedly go through bottleneck events or are locally eliminated. Surprisingly, high levels of genetic variability were observed in populations from the Great Chaco and Bolivia despite intensive insecticide application (Garcia et al., 2003; Giordano et al., 2005; Pérez de Rosas et al., 2007; Marcet et al., 2008; Piccinali et al., 2009).

In this context, two studies addressed the influence of insecticide treatment on the genetic variability of the populations, using 10 microsatellite loci. The studies were conducted at the macrogeographical scale, involving samples from several provinces in Argentina (Pérez de Rosas et al., 2007), and at the microgeographical scale (populations at the house level) in Santiago del Estero, Argentina (Marcet et al., 2008). In both cases there were no significant differences in the levels of

allelic richness (aRich) and genetic diversity (H_e) between the groups of recently (1−5 years) insecticide-treated populations (average aRich = 3.8; $H_e = 0.6$) and those not previously treated, or that had not been treated for more than 9 years (average aRich = 3.8; $H_e = 0.7$).

Moreover, high levels of haplotype diversity (h) for the *cyt b* gene were obtained for samples from four locations in Bolivia (Giordano et al., 2005) that had not been sprayed for at least 3 years ($h = 0.29-0.67$). Consistently, results of the 12S and 16S genes (Garcia et al., 2003) in four provinces of Argentina showed the highest haplotypic diversity and private haplotypes in the treated locations of El Jardín and Chancaní (La Rioja province) compared with other untreated areas.

However, although variability seems not to have been severely affected by the control measures, an important signature in the populations subjected to insecticide treatment was observed. Genetic structure was compared between villages under long-term control and followed by surveillance and villages that were randomly treated and not subject to surveillance (Cardinal et al., 2007). High levels of genetic structure was observed in the area with high vector control pressure, represented in significant differentiation among most houses ($F_{ST} = 0.04-0.235$; $P < 0.00023$) and among all villages ($F_{ST} = 0.03-0.14$; $P < 0.0009$) and the presence of eight significant genetic clusters within this area. On the other hand, in the randomly treated area, with populations from houses located 2−60 km apart, 31% of the houses were not significantly differentiated, the variation on the village level was not significant, and all samples from this area fell within a single genetic cluster (Marcet et al., 2008).

A plausible explanation for this pattern is that the genetic structure of *T. infestans* subpopulations recovering from heavy insecticide spraying is molded by the recrudescent subpopulations. Such "founder effects" would randomly preserve in each surviving subpopulation, subsets of the variability originally detected. Moreover, each subpopulation would be independently subjected to the effects of genetic drift, further promoting genetic differentiation (Pérez de Rosas et al., 2007; Marcet et al., 2008).

The process of reinfestation by either local survivors, migrants from neighboring sites, or the occasional importation from distant areas by passive dispersion would gradually mix the different genetic variants. The speed of this process would depend on the degree of genetic exchange among populations. This would not only depend on the geographic distance among them, but also on local conditions that determine the dispersion rates (i.e., microclimatic, environmental, host availability), the number of reinfesting individuals, prespraying abundance, and effective population sizes (Lehane and Schofield, 1982; Lehane et al., 1992; Cecere et al., 2004, 2006; Vazquez-Prokopec et al., 2004; Ceballos et al., 2005; Cardinal et al., 2006; Marcet et al., 2008). If vector control is interrupted in such areas, given enough time, gene flow would increase among neighboring *T. infestans* populations (either by active migration or by passive dispersal), which would eventually homogenize the gene frequencies, reducing the high levels of genetic structure initially observed.

It is therefore highly recommended that all results be analyzed within the context of the epidemiological history of the populations being studied. The levels of genetic structure, variability patterns, and other genetic parameters estimated from

the samples considered should be discussed and interpreted depending on the scale of the populations being investigated and its insecticide spraying history.

The evidence of local processes of genetic diversification calls for exhaustive studies at microgeographical level of analysis to characterize the local genetic pattern observed in the area. A follow-up of the genetic structure pattern would be recommended to appraise reinfestation sources, or to monitor the appearance of insecticide resistance.

The high variability in terms of sampling design and lack of a precise definition of "populations" being studied are concerns. The abundance of analytical tools available today further complicates the comparison between different studies. The development of standard research strategies to address specific vector control questions is needed.

8.8 Perspective and Future Directions

Population genetics studies have provided important contributions to an understanding of the epidemiology (and to control strategies) of Chagas disease. We have learned about the identity of the epidemiologically important species, the origins of triatomine populations, and the adequacy of control strategies, among other findings. Certainly, there are many interesting questions to be addressed with new and emerging tools. With the advances in geographic information system technology, it is now possible to combine genetic with geographic and environmental data to predict the potential distribution of the vector populations.

The origin and evolutionary history of several species has begun to be studied. What are the historical, geological, ecological, and demographical conditions that resulted in the current distribution of species and differentiated subpopulations? Have populations undergone bottlenecks, population expansions, or introductions into new localities? How has this affected their epidemiological importance? The issue of hybridization and introgression, observed in many triatomine species, is very interesting. Where distinct taxa are **sympatric**, are there differences in the genome that keep species reproductively isolated? What will be the effect of human activities such as deforestation and climate change? What will be the results of the ongoing control efforts?

Comparative genomics and proteomics can help to identify genes and proteins involved in colonization of human dwellings, hematophagy, or pesticide resistance. These approaches can also help to unravel vector/parasite interactions (e.g., does parasite infection result in changes in vector behavior in ways that increase transmission, and what genes are involved in vector competence and capacity?).

While DNA-based molecular markers provided population geneticists with dramatically increased resolution over allozyme markers, development of new tools such as SNP assays and whole-genome sequencing will provide a new leap in resolution over current molecular methods used in triatomines.

Glossary

Allozymes codominant enzyme variants encoded by the same locus (i.e. alleles), used as markers to assess the genetic variability of natural populations.
Bottleneck temporary, drastic reduction in population size.
Codominance the condition in which both alleles are expressed in the heterozygote.
Cryptic species morphologically similar but reproductively isolated species.
Domestic occurs in human dwellings.
F_{ST} see **Wright's fixation index.**
Genetic drift changes in population allele frequencies that occur from generation to generation due to chance during the random "sampling" of gametes that will constitute the zygotes of the next generation. The smaller the population size, the larger will be the effects of drift.
Haplotype a set of linked single nucleotide polymorphisms (SNPs) that are typically inher ited together (or, in other words, a unique haploid DNA sequence).
Hybridization a cross between individuals of different species.
Hybrid an organism resulting from crosses between individuals from different species.
Introgression transfer of genetic material from one species into another through hybridization.
Isolation by Distance model a situation in which genetic distances among subpopulations are positively correlated with their respective geographic distances.
Microsatellites short (2 5 bp), tandemly repeated motifs.
Panmictic randomly breeding.
Peridomestic occurs in the area surrounding houses (e.g., wood piles, animal corrals, fences, walls).
Polymorphic locus a locus that contains more than one allele in the population. In order to avoid sample size biases it is usually expressed as P (0.95), meaning the most common allele must occur at a frequency lower than <0.95.
Population genetics the study of genetic variation and gene flow among populations.
Species complex a group of closely related and morphologically similar species.
Sylvatic occurs in the wild.
Sympatric occupying the same geographical area.

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9 Geographic Distribution of Triatominae Vectors in America

David Gorla[1] *and François Noireau*[2,*]

[1]Centro Regional de Investigaciones Científicas y Transferencia Tecnológica de La Rioja, La Rioja, Argentina, [2]Institut de recherche pour le développement, Centre National de la recherche scientifique, Montpellier, France

9.1 Introduction

At present, some 140 species are grouped in the subfamily Triatominae. The number of valid species in this subfamily is mainly based on the revision by Lent and Wygodzinsky (1979), and was later updated by Galvão et al. (2003). The subfamily has slightly increased since the description of new **taxa**, including *Belminus corredori*, *Belminus ferroae*, *Panstrongylus mitarakaensis*, *Triatoma boliviana*, and *Triatoma juazeirensis* (Galvão and Angulo, 2006; Bérenger and Blanchet, 2007; Costa and Felix, 2007; Martinez et al., 2007; Sandoval et al., 2007). Most triatomine species (∼125) occur exclusively in the New World, between latitude 42°N (northeast of the USA) and 46°S (Argentine Patagonia) (Carcavallo et al., 1999). One species (*Triatoma rubrofasciata*) is widespread according to reports from port areas, both in the New World (mainly northeast Brazil) and in many tropical regions of Asia and Africa (Schofield and Galvão, 2009). Seven species of *Triatoma* and six species of the genus *Linshcosteus* are known to exist only in Asia and India, respectively (Lent and Wygodzinsky, 1979; Galvão et al., 2003). Some authors suggest that the Old World species are derived from *T. rubrofasciata* and transported from North America, associated with rats on sailing ships (Gorla et al., 1997b; Patterson et al., 2001; Hypsa et al., 2002; Schofield and Galvão, 2009).

The Triatominae occurring in the Americas are customarily classified into 5 tribes and 15 genera (Lent and Wygodzinsky, 1979), including Alberprosenini (genus *Alberprosenia*), Bolboderini (genera *Belminus*, *Bolbodera*, *Microtriatoma*, and *Parabelminus*), Cavernicolini (*Cavernicola*), Rhodniini (*Psammolestes* and *Rhodnius*), and Triatomini (*Dipetalogaster*, *Eratyrus*, *Hermanlentia*, *Mepraia*, *Panstrongylus, Paratriatoma*, and *Triatoma*). Extensive information exists on their geographic distribution, and as a general review of the group we suggest the studies of Lent and Wygodzinsky (1979), Carcavallo et al. (1999), and Galvão et al. (2003).

*E mail: francois.noireau@rd.fr

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The present review on the geographic distribution of Triatominae considers the species of epidemiological importance or the groups of species in which at least one species has an epidemiological significance as a vector of *Trypanosoma cruzi* to humans. All the species considered belong to the tribes Rhodniini and Triatomini.

9.2 Limitations of Sampling Methods to Estimate the Geographic Distribution of Triatominae

Data on the distribution of triatomine species are usually obtained from the detection of peridomestic/domestic colonies, focal sampling of sylvatic populations, and information on the domestic intrusion of wild adult forms. Consequently, the more the **synanthropic** process of a Chagas disease vector is advanced, the more its geographic range may be precisely known. Thus, the past and recent changes in the geographic range of the most efficient vectors of *T. cruzi* to humans (*T. infestans* and *R. prolixus*) are well known. However, an incomplete knowledge of species distribution exists when these organisms are restricted to sylvatic environments. It is unfeasible to systematically sample over wide areas (wild populations are usually focally sampled) because it is difficult to access certain types of ecotopes, thus making it difficult to investigate these areas. Moreover, in the case of exclusively sylvatic species, the sampling is generally random and, consequently, often unproductive. Finally, the sampled subsets are considered isolated species when they may represent components of an unknown continuous population (Schofield and Galvão, 2009). With regard to triatomine species that exhibit a certain level of domestic incursion, their easy detection will depend on some behavioral traits, such as flight ability and attraction to light. The live-baited trap and light trap are the tools currently available to collect wild Triatominae. Unfortunately, both trapping systems only catch starved triatomines and, in the case of the light trap, only adult forms. Thus, the number of bugs a trap would be able to capture in a defined area would be inversely correlated to the nutritional status of the population (Noireau et al., 2002).

9.3 Pattern of Species Richness in the New World Triatominae

Of the five tribes within the Triatominae subfamily, Triatomini and Rhodniini include 88% of the recognized 140 species. Within the two most numerous tribes, the genera *Rhodnius*, *Panstrongylus*, and *Triatoma* constitute 86.5% of the species found in the Americas. Controversies about the evolution of the Triatominae still exist, although there is ample evidence supporting its **polyphyletic** constitution. The 16 *Rhodnius* species are generally associated with palm tree species found in

the geographic range from southern Amazonia to Central America. The majority of the 13 *Panstrongylus* species are exclusively sylvatic with a wide variety of habitats and wild animals. *Triatoma* is the most numerous genus, including 80 species distributed as far as the extreme northern and southern latitudes. Recent evidence suggests that *Triatoma* species of Central and North America form a group that evolved independently from the *Triatoma* species of South America (Schofield and Galvão, 2009).

A study considering 118 Triatominae species of the New World showed that groups analyzed at the continental scale had patterns of species richness that showed a significant linear latitudinal gradient with low values at the extreme latitudes and highest values near the equator (precisely on the 5° to 10° southern latitudinal band). The study also showed that species richness is significantly associated with habitable geographic area and temperature in the Southern hemisphere; in this hemisphere, there is a significant longitudinal gradient given by the Andes range, an element that influences the increase of species richness toward the east of South America (Rodriguero and Gorla, 2004).

We expanded the analysis of the data used by Rodriguero and Gorla (2004) and used a similar methodology to analyze the latitudinal range for the species (difference between maximum and minimum latitude of the species geographic distribution). The results showed a significant linear increase of the latitudinal range of the species from the equator (i.e., narrower near the equator, wider toward higher latitudes), as predicted by the **Rapoport's rule** (Figure 9.1).

When considering the species richness across the latitudinal regions of the most numerous Triatominae genus, it is clear that individual genera do not follow the

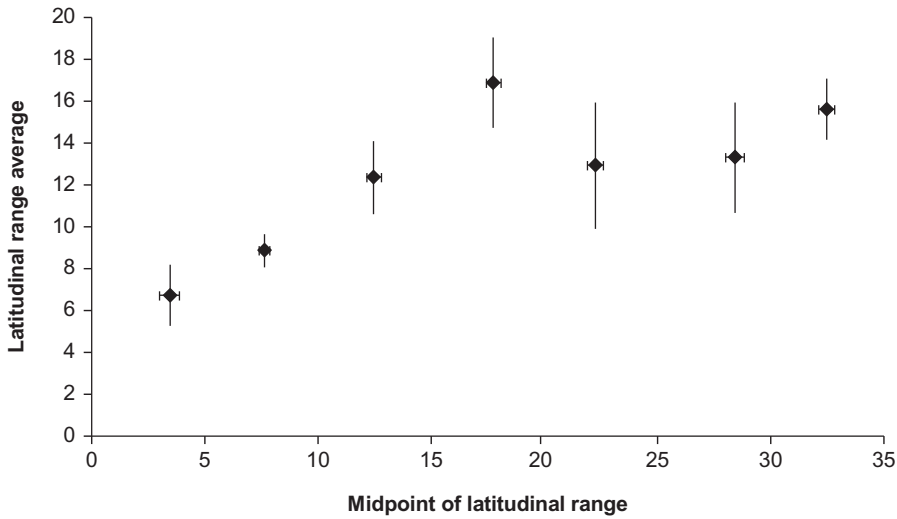

Figure 9.1 Relationship between the average latitudinal range and the midpoint of the latitudinal range for Triatominae species in the Americas. Vertical and horizontal lines over the points indicate standard errors.

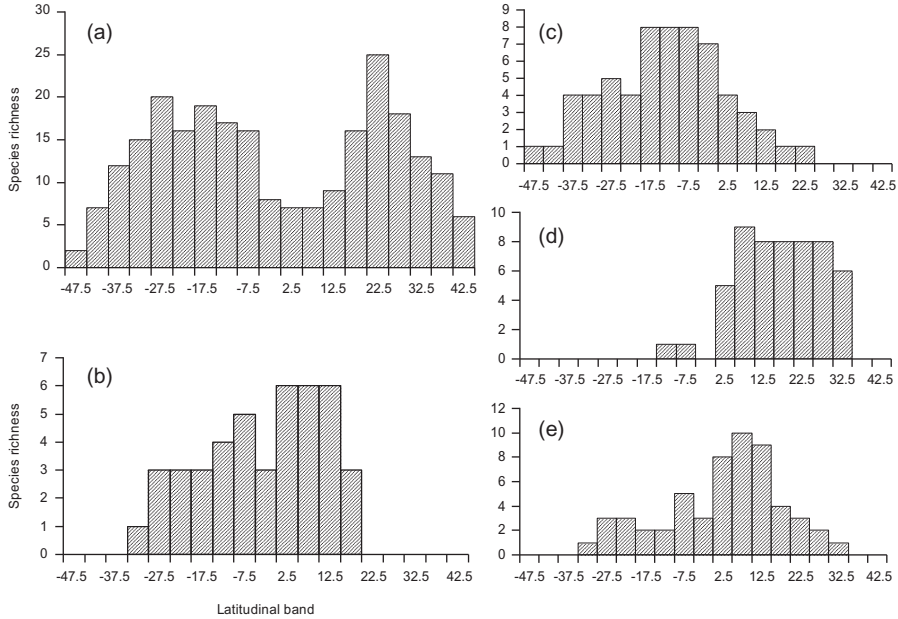

Figure 9.2 Species richness of Triatominae per 10° latitudinal band. (A) *Triatoma* species, (B) *Rhodnius* species and *Psammolestes* species, (C) *Panstrongylus* species, (D) Old World Triatominae, (E) *Dipetalogaster*, *Eratyrus*, *Hermanlentia*, *Alberproseniini*, *Bolboderini*, and *Cavernicolini*. Negative latitudinal bands are in the Southern Hemisphere.

same pattern shown by the complete set of Triatominae species. For instance, *Triatoma* shows a bimodal pattern of species richness with low frequency at low latitudes and modal maxima at around 23° north or south. *Rhodnius* shows a unimodal species richness distribution but strongly skewed with modal number of species located on the Northern Hemisphere at low latitudes and a few species on the Southern Hemisphere, some of them reaching the 30° southern latitude. *Panstrongylus* also shows a unimodal species richness distribution with the majority of species located on the Southern Hemisphere (Figure 9.2).

9.4 Distribution of Triatominae in the Americas

9.4.1 Genus Rhodnius

At the moment, the genus *Rhodnius* consists of 16 valid species (Galvão et al., 2003). All *Rhodnius* species have been primarily associated with palm trees even though some species were found in other sylvatic habitats. Thus, *R. domesticus* has been reported as colonizing bromeliads and hollow trees in Amazonia, and specimens of *Rhodnius* species have been found in the cactus mandacaru (*Cereus*

jamacaru) in the **Caatinga** of northeastern Brazil (Lent and Wygodzinsky, 1979; Gaunt and Miles, 2000; Dias-Lima et al., 2003). Some *Rhodnius* species may be associated with particular types of palms, such as *R. brethesi* with *Leopoldinia piassaba* and *R. ecuadoriensis* with *Phytelephas* species. Consequently, the distribution of sylvatic *Rhodnius* broadly coincides with the distribution of palm trees (Gaunt and Miles, 2000). Recent work suggests that deforestation and the associated loss of habitat and host diversity might increase the frequency of *Rhodnius*−human contact (Abad-Franch et al., 2009).

The genus *Rhodnius* encompasses a variety of species, including one primary domestic vector (*R. prolixus*), various synanthropic species that invade and sporadically colonize man-made ecotopes (*R. pallescens*, *R. neglectus*, *R. nasutus*, *R. stali*, and *R. ecuadoriensis*), species that invade but do not colonize houses (*R. robustus*, *R. pictipes*, and *R. brethesi*), and strictly sylvatic species (Abad-Franch et al., 2009). *Rhodnius* naturally occurs from Central America to northern Argentina, and species richness is highest in Amazonia.

R. prolixus is a domestic vector of *T. cruzi* to humans in Venezuela, Colombia, and some countries of Central America, including Honduras and Nicaragua. *R. prolixus* was likely erroneously recorded from Bolivia, Brazil, Ecuador, French Guiana, Guiana, Panama, and Suriname due to confusion with *R. robustus* (all countries) and *R. neglectus* (Central Brazil). *R. prolixus* is no longer found in its previously reported collection areas in Mexico (Oaxaca and Chiapas), Guatemala, El Salvador, and most of Honduras. Sylvatic populations have been captured in palm trees in Venezuela and the Orinoco region in Colombia but they have never been found in Central America (Guhl, 2007; Fitzpatrick et al., 2008; Guhl et al., 2009). Moreover, neither sylvatic nor domestic populations have ever been collected in Panama or south of Costa Rica. The discontinuity of the distribution area combined with the genetic homogeneity of *R. prolixus* in Central America suggest that domestic vectors from the South American populations have invaded several countries of Central America (Dujardin et al., 1998). Two explanations have been put forward. One explanation suggests that these vectors have been dispersed through passive carriage of eggs and young nymphs in the plumage of storks (*Mycteria americana*), which are known to migrate between the two regions and nest in palm trees (Gamboa, 1963). The second explanation suggests that these vectors have spread due to an accidental escape of laboratory-bred insects (Zeledon, 1996).

The species forming the *prolixus* group (*R. prolixus*, *R. robustus*, *R. neglectus*, and *R. nasutus*) are particularly difficult to distinguish. The specific status of *R. robustus*, which is virtually indistinguishable from *R. prolixus* using morphological characters, has been clarified by the use of modern techniques of species characterization, such as the sequence analysis for a fragment of the mitochondrial **cytochrome *b***. *R. robustus* currently includes four **cryptic species**. *R. robustus* I, which occurs in Venezuela (Orinoco region), is more closely related to *R. prolixus* than to the other three cryptic species found in the Amazon region (Monteiro et al., 2003). The near-sibling *R. neglectus* and *R. nasutus* are Brazilian species. The geographic distribution of *R. nasutus* is restricted to the northeastern region,

particularly to the semiarid Caatinga. *R. neglectus* has a wider distribution across the **Cerrado** and the adjacent regions of Central Brazil (Carcavallo et al., 1999; Galvão et al., 2003). Recent studies show that both species are **sympatric** in the northern Bahia State (Abad-Franch et al., 2009).

R. pallescens has been reported in Belize, Nicaragua, Costa Rica, Panama, and Colombia, where it inhabits sylvatic environments and it is often found in human dwellings although without building intradomestic colonies (Arboleda et al., 2009). The palm tree *Attalea butyracea* is its primary **biotope**. This species is characterized by its sporadic presence inside dwellings in Panama, where it is the only vector of *T. cruzi* to humans (Calzada et al., 2006). *R. stali* is a fairly unknown Bolivian species that has been historically confused with *R. pictipes*. The distribution of *R. stali* closely matches that of *Attalea phalerata* palms in the southwestern fringe of the Amazon biome. *R. stali* is able to establish colonies in domestic and peridomestic habitats, and the observation of Chagas disease seropositivity in the indigenous population of the Alto Beni region strongly suggests the presence of an ongoing **anthropozoonotic** disease transmission cycle (Matias et al., 2003). *R. ecuadoriensis* survives under a wide range of climatic conditions in Ecuador and northern Peru (Abad-Franch et al., 2009). Sylvatic populations of this species have been mainly found living in the *Phytelephas aequatorialis* palm trees in northern Ecuador (Lent and Wygodzinsky, 1979; Abad-Franch et al., 2009). The strong synanthropic behavior of *R. ecuadoriensis* and the absence of palm trees in southern Ecuador and northern Peru suggested that this species might have spread to the region through association with humans (Abad-Franch et al., 2001; Vargas et al., 2007). The recent detection of *R. ecuadoriensis* in sylvatic habitat (squirrel nests) in the southern Highlands of Ecuador contradicts this hypothesis (Grijalva and Villacis, 2009).

R. brethesi is a species occurring in the Brazilian Amazon that seems to be tightly associated with *Leopoldinia piassaba* palm trees. *T. cruzi* transmission in the Negro River region (Amazonas, Brazil) is caused by invasion of human dwellings by the vectors. Other observations attribute the attack of wild triatomines to the collectors of fronds from the piassaba palm (Coura et al., 2002). *R. pictipes* has a wide geographic distribution throughout the Amazon basin (north and northwest South America) in association with various species of palm trees (Carcavallo et al., 1999). The sporadic invasion of houses by light-attracted adult *R. pictipes* may be promoted by the presence of palm trees near households. The ingestion of palm fruit juices contaminated with crushed vectors was documented in some outbreaks of acute oral Chagas disease, which is the main transmission mechanism of *T. cruzi* in the Amazon region (Aguilar et al., 2007).

9.4.2 Genus Panstrongylus

There are 13 recognized species within the genus *Panstrongylus* that have a wide geographic distribution throughout the Neotropical region, extending from Mexico to Argentina (Curto de Casas et al., 1999). Among these species, some appear to be involved in a process of domiciliation, showing the ability of the species to

colonize human dwellings (*P. geniculatus*, *P. rufotuberculatus*, *P. lutzi*, and *P. chinai*). Other species are more opportunistic and occasionally fly from the sylvatic environment to houses without colonizing (e.g., *P. lignarius* in the Amazon basin). The **phylogeny** of the group is currently under discussion, although there is evidence suggesting the existence of a northern and a southern **clade** that is parallel to the northern and southern clade of *Triatoma* species; *Panstrongylus* species are considered to be evolved from this latter clade (Patterson et al., 2009).

Although some species can be found in palm tree crowns (e.g., *P. megistus*), all species are associated with terrestrial burrows, tree-root cavities, or hollow trees (Gaunt and Miles, 2000). *P. geniculatus* and *P. rufotuberculatus* have the widest geographic distribution, extending from Mexico to Argentina, including the Caribbean Islands (found in 18 and 10 countries, respectively). *P. lignarius* is the third most widely dispersed and it is found in seven South American countries. *P. megistus* is restricted to eastern and central South America. The remainder of the species have more limited or undetermined geographic distributions.

P. geniculatus has been considered a eurythermic species, meaning that it is adapted to several dry as well as humid ecotopes, and it is found in a great variety of sylvatic habitats (very dry forests or savannahs, dry, wet, moist, and rainy forests). This species is frequently captured in peridomestic environments and its occurrence inside houses has been cited in several countries (Valente et al., 1998; Gaunt and Miles, 2000; Feliciangeli et al., 2004; Carrasco et al., 2005). This species has epidemiological importance because of the high incidence of blood-fed specimens on humans concomitantly infected with *T. cruzi* I in Venezuela (Carrasco et al., 2005). The ingestion of fruit juice accidentally contaminated by *P. geniculatus* is thought to have caused an outbreak of infections in 2007 in Caracas possibly via oral transmission.

P. rufotuberculatus is generally considered to be a sylvatic species ranging from Mexico to Argentina. This species has been found in palms, hollow trees, and the refuges of wild mammals (Lent and Wygodzinsky, 1979; D'Alessandro et al., 1981; Miles et al., 1981). Adult insects frequently invade human dwellings as they are attracted by electric light (Lent and Wygodzinsky, 1979; Salomón et al., 1999). Breeding colonies have been found inside dwellings in Bolivia, Ecuador, and Peru (Noireau et al., 1994; Abad-Franch et al., 2001; Cuba et al., 2002). *P. rufotuberculatus* has been incriminated as a vector of Chagas disease in Andean and coastal foci of Ecuador. In the municipality of Amalfi (Antioquia, Colombia), the presence of *P. rufotuberculatus* is an epidemiological risk factor (Wolff et al., 2001). Several characteristics that could be linked to high vectorial capacity were observed for this species, including longevity, rapid response to the presence of a host, large volumes of blood ingested, and frequent defecation during the feeding process (Wolff et al., 2004).

P. megistus has a wide geographic distribution, ecological valence, and great potential for the colonization of artificial ecotopes. This species occurs in all varieties of Brazilian forests, including dry and moist humid forests in the Cerrado and Caatinga. The species is usually associated with humid forests from where adults can invade houses (Forattini et al., 1977), especially during the rainy season (Dias

and Dias, 1968). In other countries, such as Argentina, Bolivia, Paraguay, Uruguay, *P. megistus* is almost entirely sylvatic (Salvatella, 1986). On the occasions when this species is found in domestic habitats, it is usually associated with synanthropic hosts, especially opossums (Steindel et al., 1994). *P. megistus* was considered the main domestic vector in Brazil until it was progressively replaced by *T. infestans*, probably since 1930 (Dias and Dias, 1968; Dias, 1982). Following the success of the Southern Cone Chagas Disease Control Programme (INCOSUR) that achieved the elimination of *T. infestans* in many areas (Dias and Schofield, 1999), *P. megistus* reinitiated house invasion and is once again domiciliated in several states of Brazil. Thus, *P. megistus* is currently considered to be the main autochthonous vector of Chagas disease in the central, eastern, and southeastern regions of Brazil.

The most strongly synanthropic species, *P. lignarius* (formerly *P. herreri*), is considered to be the principal vector of Chagas disease in Peru (Cuba et al., 2002). Transmission of sylvatic *T. cruzi* to humans has also been associated with *P. lignarius*. In the Amazon basin, this species was observed flying from palm trees (*Attalaea phalerata)* to houses (Teixeira et al., 2001). In Colombia, this species has been found in bird nests and it is not considered of epidemiological importance (Guhl et al., 2007).

P. lutzi is one of the most important secondary vectors in Brazil. It has great capacity for invading houses through flight and shows high rates of natural infection with *T. cruzi*, likely due to the close vector association with armadillos (Dias-Lima et al., 2003). *P. chinai* may act as the vector of *T. cruzi* in sylvatic cycles in arid areas of northern and eastern Peru (Vasquez, 2005) as well as southeastern Ecuador (Abad-Franch and Aguilar, 2003). *P. howardi* is considered to be a potential vector of *T. cruzi* in the coastal region of Ecuador (Abad-Franch and Aguilar, 2003). The remaining *Panstrongylus* species (*P. diasi*, *P. guentheri*, *P. humeralis*, *P. lenti*, *P. mitarakaensis*, and *P. tupynambai*) have not been described as vectors of *T. cruzi* to humans, but most of them are probably involved in sylvatic *T. cruzi* cycles (Patterson et al., 2009).

9.4.3 *Genus* Triatoma

Triatoma is the most numerous genus of Triatominae, with 80 formally recognized species (Schofield and Galvão, 2009). Species of the genus occupy a wide array of habitats that are mainly associated with mammals and birds (Carcavallo et al., 1998). According to Gaunt and Miles (2000), the genus *Triatoma* has predominantly evolved in terrestrial, rocky habitats. However, many *Triatoma* species are specifically or preferentially **arboreal** and found in bird nests, palm trees, hollow trees, and under the barks of trees. This is the case for *T. delpontei*, *T. platensis*, *T. infestans* "dark morph," *T. pseudomaculata*, *T. sordida*, *T. guasayana* (except for the Andean populations that live among stones), *T. nigromaculata*, *T. maculata*, *T. ryckmani*, and *T. tibiamaculata.* The review dedicated to the geographic distribution of the genus *Triatoma* only addresses species of epidemiological importance, namely those that establish domestic colonies or occasionally infest houses by intrusion from peridomestic or sylvatic habitats. These invasions require innovative

control strategies to disrupt *T. cruzi* transmission and represent an important challenge for public health.

9.4.3.1 Triatoma infestans *Subcomplex (*sensu *Schofield and Galvão, 2009)*

This subcomplex includes the species *infestans*, *delpontei*, and *platensis*. The latter two species are closely associated with bird nests and have never been found colonizing intradomestic habitats. These three species are very closely related and have the same **diploid** chromosome number $2n = 22$ (20 autosomes + XX/XY). They also have several cytogenetic traits that differ from all other triatomines, including large **autosomes**, **C-heterochromatic blocks**, and meiotic heteropycnotic chromocenters formed by autosomes and sex chromosomes (Panzera et al., 1995). *T. infestans* remains the most important and widespread vector of Chagas disease in South America. *T. platensis* is a species almost exclusively present in nests of **Furnariidae** (*Anumbius* species, *Coryphistera alaudina*, *Pseudoseisura lophotes*) in northern Argentina, Paraguay, Uruguay, and southern Brazil (Carcavallo et al., 1998). This species has been occasionally found in chicken coops, where it is able to crossbreed with *T. infestans* (Pereira et al., 1996). All evidence indicates that within this subcomplex, *T. platensis* is the closest relative to *T. infestans* (Bargues et al., 2006). The status of *T. infestans* and *T. platensis* as two distinct species is almost entirely based upon their **ecological niche** separation. *T. delpontei* is another **ornithophilic** arboreal species and has a marked preference for woven stick nests of colonial monk parrots (*Myiopsitta monachus*) (Carcavallo et al., 1998). It is distributed in Bolivia, Paraguay, Uruguay, and Argentina. Despite the bird specificity, *T. delpontei* females are able to crossbreed with *T. platensis* males under laboratory conditions (Usinger et al., 1966). Their morphological similarity would be the consequence of a convergence related to a highly specialized adaptation to bird nests rather than having a common ancestry (Pereira et al., 1996). Both species have no role in the transmission of *T. cruzi* because of their specific association with birds that are not susceptible to the parasite infection.

9.4.3.2 *Triatoma dimidiata*

This species is a major Chagas disease vector found in Central Mexico, the Yucatan peninsula, Central America, northern Colombia, Venezuela, and Ecuador (Galvão et al., 2003). *T. dimidiata* is becoming the most important vector of Chagas disease in this region because the control activities to eliminate *R. prolixus* have made substantial progress. This species has extensive **phenotypic**, **genotypic**, and behavioral diversity in sylvatic, peridomestic, and domestic habitats across its geographic range. Thus, it is a domiciliated vector in most of Central America and Central Mexico where sylvatic and peridomestic populations also occur. This species may also act as vector of intrusion in the southeast of Mexico, Belize, and some parts of Guatemala. In Ecuador, where no sylvatic populations have been reported, it is an exclusively domestic vector. Across their geographic range, sylvatic populations of *T. dimidiata* have been found in a great variety of microhabitats, such as in the bark

of dead trees and hollow trees, palm trees, rock piles, Mayan ruins, caves occupied by bats, and nests of several mammals (e.g., opossums and armadillos) (Dorn et al., 2007). Recent studies strongly suggest that *T. dimidiata*, which has been historically regarded as a single species, includes several cryptic species distributed in specific geographic areas with different epidemiological importance (Panzera et al., 2006; Bargues et al., 2008; Dorn et al., 2009). More than 60 years ago, *T. dimidiata* represented an assemblage of morphologically variable populations, and Usinger (1944) had given subspecific status for some populations, namely *T. d. dimidiata* (Central American forms), *T. d. capitata* (Colombian forms), and *T. d. maculipennis* (some Mexican forms). **Cytogenetics** and molecular tools have confirmed this diversity (Panzera et al., 2006; Dorn et al., 2009) and the taxonomy adopted by Usinger (1944) has been reused (Bargues et al., 2008). Currently, Central American populations in Honduras, Nicaragua, and southern Guatemala correspond to subspecies *T. d. dimidiata*. A southern spread into Panama and Colombia gave the *T. d. capitata* form. A northwestern spread rising from Guatemala into Mexico gave the *T. d. maculipennis* form. *Triatoma hegneri* appears as a subspecific insular form (Cozumel Island). A cryptic species is confined to the Yucatan Peninsula and northern parts of Chiapas State (Mexico), Guatemala, and Honduras. Finally, the population introduced in Ecuador derives from Central America and corresponds to *T. d. dimidiata* (Bargues et al., 2008). The large intraspecific genetic variability found in *T. dimidiata s.l.* and subsequent distinction between the five different taxa have major implications for transmission capacity and vector control.

9.4.3.3 *Other* Triatoma *of Epidemiological Importance*

Some autochthonous species of the genus *Triatoma* that were originally restricted to the wild environment are increasingly found as domiciliated colonies. Studies of these species are relevant because such species may act as vectors of *T. cruzi* to humans and are generally not targets of control actions. In the Southern Cone of South America, four species may be considered as emerging vectors; these species include *Triatoma brasiliensis*, *Triatoma pseudomaculata*, *Triatoma sordida*, and *Triatoma guasayana*.

T. brasiliensis is a species complex consisting of two subspecies (*T. b. brasiliensis* and *T. b. macromelasoma*) and two other taxa recently identified as different species (*T. melanica* and *T. juazeirensis*). This species complex is found under large piles of rocks in the sylvatic environment and is native of the Caatinga, a **xerophytic** region in northeastern Brazil. The four members of this complex present varying rates, of epidemiological importance. The most significant is *T. b. brasiliensis*, given its geographic range covering five states in Brazil, high *T. cruzi* infection rate, and ability to form abundant domestic colonies (Costa et al., 2002). *T. pseudomaculata* is another species native to xerophytic ecosystems in northeastern Brazil. Its geographic range covers 13 states in Brazil in the Caatinga and the Cerrado. In the sylvatic environment, *T. pseudomaculata* is strictly arboricolous, found in hollow trees and bird nests. It often invades peridomestic structures but

does not display a significant ability to colonize human dwellings (Carbajal de la Fuente et al., 2008).

T. sordida and *T. guasayana* are considered potential substitutes for *T. infestans* in some areas of the Southern Cone, where they are particularly prevalent in peridomestic habitats and frequently found to be infected by *T. cruzi.* They occasionally invade human habitations and feed on humans and synanthropic animals. Nevertheless, there is still no evidence of vector transmission of *T. cruzi* to humans by these vectors (Noireau et al., 1997; Vazquez-Prokopec et al., 2005). Both species may be occasionally found in the Andean valleys of Bolivia at altitudes as high as 2800 m above sea level for *T. sordida* and 1800 m above sea level for *T. guasayana*. However, the two species are more prevalent in the lowlands. *T. sordida* occurs in the Cerrado and Chaco ecoregions whereas *T. guasayana* is restricted to the Chaco. In addition to some Andean valleys of Bolivia, their distribution overlap throughout northern Argentina and parts of the Chaco region in Bolivia and Paraguay. In the highlands, both species can be collected in rupicolous ecotopes or hollow trees. In the lowlands, *T. sordida* is arboricolous, found in hollow trees and bird nests, whereas *T. guasayana* is mainly found in dry cacti, bromeliads, and fallen logs (Carcavallo and Martinez, 1985).

T. maculata and *T. venosa* may be considered as emerging vectors in the northern Andean countries (Venezuela and Colombia). In some areas of Venezuela and Colombia, *T. maculata* has the capacity to colonize human dwellings and may be involved in Chagas disease transmission (Feliciangeli et al., 2003; Guhl et al., 2007). In the sylvatic environment, this species has been found in palm trees of the *Attalea* complex (genera *Attalea* and *Scheelea*), bird nests, bromeliads, and dead trunks (Carcavallo et al., 1998). Wild and peridomestic *T. maculata* is also found in Brazil (Roraima state). In Guiana, French Guiana, and Suriname, this species has a distribution in only the sylvatic environment (Galvão et al., 2003). *T. venosa*, which occurs in Costa Rica, Ecuador, and Colombia, is considered as a secondary vector of Chagas disease in Colombia where it is frequently found in houses and peridomestic structures in active *T. cruzi* vectorial transmission areas. However, its sylvatic habitat is unknown (Vargas et al., 2006).

Some *Triatoma species*, such as *T. barberi* and species of the *Phyllosoma* complex, are restricted to Mexico and are regarded as locally important vectors (Guzman-Bracho, 2001). Currently, *T. barberi* is considered to be the most important vector in Mexico. This insect is confined to the central valleys that are south of the Tropic of Cancer. This species has only been observed in domestic and peridomestic habitats, but it is assumed to have wild habitats in rock piles. Domestic population density is generally low (Guzman-Bracho, 2001). The *Phyllosoma* complex is composed of nine species, including several of epidemiological importance in Mexico: *T. longipennis*, *T. mazzotti*, *T. mexicana, T. pallidipennis*, *T. phyllosoma*, and *T. picturata*. These species dominate the central and northwestern part of the country in both tropical and subtropical areas. They additionally display different degrees of synanthropism, showing a behavioral gradient from household occasional invasion by adult triatomines to the stable colonization of artificial structures (Ramsey et al., 2000; Guzman-Bracho, 2001).

9.5 Environmental Variables as Indicators of Triatominae Geographic Distribution

Because of their hematophagous habit, Triatominae species are generally associated either with their blood sources and/or specific habitats where there is a significant chance of finding a blood meal. Some species are host specific and others are habitat specific. As an example, *T. delpontei* is exclusively found in *Myopsitta monachus* colonial nests. The nest is occupied over several years, and individual nests are added every year to the colonial structure. During this time, the triatomine population has a relatively stable population of *M. monachus* to feed upon. A closely related species to *T. delpontei*, *T. platensis*, is associated with nests of various species of furnariid birds, which are only occupied during the breeding season when the species has the opportunity to feed on brooding adults and chicks. During the nonbreeding season, birds abandon the nest, and this nest is eventually occupied by other birds and/or mammals (especially rodents) that will become hosts for the triatomines. *Rhodnius* species are well-known generalists among the triatomines that occupy palm tree crowns. Different *Rhodnius* species are associated specifically (or preferentially) with a palm species, where the triatomines will feed on the rich fauna of birds and mammals nesting in the crown (e.g., *R. brethesi* with *Leopoldinia piassaba*). Habitat availability for sylvatic populations of particular triatomine species strongly depends on the community structure and environmental conditions. Given this close association between habitat and Triatomine species, a number of studies have used sets of environmental variables at continental and subcontinental scales to analyze the geographic distribution of Triatominae species. The basic hypothesis of these studies investigates if places with similar environmental conditions would potentially represent places of additional occurrence of the species based on other areas with known occurrence of a particular Triatominae species. The idea is mainly applicable to studies on the geographic distribution of species at regional scales, where extrinsic factors of the studied populations are more important than intrinsic factors (i.e., climate variables and vegetation versus intra/interspecific competition and predation). For these types of studies, discriminant analysis and ecological niche modeling were used. The distribution of Triatominae species was studied using climate variables data that were collected at the ground level by meteorological stations available at continental scales (i.e., Worldclim database) and land cover and climate variables data recorded by earth observation satellites. Studies showed that environmental variables are good indicators of the geographic distributions of sylvatic, peridomestic, and domestic species of Triatominae.

9.5.1 Environmental Variables and the Distribution of T. infestans

Gorla (2002) described the maximum potential expansion of the geographic distribution of *T. infestans* using a 20-year time series of satellite imagery produced by the advanced very high resolution radiometer (AVHRR). From these images, series

statistics (amplitude and phase of 1-, 2-, 3-annual cycles, maximum, minimum, average, variance) of the normalized difference vegetation index (NDVI), estimations of land surface temperature (LST), air temperature (AT), vapor pressure deficit (VPD), and a measure of middle infrared radiation (MIR) were estimated. The maximum potential distribution was derived from a multivariate discriminant analysis and then compared with the *T. infestans* distribution determined using data collected by field teams of vector control programs of the Southern Cone countries and academic reports. This analysis resulted in an overall 90% correct classification of either presence or absence of *T. infestans*. The model identified the variability of the AT, air temperature average, and amplitude of the AT annual cycle as the main variables defining the geographic distribution of *T. infestans*.

The maximum expansion of *T. infestans* geographic distribution probably occurred between 1970 and 1980 when the presence of the species was first reported to have crossed the São Francisco River in northeastern Brazil (Barrett et al., 1979; Silveira et al., 1984). Current knowledge of the species distribution originated from several studies using different methodological approaches (i.e., population genetics, morphometric geometry, cytogenetics) supports the hypothesis that the Andean valleys in Bolivia were the center of origin and expansion of *T. infestans* throughout the southern countries of South America (Schofield, 1988; Panzera et al., 2004; Bargues et al., 2006). The present geographic distribution of domestic populations differs for their reduced habitat ranges (Schofield et al., 2006) from the one described at the maximum geographic distribution of the species (Gorla, 2002). This reduction was caused by the activity of the concerted actions of the vector control programs of the Southern Cone countries together with living conditions improvement of previously affected communities and the generalized migration from rural to urban settings in many areas.

Recent studies showed sylvatic populations of *T. infestans* occurring more frequently than previously expected in the Andean and the Chaco regions of Bolivia and Argentina (Noireau et al., 2005; Noireau, 2009). Taking into account the existence of the Andean and non-Andean (Chaco) cytotypes described by Panzera et al. (2004), an analysis of the distribution of Andean and Chaco sylvatic populations of *T. infestans* was conducted using field-collected data of presence and absence of the target populations and environmental variables to identify potential areas of the geographic distribution of both types of sylvatic populations. The methodology and environmental data are the same as those used by Gorla (2002) to describe the maximum potential distribution of *T. infestans* in the Southern Cone countries of South America (see above). The geographic distribution models derived from linear discriminant analysis showed that the Andean population of sylvatic *T. infestans* is restricted mainly to the southern part of La Paz department, southern Cochabamba, and the boundary between the departments of Chuquisaca and Tarija with Potosi (Figure 9.3).

Areas with potential presence of the Andean sylvatic *T. infestans* are largely determined by the MIR average, the terrain altitude, and the phase of the annual cycle and minimum of the NDVI. The Chaco populations of sylvatic *T. infestans* have a wider area of potential distribution around the Chaco region of Argentina, Bolivia, and Paraguay (Figure 9.3). The distribution of the sylvatic Chaco

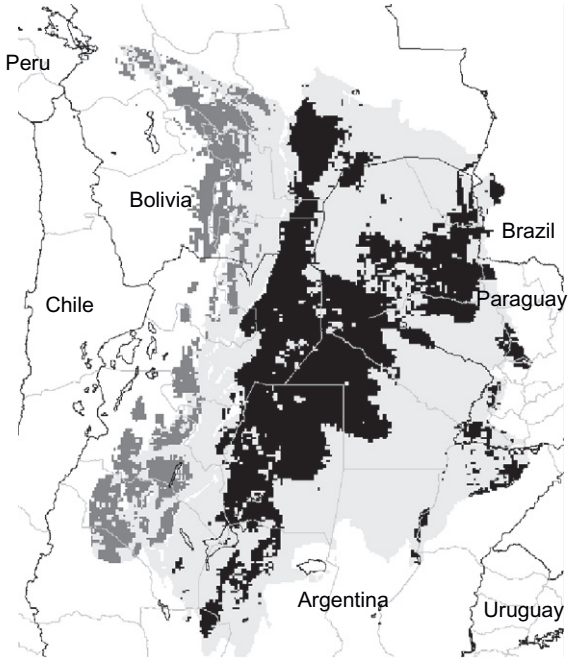

Figure 9.3 Potential distribution of sylvatic *T. infestans* populations of the Andean and Chaco regions. Areas in black and medium grey are predictions of presence for the sylvatic Chaco an Andean types, respectively. Areas in light grey are predictions of absence of *T. infestans*.

populations is determined by the variability of the AT, the amplitude of the annual cycle of MIR, and the phase of the annual cycle of the AT (see Gorla, 2002 for details). The model for the Andean populations has a 100% correct identification for presence and absence sites, whereas the model describing the distribution of the Chaco populations has a 94.4% correct identification.

9.5.2 Environmental Variables and the Distribution of Other Triatominae

Costa et al. (2002) described the geographic distribution of *T. brasiliensis* in the northeast of Brazil. These authors used 22 environmental variables, including climate data (temperature, precipitation, vapor pressure, etc.), terrain elevation, and land cover data, which were analyzed with the genetic algorithm for rule-set production (GARP) tool. The results showed the **allopatric** and **parapatric** distribution of four *T. brasiliensis* populations. *T. b. brasiliensis* occupies regions at 16.5−22.5°C that experienced 10−35 mm of precipitation. *T. b. juazeirensis* was similar but had a narrower ecologic altitude. *T. b. macromelasoma* was found at the highest altitude, occupying two potentially disjunct ecological zones where annual mean temperature ranges from 11.0°C to 23.5°C and annual mean precipitation of 10−45 mm. *T. b. melanica* appears restricted to a narrow ecological zone with annual temperatures between 18°C and 19°C and precipitation of 20−35 mm.

Carbajal de la Fuente et al. (2009) showed that the sylvatic triatomine species of eastern Brazil, *T. pseudomaculata* and *T. wygodsinskyi*, have allopatric but not partially sympatric populations as previously accepted. This was discovered after studying their geographic distributions characterized by temperature, VPD, vegetation, and altitude estimated from information provided by the satellite remote sensors AVHRR and moderate-resolution imaging spectroradiometer (MODIS). In this study, the 8×8 km spatial resolution of the AVHRR imagery more accurately described the species distributions compared to the MODIS imagery. Both satellites were able to produce a >85.7% correct classification for presence and absence from point data.

Peterson et al. (2002) studied the joint distribution of *Triatoma* species of the *Protracta* complex with packrat species (*Neotoma* species) using terrain elevation, hydrological, and climate data, which were analyzed with the ecological niche modeling GARP tool. This study showed a close association between the distribution of *T. barberi* (one of the main vector species for *T. cruzi* transmission in Mexico) and *N. mexicana*, a finding that led these authors to suggest a specific interaction between the species.

Gurgel-Gonçálvez and Cuba (2009) studied the distribution of *R. neglectus* in Central Brazil, using data on biophysical variables (altitude, temperature, vegetation, and rainfall) obtained from the Worldclim database with information about the distribution of palm tree and bird species. This study showed that this *Rhodnius* species is closely associated with dry areas of the Cerrado−Caatinga corridor and partially overlaps with the considered palm tree and birds species.

Arboleda et al. (2009) studied the geographic distribution of *R. pallescens* using the 1982−2000 monthly time series of AVHRR imagery at the 8×8 km spatial resolution mentioned above. The analysis showed that the minimum VPD is the most important variable for determining the geographic distribution of the species, along with its **stenohydric** status. The study also showed the potential distribution of most species of *Rhodnius*, except *R. domestic*, *R. neglectus*, and *R. nasutus*, a result probably derived from the evolution of common ancestors among the species groups. Using correlation analysis and logistic regression, Bustamante et al. (2007) studied the distribution of domestic Triatominae species in Guatemala. Results of this study showed that the distribution of *T. nitida* is positively associated with places of lower average minimum temperature, whereas the distribution of *R. prolixus* corresponded with areas of maximum absolute temperature and relative humidity.

9.6 Global Warming and Expansion of the Geographic Range of *T. infestans*

The Intergovernmental Panel on Climate Change (IPCC) synthesis report of 2007 concluded that local maximum temperatures will only modestly increase while minimum temperatures will increase dramatically under climate change scenarios

(Ostfeld, 2009). Because of the predicted increase in temperature and the known effect of temperature on insect development and reproduction, a number of authors have predicted a global increase in the transmission of vector-borne diseases (VBDs) (Epstein, 2000; Campbell-Lendrum et al., 2003). However, not all experts agree on these predictions because of the simplistic linear relationship that underlies the former argument. In the latter case, authors argue that the epidemiology of each VBD is a system-specific product of complex, commonly nonlinear interactions between many disparate environmental factors. These factors include climate and other abiotic conditions (e.g., land cover), the physical structure of the environment, host abundance and diversity, socioeconomic factors driving human living conditions and behavior that determines the degree of exposure to vectorial transmission risk, and the nutritional status and concomitant immunity that determine resistance to infection (Randolph, 2009). In addition to the complexity argument, these authors state that there is no single infectious disease with increased incidence over recent decades that can be reliably attributed to climate change (Lafferty, 2009). For the case of Chagas disease vectors, there are no specific studies on their relationship with the expected climate change, except for an early article by Gorla et al. (1997a) which discussed the potential changes in the geographic distribution of *T. infestans* and Chagas disease transmission under a global temperature increase scenario. Based on 3-year field data on the population ecology of *T. infestans* under the natural climate conditions of the southern Gran Chaco (reported by Gorla, 1992), Gorla et al. (1997a) estimated the effect of a generalized temperature increase on the maximum potential population growth rate (r) of *T. infestans* and *T. cruzi* transmission risk (based on the relationship between temperature and *T. infestans* biting frequency) over the Argentinian territory. At a hypothesized 2°C or 4°C temperature increase, the vector populations may potentially expand the southern limit of the geographic distribution and have a greater capacity to increase its population abundance in the Gran Chaco region, which corresponds with a parallel increase in the *T. cruzi* transmission risk. The authors stated that the distribution and abundance of *T. infestans* and the vectorial transmission of *T. cruzi* depend on factors other than just temperature. Although the study predicts the species-potential expansion of the southern limit and an eventually increased transmission risk, the vector control activities and the improved living conditions of rural communities in previously infested areas were able to eliminate domestic infestations by *T. infestans*. Additionally, interruption of the vectorial transmission of *T. cruzi* in several provinces outside the Gran Chaco region by 2001, as certified by the Pan American Health Organization (INCOSUR, 2002), has significantly contributed to a decrease in disease prevalence.

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David E. Gorla is a senior researcher of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina.

Glossary

Some of the specialized words are explained in the text; others are defined here.

Allopatric geographically isolated and thus unable to crossbreed.
Anthropozoonotic transmissible from animals to humans.
Arboreal living in trees.
Autosome chromosome that is not a sex chromosome.
Biotope area of uniform environmental conditions providing a living place for a specific assemblage of plants and animals.
C-heterochromatic blocks densely staining chromosomal material that appears as nodules and contains relatively few genes.
Caatinga dry forest region in the northeastern part of Brazil.
Cerrado extensive woodland savannah in Central Brazil.
Clade group of biological taxa (as species) that includes all descendants of one common ancestor.
Cryptic species species that appear morphologically identical but are genetically isolated from each other.
Cytochrome *b* mitochondrial gene.
Cytogenetics study of the structure and function of the chromosomes.
Diploid having a pair of each type of chromosome.
Ecological niche biological space occupied by a species.
Furnariidae family of ovenbirds.
Genotypic relating to the genetic makeup of an individual.
Ornithophilic that feeds on bird.
Parapatric having contiguous geographic ranges.
Phenotypic relating to the observable traits of an organism.
Phylogeny evolutionary relationships within and between groups.
Polyphyletic derived from two or more distinct ancestral lineages; the alternative is "monophyletic."
Rapoport's rule relationship between the average latitudinal range and the midpoint of the latitudinal range.
Stenohydric tolerant of a narrow range of humidity.
Sympatric occupying the same or overlapping geographic areas without interbreeding.
Synanthropic ecologically associated with humans.
Taxon (*pl.* taxa) taxonomic category or group such as a phylum, order, family, genus, or species.
Xerophytic adapted to a dry environment.

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10 Control Strategies Against Triatominae

David Gorla[1], *Carlos Ponce*[2], *Jean-Pierre Dujardin*[3] *and C.J. Schofield*[4,*]

[1]Centro Regional de Investigaciones Científicas y Transferencia Tecnológica de La Rioja, La Rioja, Argentina, [2]Laboratorio Central de Referencia, Tegucigalpa, Honduras, [3]Institut de recherche pour le développement, Centre National de la recherche scientifique, Montpellier, France, [4]ECLAT network, London School of Hygiene and Tropical Medicine, London WC1 E7HT, UK

10.1 Introduction

Control of Triatominae (Hemiptera, Reduviidae) is a primary component of strategies to halt the transmission of Chagas disease, along with serological screening of blood donors to reduce the likelihood of transmission through infected blood transfusions. In the early 1990s, an estimated 80% of Chagas disease cases were attributed to transmission from triatomine vectors (Schofield, 1994). Since 1991, a series of multinational initiatives have focused on elimination of the domestic vector populations throughout the endemic areas of Latin America. Largely as a result of these initiatives, transmission rates have been steadily reduced, with corresponding reductions in infection prevalence. Current estimates suggest that around 7 million people are infected, down from the 1984 estimate of 24 million (Walsh, 1984); annual transmission rates are probably fewer than 50,000 new cases per year (OPS, 2006; Schofield and Kabayo, 2008). The geographical distribution of domestic vector populations has been drastically reduced, especially *Triatoma infestans* in Southern Cone countries and *Rhodnius prolixus* in Central America. Uruguay, Chile, and Brazil, together with several provinces and departments of Argentina and Paraguay, have been formally declared free of transmission due to *T. infestans*, and Guatemala has recently been declared free of transmission due to *R. prolixus*. In addition, there has been steady progress in blood donor screening, with coverage now approaching 100% in most of the endemic countries (Schmunis and Cruz, 2005).

But this is not to say that the task of controlling Chagas disease is concluded. Rates of transmission due to domestic vectors remain high in several regions, most notably

*E mail: cj.schofield@lshtm.ac.uk

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the Gran Chaco region of Argentina, Bolivia, and Paraguay, and also parts of the Andean Pact countries and Mexico. Even in those regions where the main domestic vector populations have been eliminated, there is a consistent requirement to maintain surveillance with focal interventions against potential new domestic infestations. There is not only a risk of repopulation by the main vector species but also a risk of other species invading domestic habitats and either establishing new domestic colonies or provoking "accidental" transmission (e.g., through contamination of food or drink) without necessarily establishing new colonies. This, in conjunction with declining public health interest in Chagas disease as the apparent control successes become more widely discussed, imposes a need for additional strategies in vector surveillance and control that can be sustained as a matter of routine over the long term. Current strategies, based on large-scale campaigns of indoor residual spraying (IRS) against established vector populations, are still required in many areas, but will need to become progressively more focal and guided by a sustainable surveillance system integrated with routine public health vigilance. Such techniques are available, and the strategies are being developed (Schofield et al., 2006). To paraphrase Sir Winston Churchill from another context, "This is not the end; it may not even be the beginning of the end; but we believe it may be the end of the beginning. . ."

10.2 Elimination of Domestic Populations of Triatominae

Since the pioneering work of Carlos Chagas and colleagues in Brazil, and Salvador Mazza and colleagues in Argentina, a very wide range of vector control methods have been trialed with a view to eliminating domestic infestations of Triatominae, including biological control and insect pathogens, as well as a range of physical and chemical methods. The resulting experience accumulated from experiments and field trials in most countries of Latin America has led to a basic vector control approach with three main components: (1) IRS by trained professionals, (2) householder and community participation in monitoring and surveillance, and (3) rural house improvement and health education.

In general, a well-applied IRS campaign is sufficient to eliminate existing domestic bug populations, although repeat treatments are sometimes given after 3–6 months (Hashimoto et al., 2006). Since the 1980s, wettable powder (WP) or suspension concentrate (SC) formulations of pyrethroids have been the products of choice; other classes of insecticide are not generally used except when donated (or against some recently reported foci of pyrethroid-resistant *T. infestans* in southern Bolivia and northwestern Argentina). Throughout Latin America, IRS campaigns followed by long-term surveillance have been the primary component of Chagas disease control programs, together with improved blood-bank screening to reduce the likelihood of transfusional transmission from infected blood donors, and improved patient care, counseling, and treatment for those already infected.

By itself, rural house improvement seems insufficient to eliminate an established domestic bug population (Guillén et al., 1997) and it tends to be a relatively slow

process that rarely reaches all the poorest householders. In addition, house improvement programs need not be guided by the presence or absence of a particular vector, nor do they necessarily rely on health sector professionals. By contrast, well-managed IRS programs can reach all domiciles and can usually treat 2–10 houses per worker per day, depending on terrain, size, and distribution of houses and the extension of peridomestic structures that are included in the treatment. However, community agreement and householder participation are essential, both to assist in preparing the premises for spraying and also to participate in postcontrol monitoring and surveillance to ensure that the domestic bug population has indeed been eliminated and to provide early warning if any subsequent infestations are found.

Education is seen not only as a way of improving the general culture of future adults that would make better efforts to avoid the presence of triatomines within a house in the long term, but also as a way of encouraging schoolchildren, teachers, and the community to participate actively in the vector surveillance activities after the insecticide application in the endemic areas (Sanmartino and Crocco, 2000). Although difficult to achieve because of the inherent complexities, the more integrated the vector control and vigilance program (vector control, community education, house improvement, productivity of domestic animals), the more efficient and long lasting would be the eventual elimination of intradomestic infestations.

Integration of vector surveillance activities with the primary educational system is also seen as a crucial component for long-term sustainability. The essential difficulty for dedicated Chagas disease control programs is that the more successful they are in reducing the epidemiological indices, the less the perceived demand for their expertise. Consequently, successful Chagas disease control programs become essentially "self-limiting" the so-called punishment of success and must therefore adapt the surveillance and control strategy in accordance with the changing epidemiological patterns (Wanderley, 1994). An effective way to do this involves a routine program of interview and serological surveillance of schoolchildren; for example, as part of a routine program of school health care such that absence of reports of domestic Triatominae, combined with absence of serologically positive children, can be used as evidence that there is no currently active transmission within the school catchment area. Similarly, the presence of serologically positive children can guide both specific treatment for those children and entomological inspection of their houses with selective insecticidal treatment where necessary (Schofield et al., 2006).

10.3 Multinational Initiatives

Following a great many trials during the postwar decades and national control campaigns in Venezuela, Argentina, and Brazil, the enlightened response to the geographical scale of Chagas disease control came as a series of multinational initiatives, beginning with the Southern Cone countries in 1991, followed by

initiatives of the Andean Pact (IPA) and Central American countries (IPCA) launched in 1997, and the Amazon Initiative (AMCHA) launched in 2002. The Southern Cone Initiative (INCOSUR) involved six countries (Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay) which, with southern Peru, was designed to cover the entire distribution of the main vector, *T. infestans*. At the time, it was believed that *T. infestans* was almost entirely domestic throughout its range (except for small sylvatic foci in the Cochabamba−Sucre region of central Bolivia); the aim of the INCOSUR program was to halt Chagas disease transmission by eliminating all domestic populations of *T. infestans* (with concurrent elimination of any other domestic vector populations in the same area) and by improving screening of blood donors to reduce the risk of transfusional transmission. The idea was that simultaneous vector control programs throughout the area would prevent reinfestation of treated premises by *T. infestans* being accidentally transported from non-treated regions. In addition, the multinational nature of the program coordinated by the Pan American Health Organization (PAHO) should give political continuity to the interventions, making it less likely that a country would suddenly divert resources away from the Chagas disease control program (Schofield and Dias, 1999).

The Andean Pact and Central American Initiatives had similar aims and rationale, focusing on the elimination of domestic populations of their main vector, *R. prolixus*, together with control of other vectors in the region, particularly *Triatoma dimidiata*. There was strong evidence that *R. prolixus* had been accidentally imported from Venezuela into Central America at the turn of the last century (Dujardin et al., 1998; Zeledón, 2004); consequently, it appeared that *R. prolixus* could be completely eliminated in Central America. Similarly, there was evidence that *T. dimidiata* had been accidentally transported from Central America to Ecuador and northern Peru during pre-Colombian times (Abad-Franch et al., 2001) so that it could potentially be eliminated from there even if not from Central America, where it retains extensive sylvatic populations.

The INCOSUR, IPA, and IPCA initiatives were designed primarily as vector elimination programs and, at the time of writing, Brazil, Chile, Uruguay, and Guatemala have been formally declared free of Chagas disease transmission due to their main vectors. Similar declarations have been made for various provinces and departments of Argentina and Paraguay.

The distribution of *T. infestans* has been reduced from its predicted maximum of 6.28 million km^2 (Gorla, 2002) to under 1 million km^2 (Dias et al., 2002; Schofield et al., 2006; Schofield and Kabayo, 2008), while *R. prolixus* appears to have virtually disappeared from Central America except for a few remaining foci in Honduras. In all countries of Latin America, screening of blood donors has been improved, with coverage now close to 100% in most countries (Schmunis, 2007). Costs averaged around US$30 million per year for the Southern Cone, and around US$4 million to $7 million per year for the Central American countries, but studies in Argentina and Brazil indicate economic returns equivalent to over US$7 for every dollar invested in the Chagas disease control programs (Basombrio et al., 1998; Akhavan, 2000). Benefits have also accrued to those already infected, as

clinicians throughout the intervened regions report reductions in the severity of the chronic lesions (Dias et al., 2002), which, from studies in mouse models, seems to be largely due to lack of reinfection once the domestic vectors have been eliminated (Bustamante et al., 2002, 2007).

By contrast, the Amazon Initiative (AMCHA), which includes parts of nine countries, was designed primarily as a surveillance program because domestic vector populations are rare in most of the Amazon region (except for *Triatoma maculata* in parts of Roraima and southern Venezuela) (Guhl and Schofield, 2004). Instead, vector-borne transmission in the Amazon region is attributed primarily to adventitious sylvatic bugs (mainly species of *Rhodnius* and *Panstrongylus*) flying into houses and contaminating food and drink (Coura et al., 2002). Such transmission is often described as "oral-route transmission" and has resulted in a series of so-called family microepidemics of acute Chagas disease in various parts of the Amazon region (and elsewhere). In such circumstances, there is little role for vector control programs; instead emphasis is given to detection and treatment of those occasional outbreaks of acute disease, a task in which malaria slide microscopists are playing an increasing role by identifying trypanosomes in the peripheral blood smears of febrile patients originally suspected of malaria. In a sense, the Amazon Initiative may also be revealing aspects of how the future of Chagas disease control could proceed throughout the Americas once the existing domestic vector populations have been eliminated.

10.4 The Beginning of the End?

With these apparent successes, a much debated question then becomes "can Chagas disease be eliminated?" We must be clear on terminology: the causative agent, *Trypanosoma cruzi*, will not be eliminated it is a widespread parasite of small mammals and marsupials throughout the Americas; the vectors, Triatominae, will not be eliminated there are over 140 species distributed in the Americas (and some also in India and southeast Asia). As a consequence, the disease will not be eliminated, in the sense that the ubiquity of parasites and vectors in Latin America will always pose a risk of occasional transmission to humans. These, without prompt diagnosis and treatment, can in turn pose a risk of onward transmission through nonvectorial routes, such as blood transfusion, organ transplant, and occasional congenital cases.

But some vector populations can be eliminated: *T. infestans* over most of its original distribution in Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay, and southern Peru; the central American form of *R. prolixus* and the South American form at least from the central valleys of Colombia; *R. ecuadoriensis* from northern Peru, and *T. dimidiata* from Ecuador. All these populations appear to have been imported as domestic variants from elsewhere, mainly by accidental carriage by humans, and mostly within the last 150 years. In a sense, their presence outside their original foci is aberrant, due to human accidents that should be corrected.

These populations have probably accounted for over 80% of Chagas disease transmission, but they are not the only vectors. All populations of all species of

Triatominae should be considered at least as potential vectors, although without human contact they can play no epidemiological role. Perhaps the focus should be to minimize that contact, and then to minimize the risk of that contact. With this perspective, outline strategies become clear. All existing domestic populations of Triatominae, of whatever species, should be eliminated, and experience accumulated from control trials and programs throughout Latin America shows that this is possible. But then how do we sustain this absence of domestic Triatominae, knowing that the previously infested houses may remain susceptible to reinvasion? Promotion of rural house improvements may help, but for reasons already mentioned, this is unlikely to reach all communities that may merit such development. The other technical response is to improve insecticide formulations, in an attempt to give longer protection to the treated premises. But recognizing that no treatment can last for ever, the strategic response, as illustrated by the multinational initiatives, is to try to remove source populations to make reinfestation unlikely. Successful when dealing with an imported domestic variant (such as *R. prolixus* in Central America), this strategy is much less successful when dealing with domestic populations that also occupy extensive peridomestic habitats (such as *T. infestans* in the Chaco region of northwest Argentina and southern Bolivia) or that retain local sylvatic ecotopes (such as *T. dimidiata* in parts of Central America).

The control of peridomestic populations of Triatominae is seen as a major technical challenge. Conventional spraying with WP or SC pyrethroids, such as those used inside houses, tends to have reduced impact as the superficial deposits can be degraded by sunlight or quickly covered with dust or animal dejections. Some authors report better results using a double spray (Cecere et al., 2006) or using slow-release polymer formulations (Dias and Jemio, 2008; Amelotti et al., 2009a). Others prefer physical modifications to the peridomestic habitat, for example, by using higher standard fencing materials instead of piled brushwood for goat corrals in the Argentine Chaco, which can greatly reduce the habitat available for peridomestic *T. infestans* and *Triatoma guasayana* (Gorla et al., 2007). Other approaches involve the concept of "xenointoxication," or treating domestic animals with a pour-on or powder formulation of insecticide, in order to kill any bugs that may attempt to feed on them (Amelotti et al., 2009b). Insecticide-impregnated dog collars have been used for a similar purpose (Reithinger et al., 2005, 2006) and it seems likely that further technical developments will lead to improvements in the ways to control peridomestic Triatominae. But perhaps a strategic response needs also to be considered. The importance of peridomestic Triatominae is primarily as a potential source for reinfesting the domestic habitat. Domestic reinfestation from peridomestic populations after 1−4 years of insecticide application is a well-established concept, based on long-term studies mainly in Santiago del Estero (Argentina) carried out by Gurtler and colleagues (Gürtler et al., 2007). Studies based on population genetics and morphometry suggest that populations are highly structured, even at the habitat level (Schachter-Broide et al., 2009; Hernandez et al., 2010). The latest studies, showing spatial structure at the habitat level (i.e., habitat-specific populations that do not show evidences of individual flow between populations), raise questions about the speed at which peridomestic populations

actually reinfest intradomestic structures. The latest questioning is supported by a 10-year observation on house infestation in the Department Castro Barros (La Rioja) and field data collected from the region of Los Llanos by the Chagas vector control program of La Rioja.

During the last 12 years, house infestation by *T. infestans* in the Department of Castro Barros (north of La Rioja-Argentina, 743 houses distributed in 10 villages) was periodically evaluated either by research teams or provincial or national Chagas vector control programs (Zerpa and Catalá, 2001; Servicio Nacional Chagas Catamarca and Programa Provincial Chagas La Rioja, unpublished data). Repeatedly, evaluations of peridomestic infestation ranged between 25% and 30% and intradomestic infestation between 0% and 4%. But in spite of the relatively high infestation of peridomestic structures, intradomestic infestation remained low, with very low abundant vector populations. The virtual absence of vectors inside the rooms, where the parasite transmission by vectors usually takes place, was associated with zero infection in children younger than 15 years of age evaluated twice between 1998 and 2009 (except four cases associated with *T. cruzi*−infected mothers, which were identified as congenital cases and treated accordingly).

House infestation in the Department San Martin (south of La Rioja, within the highly endemic region of Los Llanos) was 34.4% and 48.4% (intradomestic and peridomestic, respectively) on average until 2004 (Porcasi et al., 2007). After systematic vector control interventions during the last 6 years, intradomestic infestation dropped to 3%, although peridomestic infestation averages 30%. In spite of the relatively high peridomestic infestation, no children below 6 years of age were found to be infected with *T. cruzi*, except for a few congenital cases (Programa Chagas La Rioja, unpublished data).

T. cruzi vectorial transmission seems to be interrupted in the Departments of Castro Barros and San Martin, even though there has been a sustained 25−30% peridomestic infestation during the last decade. So, in at least these two departments of La Rioja, the sustained interruption of vectorial transmission of *T. cruzi* did not require the elimination of peridomestic infestations of *T. infestans*. Although it would be risky to make extrapolations, it seems that there is at least some heterogeneity about the speed at which the intradomestic infestation from peridomestic populations of *T. infestans* takes place (faster in Santiago del Estero, slower in La Rioja). One possibility that has to be considered is the origin of the individuals that produce the intradomestic colonization. Because of the imperfect methods to detect intradomestic infestation, the origin of the population producing the intradomestic colonization after a vector control intervention with residual insecticide (individuals coming from peridomestic structures or intradomestic survivors reconstituting the original population) has not been routinely analyzed. If the newly apparent intradomestic colonization in fact results from survivors of the original intradomestic residual population, then evaluation of the spraying method quality is crucial.

Where possible, peridomestic populations of Triatominae should be reduced or eliminated (not least, for their effects on the productivity of peridomestic animals), but from a public health standpoint they can also be viewed as akin to sylvatic

populations, some of which are also potential sources for reinfesting the domestic habitat. Seen in this light, the strategy changes. It is both impractical and ecologically unacceptable to contemplate large-scale interventions against sylvatic populations of Triatominae. It is also irrelevant in terms of transmission control. Only by coming into contact with humans, for example, by entering a house, does a sylvatic bug assume possible epidemiological significance, either by causing direct transmission or by establishing a new domestic colony. But a newly established domestic colony can be eliminated, and a transmission event can be treated, which is the basis of the Amazon surveillance strategy. Perhaps even elsewhere, peridomestic and sylvatic populations should be considered similarly, focusing on the vectors only when incipient domestic colonization is apparent, but otherwise focusing only on diagnosis and treatment of possible new cases of infection.

10.5 Criteria for Stratification of Vector Control Priorities

Often, the available resources for the vector control interventions do not allow the coverage of all the endemic areas. In these cases, some sort of prioritization is needed, and knowledge to build priorities under these circumstances has been accumulated from a number of operational research studies. Two cases, with different approaches, are presented below showing possibilities to carry out the interventions under tight budgets.

The first case is the control of *R. prolixus* in Honduras, the main vector species for *T. cruzi* in Venezuela, Colombia, and Central American countries (Ponce et al., 1995; Paz-Bailey et al., 2002). In the latter countries, the species is exclusively domestic, and it is believed to have been accidentally transported from Venezuela, before 1915 (Dujardin et al., 1998; Zeledón, 2004). This hypothesis is supported by the similarity of genetic data when specimens from Venezuela and Colombia are compared with the Central American specimens, showing low genetic variability and a reduced genome in terms of the number of bands revealed by RAPD comparisons. The Central American populations of *R. prolixus* seem unable to live outside the domestic habitats and are highly susceptible to available insecticides. The Chagas control program of the Honduras Ministry of Health successfully developed a system to collect data over regions where no previous information existed on Chagas disease, implementing the use of a rapid immunochromatographic test applied by field personnel. A serological screening with this test allows rapid and reliable data collection for the stratification of endemic areas, to identify transmission foci, and obtain baseline data on the children population. It can be used to predict areas where domestic infestations can be expected, particularly with *R. prolixus*. The experience in Honduras allowed shortening the response time and reduction of the costs of interventions, giving support to affected communities with objective criteria of priority and cost-effectiveness in public health. The approach allowed a rapid identification of areas infested by *R. prolixus* in regions where no previous information existed.

The second case refers to vector control activities in La Rioja province (Argentina) historically highly endemic for Chagas disease with active vectorial transmission (Segura, 2002). From 2004, a new structure for the vector control program of the province was organized. The new vector control activities, besides the normal entomological evaluation and insecticide application, included the individual coding of rural houses and geolocation using a global positioning system device and the organization of a regularly maintained information system. After 6–12 months, the vector control field teams returned to the previously reported intradomestic infested houses to carry out a new entomological evaluation and respray the houses (intra- and peridomestic application) if still infested. In parallel, using the geographic information collected in the field, a spatial analysis was carried out of house infestation to identify spatial aggregates, where the activities of the field teams could be reinforced. Using this simple strategy, with a modest number of personnel and field vehicles during 5 years of uninterrupted activities, the intradomestic infestation by *T. infestans* of rural houses dropped from 25% to less than 1%, and no acute cases of Chagas disease were reported. Peridomestic infestation is still relatively high in some provincial departments (>20%), and continued efforts integrating other vector control methods (e.g., modification of peridomestic structures for animal shelters) are currently under way in the affected areas (Porcasi et al., 2006, 2007; Gorla et al., 2009).

10.6 Insecticide Resistance

During 2000, a reported control failure after a pyrethroid application for *T. infestans* control in Salvador Mazza (Salta, Argentina) appeared (Picollo et al., 2005). This was attributed to pyrethroid resistance of the S. Mazza population of *T. infestans*. Subsequent studies have shown the occurrence of other control failure events of *T. infestans* populations, that were shown resistant to pyrethroids in the Bolivian Chaco region (Picollo et al., 2005; Santo Orihuela et al., 2008) and more recently in Pampa del Indio (Chaco, Argentina) (Gurtler, personal communication). For the moment, the mechanism of insecticide resistance appearance although it appears to involve changes in the penetrability of the insect cuticle (P. Juarez et al., personal communication) and the geographical extent is not clear. Studies reported so far on the *T. infestans* populations of the Bolivian Chaco and S. Mazza indicate a resistance rate to deltamethrin higher than 400 in some places, with cross-resistance to other pyrethroids and to fipronil, but generally susceptible to organophosphates and carbamates. Organophosphate and carbamate compounds have been sprayed by professional field teams of Argentina and Bolivia to control domestic and peridomestic populations of *T. infestans* in S. Mazza and various localities in Bolivia. Although postspraying reports indicate a decrease of vector abundance and house infestation, the resistant *T. infestans* populations are still present in the area. A different intervention type, using insecticidal paints based on organophosphate compounds, was applied to domestic and peridomestic structures in southern localities of Santa Cruz

in Bolivia (intervention applied to over 2000 houses), where *T. infestans* is resistant to pyrethroids. In this case, houses show no infestation by *T. infestans*, either intradomestic or peridomestic populations, even 3 years after the application of the insecticidal paint (Dias and Jemio, 2008; Gorla et al., unpublished data).

10.7 The Political Commitment

The experience accumulated over the last decades illustrated by the examples given earlier seems to suggest that control of Chagas disease vectors is feasible, and results in strikingly reduced rates of infection incidence and prevalence. The end point for elimination of Chagas disease as a public health problem can be then described when all existing domestic infestations of Triatominae have been eliminated, and local health authorities are structured and equipped to diagnose and treat occasional new infections, and to eliminate perhaps through contracts with local pest control operators any incipient domestic vector infestation. Epidemiologically, the situation might then resemble that of Lyme disease in Europe the vector ticks (*Ixodes ricinus*) are present in gardens (which may be said to comprise both peridomestic and sylvatic habitats), and there is a risk of *Borrelia* transmission; however, the ticks do not enter houses (and if they did, would be rapidly dealt with), and if a new infection occurs, it is relatively simple to diagnose and can be treated.

Although Chagas disease will not be eliminated in the sense of ceasing to exist as a human disease, we believe that it could be eliminated as a serious public health problem when all existing domestic vector populations have been eliminated, and all aspects of current control programmes are adequately incorporated into routine local health programs. The products, equipment, and experience are available for this, and strategies have been developed both for the initial campaigns and their consolidation through active vigilance, and for subsequent integration of the surveillance activities into routine public health activities (Schofield et al., 2006). But all comes to nought without political commitment and leadership, which in turn liberates the required resources. In a few countries, there is still no coherent national program; in others the national program is in disarray, with spraymen and vehicles idle as they lack the minimum resources to mobilize. Perhaps the initial successes of the multinational initiatives were too widely hailed, but relieving some 60 million people from the molestation of Triatominae and risk of disease (as some have claimed) still leaves some 40 million with little protection which is both inappropriate and unethical, given the demonstrated feasibility of the large-scale control interventions. Paradoxically perhaps, a renewed urgency to complete the control interventions may come from the previously nonendemic countries now receiving migrants from Latin America some of whom require treatment for their chronic Chagas infection, and some of whom pose a new risk for onward transmission by blood transfusion or organ transplant (cf. Schmunis, 2007). It is to be hoped that the domestic Latin American vectors can be eliminated before they too begin to arrive in Europe and elsewhere (Schofield et al., 2009).

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11 Domestic and Wild Mammalian Reservoirs

Ana Maria Jansen and André Luiz Rodrigues Roque*

Laboratory of Trypanosomatid Biology Oswaldo Cruz Institute, Rio de Janeiro, Brazil

11.1 Introduction

It has been suggested that *Trypanosoma cruzi* originated around 80 million years ago in a southern "supercontinent" of what are now South America, Antarctica, and Australia, which remained connected after its separation from Africa. Marsupials and xenarthrans, which represented the autochthonous mammalian fauna, were *T. cruzi*'s first hosts. Since no hematophagous insects existed at that time, the parasite was transmitted most probably by: (i) predation of infected mammals and (ii) material from anal glands of infected opossums (by ingestion and/or contact with mucosal and injured skin), since these glands can maintain the extracellular multiplication cycle of *T. cruzi* and eliminate infective metacyclic forms (Deane et al., 1984; Schofield, 2000). The scenery in South America remained unchanged up to the arrival of the first caviomorph rodents and primates from Africa (35 million years ago), which represents the first of several migration waves that occurred up to 5 million years ago and resulted in the subsequent diversification of the fauna observed nowadays in the whole continent (Flynn and Wyss, 1998). In this sense, marsupials and xenarthrans may be considered the oldest *T. cruzi* hosts (Briones et al., 1999; Stothard et al., 1999; Buscaglia and Di Noia, 2003).

Subsequently, *T. cruzi* adapted to new hosts as they arrived in South America: initially rodents and primates, and later, bats and carnivores. The diversity of mammalian hosts species introduced in the *T. cruzi* transmission cycle provided different selective pressures that resulted in the huge variety of parasite subpopulations observed today, which have distinct biological and molecular patterns. These differences are discussed in Chapter 19. Since the majority of studies on wild *T. cruzi* reservoirs were performed before knowledge leading to the current separation of the parasite subpopulations based on discrete typing units (DTUs) (Brisse et al., 2000), we adopted here the previous consensus proposed by experts in 1999 that recognizes two major genotypes, TCI and TCII, and a group of subpopulations of

*E mail: jansen@ioc.fiocruz.br

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parasites that do not fall within these two genotypes and continued to be known as Z3 (zymodeme 3) (Anonymous, 1999).

The proposed ancient coevolutionary history of *T. cruzi* with its mammalian hosts is based on the relatively recent acquisition of hematophagic habits by the triatomine vectors (Schofield and Galvão, 2009). Although the origin of their Reduviidae ancestors dates from about 230 million years ago, the vast majority of its subfamilies consist of predatory insects and so far, only one supposedly hematophagic fossil species was found (Poinar Jr., 2005). Thus, the digenetic cycle observed today, which involves triatominae vectors and mammalian hosts, seems to have started only after the acquisition of hematophagic habits by triatomines, which is supposed to have occurred approximately 5 million years ago, and after the adaptation of the parasite to the gut of these vectors (Carcavallo et al., 1999; Schofield, 2000).

11.2 The Complex *T. cruzi* Transmission Cycle

Currently, *T. cruzi* circulates in more than 100 mammalian species dispersed in eight different orders and dozens of species of insect vectors, which are bugs of the Reduviidae family (Noireau et al., 2009). Birds and cold-blooded vertebrates are refractory to the parasite. *T. cruzi* transmission cycles are found in all phytogeographical regions in the Americas, from North Argentina to the southern USA. The diverse ecological niches where the parasite can be found consist of numerous microfoci of transmission that display different epidemiological profiles. *T. cruzi* is also able to colonize almost all tissues in its many mammalian hosts, including unconventional sites, such as the scent glands of *Didelphis* sp. (Deane et al., 1984) and cornea of *Thrichomys apereoides* (Herrera et al., 2007b). The multiplicity of host−parasite interactions in the wild is reflected in transmission cycles that can be characterized as complex and multivariable systems (PAHO, 2009).

Triatomines transmit the parasite only if infected, which occurs when they feed on a infected mammalian host and ingest, with their blood, the trypomastigote forms of the parasite. In the digestive tract of triatomines, the parasites differentiate into epimastigotes (multiplicative form) and then to metacyclic trypomastigotes in the final portion of the intestine. Infection of mammals occurs when they come in contact with the infective metacyclic forms of the parasite that are eliminated with the feces of triatomines after their feeding. This contact occurs through the mucosa or through injury, preexistent or resulting from the bite of the bug. The oral route (i.e., ingestion of infective forms of the parasite) occurs when the animal scratches with his mouth the place on its body where the bugs' feces were deposited, eats food contaminated with the parasite, or preys on bugs or other infected mammals. Currently there is a great concern about human congenital transmission. In fact, mother to child *T. cruzi* transmission represents a major public health problem in Bolivia, a highly endemic country. Moreover, due to migration of people from endemic areas, congenital Chagas disease is also becoming a problem in nonendemic countries (Dias, 2009). Thus, a great effort is presently being spend with the

objective to clarify what variables (host and/or parasite related) determine vertical transmission.

11.3 What Are the *T. cruzi* Reservoir Hosts?

Since the discovery of *T. cruzi* a century ago, several researchers have sought to know which mammalian species were the main source of infection for the triatomines and, hence, to man. Numerous species of wild mammals were found infected by *T. cruzi* and named "natural reservoirs" of the parasite (Barreto and Ribeiro, 1979). Subsequent studies showed that, for multihost parasites such as *T. cruzi*, several mammalian species could be identified as the bugs' source of infection in a given place. The researchers started to understand that these mammalian species differ in their importance as source of infection for the triatomine vector, and that the same mammalian species can play different roles in the maintenance of the parasite in different regions and time schedules. Under this focus, we now consider "reservoir" not as a single species of mammal found infected in a given locality, but a system that includes one or more species of mammals that are responsible for maintaining the parasite in nature. In each ecological system, the mammalian species may play different roles in the maintenance of the parasite, which means that the reservoir system varies and should be considered as unique within a certain spatiotemporal scale.

As in any other host−parasite system, the *T. cruzi* infection patterns in any mammalian host species are determined by host factors (species, sex, age, behavioral patterns), parasite traits (generation time, dispersion strategies, molecular and biochemical characteristics of its subpopulations), and by the environmental conditions (stress, host coinfections, availability of natural resources) where the host−parasite interaction takes place. We consider maintenance reservoirs as those able to be infected and to retain the infection of a given parasite. Amplifier reservoirs are those that display a characteristic of infection that favors the transmission of this parasite (i.e., high transmissibility). It is worth mentioning that these characteristics are interchangeable, so maintenance reservoirs may act as amplifier reservoirs according to, among other factors, health conditions including immune suppression and concomitant parasitic infections. Concerning *T. cruzi*, the transmissibility from a given mammalian species to the triatominae vector is guaranteed if this host displays circulating infective forms of the parasite in its blood. This feature is demonstrated by the detection of parasites in the mammal's blood by parasitological techniques that include fresh blood sample examination, hemoculture, or xenodiagnosis. In contrast, transmission of parasites between two mammalian species (predator−prey) can be attained even in the absence of circulating infective forms in blood, since amastigotes present in the tissues of the preyed mammalian host can also be a source of infection. Molecular diagnosis by polymerase chain reaction (PCR) can be considered an enriched direct parasitological assay, since it detects constitutive parts of the parasites, in this case fragments of DNA.

This technique can be applied to diagnose *T. cruzi* in the blood, but an important feature should be taken into account: the inhibitor property of the iron ions present in the mammal's blood during PCR reaction, which can induce false-negative results (Lachaud et al., 2002; Piron et al., 2007). Even for the positive ones, we have to consider that this test indicates the presence of DNA fragments, and its high sensitivity does not allow us to conclude whether the parasite load detected would be sufficient to ensure the transmissibility of that parasite (Castro et al., 2002; Britto, 2009). Alternatively, a quantitative reaction (real-time PCR) may in future indicate the minimum parasite load capable to ensure transmissibility to the vectors, but this kind of reaction still needs to be standardized (Schijman et al., 2003; Britto, 2009). The diagnosis of *T. cruzi* infection obtained by indirect tests, such as the serological ones, not only shows the exposure of that mammalian species to the parasite, but also does not reflect its transmissibility to the bugs or other mammals. Serum-positive mammals shown to be exposed to *T. cruzi* infection are indeed hosts of this parasite, but are not necessarily important for the maintenance of that parasite in nature (i.e., are not necessarily reservoirs of the parasite).

In this sense, an epidemiological investigation on *T. cruzi* wild hosts should include, essentially, a representative sampling of the most abundant mammalian species in the area and a broad methodological approach that includes the diagnosis of infection by direct and indirect parasitological tests (Roque et al., 2008). Nevertheless, most studies are geographically restricted and do not include data on the broader environmental conditions, such as local fauna diversity, relative abundance of infected mammalian species, or the population structure. The restriction of studies to some specific mammalian species or specific areas, such as the peridomestic ones (which are not representative of the whole area), results in misinterpretation of data and description of the most common species in a given habitat as the main (or even the unique) reservoirs of the parasite. Some mammalian species such as the synanthropic *Rattus rattus*, *Mus musculus*, and *Didelphis* sp. are often described infected in studies, but do not always represent the most important species implicated in the maintenance of the parasite in the area (PAHO, 2009). It is important to note that a given mammalian species may display a high prevalence of *T. cruzi* infection, but should still not be characterized as a reservoir host. As pointed out before, a reservoir host must be able to maintain the transmission cycle of the parasite and for that, this mammalian species should be abundant in the area. If this species represents only a negligible relative abundance of the local mammalian species, its importance in the transmission cycle of the parasite is lower than the importance of a second mammalian species that displays a lower prevalence of infection but is much more abundant in the area. Likewise, in some scenarios, an infected species that displays a high relative abundance is more competent as reservoir host in a given area than in another, where its abundance and total biomass are not very significant (Ashford, 1997; Roque et al., 2008).

Due to this complex epidemiology, it is clear that unlike linear systems, the transmission cycles of *T. cruzi* in nature have to be understood as dynamic webs, with parasite transmission by different routes in transmission cycles in the forest floor, understory, or canopy that may or may not overlap. These transmission nets

include different species of mammals and bugs, each one displaying peculiarities of their interaction with *T. cruzi* that are reflected in their ability to transmit the parasite. This is the context in which we present some of the mammalian species able to maintain the parasite in nature (and, therefore, potential reservoirs), and some of the most relevant studies in each one of the eight orders of mammals responsible for maintaining *T. cruzi* in nature. Domestic animals are discussed in a separate section at the end of this chapter.

11.3.1 Order Marsupialia

Representative of the autochthonous fauna, the order Marsupialia displays its greatest diversity of species in the Australian continent, while only one family is recognized in the Americas, the Didelmorphidae. Its ancestors are regarded as the oldest hosts of *T. cruzi*. The genus *Didelphis* (Figure 11.1) is the most spread on the continent, occurring from southeastern Canada to southern Argentina, and is recognized by many authors as the most important mammalian reservoir of the parasite (WHO, 1991; Yeo et al., 2005).

Its wide distribution is mainly due to its remarkable adaptability to different ecological niches, especially environments with a high degree of human action. These animals can colonize ceilings of houses and other shelters in domestic and peridomestic areas, where they survive feeding on human food garbage. Because of that, *Didelphis* sp. are currently recognized as synanthropic mammalian species and their presence is a strong indicator of environmental disturbance by human action

Figure 11.1 Opossum *Didelphis albiventris* from São Borja, Brazil. *Source*: Photograph by Ana Maria Jansen.

(Olifiers et al., 2005). *Didelphis* sp. are nomadic, solitary (especially males), excellent climbers, and their main refuge are holes and foliage of trees. Their importance as reservoir varies in time and space, as observed in the infection rates of *D. aurita*, which range between 11% and 90% among animals collected in different localities in the state of Rio de Janeiro, Brazil (Fernandes et al., 1999). Besides vector transmission, its omnivorous habits favor the oral acquisition of infection by predation of infected bugs or small mammals.

When infected by *T. cruzi*, these marsupials exhibit some characteristics that make them unique when compared to other hosts of the parasite: the ability to act both as hosts and vectors of the parasite. In infected *Didelphis* sp., some parasites reach the scent gland's lumen where the bloodstream trypomastigotes differentiate into epimastigotes that multiply by binary division. They then differentiate into metacyclic trypomastigotes that are the infective forms, which are released with the secretion of this gland (Deane et al., 1984). This stage simulates the multiplication cycle of the parasite observed in the digestive tract of the bugs and occurs simultaneously with the intracellular multiplicative cycle that is driven by amastigote forms in various tissues of the marsupial. The vector competence of these glands and, therefore, their epidemiological importance remains unknown, regardless of the elevated rate of metacyclogenesis (50%) that points this way as an efficient route of spreading the parasite. Notwithstanding, monogenetic trypanosomatids (*Leptomonas*, *Crithidia*, and *Herpetomonas*) may also efficiently colonize the scent glands of *Didelphis aurita* under experimental conditions when the axenic culture form is directly inoculated into these glands (Jansen et al., 1988). Besides *Didelphis* spp., the *T. cruzi* multiplication cycle in scent glands has also been demonstrated in experimentally infected *Lutreolina crassicaudata* (Steindel and Pinto, 1988).

Experimental studies showed the absence of neonatal transmission of the parasite in *D. aurita* and that they are able to maintain stable infections by subpopulations of the genotype TCI and control or even eliminate subpopulations of genotype TCII. However, these studies were performed with a small number of isolates that are not representative of the heterogeneity observed among parasite subpopulations of the genotype TCII (Jansen et al., 1991). In fact, although these animals are most commonly associated to genotype TCI, the TCII genotype has also been isolated (Pinho et al., 2000). Although often associated with the arboreal strata, *Didelphis* sp. move among all ecological strata and may act as a bridge of independent transmission cycles, since they can become infected at soil level and be a source of infection for another bug in the crown of a tree (and vice versa) (Jansen et al., 1999; PAHO, 2009).

In nature, *Philander frenatus* and *P. opossum* may also play an important role in the transmission of the parasite, since they are found displaying a prevalence of positive hemocultures that can reach 80%. Distinct from *D. aurita*, the experimental infection of *P. frenatus* resulted in high parasitemia and elevated antibody titers when infected by both TCI and TCII genotypes of the parasite. The patterns of experimental infections of *D. aurita* and *P. frenatus* show that these species may exert different selective pressures for distinct *T. cruzi* subpopulations, and thus play different roles in the transmission of the parasite in nature (Legey et al., 1999).

Both species are mainly associated to gallery forests and do not show the synanthropic behavior observed in *Didelphis* sp, which may indicate a more limited importance as *T. cruzi* vector to men.

Monodelphis domestica is a terrestrial marsupial species commonly found infected with *T. cruzi* and has already been identified as the main reservoir of the parasite in a recent outbreak of orally transmitted Chagas disease in northeast Brazil (Roque et al., 2008). *Caluromys lanatus*, *Lutreolina crassicaudata*, *Marmosa* sp., *Metachirus nudicaudatus*, and *Monodelphis brevicaudata* are other species of marsupials already found naturally infected by *T. cruzi* (Barretto and Ribeiro, 1979; Herrera et al., 2005; Marcili et al., 2009a).

Tissue lesions of experimental *T. cruzi* infections in *D. aurita* are characterized by mild inflammatory infiltrates mainly composed by lymphocytes and macrophages; more severe infiltrates by the same cell type have been described in naturally infected *D. aurita*. Moreover, in both cases, these lesions were demonstrated to be significantly milder in comparison to Swiss mice. In fact, mortality was observed only in 24-day-old still pouch-dependent, experimentally infected opossums (Carreira et al., 1996).

Opossums are fascinating mammals due to their unique reproduction strategy: a newborn opossum is in fact still a fetus and unable to control body temperature; it does not produce immunoglobulins and does not reject allografts; its eyes and ears are sealed; its hind limbs are still not differentiated; and its mouth displays a small aperture, sufficient only to allow the attachment to the nipple, that after attachment almost reaches the stomach of this newborn. Struggle for life starts at the very beginning of the opossum's life, since there are generally more newborns than available nipples. Moreover, a newborn must literally crawl with the aid of its forelimbs over its mother's belly toward the maternal pouch and attach to a nipple, where it will remain until around 45 days of age without detaching. Total independence from the mother opossum will be reached by 100−120 days after birth. Neonatal or vertical transmission was not observed in experimentally infected opossums, even during the acute phase.

11.3.2 Superorder Xenarthra

Xenarthrans were previously classified in an order termed Edentata, recently found to be polyphyletic, that also included anteating Pangolins. With the reclassification of the latter to the Pholidota order, the orders Cingulata (armadillos) and Pilosa (anteaters and sloths) were classified together in the superorder Xenarthra (odd joints). Xenarthra display fascinating biological peculiarities such as fused pelvic bones, spine-reinforcing bones, and a peculiar blood vessel structure that allows its energy-sparing extremely low metabolic rate. These adaptations did not prevent a massive extinction of a huge number of representatives of this taxon so that the current extant genera represent only a minor part of those found in the Tertiary. In modern times, the specific niche destruction due to human action certainly contributed expressively, but the overall causes of this massive extinction are still under debate (Bugge, 1979; Gaudin and Biewener, 1992).

Armadillos, sloths, and anteaters, currently the main representatives of this superorder, have a long coevolutive history with trypanosomatids. So, besides the bizarre genus *Endotrypanum*, sloths may harbor several *Leishmania* and *Trypanosoma* species (Rotureau, 2006). Armadillos, sloths, and anteaters represent, besides marsupials, the most ancient hosts of *Trypanosoma cruzi*. The first description of a wild reservoir of this parasite was done by Carlos Chagas in 1912, when he found tripomastigote forms similar to *T. cruzi* in the wild nine-banded armadillo *Dasypus novemcinctus*, which he classified as a "depository of the agent of Brazilian trypanosomiasis in the outside world" (Chagas, 1912). In its underground refuges, armadillos are usually associated with triatomines from the *Panstrongylus* genus and some studies suggest an association between this mammalian host and the parasite genotype TCII (Yeo et al., 2005). Nevertheless, these animals have been found infected with the other genotypes of the parasite, TCI and Z3, showing its putative importance in the maintenance of distinct transmission cycles in nature (Acosta et al., 2001; Marcili et al., 2009a).

Armadillos are widely distributed and are found naturally infected by *T. cruzi* in a prevalence of infection that ranges from 4% to 50%, from the southern USA to Uruguay. In Louisinana, in the USA, infection by *T. cruzi* was detected in 26% of 80 armadillos (Yaeger, 1988). In another region of the same state, only 3.9% of 415 *Dasypus novemcinctus* were infected (Paige et al., 2002). In a retrospective study conducted in French Guiana, *Dasypus novemcinctus* were identified as an important reservoir of *T. cruzi*, following *Didelphis marsupialis* (Raccurt, 1996). Other species found to be infected by *T. cruzi* are *Cabassous unicinctus*, *Chaetophractus vellorosous*, and *Euphractus sexcinctus* (Figure 11.2).

It is worth mentioning that, in rural areas, *E. sexcinctus* regularly invade chicken facilities to prey on eggs and/or chicks. This behavior favors the nearness of this

Figure 11.2 Armadillo *Euphractus sexcinctus* from Axixá, Brazil.
Source: Photograph by Ana Maria Jansen.

armadillo species with peridomestic areas, where it can be a source of infection for triatomine bugs that nest there.

Concerning the arboreal xenarthrans, sloths are usually associated to triatomines from the genuses *Belminus*, *Panstrongylus*, and *Rhodnius* (Carcavallo et al., 1998), and some sloth species, such as the three-toed sloth (*Bradypus torquatus*), have been found naturally infected (Fernandes et al., 1999). Two different species of anteaters, the lesser anteater (*Tamandua tetradactyla*) and the silky anteater (*Cyclops didactylus*), have also been considered as natural hosts of *T. cruzi* (Bento et al., 1992; Yeo et al., 2005).

The epidemiological importance of these mammalian species is enhanced as sloths, anteaters, and armadillos are hunted and eaten in some areas of South America, such as the Amazon region. The careless handling of the carcass or the ingestion of undercooked meat from infected animals can be a source of *T. cruzi* infection for humans.

11.3.3 Order Rodentia

Rodents represent perhaps the most diverse and spread-out of mammalian taxa. A common quality of rodents is the continuously growing single incisor tooth pair that does not present enamel on its posterior face. The morphological diversity in the taxon is exemplified by the contrast between a tiny 5 g pigmy mouse and the huge capybara that may reach 70 kg in weight. Rodents may be found in desert areas, adapted to aquatic media, digging long and interconnected tunnels as well as on forest canopies. In spite of being so diverse, there are several groups whose systematic position is still under debate and that may only be separated by karyotyping. Reproduction strategies are also quite distinct; thus, reproduction seasonality, gestation time, and number of offspring may differ significantly among the genera of this order (Wilson and Reeder, 2005).

Rodents are not autochthonous from Americas and the first animals (Hystricognathi-caviomorphs) arrived along with the first primates originating from Africa about 45 million years ago. The second great migration wave of rodents to the Americas (Sciurognathi cricetids) is much more recent and appears to be related to the diversification of murids and cricets in Africa with arrival by a migration route that included an initial establishment in North America. Since their arrival, rodents have adapted well and diversified into a large number of species in the Americas. They colonized various types of habitats, from rain forests to deserts, high-altitude plateaus to floodplains, wild to urban environments; moreover, they colonize the diverse vegetation strata in which can be found from fossorial up to arboreal and semiaquatic rodents (Wilson and Reeder, 2005).

In nature, rodents are ubiquitous mammalian species, and their diverse microhabitats are often shared with bugs from genus *Triatoma* (associated with rocky terrestrial refuges) and *Panstrongylus* (associated with holes in the ground) (Carcavallo et al., 1998). Among mammalian species, rodents are the main targets of predation, and this is an important feature since it enables the transmission of *T. cruzi* by the oral route. Their epidemiological importance is even more evident when we consider that

many rodent species, although predominantly wild, can frequent human dwellings and participate in the *T. cruzi* transmission cycle in peridomestic areas (Mills and Childs, 1998; Roque et al., 2008).

Despite being a widespread mammalian order, the natural infection of wild rodents by *T. cruzi* is little reported, which may be the result of the limited methodology employed for animal sampling in several studies, frequently restricted to peridomestic areas. This is confirmed by the fact that the most common rodent species found infected by *T. cruzi* is the rat, *Rattus rattus*, a synanthropic species highly abundant in most urbanized cities in South America, but rare inside rain forests. Although abundant in experimental studies, natural *T. cruzi* infection in the mouse *Mus musculus* is little reported. Other rodent species already found naturally infected by *T. cruzi* are: *Agouti paca*, *Akodon* sp., *Bolomys lasiurus*, *Calomys expulsus*, *C. callosus*, *Cavia* sp., *Clyomys laticeps*, *Dasyprocta* sp., *Echymis chrysurus*, *E. dasytrix*, *Galea spixii*, *Holochilus brasiliensis*, *Kerodon rupestris, Nectomys squamipes*, *Octodon degus*, *Octodontomys* sp., *Oecomys mamorae*, *Oligoryzomys stramineus*, *Oryzomys capito*, *O. scotti*, *Proechimys* spp., *Rhiphidomys macrurus*, *Sigmodon hispidus*, *Thrichomys* spp., and *Tylomys mirae* (Herrera et al., 2005, 2007a; Yeo et al., 2005; Vaz et al., 2007; Roque et al., 2008).

Among these, the wild rodents whose participation in the transmission cycles of *T. cruzi* is more empirically proven are the caviomorphs from *Thrichomys genus*. This genus comprises at least five sibling species distributed in the Pantanal/Chaco (marshlands), Cerrado (savannah), and Caatinga (white shrub) biomes (Bonvicino et al., 2002). *Thrichomys laurentius* (Figure 11.3) was found infected by *T. cruzi* in prevalences that can reach 44% (positive hemocultures) in some localities surrounding the Serra da Capivara National Park in Brazil (Herrera et al., 2005).

This rodent species was also found infected in an area of orally transmitted Chagas disease in a recent outbreak in the Ceará state of Brazil (Roque et al., 2008). *T. pachyurus* and *T. apereoides* are other species from the same genus also found infected by *T. cruzi.* The three different genotypes of *T. cruzi* (TCI, TCII, and Z3) were already reported in natural *Thrichomys* spp. infections, including a mixed infection (TCI/Z3) (Herrera et al., 2007a).

Experimental studies have shown that *T. apereoides* are able to maintain stable infections by both genotypes TCI and TCII of the parasite (Herrera et al.,

Figure 11.3 Punare rodent *Thrichomys laurentius* from São Raimundo Nonato, Brazil. *Source*: Photograph by Ana Maria Jansen.

2004). Two other *Thrichomys* species (*T. laurentius* and *T. pachyurus*) were also able to maintain a controlled experimental infection with *T. cruzi*, displaying patent parasitemia, effective humoral immune response, and important tissue damage (Roque et al., 2005). In the latter study, the differences observed in *T. cruzi* infection patterns showed that *T. laurentius* was more resistant to infection than *T. pachyurus*, expressed by lower parasitemia and less tissue damage. The differences observed in these experimental infections may influence the ability of these different rodents to act as *T. cruzi* reservoirs in their respective biomes.

Taken together the results from the experimental infection, the wide distribution of *Thrichomys* spp. in nature and the prevalence of natural infection by *T. cruzi*, these rodents can act as (i) maintenance hosts, due to the ability to maintain long-lasting subpatent parasitemias, and (ii) amplifier hosts, demonstrated by the long period of patent parasitemia in experimental conditions and positive hemocultures in natural infections (Roque et al., 2005).

Finally, studies involving *T. cruzi* and caviomorphs showed that a long historic host−parasite relationship does not necessarily evolve into a harmonic interaction; but may evolve into one that favors the transmission of the parasite. In *Thrichomys* sp., experimentally infected rodents presented significant heart damage, which is certainly reflected in its ability to provide tissue oxygenation. This means that an infected rodent would have a diminished ability to avoid predators, predisposing them to transmit the parasite to other mammalian species.

11.3.4 Order Primata

A marmoset *Callithrix penicillata* was the first host identified by Carlos Chagas when he discovered a new trypanosome in Lassance (Minas Gerais state, Brazil) named *Trypanosoma minasense*. Later, investigating the local triatomines, Chagas found flagellates that he inferred to be the intermediate forms of the trypanosomes diagnosed in marmosets. To confirm this hypothesis, Chagas sent some infected bugs to be used to infect groups of *Callithrix jacchus* that were kept in captivity. Performed by Oswaldo Cruz, this study resulted in the visualization of flagellates in the peripheral blood of marmosets displaying morphology quite distinct from *T. minasensis*: it was the discovery of *Trypanosoma cruzi* (Dias and Coura, 2009).

Since then, different species of Neotropical primates included in Cebidae (monkeys) and Callitrichidae (marmosets) families are commonly found naturally infected by *T. cruzi*. Widespread in the Americas, these primates occupy different forest strata and have varied feeding habits, including species that feed on invertebrates and small mammals, which facilitates *T. cruzi* transmission by the oral route. Their nightly refuges in hollow trees are often shared with triatomine bugs, which allows (or propitiates) vector transmission of the parasite to these mammalian species (Carcavallo et al., 1998).

The infection prevalence in primates varies between 4% and 88% and can be quite elevated in tamarins (Lisboa et al., 2004). Both the prevalence of infection and the transmissibility potential of the parasite by the host (attested by positive hemoculture) may vary according to gender, age, species, coinfection with other parasites

Figure 11.4 Gold lion tamarin *Leonthopitecus rosalia* from Silva Jardim, Brazil. *Source*: Photograph by Rodrigo Mexas.

and general health, ecological characteristics, and origin of the studied population. An association between the presence of trichostrongilid nematode infection and prevalence of positive hemocultures, for example, has already been described in lion tamarins *Leonthopitecus rosalia* and *L. chrysomelas* (Monteiro et al., 2007).

In the Poço das Antas National Park (Rio de Janeiro, Brazil), the golden lion tamarin (*L. rosalia*) (Figure 11.4) acts as an important *T. cruzi* reservoir since it displays a high prevalence of seropositive animals and high rates of positive hemoculture (46%). Tamarins live in quite stable social groups and are extremely territorial. The components of the groups share tasks like carrying the newborns. The *T. cruzi* infection prevalence is not homogeneously distributed among the tamarin social groups; in fact, the prevalence varies among the different social groups of the Poço das Antas National Park. The aggregated character of the *T. cruzi* infection among the tamarin groups is probably due to microenvironmental peculiarities, since these tamarins display no expressive genetic heterogeneity (Lisboa et al., 2000). This prevalence of infection was found to be quite distinct from other areas, adjacent to the park, where the same species *L. rosalia* presents a low prevalence of *T. cruzi* infection, reinforcing the nidal characteristic of the parasite maintenance in nature.

High positive hemoculture prevalence was also observed in the sibling tamarin species *Leontopithecus chrisomellas* in a biological reserve located in Una, Bahia

state, in a Brazilian northeastern fragment of the Atlantic coastal rain forest. The absolute majority of the *T. cruzi* isolates derived from both tamarin species were characterized as TCII. The genotype TCI was observed in other species of nonprimate mammals from the Poço das Antas National Park and in a dozen tamarins of both biological reserves (Lisboa et al., unpublished data).

The infection of primates is not primordially by the TCII genotype of the parasite, since many other primate species have been found naturally infected with the TCI genotype of the parasite in different regions of Brazil, from the Atlantic to the Amazon region (Lisboa et al., 2006). The genotype Z3 of the parasite has also been described in primates, but this finding is still restricted to the Amazon region (Marcili et al., 2009b).

An important feature that has to be taken into consideration is that several Neotropical primates are threatened species submitted to conservation programs, such is the case of the golden lion tamarin. These programs often include exchange, translocation, and reintroduction of animals without considering the prevalence and pattern of infection by parasites such as *T. cruzi*. Such programs can lead to an introduction of infected mammals in untouched areas and trigger the establishment of new transmission cycles in other areas.

Experimental studies in Neotropical (*Cebus* sp., *Callithrix* sp., and *Saimiri* sp.) and African (*Macaca mulatta*) primates show that infection by *T. cruzi* in these mammalian species presents some similarities with Chagas disease, such as a low frequency of cardiac abnormalities and the rare occurrence of megasyndromes and systemic changes. Electrocardiographic alterations observed were the low voltage of T- and R-waves and high voltage of V3 waves, all of them in DII (Monteiro et al., 2006).

11.3.5 Order Carnivora

Carnivores are also a very heterogeneous group that includes all meat eaters, thus the grizzly bear, skunks, weasels, and the domestic cats are included in the taxon. Besides predating other vertebrates, carnivores complete their diet feeding also on plant insects, among other things. Its common trait is the presence of five-finger feet presenting claws and teeth adapted to tear. Highly persecuted due to their livestock predation potential and other reasons, many of the big carnivore species are near extinction. Wild carnivores such as the coati (*Nasua nasua*), the raccoon (*Procyon lotor*), the weasel (*Eira barbara*), and crab-eating fox (*Cerdocyon thous*) have been found naturally infected with *T. cruzi* (Barretto and Ribeiro, 1979; Yeo et al., 2005). Some of them, like the coati and the weasel, are found both on the ground and in the canopy of trees, favoring parasite spread among different forest strata. Carnivores have important body mass and large life area, important aspects to successfully spread parasites and act as amplifier reservoirs. Medium and large carnivores are known to be top predators of their food chain that usually includes smaller mammals that may be infected by *T. cruzi*. Thus, although vector transmission also occurs, the most common way of infection for these mammalian species, as to any other predator in nature, seems to be the oral route through ingestion of infected mammals.

In the Pantanal/Chaco region (marshland), coatis (Figure 11.5) seem to be the main *T. cruzi* reservoirs and were already found infected by all genotypes of the parasite (TCI, TCII, and Z3) in single or mixed infections (TCI and TCII, TCI and Z3, Z3 and TCII) (Herrera et al., 2008b). The monitoring of recaptured mammals showed that *T. cruzi*−infected coatis can present high and long-lasting parasitemias. Due to their high biomass and high relative abundance among mammalian species, coatis certainly play a role in the amplification and dispersal of the main *T. cruzi* subpopulations in this region, demonstrating that predator−prey links may be excellent mechanisms for *T. cruzi* transmission and perpetuation in the wild (Herrera et al., 2008b). In Pantanal, the presence of *Didelphis* sp., considered as the main reservoir host of *T. cruzi*, was only negligible. In this area, coatis were acting as the main reservoir host. This finding reinforces the importance of not aprioristically electing any target species as a reservoir.

The Z3 genotype of the parasite was also isolated from a ferret (*Galictis vitatta*) in the Atlantic Forest of Rio de Janeiro, Brazil (Lisboa et al., 2009). Skunks (*Conepatus chinga*) in Argentina (Pietrokovsky et al., 1991), and gray foxes (*Urocyon cinereoargenteus*) in Central America and southern United States (Rosypal et al., 2007) are examples of other wild carnivores often found infected with *T. cruzi.*

Figure 11.5 Coatis *Nasua nasua* from Brazilian Pantanal.
Source: Photograph by Rita de Cassia Bianchi.

11.3.6 Order Chiroptera

Bats are the only flying mammalian species and, besides birds, the only animals that perform seasonal migration, a trait that is suggested to have evolved several times and independently in the distinct bat lineages. These nocturnal mammalian species perform true flapping flight, a trait that probably was inherited from their gliding ancestors and that also apparently evolved in several and distinct times within the lineages of this taxon. Except the Old World fruit bats, Chiroptera display a sophisticated echolocation system that allows them to identify the environment. Both the flight and this sensitive orientation system resulted in their high dispersion capacity. In spite of their high diversity, currently bats are considered as a monophyletic group (Jones and Teeling, 2006; Bishop, 2008; Bisson et al., 2009).

Bats are commonly found infected with several trypanosomatids, including *T. cruzi*. Their refuges include hollow trees, canopies of palm trees, and ceilings of human houses and other rural buildings, which can be shared with triatomine bugs. These mammalian species may be important reservoirs of the parasite, since they are abundant, well-adapted to anthropized environments and may display high prevalence of infection in certain areas.

T. cruzi infection of bats occurs by different ways, but oral transmission due to ingestion of infected bugs certainly plays an important role. Bats from genus *Carollia*, *Artibeus*, and *Molossus* became infected after experimental feeding on infected *Rhodnius prolixus* (Thomas et al., 2007). Furthermore, the omnivorous species *Phyllostomus hastatus* became infected after preying on infected mice (Thomas et al., 2007). Even predominantly frugivorous bats such as *Artibeus* sp. (Figure 11.6), *Carollia* sp., and *Glossophaga* sp. often feed on insects and can become infected in this way (Gardner, 1977).

Although feeding on mammalian blood that may contain infective forms of the parasite, *T. cruzi* infection in hematophagic bats is rarely documented. This feature

Figure 11.6 Bat *Artibeus planirostris* from Cachoeira do Arari, Brazil.
Source: Photograph by Ana Maria Jansen.

can be explained by the fact that these bats feed preferentially on cattle and horses, two groups of mammalian species almost never associated with infection by *T. cruzi*.

Among the triatomines that share refuges with bats are bugs from the genus *Cavernicola* (*C. lenti* and *C. pilosa*), often found in caves, and genus *Rhodnius* and *Panstrongylus*, found in hollow trees (Carcavallo et al., 1998). *R. prolixus* kept in contact with *Carollia perspicillata*, *Glossophaga soricina*, and *Desmodus rotundus* were able to perform a complete blood meal, suggesting that this vector route may also be effective for the transmission of the parasite in nature (Thomas et al., 2007). *T. cruzi* congenital transmission was already described in *Molossus molossus* and was pointed as another important way of parasite spread (Añez et al., 2009), which contrasts with the observations made in primates and marsupials (Jansen et al., 1994; Lisboa et al., 2004) that did not evidence congenital transmission. These data, apparently contradictory, reinforce the extreme complexity of the transmission cycle of *T. cruzi* among wild mammals.

A survey in 93 bats captured in different regions of Brazil showed that 15% of them, included in four different families (Molossidae, Noctilionidae, Phyllostomidae, and Vespertilionidae), displayed positive hemocultures that were further characterized as *T. cruzi* (Lisboa et al., 2008). The majority of the isolates (80%) derived from the generalist species, *Phyllostomus hastatus*, which can become infected eating infected bugs or small mammals. The family Phyllostomidae is the most prevalent in Brazil (57% of bat species) and their high prevalence of infection points to the putative importance of these mammalian species in the parasite dispersal. *M. molossus*, found in different environments and usually associated with human settlements, is another bat species frequently found infected by *T. cruzi*. No firm associations between bats and *T. cruzi* subpopulations has been established yet, as all genotypes of the parasite have already been described in these mammalian species.

11.3.7 Order Artiodactyla

Mammals from this order are rarely studied in the wild concerning the putative role played in the transmission cycle of *T. cruzi.* Despite that, an interesting situation is represented by the feral pig (Figure 11.7), domestic pigs (*Sus scrofa*) that returned to the wild environment in the Brazilian Pantanal.

These animals were infected with the TCI genotype of *T. cruzi* and described as important maintenance reservoir hosts of the parasite in nature (Herrera et al., 2008a). In the same study, two sympatric species of wild boar (*Tayassu tajacu* and *T. peccary*) were also found infected, as demonstrated by the presence of anti-*T. cruzi* antibodies in the serum of these animals.

11.4 Importance of Wild and Synanthropic Mammals on Public Health in Brazil

Since June 2006, Brazil is considered free from Chagas disease transmission due to *Triatoma infestans* (Schofield et al., 2006). This statement means that the

Figure 11.7 Feral pig *Sus scrofa* from Brazilian Pantanal.
Source: Photograph by Rita de Cassia Bianchi.

maintenance of a domiciliary *T. cruzi* transmission solely by infected men and domiciliary bugs is not encountered anymore. Human infection nowadays occurs due to vectorial transmission outside the houses, nondomiciliated bugs that invade the houses, or by ingestion of food contaminated with feces of infected bugs (Dias, 2007). In all of these cases, infected wild and synanthropic mammals play a crucial role in the maintenance of parasite circulation.

In a given environment, several mammalian species frequent different forest strata, which favor the parasite exchange among them. Opossums, for example, are predominantly arboreal, but can easily be found on the ground. Several carnivores, such as coatis and weasels, are terrestrial mammalian species that can use tree trunks as refuges. That means that a given mammal infected through the ingestion of an infected bug on the ground may be later a source of infection for other triatomine species in a hollow tree refuge. In fact, the multiplicity of vertebrate hosts and possibilities of parasite transmission is probably the main responsible factor for the extremely well-succeeded dissemination of the parasite in the Americas. It is difficult to find a forest fragment in South America where triatomines and potential mammalian hosts cohabit absolutely free from *T. cruzi* infection. For this reason, human exposure to wild environment, during extractive activities, for example, common in several areas, but especially in the Amazon basin, should always be considered as a risk to come in contact with infected triatomines and in acquiring human infection. Human cases of disease, as a consequence of this kind of exposure, seem to be very common in Pará state, inside the Brazilian Amazon (Roque et al., 2008; Roque et al., unpublished data).

In nature, *T. cruzi* transmission cycles assume different profiles that are mostly dependent on the local mammalian fauna and their ability to disseminate the parasite. Since this ability varies according to the different conditions of the environment in which they are inserted, it is not possible to predict the intensity of the enzootic cycle before examining the local fauna. Moreover, due to the dynamic character of the *T. cruzi* transmission net, even surveillance like this should be considered as restricted to a given period of time. Modifications of local fauna and dynamics of *T. cruzi* infection may result in higher or lesser prevalence of infection in the local fauna, in a process that is called "amplifier or dilution effect" (Ostfeld and Keesing, 2000). These kind of effects were first described for Lyme disease, and since then applied to other parasitic interactions: Cutaneous Leishmaniasis (Chaves and Hernandez, 2004), Hantaviruses (Dobson et al., 2006), West Nile Fever (Kilpatrick et al., 2006), and also Chagas disease (Vaz et al., 2007; Roque et al., 2008).

The dilution effect occurs when the number of infected and competent *T. cruzi* reservoirs (those that display a pattern of infection that favor the parasite transmissibility to the vectors) is low when compared to other possible sources of blood meal for triatomines. As a result, the probability of an encounter between one infected and competent reservoir and the triatomine bug is limited, resulting in an overall low prevalence of infection in the local triatomine fauna. The amplifier effect is observed in reverse when some modification of the environment results in a positive selection of infected mammalian hosts that are competent *T. cruzi* reservoirs. In this case, the probability of an encounter between infected mammals and triatomine bugs is enhanced, resulting in a higher prevalence of *T. cruzi* infection in the local triatomine fauna. When such modifications are triggered by men who colonize adjacent forest areas, synanthropic mammalian species especially marsupials, rodents, and bats can start to frequent peridomestic areas where they can be a source of a blood meal (and infection) for triatomines. Hence, environmental modifications imposed by men on areas adjacent to their dwellings, besides all the ecological consequences, could also result in a higher risk of infection by *T. cruzi.* Infected triatomines in peridomestic areas are attracted by light and invade the houses where human infection can take place both during the bug's blood meal or accidentally by the ingestion of food contaminated with feces of infected triatomines. In fact, the latter process seems to be involved in recent outbreaks of Chagas disease in Brazil (Roque et al., 2008).

11.5 Domestic Animals

11.5.1 Domestic Mammalian Species

Dogs, cats, pigs, and goats are the main domestic mammalian species investigated for the *T. cruzi* infection. Dogs and cats represent the first domestic *T. cruzi* hosts studied by Carlos Chagas: a cat in Lassance (Minas Gerais state, Brazil) was the first mammalian host in which he found trypomastigote forms of the parasite in the

blood, while dogs were among the first experimental models used by him. Since then, several studies have shown that dogs and cats can be competent *T. cruzi* reservoirs, but as described for the other taxa of mammals, their importance in the transmission cycle of the parasite varies between different regions and local characteristics.

In the Argentinean Gran Chaco, both dogs and cats are epidemiologically important and described as highly infective to the triatomine vectors (Gurtler et al., 2007). A similar profile was found in Venezuela, where both genotypes TCI and TCII of the parasite have already been described in dogs (Crisante et al., 2006). The third genotype of the parasite (Z3) was also described in dog infection in Paraguay (Chapman et al., 1984), Argentina (Cardinal et al., 2008), and Brazil (Marcili et al., 2009a). Active transmission, which includes symptomatic dogs, was also observed in the southern USA (Kjos et al., 2008). An opposite scenario is observed in Brazil where, despite being exposed to parasite infection (as evidenced by the presence of anti-*T. cruzi* antibodies in the serum), isolation of parasites from dogs is rarely documented, either by hemoculture or xenodiagnosis (Roque and Jansen, 2008; Noireau et al., 2009). Our experience includes more than 800 dogs examined in different regions of the country, from the Amazon region to Southern Brazil, including several states from the Northeast and Southeast regions of the country, and the patterns of infection in dogs in all of these areas were quite similarly subpatent.

Concerning domestic pigs, these animals may be attractive to bugs, especially for species from genus *Panstrongylus*, as observed in some parts of the Amazon region (Valente et al., 1998). Despite being exposed to the *T. cruzi* transmission cycle, there are few reports on isolation of parasites from domestic pigs (Salazar-Schettino et al., 1997; Valente et al., 1998) and their role as reservoir still needs to be further studied. Their exposure to the parasite's transmission cycles in peridomestic areas and the possibility of diagnosing the infection by serological tests point to the importance of these mammalian hosts as sentinels in surveillance and control programs (Roque and Jansen, 2008).

The narrow number of studies in goats suggests that the role played by these mammalian species in the peridomestic transmission of *T. cruzi* is of minor importance, although they are frequently found to be exposed to the parasite (Noireau et al., 2009). High parasitemias in naturally infected goats have never been reported and the diagnosis of infection was only performed with highly sensitive molecular methods (PCR) or indirect serological methods (Indirect Immunofluorescence Assay [IFA]) (Herrera et al., 2005; Rozas et al., 2007). Isolation of *T. cruzi* from goats by xenodiagnosis or hemoculture has been achieved only from experimentally infected animals (Fernandes et al., 1994). This is probably also the case with bovines, equines, and rabbits.

11.5.2 Domestic Nonmammalian Species

Amphibians, reptiles, and birds are refractory to *T. cruzi* infection since they are capable of destroying the trypomastigote forms of the parasite due to a complement-mediated lytic effect of their blood (Kierszenbaum et al., 1981). Despite that and in

the case especially the birds, these domestic animals may play an indirect but considerable role in the transmission cycle of *T. cruzi* in both wild and peridomestic areas since they represent important feeding sources for the triatominae vectors. It is known that the amount and frequency of blood intake by infected bugs influences the *T. cruzi* development. Hence, blood ingestion in shorter intervals due to availability results in an increase in the total parasite population, while starvation reduces the density of this population and percentage of trypomastigotes in the bug's digestive tract (Kollien and Schaub, 2000).

In peridomestic areas, poultry shelters may be very attractive to triatomines and are frequently constructed close to human dwellings. This practice certainly results in an increase in the local bug population, but the consequences for the *T. cruzi* transmission may be completely antagonistic. The availability of blood sources to triatomines may prevent their evasion of chicken coops while searching for other blood sources, acting as a barrier against domiciliary invasion by the bugs. Without taking a blood meal on an infected mammalian host, these insects will never come in contact with *T. cruzi.* In this case, even accidental contact of these insects with men would not result in human infection. However, the continuously offered blood sources for triatomines may result in an overpopulation of insects, which in a given moment may evade this poultry and start to colonize other adjacent rural buildings. In this second situation, the increase in bug's population enhances the chances of contact between the triatomine vectors, domestic and synanthropic mammalian species (that may be infected), and humans. The higher the abundance and prevalence of *T. cruzi* infection in the triatomine bugs that live in peridomestic areas, the higher the probability of transmission of the parasite to humans.

11.6 Importance of Infected Domestic Mammals on Public Health in Brazil

The detection of *T. cruzi* infection in domestic mammalian species reveals the presence of parasites in areas where these animals circulate. The main objectives of the surveillance of *T. cruzi* infection in domestic animals are: (i) to identify mammalian species that can act as amplifiers of parasite populations and (ii) to identify mammalian species that can act as bioindicators (sentinels) of *T. cruzi* transmission risk to humans. It is known that in areas that present a high prevalence of *T. cruzi* infection in wild mammalian and triatomine hosts, domestic and peridomestic mammalian species are exposed to infection and this infection usually precedes the establishment of human disease in that area.

The characteristics of the domestic mammalian species management differ in distinct areas, which means that different mammalian species have greater or lesser importance in surveillance programs depending on the area. In Cachoeiro Arari (Para state, Brazil), dogs are used for house protection, and thus are more restricted to domestic areas. In the same area, pigs are managed in a semiextensive manner, which

results in mammals that forage freely in the wild environment. For this reason, in this area, pigs are more exposed to the *T. cruzi* wild transmission cycles and, therefore, display the highest prevalence of infection (Roque and Jansen, 2008).

An opposite scenario can be observed on the periphery of Abaetetuba (Para state, Brazil). Here, pigs are raised in confined pigsties while dogs move freely between the houses (almost all without external walls) and the remaining forest fragments. In this area, the prevalence of *T. cruzi* infection is greater in dogs, consequence to their higher exposition to the wild transmission cycles (Roque et al., unpublished data). The prevalence of *T. cruzi* infection in domestic mammalian species that display completely different behavior reflects the most probable source site of mammalian infection and its proximity to human habitation. *T. cruzi* infection in pigs from Cachoeiro Arari and dogs from Abaetetuba reflect the presence of wild transmission cycles near peridomestic areas. In a reverse situation, dogs from Cachoeiro Arari and pigs from Abaetetuba reflect the presence of the parasite inside peridomestic areas and, therefore, a greater risk of *T. cruzi* transmission to humans. The results obtained in our studies in outbreak areas of acute Chagas disease in Brazil (Roque et al., 2008; Roque and Jansen, 2008), besides others (Herrera et al., 2005, 2007a), show that in areas where an effective *T. cruzi* wild transmission cycle (with high prevalence of positive hemocultures) can be observed, domestic mammalian species are exposed to infection and their infection usually precedes the cases of human Chagas disease. In Brazil, in most cases, the infection of domestic mammalian species has been restricted to serological tests. In this scenario, these animals serve as biological barriers (or "shields") since they are exposed to the transmission cycle, but with no role in the amplification of parasite populations, since they almost never show parasites in their blood (low percentage of positive hemocultures). This means that these mammalian species have come into contact with *T. cruzi*, are most probably still infected, but are not sources of infection for the bug vectors. In our experience, in Brazil, from more than 800 examined dogs, we achieved only one isolation from a dog from Ajuaí village (Abaetetuba, Pará state, Brazil), from which we were able to isolate the parasite twice, in April and November 2008 (Roque et al., unpublished data). In fact, positive hemoculture or xenodiagnosis in domestic mammalian species from Brazil is still rarely documented.

A proposal from our group that has received increasing attention from the Brazilian Health Authorities is the longitudinal serological survey of domestic and peridomestic mammalian species to determine the prevalence and/or incidence of *T. cruzi* infection (Roque and Jansen, 2008; PAHO, 2009). This is because areas that present a high prevalence of infection among these mammalian species are probably those that display the highest rates of infection among wild and synanthropic mammalian species and vectors and, therefore, are at elevated risk for the emergence of human cases. This proposal was based on the epidemiological profile of infection observed in different areas from Brazil, including areas that recently faced Chagas disease outbreaks. Moreover, this is a measure that can be immediately applied in order to identify risk areas.

Similar strategies were already proposed and applied in Argentina (Gurtler et al., 2007), Venezuela (Crisante et al., 2006), Mexico (Estrada-Franco et al., 2006), and the USA (Shadomy et al., 2004). Among domestic mammalian species, the importance of dogs in such investigations is greater, since dogs are easy to handle and their shifts may be monitored most of the time. Blood collection and the subsequent serological assay (or sending of serum samples to governmental central laboratories for diagnosis) does not require great cost and/or structure. Moreover, in Brazil, collection of dogs' blood samples are routinely performed for diagnosis of *Leishmania* sp. infection in many areas of the country, and this serum could also be used for the diagnosis of *T. cruzi* infection. Alternatively, vaccination campaigns against rabies occur once a year in the whole country and provide a good opportunity to collect blood samples from a representative amount of dogs in a given area (Roque and Jansen, 2008).

Whichever strategy is employed, the pivotal importance is that seropositive dogs reflect exposure to *T. cruzi* and indicate the presence of parasites in areas where these animals visit. The knowledge of the prevalence of *T. cruzi* infection in these hosts may direct epidemiological measures to risk areas even before the occurrence of human cases. Once this measure is implemented, health authorities will have an important indicator for the selection of areas that present a higher risk of *T. cruzi* transmission to humans and therefore need more urgently the implementation of epidemiological control measures and health education to present the situation and correctly inform the residents of that area (Roque and Jansen, 2008).

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12 Veterinary Aspects and Experimental Studies

Marc Desquesnes[1,*] *and Marta de Lana*[2]

[1]Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France; Kasetsart University, Bangkok, Thailand, [2]Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil

12.1 Introduction

Trypanosoma cruzi is a human parasite but is also found in many other animal species, both wild and domestic; human infection can occur in rural as well as in urban areas, which reveals various roles of insects and other mammals in its epidemiology (Medrano-Mercado et al., 2008). Even if the main mammalian hosts and reservoirs are considered to be humans, marsupials, dogs, and cats (Woo and Soltys, 1970), researchers have compiled a nonexhaustive list of 150−200 wild animal species (including the vampire bat *Desmodus rotundus*) and some 10 domestic or peridomestic animals that have been found to be infected (Anonyme, 1991; Roellig et al., 2009a). In particular, *T. cruzi* can be naturally found in dogs, cats, cattle, goats, sheep, rabbits, and equines (Correa et al., 1982; Anonyme, 1991; Fujita et al., 1994; Salazar-Schettino et al., 1997). Among susceptible domestic animals, guinea pigs may play an important epidemiological role, particularly in Peru where they are bred for meat. In Paraguay, it was suggested that cattle, pigs, dogs, and cats provide reservoirs for *T. cruzi* (Fujita et al., 1994). In French Guiana, studies have shown that *Didelphis marsupialis* and *Philander opossum* are frequently infected (Dedet et al., 1985), as are domestic dogs (Raccurt, 1996); however, *T. cruzi* was not reported in livestock and humans (Desquesnes, 2004) in this region. Indeed, *T. cruzi* is rarely reported in livestock, but is it due to a lack of presence or a lack of investigation and a lack of efficient diagnosis method?

From a veterinary point of view, it is difficult to classify these various categories of animals since their role is variable from one situation to another. In some instances, their role may be underestimated. As a first attempt to gather animals in categories, we can consider (i) wild (e.g., opossum, armadillo, raccoon, rat, mouse) or domestic (e.g., dog, cat, guinea pig; see Chapter 11) animal species known to

*E mail: marc.desquesnes@cirad.fr

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have a potential role in the epidemiology of the disease; (ii) wild (e.g., monkey, deer, wild pig) or domestic (e.g., horse, cow, ox, sheep, goat, pig) animals that are receptive but are not known to be reservoirs, although they may have a role in the epidemiology; and (iii) experimental animals that have been very useful to the study of human disease, such as mice, rats, guinea pigs, hamsters, rabbits, dogs, and monkeys.

Wild and domestic animals as reservoir are studied in Chapter 11, so this chapter will focus on experimental models and on livestock infections; however, due to the lack of knowledge of the latter, it is important to provide an overview of the potential ways of infection of livestock and the difficulties encountered while trying to establish a reliable diagnosis in these animal species. Indeed, a study aiming at establishing the seroprevalence of *T. cruzi* infection in livestock or dogs would meet the problem of species specificity due to the interference of *Trypanosoma vivax* or *Trypanosoma evansi* in the detection of *T. cruzi* infection; conversely, *T. cruzi* can interfere with the diagnosis of trypanosomoses in livestock, particularly serological diagnosis of *T. vivax* in ruminants and *T. evansi* in equines (Desquesnes, 2004).

In rodent, monkey, and dog infections observed under natural and experimental conditions (described hereafter), information on the pathogenicity and evolution of the infection is available, but information on the livestock aspects of *T. cruzi* infection is very limited. This paucity of data is probably due to the following reasons: (i) *T. cruzi* infection is not suspected to be of medical and economical significance in livestock; (ii) some of the symptoms are greatly delayed from the infection, far beyond the duration of most of the experimental designs in livestock species; (iii) the lack of specificity of the diagnosis tools; (iv) the existence of a high risk of human infection when handling experimentally infected animals, especially large animals, such as horses and bovines; (v) the existence of a great number of other obviously more important hosts and reservoirs of the parasite.

This chapter will present the data generated by the experimental infection of dogs, monkeys, and laboratory rodents, and will gather the scarce information of *T. cruzi* natural and experimental infections in livestock. It will also focus on the specific natural way of infection of these animals and the potential and possible risks, as well as the history of the infection spreading from these animals to other animals, including humans. This will be partly speculative due to the very poor information available on the real status and role of livestock in the epidemiology of the infection.

12.2 The Various Ways of Infection of Animals (and Humans)

T. cruzi is not a livestock trypanosome as such, but it is sometimes found in domestic ruminants, pigs, and horses; however, the ways of infection of livestock have not been studied. Hypotheses can be suggested based on the various ways of transmission of the parasite already known and briefly reviewed here.

In humans, the transmission of *T. cruzi* is mainly due to the triatomine, or reduviid bugs, of the genera *Rhodnius*, *Panstrongylus*, and *Triatoma*. The metacyclic trypomastigote infective form (metatrypanosome) present in the excrement of the bugs contaminates bitten skin wounds or mucous membranes, particularly the eye; in this cycle, the best vectors are the bugs that defecate soon after having their blood meal. This way of contamination to livestock and pets is possible if they share a close habitat with humans.

T. cruzi, a blood parasite, can be transmitted through the blood by several ways with variable probability and consequences, depending on the host considered. These routes include laboratory accidents, blood transfusion, and human organ transplants (Monteon-Padilla et al., 1999); congenital transplacental (*in utero*) or perinatal infection (Myriam et al., 2005); surgery or any other iatrogenic transmission (human and animals); and mechanical transmission by tabanids and stomoxes (animals). Mechanical transmission by some other vectors should also be considered, such as lice, which is a potential vector in monkeys (Arganaraz et al., 2001). The most important secondary mode of transmission of *T. cruzi* is by peroral route (Coura, 2006). In humans and animals, this may occur by (i) ingestion of infected flesh or blood or (ii) ingestion of food infected by triatomine or opossum feces; in animals, transmission may also occur through ingestion of infected triatomine bugs themselves.

Indeed, the transmission of the infective forms found in the feces of the bugs can take the peroral route (Coura, 2006). Recent work in mice that ingest infected bugs or their excreta indicates that the parasite gains entry through the gastric mucosae and produces local immunity (Hoft et al., 1996). This may explain how many family infections arise from food contaminated by the excreta of the bugs (Lainson et al., 1980). Ingestion of infected bugs is also thought to be a significant cause of contamination of dogs, cats, and livestock. The peroral infection may have a significant role in the epidemiology of *T. cruzi* disease, especially in livestock but also in humans. This theory is supported by the recent outbreaks in Brazil where human infections were traced to ingestion of either fruit juice or sugar cane juice in Catarina (Steindel et al., 2008) and palm fruit juice in Pará and Amapá (Cardoso et al., 2006). Experimental work proved that *T. cruzi* can survive at least 24 h in the sugar cane juice (Cardoso et al., 2006).

If transmission does not occur conventionally through the transcutaneous route, livestock may become infected by ingesting reduviids or feed contaminated with reduviids or their excrement, or with *D. marsupialis* feces. Cattle and horses rarely share the same habitat as reduviid bugs; for this reason and except in very unusual circumstances, contamination of cattle and horses by bugs would be limited. However, small ruminants and pigs are more likely to share the same habitat as these vectors, in particular under conventional stock-farming conditions. The opossum is perhaps another important link in the chain of contamination from bugs to livestock and will be explored in greater detail later in this section.

Carnivores and omnivores are exposed to a specific risk of peroral infection. Just as with *T. evansi* (Raina et al., 1985), very early research confirmed that the bloodstream form of *T. cruzi* was transmissible perorally (Dias, 1940), even if

the amastigote form is not infective (Roellig et al., 2009b). Dias was able to achieve contamination of a cat by ingestion of infected rats. It can therefore be concluded that ingestion of the fresh raw flesh of infected animals can cause infections in humans and animals, particularly in dogs (oro-digestive transmission). Although this observation was not possible in raccoons fed with amastigote infected tissues, they were susceptible to trypomastigote ingestion; consequently, ingestion of infected fresh flesh should still be considered in the epidemiology of *T. cruzi* in carnivores and also in pigs and other livestock known to be able to eat flesh, notably that of rodents. Other trypanosomes in the subgenus *Herpetosoma* have also been shown to be orally transmitted in rodents, including in the trypomastigote form: *T. lewisi*, *T. microti*, *T. evotomys*, and *T. grosi* (Maraghi et al., 1995).

As a stercorarian parasite, *T. cruzi* is cyclically transmitted by biting bugs; however, triatomine insects are not the only cyclical vectors of *T. cruzi.* Indeed, the life cycle described in the gut of triatomines has also been observed in the lumen of the anal scent glands of *D. marsupialis* (southern opossum) in which the parasite multiplies as an epimastigote and differentiates into metacyclic form (Deane et al., 1984). The parasites extracted from the scent glands have the same features as the metacyclic forms in insects and are infective via the subcutaneous, intraperitoneal, peroral, and transconjunctival routes (Urdaneta-Morales and Nironi, 1996). Because infective forms of *T. cruzi* are present in the excreta of marsupials, contamination between marsupials is highly likely, making the opossum a true reservoir for the parasite. It also means that the parasite can be transmitted in the absence of insect vectors (e.g., in urban areas or in wild) through the contamination of food by opossum feces. All mammals can be contaminated via this way by the oral route.

On the basis of these observations, *D. marsupialis* can be classified amongst the biological reservoirs/hosts/vectors for cyclical transmission of *T. cruzi*; it is remarkable that the typical stercorarian cycle described for insects occurs in an analogous way in the distal portion of a mammal's gut (Desquesnes, 2004). Finally, triatomines and opossums may both contaminate the human and animal food, making Chagas disease not only a vector-borne disease but also a food-borne disease. The role of the opossum suggests that the extension of *T. cruzi* infection may even emancipate from the triatomine bug and would be possible via the marsupials alone.

12.3 The Problem of Diagnosis in Animals

If the diagnosis of a pathogen in its favorite host is generally made easy by a naturally high proliferation of the pathogen or a strong immune response, this diagnosis is of lower efficacy (sensitivity, especially) in hosts of lower susceptibility because of lesser pathogenemia or immune response. In the case of *T. cruzi*, the diagnosis is difficult in humans and even more so in other animals in terms of both sensitivity and specificity due to very low and transient parasitemia. Indeed, Latin America is nowadays an endemic region for a number of *Trypanosoma* species, some of which

can interfere in this detection, such as *T. rangeli*, *T. vivax, T. evansi, T. equiperdum,* and *T. theileri*.

In the early stage of the infection, *T. cruzi* is classically found in the host in circulating bloodstream forms. Typically 16−22 μm in length, the parasite may be C- or S-shaped with a 1.2-μm kinetoplast that sometimes forms a "stain" that overlaps over the edge of the body. The flagellum forms a stunted, undulating membrane, and the nucleus is located in the central or front portion of the body (nuclear index: 0.9−1.9 or more). In fresh samples, slender forms move rapidly while stumpy forms move slowly. Rather than replicating, this stumpy form invades the tissues and gives rise to an intracellular form of the *Leishmania* type that replicates in the amastigote, epimastigote, and trypomastigote forms located in the reticuloendothelial cells, muscles (including the heart), liver, and nervous system (Hoare, 1972); bloodstream and tissue forms alternate periodically. From that stage, in terms of routine parasitological diagnosis, it becomes almost undetectable.

During the early stage, detection of the blood forms of *T. cruzi* in animals can be achieved by direct examination of blood, preferably after enrichment by centrifugation (hematocrit centrifuge technique) as used for African animal trypanosomes (Woo, 1969). Using this method, positive cases in livestock are rarely reported (Fujita et al., 1994), most probably because the parasitemia is low and transient. Minianion column techniques are generally considered too expensive for veterinary studies. More sensitive methods can be applied, such as mouse inoculation, but they are no longer popular since they are using living animals and present a risk of handling highly infective material for humans. The use of xenodiagnosis and hemoculture are also limited in animals.

Nowadays, DNA detection is the most sensitive and specific method for diagnosis of active infection. Since the first specific primers for the detection of *T. cruzi* were described (TCZ1 and TCZ2) (Moser et al., 1989), a number of primers with variable levels of specificity have been published (Desquesnes and Dávila, 2002). However, very few studies report their use for demonstrating the presence of the parasite in livestock. In fact, as indicated earlier, very few studies focus on *T. cruzi* in livestock.

Serological tests, such as immunofluorescent assay (IFA) or enzyme-linked immunosorbent assay (ELISA), can be used for detection of antibodies directed against *T. cruzi* in livestock; however, the species specificity of these tests is not high. With African trypanosomes, the evaluation demonstrated a strong cross-reactivity between the various species (*T. brucei*, *T. vivax*, and *T. congolense*), which could even allow using heterologous antigens for diagnosis (Desquesnes et al., 2001). The complement fixation test (CFT), indirect hemagglutination test, or the card agglutination test for trypanosome (CATT test/*T. evansi*), which cross-reacts with other trypanosomes, such as *T. congolense* and *T. vivax*, would most probably not be species-specific, but few have been evaluated for their specificity toward *T. cruzi*. For example, animals infected with *T. vivax* or *T. congolense* produce up to 85% positive results using IFA for *T. brucei*. Cross-reactions between *T. evansi* and *T. cruzi* have been reported in horses (Bakos, 1982; Monzón, 1986; Monzon and Colman, 1988) and between *T. evansi* and *T. vivax* in cattle

(Ferenc et al., 1990). ELISA using *T. evansi* antigens demonstrated the same level of sensitivity and specificity as assays using *T. cruzi* antigen for detection of *T. cruzi* infection in humans (Desquesnes et al., 2007). *T. evansi* is antigenically a very rich parasite in Latin America, indirect-ELISA *T. evansi* is probably able to detect infections induced by *T. vivax*, *T. evansi*, *T. equiperdum*, and *T. cruzi* with almost equal effectiveness.

In fact, cross-reactivity among salivarian and stercorarian trypanosomes have been recorded with all serological methods evaluated so far, and it should finally be concluded that none of them is able to detect specifically the pathogenic trypanosomes. Since studies on *T. cruzi* are scarce in livestock, there are very few positive reference samples from livestock experimentally infected by *T. cruzi*. This obviously limits the evaluation of cross-reactions, but based on the experience of African trypanosomes and on what has been achieved so far by Monzon and colleagues (Monzón, 1986; Monzón and Colman, 1988), it can be speculated that the species specificity of *T. cruzi* serological tests would be very low in endemic areas of *T. evansi* and *T. vivax*.

T. vivax, *T. evansi*, *T. cruzi*, and *T. equiperdum* have been reported in practically all Latin American countries. Given that all of the serological tools are unable to discriminate these species, knowledge on the distribution, prevalence, and medical and economic impact of *T. cruzi* in livestock is therefore very limited. Improving diagnostic tools is a priority if better knowledge of livestock trypanosomoses in this region of the world is to be achieved. Serological studies carried out with *T. cruzi* antigens would be very risky to analyze in areas potentially infected by other *Trypanosoma* species, especially if *T. evansi* is highly endemic and the status of *T. cruzi* is really unknown and unsuspected. Interpretation of such surveys should be made with high care. Moreover and reciprocally, leishmaniosis could also strongly interfere in such serological studies (Grosjean et al., 2003; Savani et al., 2005). Finally, little can be concluded from studies strictly conducted with serological tools in potentially mixed infected hosts and areas (other *Trypanosoma* and *Leishmania* species) unless they are completed by DNA tools specific for *T. cruzi*.

12.4 Natural Infections in Domestic Animals and Livestock

The role of animals in the epidemiology of Chagas disease is variable, depending on the type of the 200 species found infected (Roellig et al., 2009a). Thus, we can globally divide them into three categories, which may be involved in the three mammalian cycles (domestic, peridomestic, and wild).

Domestic species include dogs, cats, guinea pigs, hamsters, and rabbits. *T. cruzi* is markedly pathogenic in dogs (and in a lesser extent in cats), in which it produces cardiac signs with a potentially fatal outcome. It is speculated that dogs and cats can get the infection not only by the bug's bite but also by eating infected bugs or freshly infected preys (especially mice for cats). This finding may have an important role in clarifying the epidemiology of the human disease in some

circumstances, like in Argentina (Gurtler et al., 2007) or in newly settled human populations of the Amazon (Briceno-Leon, 2007). The prevalence of infection in domestic carnivores is around 10% in Chile, 9−24% in Mexico (Garcia-Vazquez et al., 1995), 20% in Brazil, 37% in dogs and cats in Paraguay (Fujita et al., 1994) and 42% in Argentina (Gurtler et al., 2007), and up to 50% in dogs in some areas of Venezuela.

An expert report published by the World Health Organization (WHO) on Chagas disease (Anonyme, 1991) mentions that the studies conducted in Argentina, Brazil, Chile, Bolivia, and Venezuela yield highly variable rates of infection by *T. cruzi* that range, in humans, from 0.5% to 2% in large cities to between 20% and 63% in highly endemic areas; these ranges vary from 4.5% to 100% in dogs and from 0.5% to 60.9% in cats. In Chile, serological studies in the provinces of Elqui, Limari, and Choapa revealed the presence of antibodies for 12−24% of dogs, 0−15% of cats, and 4−26% of rabbits (Correa et al., 1982). In guinea pigs in Bolivia, prevalence ranges from 10% to 60% (Acha and Szyfres, 2005). In the USA, dogs are sometimes found infected and are most often related to human cases (Newsome and McGhee, 2006).

Peridomestic animals include the livestock species, such as cattle, buffalo, horses, sheep, goats, and pigs. None of them is considered to be highly susceptible to the infection by *T. cruzi*. In Chile, serological studies in the provinces of Elqui, Limari, and Choapa revealed the presence of antibodies in 5−12% of goats and 4.8% of sheep (Correa et al., 1982). In another study in Chile, the seroprevalence by IFA and ELISA in goats ranged from 6.5% to 38.3% (Alcaino et al., 1995). In Paraguay, a survey demonstrated that antibodies raised against *T. cruzi* were found in 8% of cattle and 10% of pigs (Fujita et al., 1994). Oxen and pigs were found naturally infected in Mexico (Salazar-Schettino et al., 1997). In the other countries, very little, if any, information is available. In some instances, *T. cruzi* could be isolated from these animals, but in serological surveys conducted in areas endemic for other *Trypanosoma* species, the seroprevalence observed cannot be attributed to *T. cruzi* with certainty.

Because appropriate diagnostic tools are lacking, the prevalences in livestock cannot really be determined. Except for *Trypanosoma theileri*, which does not induce serological cross-reaction with other *Trypanosoma* species, there is a reciprocal interference of all trypanosomes potentially present in livestock: *T cruzi*, *T. equiperdum*, and *T. evansi* in equids, and *T. cruzi*, *T. evansi*, and *T. vivax* in others. For example, in 1995, positive antibody serologies (by CFT) for *T. equiperdum* were found healthy-appearing horses and mules intended for export from the state of Chihuahua (Mexico) to the USA. The same samples tested for *T. cruzi* with hemagglutination inhibition also turned out positive. The positive animals were slaughtered, but infection was never demonstrated. Furthermore, investigations conducted on 3000 Equidae in that state were never able to isolate the pathogen or discover clinical signs of dourine. Interference from *T. cruzi* or *T. evansi* may be the cause of serological cross-reactions. *T. cruzi* has been found in circulation in Mexico even in urban areas; a recent serological survey has shown that 8.8−24.2% of dogs are infected (Garcia-Vazquez et al., 1995). Another example is in

Argentina, where diagnosis in horses cannot distinguish *T. evansi* from *T. cruzi* infection, although both of them are equally probable (Bakos, 1982; Monzón, 1986; Monzon and Colman, 1988). Hence, it would seem that the incidence of *T. cruzi* infection of livestock is by no means negligible. Little research has been conducted either in the field, where no specific diagnostic tool is available, or under experimental conditions owing to the limited risk of human infection and/or a lack of interest in this work because its pathogenicity is presumed to be low and because of the short economic life expectancy of farm animals.

Synanthropic species, which may be peridomestic or sylvatic, are mice, rats, armadillos, raccoons, coyotes, and marsupials; the latter are considered to be the oldest and most important reservoir of the parasite (Jansen et al., 1999). In a review made in Mexico in 1997, 12 peridomestic mice, rats, squirrels, armadillos, bats, and marsupials were found to be infected. A huge number of species have been found infected, but few studies indicate the prevalence of the infection; it ranges from 13% in octodon (*Octodon degus*) to 28% in rats (*Rattus rattus*) in Chile (Galuppo et al., 2009) and from 6% in *Philander opossum* to 43% in *D. marsupialis* in French Guiana (Dedet et al., 1985). Complex epidemiological features may include marsupials, armadillos, monkeys, and raccoons as reservoir and bugs as active or passive vector toward same or other host species, including mice and rats that may be responsible for the infection of cats and dogs. The epidemiological studies carried out in diverse circumstances tend to show how complex and unique the situations can be; therefore, no generalization or prediction should be made, and each ecotope should be considered as unique system (Jansen et al., 1999). In a sylvatic area of Brazil, the collared peccary (*Tayassu tajacu*), white-lipped peccary (*Tayassu pecari*), and feral pig (*Sus scrofa*) are maintenance hosts for *T. cruzi* and *T. evansi* (Herrera et al., 2008). In wild rabbits, prevalence reaches 38% in Chile (Botto-Mahan et al., 2009). In the USA, *T. cruzi* is frequently found in raccoons; in Tennessee, a higher seroprevalence is observed in rural habitats (35%) than in suburban habitats (23%) with an average of 29% (Maloney et al., 2009); rates can reach as high as 63% in Oklahoma (John and Hoppe, 1986). Autochtonous human cases seem to be related to both raccoons and dogs (Newsome and McGhee, 2006).

12.5 Experimental Studies in Animal Models

An important requisite to studying any disease and the mechanisms involved in its pathogenic process is a good experimental model to reproduce the different phases and clinical forms observed in humans. In this context, several experimental models have been used to study Chagas disease. Carlos Chagas was the pioneer who discovered the natural hosts of *T. cruzi*, such as opossums (*D. marsupialis*) and armadillos (Chagas, 1909). Since the beginning of these studies, Chagas sent the first isolates of *T. cruzi* to Oswaldo Cruz Institute to infect several animal species (including dogs, cats, monkeys, rabbits, guinea pigs, and some rodents) to try to reproduce the disease. At the present time, these animals are still used as laboratory experimental models for Chagas disease.

According to the WHO's Chagas Disease Committee of Training Special Program and Research of Parasitic Disease (1984), a good experimental model to study Chagas disease is characterized as follows:

- Allows the isolation of the parasite throughout the course of the infection;
- Presents positive serological reactions indicative of the infection;
- Presents the diverse clinical manifestations of chronic Chagas disease;
- Develops myocarditis, myositis, and others pathological alterations characteristic of the disease;
- Induces the immune response against the host tissue;
- Offers easy maintenance and an accessible price.

12.5.1 Mice

Mice have been more frequently used as experimental models to study Chagas disease for several reasons (Figure 12.1). They are easily reproduced, of low cost, easy to handle, easy to be experimentally infected, and maintained in experimental

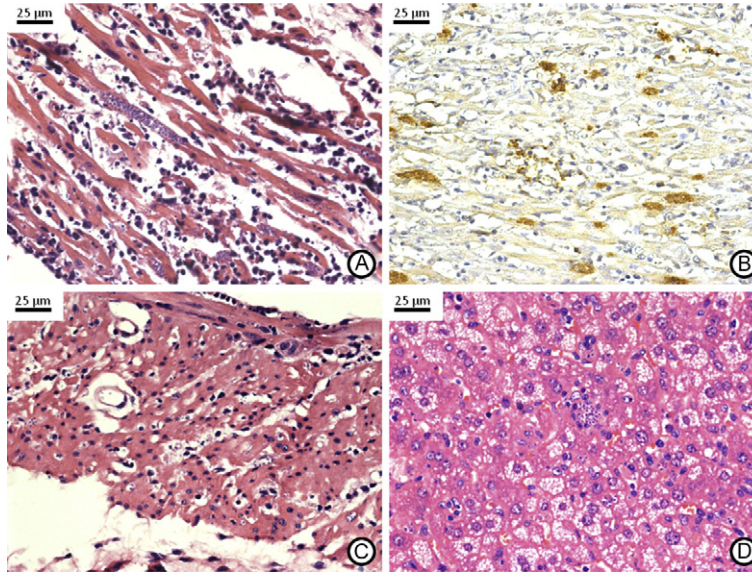

Figure 12.1 Several aspects of experimental Chagas disease in murine model during the acute phase of the infection (A, C, and D: hematoxylin and eosin counterstained). (A) Acute myocarditis with presence of amastigotes nest and mononuclear cells dissociating the cardiac fibers. (B) Immunohistochemistry showing acute myocarditis and intense presence of amastigotes. (C) Muscular layer of the intestine with presence of diffuse inflammatory infiltrate and amastigotes. (D) Liver showing amastigotes nest in Kupffer cells and diffuse inflammatory infiltrate.
Source: Photographs by M. Lana and colleagues.

conditions. The existence of a great number of strains and isogenic species, appropriated to study different aspects of the disease make this species the most commonly used in experimental studies. Different strains of mice present distinct patterns of susceptibility to *T. cruzi* infection, chosen according to the virulence of the parasite strain or the objective of the study. Moreover, nowadays the existence of several knockout lineages has facilitated the study of different immunological aspects of *T. cruzi* infection.

The acute phase is easily reproduced in this model (Collier et al., 1942; Federici et al., 1964) making this species very useful to isolate and also to maintain the parasite in the laboratory through blood successive passages. Young mice are very susceptible to infection, and parasite strain, inoculum, and route of inoculation influence the development of the infection. Different aspects of acute disease have been reproduced in mice, including symptoms (anorexia, elevated temperature, weight loss, decrease of general activity, patent parasitemia, general edema, and mortality) and histopathological lesions (diffuse myocarditis, myositis, lymphadenopathy, and congestion with infiltrate of mononuclear cells). Tafuri (1969) verified that while the parasites are intact in the host cell, no inflammation was observed, because this phenomenon caused the disintegration of both parasites and host cells. Tafuri and Raso (1962), Tafuri and Brener (1966, 1967), and Andrade and Andrade (1968) studied lesions of the autonomous nervous system of mice infected with different *T. cruzi* strains to verify the parasite's presence in the lumbar nerves, cardiac superficial plexus, and the celiac, sympathetic, and myenteric ganglia. The lesions were irregular and dependent of the parasite strain, demonstrating an important role for the inflammatory process (Andrade and Magalhães, 1996). The neuronal changes are observed in ganglia of the myoenteric plexus as well as in intracardiac parasympathic, celiac, and sympathic ganglia. Using electron microscopy, researchers have observed tumefaction and vacuolization of mitochondrias, hypertrophy of Golgi complex, dilatation of the endoplasmic reticular system, chromatolysis and vacuolization of numerous lysosomes and neuronal osmiophilic granulations (Tafuri, 1971).

The inflammatory process has an important role in neuronal lesions. Apparently the neuronal destruction occurs in the presence of great concentration of tissue parasitism during the acute phase. Autoimmune mechanisms probably participate in this process and are likely responsible for the inflammation that involves sympathic and parasympathic ganglia. Vago et al. (1996), using the PCR technique in tissue, identified the presence of parasite kDNA in fragments of biopsy from chagasic patients with megaesophagus. These findings suggest that the presence of the parasite or its DNA is important in the genesis of the inflammatory process, triggering also the autoimmune process present in Chagas disease. The role of the parasite strain on denervation was suggested when 138 *T. cruzi* strains of three Brazilian regions were characterized. They were genetically characterized by isoenzymatic profile, and the association between the presence of "megas" and zymodema II of *T. cruzi* was observed (Luquetti et al., 1986). However, it is important to consider the level of virulence and tissue tropism of the parasites since the inflammatory process they trigger is involved with the denervation and lesions of neuronal

structures. Cardoso and Brener (1980) evaluated the hematologic alterations in mice infected with different *T. cruzi* strains, verifying anemia, decrease of platelets, and leukopenia, with these changes more intense in mice with higher parasitemia.

The chronic phase is also reproduced in mice, especially regarding myocardiopathy. Schlemper et al. (1983) studied acute and chronic infection in mice infected with 12 different *T. cruzi* strains isolated from Virgem da Lapa, Minas Gerais. Infectivity, parasitemia, and tissue parasitism were evaluated during the acute and chronic phases of the infection. Except for fibrosis, the lesions were stable during the evolution of the infection. Arteritis with necrosis was observed in skeletal and heart muscles. Molina et al. (1988) studied the morphology of the heart conduction system in C3H mice infected with Tulahuen strains and verified no evolution of the lesions during the infection. No correlation with the morphological aspects and electrocardiogram (ECG) alterations were observed with the human chronic chagasic cardiopathy. In this same strain of mice infected with Colombian strains, Rossi et al. (1984) verified cardiomegaly with hypertrophy, ventricular dilatation, and ventricular aneurysm. Necrosis and myocardium degeneration, inflammatory reaction with predominance of mononuclear cells, interstitial fibrosis, and the presence of occasional pseudocysts were observed in myofibrils. These animal studies demonstrated the involvement of microcirculation and ischemia on chronic chagasic cardiopathy; cellular degeneration and necrosis were the principal findings, similar to that observed in humans.

Andrade and Grimaldi (1986) studied the mechanisms of cardiac fibrosis in mice infected with *T. cruzi* strains that were very virulent and pathogenic. The ultrastructural analysis revealed predominance of monocytes, macrophages activated, fibroblasts, myofibroblasts and intense deposition of collagen suggesting the association of inflammatory process with fibrogenesis on chronic chagasic cardiopathy. Immunotyping revealed the different types of collagen during the infection evolution and the possibility of fibrosis reversion during the later infection.

The humoral immune response of mice was studied by Peralta et al. (1980), Jeng and Kierszembaum (1984), and other authors without a clear definition of the serological profile, which changed in relation to different parasite and host strains. IgM, IgG, and isotypes IgG1, IgG2a, IgG2b, and IgG3 were observed, with the IgG2a more associated with protection. Andrade et al. (1985a) conducted a similar study with the same *T. cruzi* strains but using five different strains of mice, and results were similar. No correlation was observed between the level of antibodies and the protection of the infection. Later, it was discovered that IgG1 and IgG2 are the most important IgG isotypes with participation on the phenomenon of lyses mediated by complement, the most important humoral process of protection in chronic chagasic infection (Krettli et al., 1984).

Several pathological phenomena of *T. cruzi* infection were first studied in murine models, such as cellular damage, inflammation, fibrosis, and denervation, which explain the disease evolution and characterize the different clinical forms of the disease. However, the digestive forms of Chagas disease named “megas” have not been observed or reproduced in mice. So the related data described in the literature remains controversial.

Besides all the aspects of experimental Chagas disease in mice, the murine model has been used for the biological characterization of *T. cruzi* strains because and the results are correlated with the genetic classification of the parasite characterized by isoenzymatic profiles. This gave origin to the "biodemes" when Andrade and Magalhães (1996) classified the *T. cruzi* strains in biodemes I, II, and III, considering the global results of curve of parasitemia, tissue parasitism, histopathological lesions, and mortality during the acute phase of the infection. These "biodemes I, II, and III", respectively, correspond to groups *T. cruzi* I, *T. cruzi* II, *T. cruzi* V and *T. cruzi* VI of the current classification of *T. cruzi* populations decided during the Satellite Meeting in Arraial dos Búzios, Rio de Janeiro, 2009 (Zingales et al., 2009). The association between biological aspects observed in mice and the genetic characterization of *T. cruzi* has been confirmed by other authors, even using different genetic markers that confirm the clonal character of this parasite (Toledo et al., 2002). Camandaroba et al. (2001) clearly demonstrated that *T. cruzi* strains could represent a clonal complex and that its behavior in experimental model depends on the characteristics of the predominant clones.

The pioneer studies of chemotherapy of Chagas disease were also performed in mice (Brener, 1961). At the present, this is still the first experimental model used for this purpose when new drugs or compounds are tested because mice develop both phases of the infection and are inexpensive and easy to handle (Andrade et al., 1991; Molina et al., 2000; Urbina et al., 2003; Ferraz et al., 2007).

The use of isogenic lineages of mice for experimental studies in Chagas disease has been emphasized, as well as with other infectious agents to guarantee more standardized results. Trischmann et al. (1978) used different lineages (C57BL/10 [B10], CBA, AKR, C3H/He/ CBA/2 and BALB/c), including thymectomized mice in experimental *T. cruzi* infections, to verify that C3H was the most susceptible, BALB/c of intermediary susceptibility while B10 was the most resistant to infection. The thymectomized animals consistently presented higher parasitemia. Andrade et al., (1985b) also verified in mice of different isogenic lineages (AKR, A/J, CBA, BALB/c, C3H and B10) infected with three *T. cruzi* strains typical of the three Biodemes of Andrade and Magalhães (1996) that the parasitemia and mortality index were different among the lineages. It was verified that the same lineage may be more resistant or susceptible in function of the *T. cruzi* strain considered. In general, CBA and B10 mice were more resistant and A/J and AKR more susceptible.

More recently, the existence of different knockout lineages of mice have allowed better studies about different humoral and cellular immunological process during the acute and chronic phases of *T. cruzi* infection and its genetic regulation (Trischmann, 1986; Minoprio et al., 1991, 1993). Although the immune response had an important role in the resistance, the survival is more related to the genetic pattern of the mice than with the H-2 locus (mice histocompatibility system). The resistance of mice to acute infection is therefore regulated by multiple genetic components present in or out of the H-2 locus, and the final resultant infection and evolution depend on the parasite characteristics and the allele combination of each

mouse lineage, including immunological and nonimmunological factors. According to Minoprio et al. (1989), the consequence of acute infection by *T. cruzi* is the intense and polyclonal lymphocyte activation with the majority not driven to *T. cruzi* antigens, leading to a fast blastic activation and proliferative activity of CD4+, CD8+, and LyB. The infection evolution is dependent on the lymphocyte response and the patterns of cytokines able to modify this response with the predominance of Th1 or Th2 response. However, the paradigm type I and type II immunological response in Chagas disease is not very well established.

Several attempts to study vaccines for Chagas disease were also performed in murine model, especially because the great majority of the immunological phenomena of the acute and chronic phases of the disease were described in this animal model (Basombrio and Besuschio, 1982; Wizel et al., 1998; Sepulveda et al., 2000; Dumonteil et al., 2004).

One of the principal limitations of mouse model is the short life span of this species (approximately 2 years) which probably makes impossible the reproduction of the later clinical forms of the disease, such as intense fibrosis, dilated myocardiopathy, aneurysm, and the digestive clinical forms, specially megaesophagus and megacolon, all of later evolution.

Finally, consider that mice easily reproduce several aspects of the acute and chronic phases of Chagas disease. However, the ECG alterations observed in mice do not correspond with the typical patterns verified in humans. Evidence of fibrosing chronic chagasic cardiopathy, the most remarkable lesion of later human chronic disease, is also generally absent. However, the recent advances obtained in the study of several aspects of Chagas disease immunology in this animal, as well as the use of murine model for test of new drugs, together with ease of use, fully justify the use of this model.

12.5.2 Rats

Although easy to handle and maintain in the laboratory, rats have not been often used as experimental model for the studies of Chagas disease because they are more resistant to *T. cruzi* infection. In the acute phase, parasitemia is generally very low or subpatent, making the infection difficult to evaluate (Pizzi et al., 1954). On the other hand, this limitation makes this species useful for studies of the chronic phase of the infection since the mortality during the acute phase is not so frequent.

Considering the unpredictability of the disease evolution in this model, as well as the difficult demonstration of parasite persistency throughout long-term infection, several authors have tried to decrease the resistance of rats to *T. cruzi* infection by blocking the phagocytic mononuclear system with cortisone therapy or thymectomy (Denilson, 1943; Pizzi et al., 1954; Rubio, 1954). Bottasso et al. (1993) found that thymectomized animals had increased myocardial chronic lesions, suggesting that the thymus plays a role in the immunoregulatory process during the infection.

On the other hand, other authors have reproduced the acute phase of the infection in rats with several *T. cruzi* strains of higher virulence and using great inoculum. Brand et al. (1949) studied the course of the infection of five strains and verified that the acute phase develops later (around 3 weeks) with higher parasitemia or with low parasitemia followed by chronic infection, depending on the pathogenicity of the strain; death may also occur. Alcântara and Oliveira (1964a,b) studied several viscera of Wistar rats in the chronic infection with Y strain and verified that the cardiopathy was very frequent, occurrence of "megas" of colon, bladder, seminal vesicle and uterus. Neurons of the Meissner complex of the stomach, transverse and descendent colon were reduced as well as those of the Auerbach plexus from the stomach to rectum

Machado and Ribeiro (1989) studied the sympathetic denervation in rats infected with Y strain, verifying that this process begins very early (6 days after infection) in parallel with intense parasitism and myocardial inflammation. These authors also verified that, in the 20th day after inoculation, no nervous structure was observed in the myocardium or vases of these animals. Myocardial lesions with ganglionitis and periganglionitis of the autonomous nervous system were also verified by Scorza and Scorza (1972) when they studied rats infected with Y strain. Bestetti et al. (1987) evaluated the ECG changes in Wistar rats infected with the Colombian strain, detecting some alterations similar to those observed in humans except with more severe as Right-Block of His Branch. Junqueira et al. (1991) verified discrete ECG alterations of later occurrence in rats infected with Y, 12-SF, and Colombian strains. In histopathological evaluations of these same animals, Chapadeiro et al. (1988) verified chronic myocarditis with fibrosis in 15% of the animals, recommending this model for the cardiac study of Chagas disease.

To better understand the immunological resistance of the rats to acute *T. cruzi* infection, the contribution of mononuclear phagocytes was studied in animals inoculated with *T. cruzi*, Y strain (Melo and Machado, 2001). Acute *T. cruzi* infection triggered a dramatic increase (93.7%) in peripheral blood monocyte number at day 12 of infection. At this point, histological analysis of the heart showed high parasitism, and a moderate to intense mononuclear inflammatory process. Ultrastructural study revealed a large number of macrophages, in addition to lymphocytes and undifferentiated cells. Clusters of macrophages exhibited different morphological phenotypes, with evident signs of activation. The present findings indicate that the early phase of infection with *T. cruzi* induces rapid production, maturation, and activation of the monocyte/macrophage system so as to control *T. cruzi* replication, emphasizing the crucial role for macrophages in the rat resistance to Chagas disease. Immunological studies of Chandrasekar et al. (2000) in rats infected with Sylvio X10/7 strain during the acute phase of the infection revealed that by 1.5 days postinfection, when no parasite or immune cell infiltration could be detected, the myocardium expressed high levels of nitric oxide synthase (NOS) and NO metabolites. Nevertheless, the early production of NO in the myocardium was not sufficient to clear the parasites.

Taking these results together, the rat model, despite the relative resistance to *T. cruzi* infection, has important applicability to study certain aspects especially of

the chronic infection of Chagas disease. Furthermore, rats are easily reproduced, relatively of low cost, and easy to handle.

12.5.3 Dogs

The dog has been used as experimental model to study Chagas disease since its discovery by Chagas (1909) when he sent to Oswaldo Cruz Institute in Rio de Janeiro, the first isolates of *T. cruzi* to infect different laboratory animals. Young dogs, differently from the older, are highly susceptible to infection, and reproduce with great facility the acute phase of the disease when usually the mortality is very high. This high mortality observed during this phase disability the systematized studies of the chronic phase in experimental conditions and the reproduction of the later different clinical forms of the disease.

One of the most important advantages of the dog in relation to other animal models is the advanced knowledge and the similarity of the cardiac morphology and physiology of the heart conduction system with humans (Lumb et al., 1959; Mirowski et al., 1970). This makes this model ideal for the studies of ECG changes that are so important in the cardiac form of Chagas disease, allowing comparison with those observed in humans. Another advantage is the long life span of this animal (15−25 years), which naturally allows the evolution of the later clinical forms of the disease, besides its easy reproduction and handling.

Different sources of inoculum and routes of infection may be successfully used to infect dogs. The survival of the animals to the acute phase is very dependent on the parasite virulence, source and size of the inoculum, and route of infection.

Several authors have easily reproduced different morphological and clinical aspects of the acute phase in dogs. Goble (1952) reproduced for the first time the "chagoma" of inoculation (one of the signals of parasite entry in the vertebrate host), as well as neurological symptoms, such as paraplegia describing lesions on the brain and cerebellum of the animals. Other signs of acute infection were also reproduced by Pellegrino (1946) and Laranja (1953) in dogs, such as ascites, neurological symptoms, and signs of congestive cardiac insufficiency. Kramer Jr. (1972) also verified the "chagoma" of inoculation, hyperemia, enlargement of the lymph nodes, bilateral conjunctivitis, prostration, paraplegia with lesions, and parasitism of the central and peripheral nervous system (encephalitis and meningitis), heart, and skeletal musculature signs very similar to those observed in humans.

The first neurological symptoms and lesions were also observed by Villela and Torres (1926), Campos (1927), and Koberle (1958). This last author demonstrated also the evolution of intense myocarditis in young dogs followed by mortality. Marsden and Hagstron (1968) infected young dogs with various inocula of metacyclic and blood trypomastigotes by subcutaneous and conjunctival routes, simulating with this last inoculation the natural mechanisms of infection. These authors verified intense infection and parasites were observed in blood, urine and saliva suggesting that probably the conjunctival route facilitated the brain parasitism observed in animals inoculated by this last route. Later, Bahia et al. (2002),

studying comparatively dogs infected with metacyclic and blood trypomastigotes from the Be-62 and Be-78 strains via intraperitoneal and conjunctival, also verified that all dogs inoculated intraperitoneally became infected independently of the *T. cruzi* strain and source of trypomastigotes used. The results suggested that the source of the inoculum and the route of inoculation remarkably influenced the evolution of the infection even when the same strain of the parasite is used.

In the acute phase, dogs developed intense myocarditis with accentuated parasitism of myocytes and necrotic lesions of cardiac cells that were associated with parasites, disintegrated or not, in parallel with intense inflammatory process. These lesions began in atria, especially the right one, and migrated to the ventricles. The sinoatrial node (SAN) and atrioventricular nodes (AVN), as well as the His bundle branches were reached. Several signs were suggestive of cytotoxic and cytolytic mechanisms mediated by immune cells: the ultrastructural aspects of cellular infiltrate with granular and agranular lymphocytes (which adherent to the cardiac cells leading to myocitolysis, separation of intercellular junctions, presence of necrosis, and apoptosis (Andrade et al., 1994). Microangiopathy with adherent granular lymphocytes to endothelium of the capillary was observed during the acute phase. ECG alterations were normally discrete (ischemic, intraventricular blocks, hemiblock of the left bundle branch of His) and reversible important only when lesions of the heart conduction system were associated with necrosis of the myocells, a signal of bad prognosis.

Since the beginning of the experimental infections in dogs, ECG alterations have been studied in the acute as well as chronic phases of the infection (Taquini, 1942; Pellegrino, 1946; Anselmi et al., 1971). These changes include the decrease of QRS complex, enlargement of P wave, changes of T wave, ST-T segment, disturbance of ventricular repolarization, arrhythmia, partial and total AV blocks, and the typical ECG changes of chagasic patients, such as total block of the right bundle branch of His, associated or not with the anterior or posterior block of the left branch, extrasystoles, or inactive zone. These three last alterations are strongly associated with the occurrence of sudden death.

Anselmi et al. (1967, 1971) and Andrade et al. (1984) were the first to study the heart conduction system of dogs with acute and chronic chagasic myocarditis. Their research correlated the histopathologic lesions and the ECG alterations, especially in the acute phase. Anselmi et al. (1971) described ventricular aneurysm in 2 out of 27 dogs experimentally infected with the Bertoldo *T. cruzi* strain; however, these alterations have not been reproduced by other authors.

Andrade et al. (1981) verified that the cardiac lesions of nine dogs infected with the *T. cruzi* strains 12-SF and Colombian were very discrete, considering that all animals were in the indeterminate phase of the infection surprising, given that the dog is considered a good model to reproduce this clinical form of the disease. Cardiac dilatation, when it occurred at all, was minimal, and the histopathology revealed the presence of mononuclear cells and focal fibrosis, especially in the right atria without presence of parasites. These lesions were considered sequelae of the acute phase. Afterward, Andrade et al. (1987) observed that these dogs in the indeterminate phase of the infection presented evolution of the myocarditis and

fibrosis after administration of minimum doses of cyclophosphamide, suggesting participatory role of the immunopathologic process in the evolution of Chagas disease lesions.

The occurrence of dilatation of hollow organs and the characterization of the typical "megas," especially of esophagus and colon, are rare in the dog model. The first description, not including esophagus and intestine, was observed by Koberle (1957) in naturally infected dogs, and suggested that neurotoxin released by disintegrated parasites could be responsible for the neuronal lesions that cause the physiological dysfunctions of the hollow organs. Okumura and Corrêa Neto (1961) described the occurrence of megaesophagus and the dilatation of the colon region in dogs. However, other authors never reproduced these findings, independent of the *T. cruzi* strain and experimental protocol used.

Andrade and Andrade (1980) highlighted the importance of the reproduction of the indeterminate phase of the infection and the unpredictability and later evolution of the typical cardiac form of the disease. Their research also noted the absence of experimental digestive form of the disease in dogs, which seriously limits the applicability of this model based on the requisites established by WHO (Andrade, 1984). However, we believe that apparently the reproduction of the characteristic cardiac lesions of the chronic phase in dogs is very dependent on the *T. cruzi* strains used. Thus, Lana et al. (1988) succeeded in developing the diffuse fibrosing chronic chagasic cardiopathy in several dogs infected with the Be-78 strain. This type of lesion was reproduced in different animals infected with this strain despite the source and inoculum, route of inoculation, and time of infection. The inflammatory process was diffuse and followed by dissociation and substitution of the cardiac cells by intense deposition of collagen. These changes were not associated with the presence of the parasite, strongly suggesting that autoimmune mechanisms participate in the pathogenesis of the heart lesions (Laranja and Andrade, 1980; Lana et al., 1988). The cellular infiltrate was predominantly lymphocytic with aggregation of plasmocytes and macrophages.

In 64 animals infected with two isolates of *T. cruzi* (Be-62 and Be-78 strains) obtained from the first human case of Chagas disease (Chagas, 1909, Salgado et al., 1962; Lana and Chiari, 1986), Lana et al. (1992) demonstrated the acute and chronic phases of the infection in dogs with several histopathological lesions, ECG alterations, and signs very similar to those observed in humans. Some instances of sudden death occurred in different periods of the chronic infection. In fact, the indeterminate and cardiac clinical forms of the disease were observed in several animals throughout the infection with the severe cardiac lesions observed only in dogs infected with Be-78 strain (Lana et al., 1988; Lana et al., 1992). These findings reemphasize the dog as a good experimental model for the study of Chagas disease. In parallel, the authors registered the parasitemia and humoral immune response (IgM and IgG profiles) since the first week of the infection up to 36 months (Lana et al., 1988). Araújo et al. (2002) later evaluated the same animals that survived the infection (5−12 years), comparing polymerase chain reaction (PCR) assays with parasitological and serological methods. Despite its low sensitivity (if considering only one reaction), the PCR analysis showed 100% positivity,

demonstrating the presence of parasite kDNA in all infected dogs. Moreover, these data validate once more the dog as a model for Chagas disease since they demonstrate the permanence of infection by PCR, parasitological and serological methods. Caliari et al. (1995, 1996) and Machado et al. (1998) studied dogs experimentally infected with the same *T. cruzi* strains to evaluate the qualitative and quantitative neurological lesions, as well as the mechanisms involved in this process. Several aspects of the experimental infection in dog model during the acute and chronic phases of the disease are shown in Figure 12.2.

Zhang et al. (1999) studied the immunopathologic phenomena that participate in the cardiac lesions in the acute phase and the participation of different types of lymphocytes during the evolution of the disease. The apoptosis was observed in myocytes, endothelial cells, macrophages, lymphocytes, interstitial dendritic cells, intra- and extracellular parasites, and its participation on Chagas disease pathogenesis was discussed. The results reinforce the participation of parasites' antigens on the maintenance of cellular immune response in infected dogs, and they confirm the important role of antigens maintained in dendritic cells in the hypersensitivity phenomena associated with the developing lesions. Caliari et al. (2002) also studied immunological aspects of the disease in dogs, verifying that animals infected by the Be-78 strain had more intense myocarditis and higher caCD8+ cell counts

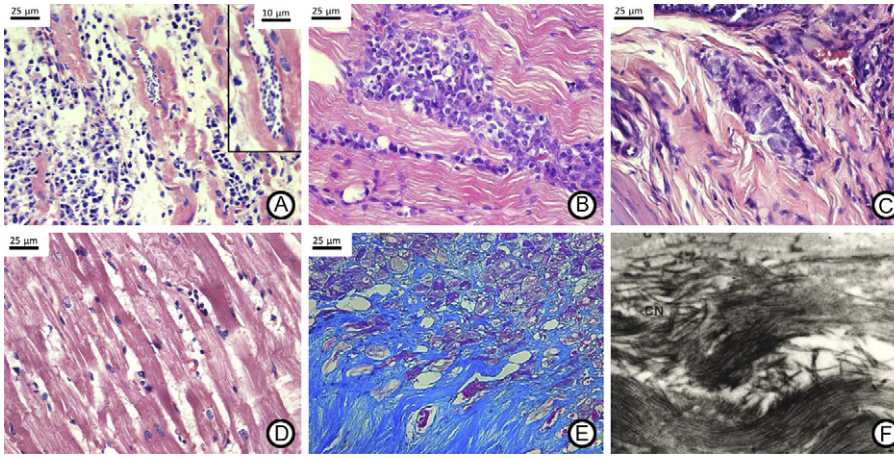

Figure 12.2 Several aspects of experimental Chagas disease in dog model during the acute and chronic phases of the infection (A−E: hematoxylin and eosin counterstained). (A) Acute myocarditis with presence of mononuclear cells dissociating the muscle's cells (Inset amastigotes nest). (B) Muscle layer of esophagus showing focal inflammatory infiltrate. (C) Ganglionitis and inflammatory cells in muscular layer of intestine. (D) Indeterminate chronic phase of the infection with discrete infiltrate of inflammatory cells. (E) Cardiac muscle with intense fibrosis during the chronic phase of the disease. (F) Collagen fibers in the heart during the chronic phase of the infection (electron microscopy).
Source: Photographs by M. Lana and colleagues.

during the chronic phase (even without evidence of tissue parasitism) than were seen in animals infected by Be-62 strain. These findings suggest the involvement of other mechanisms in the genesis of the inflammatory process (Figure 12.3).

Machado et al. (2001) studied the role of reinfection in the evolution of Chagas disease in dogs alternately infected 5 times with two *T. cruzi* strains. Animals presented a brief oligosymptomatic acute phase. The level of parasitemia decreased progressively with the number of reinfections. All parasite samples isolated during the follow-up were zymodeme B, corresponding to one of the strains but independent of the strain used in the first inoculum. The amplification by PCR of a segment of the *T. cruzi* miniexon gene showed the simultaneous presence of both strains in three of the eight animals. Antibody titers were greater among the dogs successively infected than those infected only once. Neither parasites nor kDNA was detected in the tissues of the infected dogs. However, all animals developed the indeterminate form of the disease.

Nowadays the dog model has been very used in chemotherapy studies by our team and good results were obtained when conventional drug as benznidazole was used to treat *T. cruzi* strains of different patterns of drug susceptibility (Guedes et al., 2002). The results were similar to those reported in clinical trials for treated human patients (cured and uncured) in both phases of the disease. We also showed that parasitologic and serologic tests for monitoring the parasitological cure were similar to those obtained for human trials (Guedes et al., 2002). Considering the remarkably long half-life of the triazole derivative albacanozole in monkeys and humans, more recently this compound was tested in dogs (Guedes et al., 2004) infected with susceptible and partially resistant *T. cruzi* strains. The results obtained demonstrated that this compound may be used in long-term treatment schemes (60−150 days) with minimal toxicity representing a potentially useful candidate for the treatment of human Chagas disease. Other potential triazole derivatives are still being tested in this model in our laboratory.

In conclusion, the principal advantage of the dog model is the reproduction of the distinct phases of the disease. Several clinical aspects of the disease similar to that verified in humans have been observed in dogs, offering the possibility of the ECG monitoring to enable a reliable correlation between these alterations and the cardiac excito-conduction system lesions. A disadvantage to the dog model is that dogs often need a very long term to develop the cardiac chronic clinical form of the disease, which is rare and apparently *T. cruzi* strain-dependent.

12.5.4 Rabbits

Of all experimental models to study Chagas disease, rabbits are considered the model with more contradictory results in the literature, which is the reason why they are not used very often for this purpose. Agosin and Badinez (1948) were the first authors who isolated parasites from the peripheral blood and verified tissue lesions and parasites in muscular fibers of rabbits experimentally infected with *T. cruzi*. Katzin et al. (1977) infected rabbits with metacyclical trypomastigotes, verifying low parasitemia detectable only by xenodiagnosis and humoral immune response

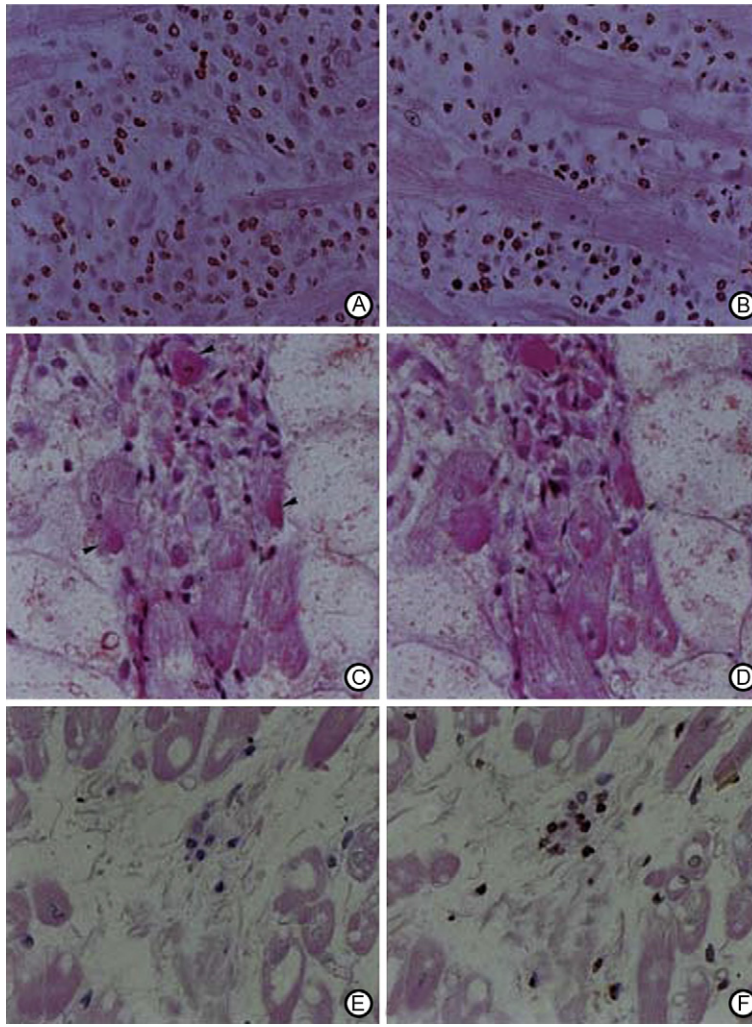

Figure 12.3 Immunohistochemistry reaction for caCD8 and caCD4 in myocardium of dogs necropsied during acute and chronic phases. (A) Acute myocarditis showing a large number of strongly labeled caCD8+ T cells. (B) Myocardium of the same dog also showing large number of intensely stained caCD4+ T cells. (C) Chronic myocarditis induced by the Be 78 strain showing a focus of caCD8+ T cells, close to damaged myocardiocytes (*arrowheads*) and without parasitism. (D) The same region contains a similar number of caCD4+ T cells. (E) Chronic myocarditis induced by the Be 62 strain showing absence of caCD8+ T cells. (F) The caCD4+ T cells in the same region (hematoxylin and eosin counterstained × 440). *Source*: From Caliari et al. (2002).

similar to that observed in humans. Teixeira et al. (1975) infected rabbits with the highly virulent Ernestina strain to reproduce the acute phase with patent parasitemia as well as the chronic phase of the infection. The animals survived the acute phase, four died with myocarditis, two presented megacolon, the majority remained asymptomatic and the lesions observed were considered similar to that observed in humans. The authors also verified that the inoculation of subcellular fractions of the parasites in mice not infected with *T. cruzi* caused similar lesions than those the infected ones being this fact a demonstration of the autoimmunity phenomena causing lesions in Chagas disease. Andrade and Andrade (1979) observed in rabbits infected with Y, Colombian and 12-SF strains subpatent parasitemia and generally absence of lesions is in both phases of the infection that were present but very discrete. These results were also confirmed by Chiari et al. (1980) when rabbits and mice were infected in parallel with Y, MR, and CL strains. In a systematic study with Ernestina and Albuquerque strains, however, Teixeira et al. (1983) achieved important results in the acute and chronic phase of the infections concerning parasitological, serological, histopathological, and clinical (ECG) aspects of the disease similar to human disease. Ramirez et al. (1987) reproduced similar results in this model when infected rabbits with Y, CL, and Ernani strains verifying the occurrence of cardiac fibrosis, aneurysm, and several ECG changes. However, the cardiac electrophysiology of rabbits is not well known, which complicates the interpretation of ECG changes in this animal model. Da Silva et al. (1996) concluded that advanced fibrosis occurred only in the late chronic phase in rabbits infected with different parasite strains. Tissue parasites occurred only in the acute phase and the digestive tract. Skeletal muscles showed mild and occasional lesions, which indicate that experimentally infected rabbits with *T. cruzi* reproduce some lesions similar to those seen in human chagasic patients, especially ones of indeterminate form. However, the development of megasyndromes was not effectively demonstrated in this model (Andrade and Andrade, 1979). Finally, the authors concluded that rabbits may be a useful, not ideal, model for study of Chagas disease.

12.5.5 Guinea Pigs

In the first years after the discovery of Chagas disease, several authors, including Chagas (1909), used guinea pigs as an experimental model (Vianna, 1911; Blanchard, 1912; Nino, 1926; Badinez, 1945). The studies that evaluated the chronic phase of the infection in this animal are rare. This model develops the acute phase of the disease with patent parasitemia in the first days of infection followed by mortality, or spontaneously evolves for the chronic phase. Knierim (1954) studied the humoral immune response of guinea pigs experimentally infected using three serological tests. Kozma et al. (1960) interpreted the histopathological findings of the chronic phase in this model as suggestive of participation of autoimmune mechanisms in the myocarditis independent of the parasite's presence. Lopes et al. (1969) demonstrated that guinea pig was a good model to study the experimental cardiac lesions in immunological and ultrastructural aspects. Franco (1990) studied the association of parasites' antigens and interstitial

inflammatory reactions independent of the parasite's presence during the first months of infection. Data were suggestive of the important participation of the infection in hypersensitivity phenomena.

Although the guinea pig is very susceptible to *T. cruzi* infections, this animal model has always been substituted by other models, such as mice, rats, and dogs, which better reproduce the aspects of acute and chronic infections observed in humans.

12.5.6 Hamsters

The Syrian hamster, *Mesocricetus auratus*, has been used as an experimental model for Chagas disease. These animals are considered to be of low immune resistance to several infections. This is one of the reasons why they have not often been used as models for Chagas disease studies. However, Ramirez et al. (1994) performed a detailed evaluation of *M. auratus* as model to study the acute and chronic phases of the infection. Both phases of disease were reproduced in these animals infected with Y and Benedito *T. cruzi* strains. A careful follow-up of the acute phase demonstrated easy recovery of the parasites by hemoculture or xenodiagnosis in all animals. An inflammatory reaction was also observed, characterized by mononuclear and polymorphous leukocyte infiltration in the majority of tissues and organs, especially in the connective loose and fatty tissues, smooth muscle myocardium, and skeletal muscle.

In the chronic phase, animals were evaluated periodically from 3rd to 10th month with the lesions observed in the same tissues and organs. The inflammatory response was less severe and characterized by mononuclear infiltration, mainly with focal or zonal fibrosis in the myocardium. Parasites were detected in 50% of infected animals in myocardium and recovered from pericardic, peritoneal, and ascitic fluids in some animals. Signs of heart failure, sudden death, and enlargement of bowel were observed regularly. The authors concluded that the hamster is a useful model for Chagas disease studies.

Persisting in the study of these same animals, Chapadeiro et al. (1999) investigated the behavior of the cardiac nervous system in the chronic phase of the infection. In the 5th, 8th, and 10th month after infection, neuronal cell counts from the cardiac autonomic nervous plexus first revealed severe neuronal destruction with characteristics similar to those observed in human Chagas disease. Cabrine-Santos et al. (2001) studied the influence of reinfections in this animal model with the VIC *T. cruzi* strain and verified that no important changes were observed. Bilate et al. (2003) used the Syrian hamster as a model for the study of chronic cardiopathy in animals infected with the Y strain. Animals were evaluated by histopathology, morphometry, and ecocardiography. More intense changes and mortality were observed in animals with higher inocula and interstitial fibrosis. Finally, the Syrian hamster develops a cardiomyopathy that resembles human Chagas disease.

However, this species, like guinea pigs, is not currently used as an experimental model for the studies of Chagas disease. More traditional models, such as mice,

dogs, and monkeys, are more able models to reproduce the disease as observed in humans.

12.5.7 Monkeys

The experimental studies of Chagas disease are not frequent in monkeys due to the great difficulties in obtaining, maintaining, and handling these animals in confinement for long periods of time. Even with these difficulties, however, the phylogenetic proximity of these animals with humans make these models more appropriate than others, particularly in immunological and pathological studies of Chagas disease or in the development of the immunological methods for the diagnosis of this disease. The literature presents several experimental studies in *Macacus rhesus* (nonautochthonous to the Americas) and in *Cebus*, *Saimiri*, and *Callithrix* genera from this continent.

12.5.7.1 Primates Autochthonous to the Americas

Chagas (1909) was the first to infect primates from the *Callithrix* genus (*C. penicillata*) with blood trypomastigotes isolated from Berenice, the first human case of the disease. Romaña (1939) reproduced the acute phase of the infection, including the Romaña sign, by inoculating the primates with feces of infected triatomines through the conjunctival route. Torres and Tavares (1958) infected *Cebus* monkeys with blood and metacyclic trypomastigotes by oral mucosa, skin, and conjunctival routes. The authors tried to verify the influence of repeated infections in the anatomopathological pictures of these animals, but this was not possible due to the distinct protocols used. Deaths in these animals occurred between 75 and 243 days, and the diffuse cardiac fibrosis observed was distinct from that verified in humans. Bolono et al. (1980) infected two exemplars of this same gender and verified that the animals did not present symptoms of the acute phase. However, 9 months later, they developed cardiac symptomatology with important anatomopathological and ECG alterations. Necropsy of these animals revealed megaesophagus, fibrosis, and inflammatory infiltrates in the myocardium. This same author, later infecting another group of 3-year-old animals, found only discrete infection with decreasing serological titers throughout the infection.

The little primate *Saimiri*, naturally infected in nature, offers relatively easier handling and maintenance in experimental conditions. Pung et al. (1988), trying to use this model for the study of Chagas disease, infected this genera with metacyclic trypomastigotes of the Brazil strain. The authors observed acute infection with patent parasitemia, ECG and hematological alterations, specific anti-*T. cruzi* antibodies, and intense lymphoproliferative response to parasite antigens.

Falasca et al. (1986) infected 18 *Cebus apella* ("prego") monkeys, each 15 months old, with repeated inoculations with three different *T. cruzi* strains in each animal. Their observations confirmed a discrete and asymptomatic acute phase of the infection. Only one animal infected with the Tulahuen strain presented patent parasitemia. The antibody level increased with each reinoculation. One case

of megacolon and one case of dolicocolon were reproduced. Various ECG and echocardiographic changes similar to those observed in humans were observed, but the histopathological lesions and fibrosis were discrete.

When the surviving animals were sacrificed 20−25 months later, the authors observed myocytolysis and leukocytic infiltrates between the muscular fibers (Falasca et al., 1990). In animals observed 36−47 months after infection, interstitial diffuse or focal fibrosis with discrete infiltrate of leukocytes was noted, predominantly in the cardiac ventricle wall or septum. This evolutionary character of the histopathological lesions suggests that this model could be more appropriate for the experimental studies of the natural evolution of Chagas disease than other non-autochthonous primate models from the Americas. Granado et al. (1983) used seven exemplars of *Cebus apella* to test drugs, demonstrating that this model could be very useful for chemotherapy trials and to evaluate the effect of treatment on several parameters of the infection. Later, Samudio et al. (1999) studied the immune response in animals of this species infected with Y strain. Parasitological, serological, and clinical parameters were monitored during a 19-month follow-up, and systemic cytokine responses were assessed sequentially in five monkeys selected according to the differential parasitemia pattern exhibited. Elevated expression of interleukin (IL)-4 was observed throughout the study in monkeys that had persistent and higher parasitemias, whereas a high level of interferon gamma (γ-INF) was seen in monkeys that controlled parasitemia soon after infection. The authors verified higher expression of γ-INF and adhesion molecules in cardiac tissue of animals 19 months, 5 years, or 10 years after infection, correlated with the persistence of kDNA of *T. cruzi* revealed by PCR.

12.5.7.2 Primates Nonautochthonous to the Americas

Macacus rhesus is one of the primate species used as a model that is not autochthonous to the Americas. This species is phylogenetically considered more similar to the human genome (98% of homology) than other species present in the American continent.

Dorland (1943) infected animals of this species by conjunctival route with metacyclic trypomastigotes from naturally infected triatomines originating from Texas and California in parallel with other animals infected with another strain isolated from humans. The infection with the wild strain was verified in all animals. The Romaña sign was observed in some monkeys, as was chronic myocarditis with tissue parasitism. Guimarães and Miranda (1961) reproduced one case of megaesophagus with the presence of parasites in the muscular fibers in monkeys of this species inoculated 10 years before. Blood of this animal was inoculated in two other exemplars, and the infection was observed in both. One died during the acute phase, and the other survived for 29 years; the latter had discrete ECG changes, discrete increase of the cardiac area, and several indicators of autoimmune response such as antibody anti-endothelium, vases, interstice (EVI) and anti-peripherical nerves (Szarfman et al., 1978).

Marsden et al. (1970a) also reproduced the infection in three exemplars of rhesus monkeys inoculated with the Peru strain by conjunctival route. Several signs of the acute phase of the infection were observed, such as patent parasitemia, ocular edema, decrease of red cells and hemoglobin, presence of IgM and IgG antibodies, tissue parasitism in several organs and tissues (including the parotid gland), and presence of parasites in ocular secretions. This author tried to vaccinate (without adjuvant) one animal with epimastigote forms of one of the *T. cruzi* strains; no evidence of protection was observed. Afterward, Seah et al. (1974) tried to vaccinate two exemplars of rhesus monkeys using adjuvant. The animals were challenged, and the results were compared with those obtained from the two unvaccinated animals; the clinical picture between the two pairs varied greatly.

De Meirelles et al. (1990) performed ultrastructural and cytochemical studies of peroxidase and acid phosphatase in skin, lymph node, and heart muscle tissue of rhesus monkeys with experimental Chagas disease. The study provided some experimental evidences that the monkey model could be used as a reliable model to characterize histopathological alterations of the human disease.

For 3 years, Bonecini-Almeida et al. (1990) evaluated acute and chronic infections in rhesus monkeys with 4 and 10 years old by subcutaneous route with little inoculum from the Colombian strain. Several clinical symptoms typical of acute phase were observed and disappeared afterward. Antibodies IgM and IgG anti-*T. cruzi* were observed since the 4th and 3rd week of infection, respectively. IgM antibodies disappeared in the 9th month of infection, and IgG remained elevated during all periods of study. Lytic and EVI antibodies appeared in the 3rd and 4th week of infection, respectively, and remained present during all periods of infection. Myocarditis and myositis were observed only in the acute and subacute phases of the infection. However, all animals that were necropsied in the later phase of the infection presented characteristics of the indeterminate clinical form of the disease without any sign of evolutionary chronic chagasic cardiopathy. These later findings put this animal model at a disadvantage in relation to *Cebus paella*, in which the evolution of the different clinical forms of the disease, including chronic cardiopathy and "megas," were observed.

The primates have been the animal model that develops an acute phase of infection most similar to that observed in human disease, but the animals are difficult to obtain, maintain, and handle in experimental conditions. These difficulties explain the scarcity of data on cardiac lesions of the chronic phase (normally of later evolution) and discourage the use of this animal model for the experimental study of Chagas disease.

12.5.8 Conclusions on Animals as Experimental Models

Although a great variety of animal models could be used as experimental models for study of Chagas disease, it has not been possible to artificially reproduce all the histopathological and clinical manifestations of Chagas disease observed in humans. The studies are not performed in standardized conditions, even when the

same animal species is considered. Variables are considerable, including race; genetic background; *T. cruzi* strain; source, number, and route of inoculations; age, sex, weight of the animal; parameters of evaluation; and length of infection. Moreover, when studies using the same *T. cruzi* strains in similar conditions in different animal species revealed that the results observed are not the same, it suggests that it is not possible to extrapolate experimental results from one model to another. Thus, the choice of an experimental model is dependent on the subject to be investigated and the previous knowledge acquired through the past century of research.

12.6 Experimental Infections in Livestock

Most of the experimental work done in *T. cruzi* was carried out in rats and mice, monkeys or dogs as shown (Guedes et al., 2002; Andersson et al., 2003; Guedes et al., 2009). In some instances other models have been used, such as rabbit or even the striped skunk (Davis et al., 1980), which appear to be also a natural host of *T. cruzi* in Argentina (Pietrokovsky et al., 1991). Very few of the experimental studies concern livestock.

Young pigs, lambs, kids, and calves were proven to be susceptible to *T. cruzi* infection in experimental conditions in the USA (Diamond and Rubin, 1958), but little is known about the pathogenicity since no clinical signs were detected; however, 10% of the animals had tissue stages, suggesting a possible role in the epidemiology of the disease. The infection persisted at least 21 days in calves, 38 days in kids, 53 days in lambs, and 57 days in pigs. In experimentally infected kids, no symptoms were visible, although a ventricular hypertrophy was detected using ECG (Alcaino et al., 1995). The long-term effects of the infection deserve more thorough investigation.

In experimental infection of four English pigs challenged with a virulent Peruvian strain of *T. cruzi*, three became infected. The course of the infection in the pigs was mild and the parasitemia too low to be detected except by mouse inoculation (Marsden et al., 1970b). In all cases, pathogenicity appeared to be fairly low according to the few experimental findings available. Certainly, duration of the observation under these experimental designs was too short to observe a complete range of pathogenicity of this slow-developing disease.

Recent studies have emphasized the importance of two types of peridomestic reservoirs for *T. cruzi* that had so far been underestimated: pets (dogs, cats, guinea pigs, and other rodents) and farm animals (horses, cattle, sheep, goats, pigs, rabbits, and guinea pigs) (Salazar-Schettino et al., 1997). Indeed, the peridomestic cycles are precisely those that present the highest risk of human contamination (Rodhain and Perez, 1985). The effective involvement of livestock in the epidemiology of Chagas disease is very difficult to estimate because of the (sometimes high) prevalence of true livestock trypanosomes (*T. vivax*, *T. evansi*, and *T. equiperdum*).

Since receptivity and susceptibility of livestock appeared to be low in these scarce experimental studies, with low and transient parasitemia, livestock infections probably play only a minor role in the epidemiology of the human disease, and livestock is probably an epidemiological dead end. However, in disadvantaged rural populations, close cohabitation between species might foster peridomestic zoonotic spread of the parasite. Disease control relies essentially on hygiene and education of the populations at risk. Veterinary practitioners who deal with pets and livestock in endemic areas and veterinarians that work in the disadvantaged areas where there is little separation between human and animal habitats should be warned. A positive diagnosis in an animal may be an indication of risk for humans. Pathogenicity of *T. cruzi* in livestock is assumed to be low, but it requires further investigation to determine the potential role of these animals in the epidemiology of the disease.

12.7 New Cycles Establish in the USA

Although Chagas disease in humans is generally considered to be present in Latin America from 38°S up to the southern part of Mexico (25°N), *T. cruzi* is present from Argentina and Chile (43°S) up to the southern part of the USA (42°N), as far as Missouri; infections are seen occasionally in humans and regularly in domestic animals. It is most common in dogs and cats (Rodhain and Perez, 1985), but also in a large range of wild animals, such as opossums, wood rats, raccoons, striped skunks, armadillos, antelope squirrels, gray foxes, and coyotes (Woo and Soltys, 1970; Kjos et al., 2008), with some high prevalences, such as 33% in opossum and 63% in raccoons (Roellig et al., 2009a).

T. cruzi is known to be present in the following states: California, Arizona, Texas, Oklahoma, Tennessee, Missouri, Louisiana, Florida, Georgia, Virginia, South Carolina, and North Carolina (Karsten et al., 1992; Yabsley and Noblet, 2002; Diaz, 2007; Dorn et al., 2007; Kjos et al., 2008; Brown et al., 2009; Maloney et al., 2009). Several vectors have been reported in the USA: *Triatoma gersaecheri* in Texas; *Triatoma sanguisuga* and *Triatoma lecticularia* in South Carolina and Georgia (Yabsley and Noblet, 2002). The very few cases of native Chagas disease were recorded in California, Texas, and Tennessee, most often by means of post-mortem PCR (Ochs et al., 1996; Herwaldt et al., 2000a). Many wild and domestic animals were found to be infected, sometimes with high prevalence such as in armadillos, badgers, and coyotes. Cattle and sheep were found to be carriers of antibodies in Texas and Louisiana; in South Carolina and Georgia, nearly 50% of raccoons (*Procyon lotor*) and some opossums (*Didelphis virginiana*) were serologically positive (Yabsley and Noblet, 2002).

The isolation of *T. cruzi* in dogs in Virginia (Barr et al., 1995) showed that a new epidemiological pattern is being established by the spread of the parasite; in this case, the mother Walker hound and seven of its eight pups were found infected. This pattern relies on a wild host/bug/carnivore cycle in which wild host or rodents

probably act as reservoir (direct transmission from rodent to rodent is also possible by biting), whereas dogs are thought to be an epidemiological *cul-de-sac*.

In Tennessee, five autochtonous human cases have been reported so far; in the last case, a child was infected without significant clinical signs and would not have been detected if the bugs had not been noticed by the mother; in this case *Triatoma sanguisuga* was captured and found to be infected. Three raccoons trapped in the vicinity of the house were also infected (Herwaldt et al., 2000). Another autochtonous case was reported from California (Deneris and Marshall, 1989), and the strain isolated could develop in two species of Triatominae native to California: *Triatoma protracta* and *T. rubida*.

The way of infection of autochtonous human cases has not been identified yet. Several hypotheses remain, including vectorial transmission, transconjunctival or peroral infection by bug feces or contaminated food, or another unidentified way. These cases in which origin and way of infection are not clearly elucidated do not preclude the possible cyclical transmission of the parasite by a vicariant vector that the parasite might have found on its way north.

One must remember that cyclical transmission of *T. cruzi* has been described in opossums (*D. marsupialis*) (Urdaneta-Morales and Nironi, 1996). Hence, in the USA, *T. cruzi* has a huge wild and domestic reservoir together with two cyclical vectors (bugs and opossum) and other potential vectors (louse). Infections in dogs are seen more and more often. It is presumed that the main cycle occurs in bugs and wild animals (raccoons and opossums). Domestic animals are infected by ingesting bugs or, in the case of carnivores, by ingesting infected wild prey. Although infected bugs may be found in the vicinity of human habitat, there are very few reports of human contamination; this is attributed to the long interval that elapses in the USA between the time when the vectors take their meals and the time when they defecate, making it unlikely for a bite wound to be contaminated (i.e., the most common mode of human contamination in South America). Furthermore, living and hygiene conditions in the USA are far less likely to foster contact between humans and vectors than those that prevail in Latin America. However, dogs and cats may be a potential link between wild reservoir and humans since they attract and maintain bugs in the vicinity of human habitat, potentially contaminating human food with bug feces.

Progression in the distribution and establishment of *T. cruzi* should be taken very seriously. *T. cruzi* is already capable of transmitting cyclically in the USA through ingestion of food contaminated by opossum or bug excreta and by entry of the metatrypanosomes present in the feces of bugs (or opossum) through bite wounds or mucosal membranes. Thus, infection focus in humans can already occur in the USA by a similar way as in Brazil, where numerous people have been infected when drinking fruit juice; the source of contamination may be the fruit stock or utensils, by bug or opossum feces. The geographical extension of *T. cruzi* in wild and peridomestic fauna is currently spreading. Furthermore, *T. cruzi* may be able to find vicarious vectors in the course of its progression, possibly establishing a new epidemiological link to humans.

12.8 Conclusions

The data brought by the veterinary aspects of *T. cruzi* are of various types. The data generated by experimental infections (animals as models) are of a very high interest, since they have been representative models of the disease in humans; however, various models are necessary to study various aspects of the disease in humans that none of them could complete alone. Mice proved to be good models for some of the immune studies and treatment assay, but only for short-term observations and not for cardiac signs. Rats and hamsters proved to be good models for chronic evolution of the disease and some cardiac signs, but only for short-term studies. Dogs are better models for long-term studies and acute signs (especially cardiac signs) since they closely correlate to human ones. The monkey model is of particular interest due to a closer relation to humans, but these animals are expensive and difficult to maintain; however, the primate is a good model for long-term studies and especially for histopathological studies (which may also be carried out in guinea pigs). Finally, depending on the strains used, all the animal models proved to be of interest but only on a particular aspect of the disease.

The data based on the observation of the parasite in insects and wild animals, as well as in such domestic animals as dogs and cats, allowed to evaluate the geographical distribution of *T. cruzi* (especially toward its northern limit, if any) and then to estimate the existence of a potential risk for human exposure outside the endemic area of typical Chagas disease.

T. cruzi occurs in livestock, but its incidence is poorly known because no adequate diagnostic tools exist. In view of its epidemiology, it can be assumed that livestock is not highly exposed to infection. However, according to recent investigations, prevalence is not insignificant. Its occurrence in livestock deserves special attention and further investigation.

Although the human disease is typically South American, the parasite is spreading northward in the USA, where the main epidemiological vehicles would appear to be dogs and wild animals (e.g., opossums, raccoons). *T. cruzi* in North America likely fulfills its complete cycle in *D. virginiana* (Yabsley and Noblet, 2002) in much the same way as has already been described for South America in *D. marsupialis* (Urdaneta-Morales and Nironi, 1996). In view of the parasite's huge domestic and wild reservoir and its various transmission modes (including the peroral route), it is reasonable to predict that *T. cruzi* will maintain and extend its area of establishment northward. In addition, the long incubation period for Chagas disease and its weak obvious clinical incidence make it a covert scourge that tends to be underestimated and belatedly discovered. Finally, just as with *T. evansi* in vampire bats, it could be that *T. cruzi* is able to call on new vectors in North America and thereby renew its geographical progression, and possibly eventually find a new epidemiological link leading to human infections. It is advisable to call the attention of the USA's sanitary authorities to this threat. The spreading of *T. cruzi* would most probably lead, sometime if not already, to the extension of Chagas disease.

Finally, it must also be emphasized that new cycles could establish themselves as they have in other countries where potential vectors of *T. cruzi* are endemic, such as in Asia (e.g., Thailand, where *Triatoma rubrofasciata* seems to be well established). Traveling of Latin American carriers to such areas could lead to the establishment of new endemic areas. Diagnosis of such infection in Asia, for instance, would not be easy and early, due to epidemiological and geographical considerations. Serological surveillance in humans could be one of the ways to ensure free status of such countries.

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13 Classification and Phylogeny of *Trypanosoma cruzi*

Patrick B. Hamilton and Jamie R. Stevens*

School of Biosciences, University of Exeter, Exeter, UK

13.1 Application of Molecular Phylogenetics to the study of Trypanosome Taxonomy and Evolution

Trypanosomes (genus *Trypanosoma*) all share vertebrate parasitism and have a characteristic morphology in the vertebrate bloodstream. Trypanosomes are highly successful, being found in all classes of vertebrate (fish, amphibians, birds, reptiles, and mammals) and in all continents. The vast majority of trypanosome species are transmitted by arthropods (mostly insects) and leeches, although a few species can be passed directly between vertebrates. Most do not appear to harm their hosts, although several species are associated with important diseases of humans and domestic livestock.

There has been considerable interest in the evolutionary origin of genus *Trypanosoma* and the relationships within the genus; in particular, the relationship between the two human pathogens, *Trypanosoma cruzi* and *T. brucei*, has received considerable attention (Haag et al., 1998; Stevens et al., 1999b; Hughes and Piontkivska, 2003b; Hamilton et al., 2004). However, in the absence of a fossil record, and with few morphological features, testing evolutionary hypotheses has only become possible within the last 20 years with the advent of molecular phylogenetics. Molecular **phylogenetic trees** (phylogenies) are constructed by comparing DNA sequences from a range of organisms. Phylogenies allow the history of a group of organisms to be traced, thus providing robust frameworks for testing evolutionary hypotheses. Phylogenetic studies have led to an improved understanding of the origins of the parasitic life history strategy, the relationship between the two trypanosomatid genera that infect vertebrates (*Trypanosoma* and *Leishmania*), and the origins of trypanosomes of medical and veterinary importance.

It is now relatively straightforward to obtain DNA sequences from trypanosomes. First the chosen gene is amplified by using the **polymerase chain reaction (PCR)**,

*E mail: p.b.hamilton@exeter.ac.uk

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followed by sequencing of purified PCR products. Computer programs can then be used to align sequences and for subsequent tree construction. Increased computing power has allowed advanced computer-intensive tree building methods, such as maximum-likelihood and Bayesian methods, to be applied to sequences from many organisms. Although the first studies of this nature relied on DNA isolated from cultured parasites, many recent studies have used DNA isolated directly from the host tissue such as blood, or from insect guts. This has led the way to large-scale surveys of parasite diversity, which have transformed our understanding of the diversity of trypanosome species and their host ranges. These studies have benefited from the availability of gene sequences from a wide range of trypanosomes and related **taxa**, which has facilitated the design of parasite-specific **primers**, enabling amplification of trypanosomal genes, while avoiding amplification of host DNA.

A range of genes have been used for phylogenetic and taxonomic studies. Most studies examining relationships between species have used nuclear **ribosomal DNA (rDNA)** markers, in particular 18S rDNA (also called small subunit [SSU] rDNA), and to a lesser extent 28S rDNA (also called large subunit [LSU] rDNA). The 18S rRNA gene has both conserved regions, suitable for primer design and resolving relationships between distantly related species, and faster-evolving regions, suitable for deducing evolutionary relationships between closely related species and at the subspecies level. The V7-V8 region of 18S rDNA is the most variable and is often called the "barcoding" region, because it is useful for species identification. The noncoding internal transcribed spacer (ITS) regions, ITS1 and ITS2, have a faster evolutionary rate, so have been useful for studying within-species diversity. More recently, protein-coding genes have been used; in particular, glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) for phylogenetic placement of newly described species and for resolving relationships within the genus. The faster-evolving kinetoplast (mitochondrial) cytochrome *b* (*Cyt b*) gene has been used for examining within-species diversity (Cavazzana Jr et al., 2010). At the time of writing, complete *gGAPDH* and 18S rDNA sequences are available in sequence databases for more than 100 taxa, from a wide diversity of different trypanosome species.

Molecular phylogenetics has also informed **taxonomy** (Stevens and Brisse, 2005). Taxonomic groups should have evolutionary relevance, and arguably names should only be applied to **monophyletic groups**. Molecular studies have questioned the existence of some species that were previously described using morphological and life-cycle data. They have also raised new questions, such as whether new species should be named on the basis of sequence information alone, and the degree of genetic divergence necessary to classify lineages as the same or different species. At the same time, the development of rapid methods for species identification (Adams and Hamilton, 2008; Hamilton et al., 2008; Adams et al., 2009) as well as sequence-based surveys of trypanosomatid diversity (Votypka et al., 2010) have led to the description of new species. While many of these new species were found in previously unexplored hosts, such as Australian marsupials (Noyes et al., 1999;

McInnes et al., 2009), some potentially pathogenic species have also been found in well-studied groups, such as the African tsetse fly-transmitted group of trypanosomes (Hamilton et al., 2008). It is now clear that much trypanosomatid diversity is yet to be discovered.

13.2 Origin of Trypanosomes—Relationship of *T. cruzi* with *T. brucei*

Molecular phylogenetic studies have provided valuable insights into the origin of trypanosomes, the relationship between *Trypanosoma* and *Leishmania* (which also includes species that are human pattosens), and the relationship between the two human pathogens, *T. brucei* and *T. cruzi*. Two groups of theories have dominated debate regarding the origin of the genus *Trypanosoma*. Vertebrate-first theories proposed that the ancestral trypanosome evolved from a gut parasite of vertebrates (Minchin, 1908), while invertebrate-first theories proposed that trypanosomes evolved from a single-host (**monogenetic**) invertebrate parasite, such as the trypanosomatids that infect insect guts (Léger, 1904; Hoare, 1972; Vickerman, 1994).

Resolving the relationships between trypanosomes and their close relatives is the key to understanding of the origin of the genus. Trypanosomes (genus *Trypanosoma*) are trypanosomatids (family Trypanosomatidae), all of which are parasitic at all stages of their life cycle. Most trypanosomatid genera (e.g., *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Leptomonas*, *Sergeia*, *Wallaceina*) are single-host (monogenetic) parasites of insects. *Trypanosoma* and *Leishmania* are two-host (**digenetic**) vertebrate parasites. The existence of another digenetic vertebrate parasite, *Endotrypanum*, has not been verified using molecular techniques, as the isolates of this genus examined have turned out to be *Leishmania* (Cupolillo et al., 2000; Noyes et al., 2002). *Phytomonas* is a digenetic parasite of plants, which is transmitted by insects.

Central to the debate of the origin of trypanosomes is whether they are monophyletic. A monophyletic group is a collection of organisms, which form a single **clade** comprising an ancestor and all its descendants. Monophyly of trypanosomes would indicate that all described taxa within the genus had a single common origin and gave rise to no other group of trypanosomatids. The monophyly debate has also been central to resolving whether the two groups of trypanosomes, that include the human pathogens *T. brucei* and *T. cruzi* respectively, evolved vertebrate parasitism independently; monophyly would indicate that these species might share some common, ancestral adaptations necessary for survival in vertebrates. Understanding such ancestral adaptations could aid the rational design of therapeutics that target a broad range of pathogenic trypanosome species. Many of the early phylogenetic trees, based on comparisons of genes encoding ribosomal RNAs, showed trypanosomes to be **paraphyletic** (Gomez et al., 1991; Fernandes et al., 1993; Landweber and Gilbert, 1994). Often the evolutionary trees obtained from these studies suggested that the clade including *T. brucei* and related parasites (the *T. brucei* clade) had an origin independent to that of the rest of the trypanosomes, including *T. cruzi*

and related species. Such a tree topology indicated an independent evolution of vertebrate parasitism in *T. brucei* and related species.

On the other hand, most later studies, also using ribosomal RNA genes, but with increased taxon-sampling, showed monophyly (Lukes et al., 1997; Haag et al., 1998; Stevens and Gibson, 1999; Wright et al., 1999; Stevens et al., 2001; Simpson et al., 2002; Hamilton et al., 2004), although several supported paraphyly (Hughes and Piontkivska, 2003a,b). The issue was resolved using taxon-rich trees based on protein-coding genes, which strongly supported monophyly of the genus (Hamilton et al., 2004, 2007; Simpson and Roger, 2004; Simpson et al., 2006), confirming earlier studies using protein-coding genes that included fewer taxa (Wiemer et al., 1995; Adjé et al., 1998; Hannaert et al., 1998; Simpson et al., 2002).

Molecular trees have also provided clues to the ancestor of trypanosomes. For example, *gGAPDH* trees, constructed using robust maximum-likelihood and Bayesian techniques, have suggested that *Trypanosoma* evolved from an insect-only trypanosomatid (Hamilton et al., 2004). The recent discovery of an insect-only trypanosomatid closely related to *Leishmania* supports the idea that the genus evolved independently from another insect-only trypanosomatid (Yurchenko et al., 2006). Thus, we do not expect *T. cruzi* and other trypanosomes to share common, ancestral adaptations to vertebrate parasitism with *Leishmania*. *Leishmania* appears to have evolved considerably more recently than *Trypanosoma*, which may partially explain its comparatively narrow vertebrate (mammals, reptiles) and invertebrate (sandflies) host range.

The evolution of trypanosomes from a monogenetic insect parasite would indicate that the first trypanosomes were insect-transmitted trypanosomes of terrestrial mammals. Therefore, trypanosomes of amphibia and fish that are transmitted by leeches must have evolved later. The position of leech-transmitted fish trypanosomes in phylogenetic trees supports this idea (Haag et al., 1998); they all fall in a subclade of the aquatic clade (Stevens et al., 1999b), indicating that they were not the first to evolve, as previously hypothesized (Vickerman, 1976). Furthermore, while the deepest split within the genus is between the aquatic clade and the terrestrial clade (Figure 13.1A), both clades contain trypanosomes that are insect transmitted. Adaptation to transmission by aquatic leeches enabled trypanosomes to colonize many aquatic vertebrates, such as freshwater and marine fish.

13.3 Relationships within the Genus *Trypanosoma*

Studies have also examined the relationships within the genus *Trypanosoma*. The composition of the clade that includes *T. cruzi* (the *T. cruzi* clade) has provided valuable clues to the origin of the species. The most taxon-rich phylogenetic trees are based on alignments of 18S rDNA and *gGAPDH* genes (Haag et al., 1998; Stevens et al., 1999b, 2001; Wright et al., 1999; Overath et al., 2001; Martin et al., 2002; Hughes and Piontkivska, 2003b; Hamilton et al., 2004, 2005a, 2007). It is clear that *T. brucei* and *T. cruzi* are in different clades and evolved human parasitism independently (Stevens et al., 1999b). While *T. brucei* falls in the *T. brucei* clade

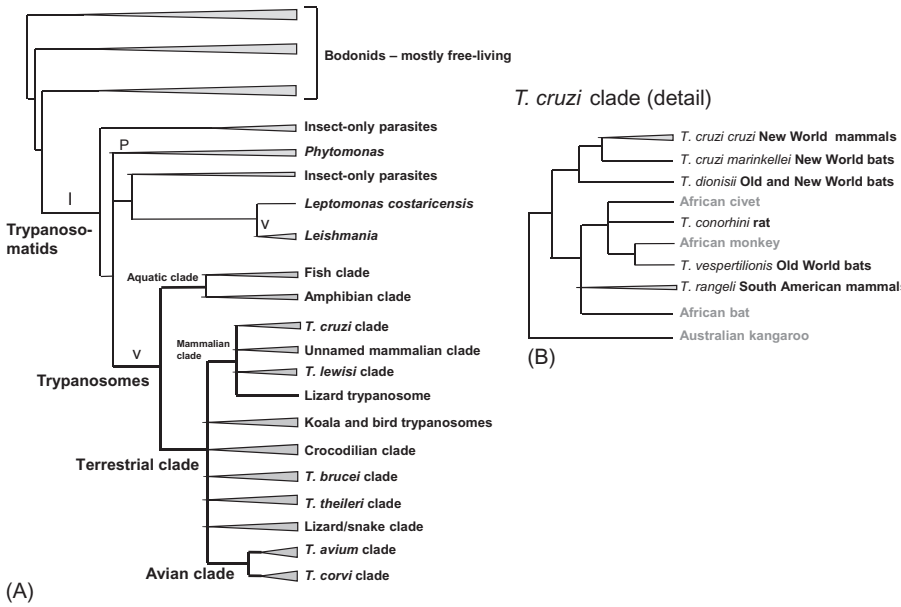

Figure 13.1 (A) Phylogenetic relationships of kinetoplastids, showing the relationships between the main trypanosomatid lineages. Groups connected by vertical lines are descended from a common ancestor. I = origin of single host (monogenetic) insect parasitism; V = origins of two host (digenetic) vertebrate parasitism; P = origin of digenetic plant parasitism. (B) Detail of relationships within the *T. cruzi* clade. Taxa in gray are hosts. *Sources*: Stevens et al. (1999b); Hamilton et al. (2004, 2007, 2009); Simpson et al. (2006); Yurchenko et al. (2006); Viola et al. (2009a,b).

with other tsetse fly-transmitted trypanosomes from Africa, indicating a long history largely confined to Africa (Stevens et al., 1999b), the evolutionary history of *T. cruzi* and other related trypanosomes in the *T. cruzi* clade is less certain and is discussed later (see Section 13.7).

There are now approximately 10 well-established clades within the genus *Trypanosoma* (see Figure 13.1A), all of which are found on more than one continent. Mapping the hosts of the trypanosomes onto the phylogenetic tree has revealed some associations between clades and certain types of vertebrate and invertebrate hosts (Hamilton et al., 2007). For example, two groups are restricted to birds, one to crocodilians, and there are several mammalian clades. Other clades appear to be largely restricted to a particular type of invertebrate, such as the *T. brucei* clade, in which most species are transmitted by tsetse flies. Overall, cospeciation with either vertebrates or invertebrates appears to have played little role in trypanosome evolution. Instead, adaptations to host types and opportunities appear to have played a more important role in determining the host range of trypanosome clades (Hamilton et al., 2007). Analyses based on combined datasets of 18S rDNA and *gGAPDH* gene sequences have provided increased resolution, combining some of these clades into

"superclades." These analyses have resolved the deepest split within the genus, which is between the aquatic clade and the terrestrial clade, comprising all the other clades. Importantly, both *T. cruzi* and *T. brucei* are in the terrestrial clade, and so are more closely related to each other than had been previously suggested; some earlier studies showed the *T. brucei* clade branching at the periphery of the main trypanosome group, indicating that they were some of the first to evolve (Haag et al., 1998). Another of these superclades, the mammalian clade, includes the *T. cruzi* clade and two other mammalian clades (see Figure 13.1A).

13.4 Molecular Phylogenetics and Traditional Taxonomy of Mammalian Trypanosomes

Traditionally, taxonomy of trypanosomes has been based on comparisons of morphology, life cycle, and disease data. The most commonly used taxonomy of Hoare (1972) divides mammalian trypanosomes into two sections. The section Salivaria comprises trypanosomes in which the infective forms are passed in the saliva of the insect. The section Stercoraria comprises species in which the developmental cycle in the insect vector is completed in the hindgut, and transmission is through contact with infective forms in the feces. In this taxonomy, *T. cruzi* was placed within the subgenus *Schizotrypanum* within the section Stercoraria. This taxonomy is given below; **type species** of each subgenus are in parentheses:

Section: Stercoraria	
Subgenera	*Herpetosoma* (*T. lewisi*)
	Megatrypanum (*T. theileri*)
	Schizotrypanum (*T. cruzi*)
Section: Salivaria	
Subgenera	*Duttonella* (*T. vivax*)
	Nannomonas (*T. congolense*)
	Pycnomonas (*T. suis*)
	Trypanozoon (*T. brucei*)

Molecular phylogenetic studies have largely supported the Salivaria and its subgenera. In contrast, the Stercoraria, and its subgenera have generally not received support. Indeed Stevens et al. (1999a) proposed that the use of the names *Herpetosoma* and *Megatrypanum* be discontinued because they are **polyphyletic** and so lack taxonomic and evolutionary relevance, whereas *Schizotrypanum* should be expanded to include all trypanosomes that fall in the *T. cruzi* clade (see Section 13.6). All trypanosomes originally placed within the subgenus *Schizotrypanum*, that have been analysed using molecular phylogenetics, fall within this suggested clade, and the clade now also includes species originally placed within *Herpetosoma* (*T. lewisi*, *T. rangeli*) and *Megatrypanum* (*T. conorhini*).

13.5 The Main Groups of Trypanosomes Recognized in Molecular Phylogenetic Analyses

A brief description of the main trypanosome clades follows (see Figure 13.1A).

- The aquatic clade: This clade comprises trypanosomes of mainly aquatic and amphibious vertebrates, including all fish trypanosomes and several species from amphibia. The vertebrate hosts also include reptiles (turtle, chameleon) and a mammal (platypus). Many of these species are transmitted by proboscid leeches (Bardsley and Harmsen, 1973; Desser et al., 1973; Khan, 1976; Martin and Desser, 1990; Jones and Woo, 1991; Siddall and Desser, 1992; Karlsbakk, 2004). There is evidence that some of the trypanosomes from amphibious vertebrates are transmitted by insects (Desser et al., 1973), and the chameleon trypanosome presumably has an insect vector.
- The *T. cruzi* clade: (see Section 13.6 of this chapter).
- An unnamed mammalian clade: This clade contains trypanosomes from Australian marsupials, the Eurasian badger and ticks from Japan (Noyes et al., 1999; Stevens et al., 1999b; Hamilton et al., 2005a; Thekisoe et al., 2007; McInnes et al., 2009).
- The *T. lewisi* clade: This clade contains trypanosomes from a wide range of rodents; it also contains trypanosomes from a lagomorph and insectivores (Hamilton et al., 2005b; Sato et al., 2005; Bray et al., 2007). The trypanosomes in this clade are thought to be specific to their vertebrate hosts. The vast majority of the vectors of these trypanosomes are fleas, although the life cycles of some species are not completely known (Hoare, 1972). The clade contains *T. lewisi*, type species of the subgenus *Herpetosoma*, thus the name *Herpetosoma* is occasionally used for this clade (Stevens and Brisse, 2005).
- An unnamed clade: This clade contains trypanosomes from an Australian marsupial (koala), an American kestrel (McInnes et al., 2009), and *T. minasense* from South American primates (red handed tamarind, *Saguinus midas*) imported into Japan (Sato et al., 2008). See Section 13.6 (*T. rangeli*) for further discussion on this species. The presence of both mammalian and avian trypanosomes within this clade may demonstrate host switching between different vertebrate classes.
- The crocodilian clade: This clade comprises crocodilian trypanosomes from Africa and South America and includes *T. grayi*, a tsetse fly transmitted trypanosome from Africa (Minter Goedbloed et al., 1993; Viola et al., 2009a).
- The *T. brucei* clade: This clade contains mostly African mammalian trypanosomes that are transmitted by tsetse flies. Two subspecies of *T. brucei* *T. b. gambiense* and *T. b. rhodesiense* are also human pathogens. Many of these species are pathogens of domestic livestock (*T. brucei*, *T. congolense*, *T. evansi*, *T. simiae*, and *T. vivax*). The majority of trypanosomes in the *T. brucei* clade are transmitted by tsetse flies (genus *Glossina*) via the saliva. The clade includes two trypanosomes from South America, which are believed to have been accidentally introduced into the continent in domestic animals relatively recently: *T. evansi* and *T. vivax* (Hoare, 1972; Cortez et al., 2006).
- The *T. theileri* clade: This clade contains trypanosomes from marsupial and placental mammals (deer, cattle, primates). It includes *T. theileri*, a trypanosome of artiodactyls that is commonly found in domestic cattle across the world (Hamilton et al., 2005a; Rodrigues et al., 2005). *T. theileri* is the type species for the subgenus *Megatrypanum*, so the name *Megatrypanum* is sometimes used for this clade (Rodrigues et al., 2005; Stevens and Brisse, 2005). *T. theileri* is known from South American cattle and buffalo, with distinct strains in each (Rodrigues et al., 2005). Tabanid flies act as the principal

vectors of *T. theileri*, although ticks also have capacity to transmit this trypanosome (Hoare, 1972; Burgdorfer et al., 1973; Bose et al., 1987a,b; Rodrigues et al., 2005). This clade also contains trypanosomes from terrestrial bloodsucking leeches (Haemadipsidae) that are closely related to a South East Asian primate trypanosome, *T. cyclops*. These leeches are common in tropical forests across Asia and Australia, and it has been sug gested that they are important vectors of mammalian trypanosomes in Asia and Australia (Hamilton et al., 2005a).

- The lizard/snake clade: This clade contains trypanosomes of lizards and snakes (Viola et al., 2009b). Sandflies are the only known vectors of trypanosomes in this clade (Ayala, 1970; Minter Goedbloed et al., 1993).
- The avian clade: This clade combines two subclades of avian trypanosomes, the *T. avium* and the *T. corvi* clades (Votypka et al., 2002, 2004). A diversity of insect vectors transmit the trypanosomes in this clade, including black flies (Desser et al., 1975; Votypka and Svobodova, 2004), hippoboscid flies, and mosquitoes (Baker, 1956).

13.6 The *T. cruzi* Clade (Subgenus *Schizotrypanum* Chagas, 1909)

This clade contains trypanosomes that are parasitic in a diverse range of mammals, including two human-infective parasites: *T. cruzi* and *T. rangeli*, which are both restricted to the New World. It also contains trypanosomes from Chiropteran (bat) hosts from both the Old and New Worlds (*T. cruzi marinkellei*, *T. dionisii*, *T. vespertilionis*). Other trypanosomes within the clade are *T. conorhini*, a rat trypanosome, two trypanosomes from African terrestrial mammals (Njiokou et al., 2004; Hamilton et al., 2009) and a trypanosome from an Australian kangaroo (Noyes et al., 1999; Stevens et al., 1999b). The only known invertebrate vectors of these trypanosomes are bugs (suborder Heteroptera; order Hemiptera), although the invertebrate hosts of several of these trypanosomes are not yet known. Three species are transmitted by triatomine bugs: *T. rangeli* by the genus *Rhodnius*, *T. cruzi cruzi* by a wide range of species and *T. conorhini* by *Triatoma rubrofasciata.* The bat trypanosomes are also thought to be transmitted by bat-feeding bugs. For instance, infections of *T. cruzi marinkellei* have been described in the bat-feeding triatomine bugs of genus *Cavernicola* (Marinkelle, 1982), while the cimicid bugs in the genus *Cimex* are frequently found infected with trypanosomes and have been implicated in the transmission of three species of bat trypanosome: *T. dionisii*, *T. incertum*, and *T. vespertilionis* (Paterson and Woo, 1984; Gardner and Molyneux, 1988a,b). The vectors of the kangaroo trypanosome, the trypanosomes of African terrestrial mammals, and other bat trypanosomes are as yet unknown.

The *T. cruzi* clade contains several trypanosomes originally placed within the subgenus *Schizotrypanum*. The close relationship between trypanosome species originally placed in the subgenus *Schizotrypanum* is not surprising, as the subgenus has well-defined morphology and development within the vertebrate host. The subgenus comprises small trypanosomes that are very difficult to distinguish

morphologically from the type species *T. cruzi.* They have a voluminous kinetoplast and typically curved bloodstream forms in the form of a C or S, with a short, pointed posterior end, which constitute distinctive morphological characters (Hoare, 1972). Within the vertebrate host, multiplication occurs within various tissues and organs, rather than in the blood (like many other trypanosome species). Nevertheless, the life cycles of several of the bat-infecting species are not completely known. Moreover, as these trypanosomes are difficult to distinguish morphologically, several of the morphologically described species may also be synonyms of *T. cruzi*. For instance, several *T. cruzi*-like trypanosomes from non-bat South American wild mammals have been described using traditional parasitological techniques: *T.* (*S.*) *lesourdi* Leger and Porry, 1918 from a spider monkey; *T.* (*S.*) *prowazeki* Berenberg-Gossler, 1908 from a Uakari (a species of New World monkey); and *T.* (*S.*) *sanmartini* Garnham and Gonzales-Mugaburur, 1962 from a squirrel monkey (Hoare, 1972). However, molecular studies have failed to demonstrate the existence of trypanosomes related to *T. cruzi* in South American terrestrial mammals, other than *T. rangeli* and *T. conorhini*. Therefore, these other morphologically described species may represent synonyms of *T. cruzi*. Likewise, a range of bat trypanosomes have been classified within the subgenus *Schizotrypanum* using parasitological techniques, including two from Australia, *T.* (*S.*) *pteropi* Breinl, 1921 and *T.* (*S.*) *hipposideri* Mackerras, 1959; and *T.* (*S.*) *hedricki*, *T.* (*S.*) *myoti,* and *T.* (*S.*) *dionisii* from elsewhere within the Old World. Morphological similarities between these bat trypanosomes have made it difficult to delineate species and to match molecular data with old parasitological descriptions. Therefore, the diversity of bat trypanosomes within this group may be considerably under- or overestimated.

Trypanosomes within the *T. cruzi* clade are listed (see Figure 13.1B).

- *T. cruzi cruzi*: (also called *T. cruzi sensu stricto*).
- *T. cruzi marinkellei*: This bat trypanosome is apparently restricted to South America and was sufficiently divergent to warrant subspecies status (Baker et al., 1978). Its close rela tionship to *T. cruzi cruzi* has been verified using sequences of 18S rDNA (Stevens et al., 1999b), *gGAPDH* genes (Hamilton et al., 2004, 2007), and kinetoplast *Cyt* b genes (Cavazzana Jr et al., 2010).
- *T. dionisii*: This bat trypanosome was first described from Europe, but recently distinct strains of this species have been found in South America (Maia da Silva et al., 2009; Cavazzana Jr et al., 2010).
- *Trypanosoma* species (civet) and *Trypanosoma* species (monkey): These trypanosomes were recently isolated from a study that examined trypanosome diversity in a wide range of wild vertebrates in Cameroon, West Africa (Njiokou et al., 2004). In that study, they remained unidentified, but later characterization by sequence analysis of their 18S rDNA and *gGAPDH* genes provided the first proof that the *T. cruzi* clade is present in non bat mammalian hosts in the Old World (Hamilton et al., 2009).
- *T. conorhini*: A trypanosome found worldwide in rats and transmitted by the triatomine bug *Triatoma rubrofasciata* (Hoare, 1972).
- *T. vespertilionis*: This is a widely distributed trypanosome of bats. The single isolate included in phylogenetic trees is from Europe (Stevens et al., 1999b; Hamilton et al., 2004, 2007).

- *T. rangeli*: This trypanosome is restricted to South America and has a wide mammalian host range including humans, although it is not pathogenic to human hosts. A high prevalence of *T. rangeli* in humans has been reported in Central America and northwestern South America (D'Alessandro and Saravia, 1992). The only known vectors are triatomine bugs of the genus *Rhodnius*. The inclusion of this species within the *T. cruzi* clade (Stevens et al., 1999a) resolved the considerable debate regarding the classification of this species; its ability to develop in saliva of triatomine bugs (although it is also transmitted via feces) had led to it being classified within the Salivaria, while resemblance of the bloodstream forms to *T. lewisi* led to classification within the subgenus *Herpetosoma*. Recent comparisons of a wide range of isolates of this species using sequences of the spliced leader gene, 18S rDNA, and ITS regions have revealed four lineages in terrestrial mammals (Lineages A, B, C, and D) and Lineage E, which is apparently restricted to bats (Maia da Silva et al., 2004, 2007). Lineage divergence appears to be associated with species of *Rhodnius*, without any clear association of trypanosome lineages with particular vertebrate hosts. Phylogenetic studies revealed some isolates previously classified as *T. minasense* and *T. leeuwenhoeki* to be *T. rangeli* (Stevens et al., 1999a). Similarly, comparison of strains of *T. saimirii* like trypanosomes (from squirrel monkeys) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) demonstrated that *T. saimirii* is a synonym of *T. rangeli* (Ziccardi et al., 2005). However, characterization of a range of isolates of *T. minasense* by SDS PAGE has indicated that it is a separate species to *T. rangeli*, as only 9 of 20 polypeptides were shared between the two species (Ziccardi et al., 2005) and 18S rDNA gene trees demonstrated that *T. minasense* isolates from South American monkeys imported to Japan were unrelated to *T. rangeli* (Sato et al., 2008).
- *An unnamed bat trypanosome*: This trypanosome was isolated from a fruit bat *Rousettus aegyptiacus* from Gabon in West Africa. Interestingly, sequence analysis based on 18S rDNA indicated that it was only distantly related to the other bat trypanosomes in the clade (Stevens et al., 1999a, 2001).
- *Trypanosome* species (kangaroo): This trypanosome was isolated in Australia (Noyes et al., 1999) and related trypanosomes have subsequently been found in a native Australian rodent, *Rattus fuscipes* (Averis et al., 2009). Analysis by Stevens et al. (1999b) provided strong support for placing this taxon within, but at the extremity of, the *T. cruzi* clade; as such, this taxon played a major part in hypothesizing the origin of the *T. cruzi* clade (see Section 13.7).

13.7 The Origin of the *T. cruzi* Clade

There are two main hypotheses regarding the origin of the *T. cruzi* clade and *T. cruzi cruzi* itself (Stevens et al., 1999b; Stevens and Rambaut, 2001; Hamilton et al., 2009). As yet, further information is required to resolve the debate.

One hypothesis is that the *T. cruzi* clade originated in the southern supercontinent comprising present day Antarctica, Australia, and South America when marsupials were the dominant mammalian fauna, more than 45 million years ago (Stevens et al., 1999b). This hypothesis is supported by the placement of a trypanosome from an Australian kangaroo on the periphery of the clade (Stevens et al., 1999b). It also receives support from the existence of two species that are found in terrestrial mammals in South and Central America: *T. cruzi cruzi* and *T. rangeli*. As both species are genetically diverse, they are likely to have had a long history

within the New World. According to this hypothesis, the ability of bats to disperse by flying is responsible for spreading bat-trypanosome lineages within the clade to the Old World (Stevens and Gibson, 1999; Stevens et al., 1999b). This hypothesis has provided some useful dates for calibrating the evolutionary tree of trypanosomes. The split between the *T. brucei* and *T. cruzi* clades has been dated using the separation of Africa and South America, which occurred approximately 100 million years ago (Stevens et al., 1999b; Stevens and Rambaut, 2001). The separation of Australia from Antarctica/South America, which occurred approximately 45−80 million years ago, has been used to date the split between the kangaroo trypanosome from the rest in the *T. cruzi* clade (Stevens et al., 2001).

Another hypothesis is that several of the lineages that infect terrestrial mammals within the *T. cruzi* clade evolved from bat trypanosomes, possibly including *T. cruzi cruzi* (Hamilton et al., 2009). This idea receives support from studies that have demonstrated that bat trypanosomes within the clade are diverse and polyphyletic and several are related to terrestrial lineages. In addition, the recent discovery of trypanosomes in the *T. cruzi* clade originating from African vertebrates, a monkey, and civet (Figure 13.1B), demonstrates that members of the *T. cruzi* clade are found in Old World terrestrial mammals (Hamilton et al., 2009). The monkey trypanosome characterized in this study was closely related to the bat trypanosome, *T. vespertilionis*. Thus, dispersal by bats and the jumping of trypanosomes between terrestrial and bat hosts could have led to the current wide distribution of this parasite clade. Although it is clear that further data are required to resolve the issue, these African trypanosomes do not represent a single lineage that was recently introduced from South America; the monkey trypanosome is most closely related to the bat trypanosome *T. vespertilionis*, while the civet trypanosome appears to be more closely related to *T. conorhini*. Thus, there could have been two relatively recent introductions, or it may indicate that this African group evolved independently in Africa for a considerable period of time (Hamilton et al., 2009). Evidence that trypanosomes within the *T. cruzi* clade frequently move between bats and terrestrial mammals has come from recent surveys of trypanosomes of bats in South America. These have revealed bat-specific strains of *T. cruzi cruzi* (Maia da Silva et al., 2009; Marcili et al., 2009; Cavazzana Jr et al., 2010) and *T. rangeli* (Maia da Silva et al., 2009). Furthermore, some *T. cruzi cruzi* lineages once thought to be restricted to terrestrial mammals have been found in bats (Lisboa et al., 2008; Cottontail et al., 2009; Maia da Silva et al., 2009; Cavazzana Jr et al., 2010), suggesting that transmission of trypanosomes between bats and terrestrial mammals is in fact relatively frequent. One implication of this hypothesis is that the clade could have evolved relatively recently, as bats could facilitate the spread of the trypanosomes in the clade between continents.

The idea that *T. cruzi* clade trypanosomes are widely distributed in terrestrial mammals in the Old World receives support from some early parasitological studies. Trypanosome species have been described in Indonesian primates that resemble *T. conorhini* (Weinman, 1977) as they developed in the *T. conorhini* vector, the triatomine bug, *Triatoma rubrofasciata* (Weinman et al., 1978). This led Hoare (1972) to argue that these Indonesian trypanosomes were primate-adapted strains of

T. conorhini. Indeed, although *T. conorhini* was thought to be restricted to the rat, *Rattus rattus*, it has been shown to be capable of transiently infecting mice (*Mus musculus*) and macaque monkeys (*Macaca* species) in laboratory experiments (Hoare, 1972). *T. rubrofasciata* infected with *T. conorhini* has been found in South America, Mauritius, India, Taiwan, and Malaysia (Hoare, 1972). On the other hand, Weinman et al. (1978) argued the Indonesian monkey trypanosomes are unlikely to be *T. conorhini*, as *T. rubrofasciata* is rat-specific and has been found in the cities, but never in the tropical rainforests of Asia. Likewise, *T. cruzi*-like trypanosomes have been described in the slow loris (a primate) in Malaysia (Kuntz et al., 1970). Thus, it is clear that molecular phylogenetic studies are required to reveal the true distribution of this clade.

13.8 Outlook

Molecular phylogenetic studies have provided a useful framework for understanding many aspects of trypanosome biology and evolution. In addition, this research can also be of direct relevance in applied studies, for example, research into diseases of humans and livestock. In particular, one goal of the trypanosomatid genome sequencing projects is to identify genes involved in pathogenicity, so comparisons with closely related, but nonpathogenic species can be informative; molecular−taxonomic studies have been instrumental in identifying such species. Knowledge of trypanosome diversity can also aid in the development of diagnostic tools (e.g., to distinguish pathogenic and nonpathogenic species); such diagnostics and the capacity they provide to distinguish species are essential for understanding the epidemiology of pathogenic species and to identify vectors and reservoirs of infection. Finally, an improved understanding of the origins of the trypanosomes that are capable of causing diseases of humans and domestic animals can provide insights into the likely sources of new and emerging pathogens. For instance, we now know that three primate-infective species are closely related to trypanosome species found in bats and thus bats may be regarded as (at least) potential sources of novel human parasites in the future. Thus, understanding the evolution and diversity of trypanosomes has the potential to deliver real benefits in applied science, epidemiology, and medicine.

Glossary

Clade a group of biological taxa or species that comprises a common ancestor and all its descendents (if the placement of all taxa within a clade is robust and no unrelated taxa are included within the clade, then the group can be referred to as monophyletic).

Digenetic a parasitic life cycle involving hosts of two different species. The two host species are essential for the completion of a particular parasite's life cycle.

Monogenetic a parasitic life cycle involving only a single species of host.

Monophyletic group a group of taxa that derive from a single common ancestor; specifically, the group includes all the descendants of a common ancestor and **no** unrelated taxa (see also C**lade**).

Paraphyletic refers to a group of taxa that derive from a single common ancestor; the group includes **all** the descendants of a common ancestor, plus additional apparently unrelated taxa.

Polyphyletic refers to a collection of taxa derived from more than one ancestor (i.e., taxa do not share a single common ancestor).

PCR (polymerase chain reaction) a method used in molecular biology to amplify a region of DNA, generating large quantities of a particular DNA region using oligonucleotide primers and a thermostable DNA polymerase.

Primer a short oligonucleotide from which DNA replication can initiate. Primers used for PCR are synthetically made and are designed to anneal to the template DNA.

Phylogenetic tree (phylogeny) a diagram (often referred to as a tree) illustrating relationships of evolutionary lineages among organisms (taxa).

rDNA (ribosomal DNA) DNA sequences encoding ribosomal RNA molecules that form subunits that together form the structure of a ribosome.

Taxon (*pl.* taxa) any grouping within the classification of organisms such as species, genus, family, order, etc.

Taxonomy the science and methodology of classifying and naming organisms based on similarities.

Type species the first recorded described specimen of a species; the specimen to which the binomial name of the species (genus name and species name) is permanently attached.

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14 Biology of *Trypanosoma cruzi* and Biological Diversity

Marta de Lana[1,*] *and Evandro Marques de Menezes Machado*[2]

[1]Departamento de Análises Clínicas, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil, [2]Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Minas Gerais, Brazil

14.1 Introduction

Trypanosoma cruzi, the etiological agent of Chagas disease, or American trypanosomiasis, is a flagellate of the order Kinetoplastida, family Trypanosomatidae, characterized by the presence of one **flagellum** and a single mitochondrion in which the **kinetoplast**, a specialized DNA-containing organelle, is situated. This protozoa was discovered by Carlos Ribeiro Justiniano das Chagas, a Brazilian physician, in 1909 (Chagas, 1909) when he was a member of the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. The genius of the still very young Carlos Chagas enabled him to describe the etiological agent, the vectors, and the principal reservoirs and mechanisms of infection, as well as several clinical stages of the disease with heart, gastrointestinal, and neurological manifestations.

Chagas disease is present only on the American continent, especially Latin America, with its vectors present from the south of USA to Argentina. The World Health Organization (WHO) currently estimates a prevalence of *T. cruzi* infection at around 13 million people in 15 countries, with an annual incidence of 200,000 cases (WHO, 2005). This disease is a great public health problem in Latin American countries due to high prevalence and geographical distribution in addition to the high morbidity and lethality observed in some clinical forms of the disease (Uchôa et al., 2002). Chagas disease ranks sixth in terms of social relevance (Hotez et al., 2006).

14.2 Evolutionary Stages

T. cruzi undergoes three distinct morphological and physiological evolutionary stages during its cycle, which are identified by the relative position of the kinetoplast in relation to the cell nucleus and the flagellum's emergence (Brener, 1973;

*E mail: delana@nupeb.ufop.br

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De Souza, 2002). All these evolutive forms can be identified in microscopy in Giemsa-stained preparations (Figures 14.1–14.3).

1. Amastigotes (Figure 14.1) are round intracellular stages in mammalian cells, displaying a short inconspicuous flagellum that is not free from the cell body on electron microscopy. These forms multiply by longitudinal binary fission. This stage can be reproduced in cel lular culture of different types of mammalian cells; they are approximately 25 μm in length and 2 μm in diameter and are infective for mammals.
2. Epimastigotes (Figure 14.2) are 20–40 μm long and are present in the intestinal tract and urine of the insect vector, where they multiply by longitudinal binary fission and present

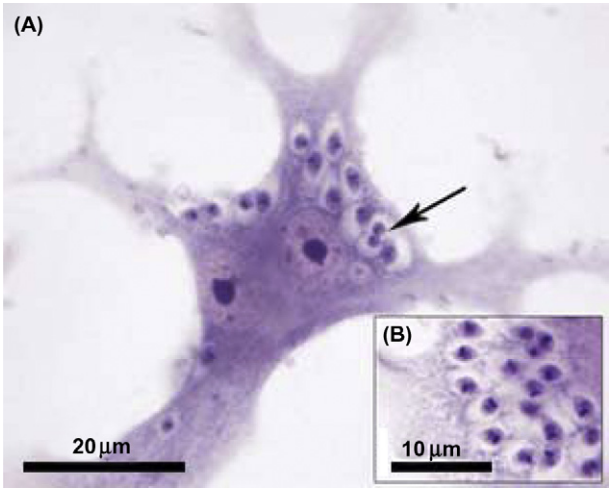


Figure 14.1 Amastigote form in cell culture stained by Giemsa (Carvalho, 2010).

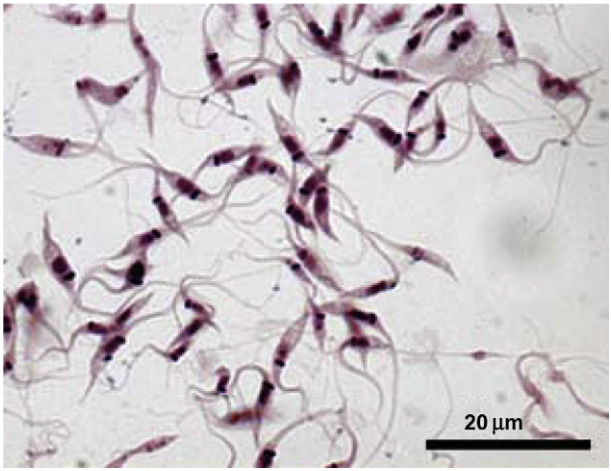


Figure 14.2 Epimastigotes forms from axenic culture (Carvalho, 2010).

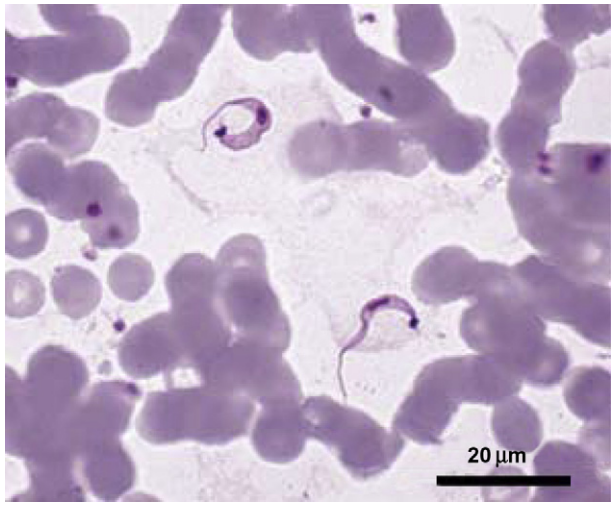


Figure 14.3 Trypomastigote forms from blood stained by Giemsa showing differential morphological aspects (Carvalho, 2010).

a free flagellum, which originates in the anterior position of the nucleus. This stage can be reproduced in liquid culture media and is not infective for mammals.

3. Trypomastigotes (Figure 14.3) present a large free flagellum originating after the nucleus. This stage is the most important, classically known as infective forms, present in the blood of mammalian hosts (called blood trypomastigotes) and able to infects triatomine vectors during blood sucking. This stage is also present in the feces and urine of the tria tomine vectors (called metacyclic trypomastigotes), where it is eliminated during blood feeding in the skin or mucus of the feeding source. They originate from the epimastigote forms by a process of metacyclogenesis, during the stationary phase of growth in axenic cultures or from amastigotes of cell culture. These forms cannot multiply.

14.3 Biological Cycle

The entire cycle of *T. cruzi* develops in two types of hosts, the mammalians of seven different orders including humans (vertebrate hosts) and several species of triatomine vectors (invertebrate host) from the order Hemiptera, family Reduviidae.

14.3.1 Biology in the Vertebrate Host

14.3.1.1 Cellular Adhesion

All *T. cruzi* stages are able to interact with vertebrate cells (Figure 14.4A). However, only some parasites remain adherent to the cells and the level of adhesion is strain-dependent. Several studies show that each infective form, but also the strain and parasite phylogeny, will determine the outcome of this interaction (Ley et al., 1990; Fernandes et al., 2007). The establishment of the infection depends on a series of events involving interactions of diverse parasite molecules with host cell

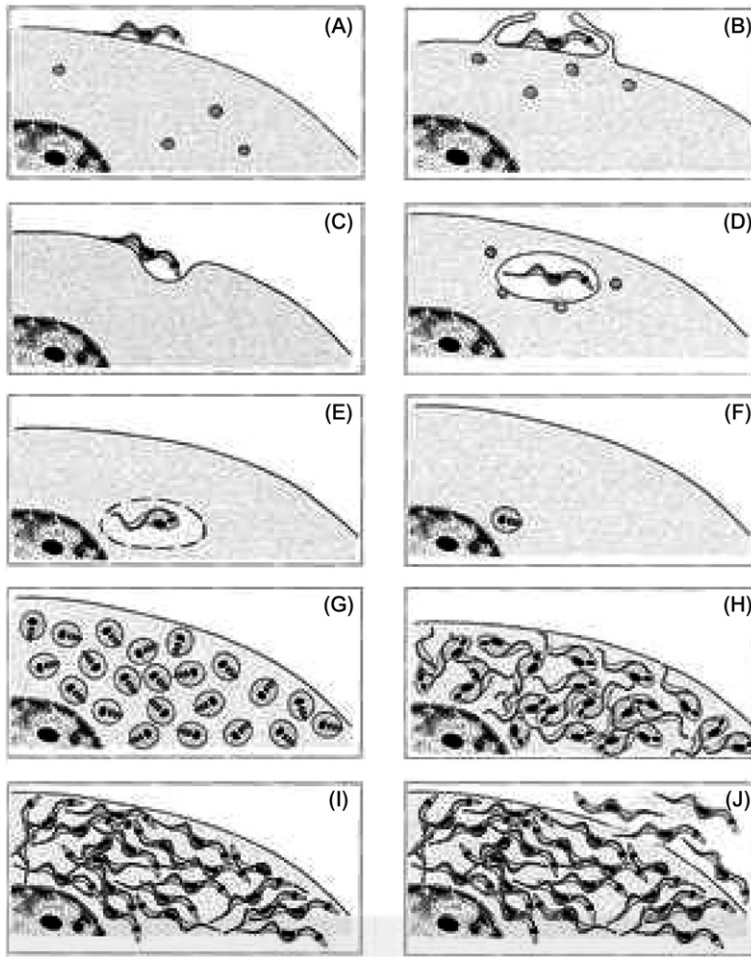


Figure 14.4 Schematic view of the various phases of the interaction of *T. cruzi* with vertebrate cells. Different phases of the parasite and vertebrate host cell interaction. (A) The parasite adheres to the host cell membrane; (B) internalization of the parasite via pseudopodes or depression (C) of the cell surface; (D) parasite inside the parasitophorous vacuole and fusion with the lysosomes; (E) change of the trypomastigote morphology and disintegration of the parasitophorous vacuole membrane; (F) amastigote free in the cytoplasm of the host cell; (G) multiplication of the amastigotes; (H) process of amastigote−trypomastigote differentiation passing by the intermediary stage of epimastigote; (I) trypomastigotes in the cytoplasm of host cell; (J) rupture of the host cell and releasing of trypomastigotes for the intercellular space (De Souza, 2002).

components. In this process, several glycoproteins, proteins with lectin activities present in both parasites and cells, are involved.

Yoshida (2006) showed that the penetration into the cell is particularly facilitated when the parasite presents surface glycoproteins with a higher gp82-kDa

concentration, as observed with trypomastigotes of the CL strain. It has been observed that parasites with a predominance of gp90 and low levels of gp82 (G strain) show a very low ability to invade mammalian cells, whereas those with gp45 and gp50 display a moderate ability to infect these cells. With the metacyclic trypomastigotes, a derivative of cellular culture, the internalization signal transduction pathways are activated both in parasite and host cells, leading to Ca^{2+} mobilization (Tardieux et al., 1992; Andrews, 2002). Some important differences are observed with these different sources of the parasite or even with different *T. cruzi* strains.

Another important factor that participates in this interaction is sialic acid, also present in both parasite and host cells. In *T. cruzi*—macrophage interaction, the presence of sialic acid in the membrane of the trypomastigote hinders the interaction process, which is facilitated when parasites are treated to remove or block this component (Meirelles et al., 1992). The trypomastigote normally presents transialidase and neuramidase in its membrane, which facilitates its interaction with vertebrate cells. Parasites that have a higher concentration of these enzymes are more invasive for the mammalian cells. Curiously, the epimastigote form, the noninfective stage, presents low levels of sialic acid. In contrast, it was demonstrated that the presence of sialic acid in the host cell membrane is also important for its interaction with *T. cruzi*.

14.3.1.2 Cellular Invasion and Formation of the Parasitophorous Vacuole

Trypomastigotes enter host cells by two mechanisms (Figure 14.4B and C), both involving an early interaction with host cell **lysosomes**. The first mechanism is mediated by a direct fusion of lysosomes with the **plasma membrane** at the parasite's attachment site, a process that produces the **parasitophorous vacuole (PV)** (Figure 14.4D) (Andrade and Andrews, 2004), which may comprise the host cell plasma membrane, either endosomal or lysosomal in origin. The second mechanism observed is the invagination of the plasma membrane, without participation of the host cell actin **cytoskeleton**. In this case, the PVs contain plasma membrane markers that rapidly mature by fusing with lysosomes (Figure 14.4D). This early fusion of the vacuole with the lysosome is critical for retaining the trypomastigotes inside host cells, further transformations, and replication.

The process of cellular invasion by *T. cruzi* is considered a particular type of endocytosis for the epimastigote or phagocytosis for the trypomastigotes. Thus, the invasion of macrophages by epimastigotes involves the polymerization of actin and formation of pseudopodes. This process is strongly blocked when the parasites or macrophages are treated with cytochalasin, which is not observed with nonphagocytic cells. In contrast, these cells undergo a process of phosphorylation of proteins with participation of phosphoinositide 3-kinase (Woolsey et al., 2003) from the parasite and host cell. Another important event during cellular invasion is the release of Ca^{2+} ions, from the parasite and host cells, which, together with lysosomes, migrate closer to the PV membrane (Andrews, 1994; Moreno et al., 1994).

14.3.1.3 Trypomastigote–Amastigote Differentiation

The parasite's time of residence inside the PV varies between infective forms, 1–2 h for amastigotes and trypomastigotes derived from tissue culture to several hours for metacyclic trypomastigotes (Alves and Mortara, 2009). Once inside the host cells, trypomastigotes and amastigotes secrete **Tc-Tox protein**, a complement 9 (C9) factor-related molecule that, at low pH, destroys the PV membrane, releasing parasites into the cytosol (Figure 14.4E) (Andrews, 1990). When the infective forms are metacyclic trypomastigotes, this process is not the same and still has not been completely studied. When the interaction is between macrophages and epimastigotes or opsonized trypomastigotes, the activation of NAD(P)H enzyme occurs, leading to the formation of free radicals of O^2 and H_2O_2 that lyse the parasite membrane with its consequent digestion (Carvalho and De Souza, 1989).

Once free in the cytoplasm, trypomastigotes differentiate into amastigotes that remain quiescent for 24–35 h (Figure 14.4F), then begin to grow by binary fission for up to nine cycles (Dvorak and Hyde, 1973). Some authors believe that a limiting factor for parasite multiplication is the cytoplasmic area of the host cell. During amastigote division, several transformations occur, such as growth of the parasite, duplication of the basal corpuscle, initial kinetoplast division, modification of the nuclear chromatin, and formation of a new flagellum. During the interphase, the nuclear chromatin is condensed in the nuclear membrane or sometimes in the central region of the nucleus with the nucleoli in a central position. With the beginning of the division process, the nucleus became less electrodense, with branches of intranuclear microtubules and electrodense plates. Afterward, the mitotic fuse is formed. Gradually the nucleus acquires an oval and later a long shape followed by a central constriction, producing a new cell with two nucleoli. At this time, the new cell has two kinetoplasts, the cell body is lengthened, the cytoplasm constricts, and two new independent amastigotes are formed. Then new successive divisions of amastigotes occur (Figure 14.4G) with a doubling time around 14 h depending on the type of parasite strain and culture conditions (Brener, 1973).

After successive amastigote divisions, these cells begin a differentiation process into trypomastigotes, the infective stage for vertebrate and invertebrate hosts, first going through an intermediary stage like epimastigotes (Figure 14.4H) (Low et al., 1992). The crucial stimuli that trigger the amastigote to the trypomastigote transformation process, which is not synchronous, are not clear. When the cell becomes filled with trypomastigotes, the plasma membrane ruptures and significant degenerative processes can be observed, probably due to the intense movement of the parasites (Alves and Mortara, 2009) that are released outside the host cell (Figure 14.4J).

Amastigote stages are also infective *in vitro* and *in vivo*. In laboratory conditions, it is possible to infect different lineages and cell types as well as laboratory animals with amastigote forms of *T. cruzi* (Carvalho and De Souza, 1986). Moreover, the transmission of Chagas disease with transplants is further clear evidence of this possibility of infection (Dobarro et al., 2008). The interaction of trypomastigotes with the host cell as well the amastigote stages on the cytoplasm are illustrated in Figure 14.5.

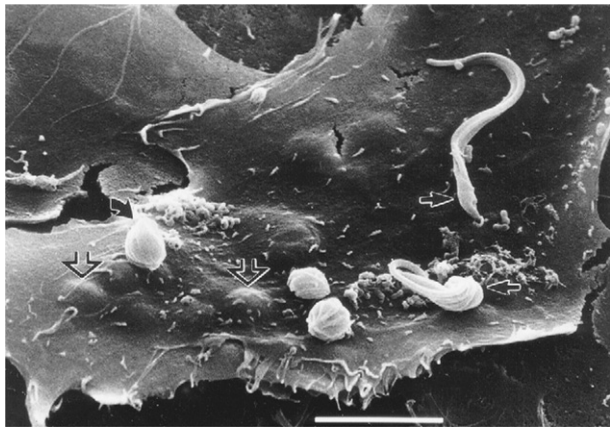


Figure 14.5 Scanning electron micrograph showing *Trypanosoma cruzi* trypomastigotes (black arrows) and extracellular amastigotes (curved black arrow) invading Vero cells. Opened downward arrows show surface protrusions compatible in size with internalized amastigotes. Bar = 5 μm (Procópio et al., 1998).

14.3.2 Biology in the Invertebrate Host

The Triatominae are defined as Reduviidae (Hemiptera, Heteroptera) that suck vertebrate blood (Lent and Wygodzinsky, 1979), several of which are vectors or potential vectors of *T. cruzi*, in contrast to the other Reduviidae subfamilies that prey on invertebrates (Schofield, 2000). There are more than 130 species classified in this Reduviidae subfamily (Galvão et al., 2003; Schofield and Galvão, 2009). Most of them are widespread in the Americas and maintain an enzootic cycle involving wild mammals in a variety of biotopes (Noireau et al., 2005). According to Noireau et al. (2005), several triatomine species have adapted to human dwellings, becoming vectors of Chagas disease. Combined with studies on orientation mechanisms and dispersal activity, they could greatly assist in understanding the process of triatomine domestication (Noireau et al., 2005). Similarly, studies on the vectorial potential of these species in the process of domestication are very important.

In the invertebrate host, the development of the parasite has been known since the original Carlos Chagas work (Chagas, 1909) and has been studied by many researchers (Dias, 1934; Brener and Alvarenga, 1976; Schaub, 1989; Schaub and Böker, 1987; Kollien and Schaub, 2000). The biological cycle in the vector initiates when the insect feeds on the mammalian host by sucking blood contaminated with trypomastigotes. A few days after feeding, the trypomastigotes form in the infected vertebrate host's blood, then transform into epimastigotes or **spheromastigotes** in the midgut of the insect vector. Then these epimastigotes divide by longitudinal binary division and can attach to the perimicrovillar membranes, which are secreted by midgut epithelial cells (Billingsley and Downe, 1986; Billingsley, 1990), to continue their establishment and development (Zeledon, 1997; Gonzalez et al., 1999; Azambuja et al., 2005). In the rectum, a proportion of epimastigotes differentiate into metacyclic trypomastigotes (metacyclogenesis), which eventually are eliminated together with feces and urine, and thus are able to complete the biological

cycle through contact of the infected Triatominae feces with the skin or mucosal tissues of the vertebrate host (Garcia et al., 2007).

Apparently, *T. cruzi* and the triatomine insect probably have not coevolved to facilitate parasite transmission (Schofield, 2000; Takano-Lee and Edman, 2002). Chagas disease vectorial transmission is dependent to a high degree on insect vector−parasite interaction (Garcia et al., 2007). Several reports on vector gut and the aggressive hemocoelic reactions that follow the establishment of *T. cruzi* infections in vectors have been described as preventing the development of parasites inside the invertebrate host, potentially making their transmission (Garcia et al., 2007) (Figure 14.6A and B).

The establishment of *T. cruzi* infection in the gut of the insect vector may depend on, and is possibly regulated by, a range of complex biochemical and physiological factors involved in the mechanism of the *T. cruzi*−vector interaction (Garcia, 1987). Phillips and Bertram (1967) suggested that the vector's genetic factors may be involved in the *T. cruzi* cycle in the invertebrate. They reported that the experimental infection rate of the progeny of a group of vectors that failed to become infected after ingesting *T. cruzi* was significantly lower than that obtained for the whole vector population.

After being ingested by the vector, the parasites encounter the components of the insect midgut and the products of blood digestion (hemolytic factor, proteolytic

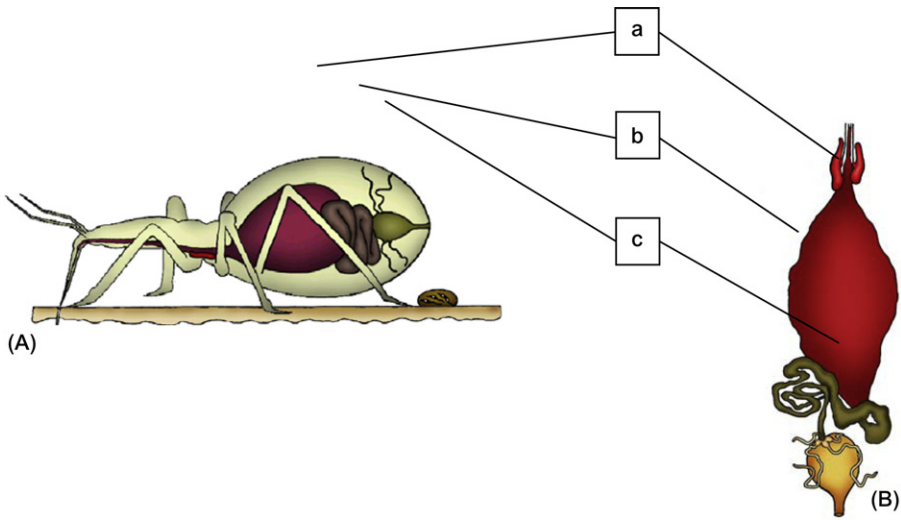


Figure 14.6 A and B: Scheme of the digestory tract of the triatominae. The insect feeds on blood infected with trypomastigote forms, which transform into epimastigotes and some spheromastigotes in the stomach (a). In the intestine, the epimastigotes multiply by longitudinal binary fission (b), increasing the population of parasites. In the rectum, the epimastigotes transform into metacyclic trypomastigotes (c), which are eliminated with the feces and urine (Azambuja and Garcia, 2010).

enzymes, peptides derived from αD-globin and lectins), bacterial symbionts, and other resident bacteria in the gut. Similarly, components of the vector's humoral immune system, such as defensin molecules, may also modulate the dynamics of establishment of *T. cruzi* transformation in the triatomine vector gut, illustrating the complexity of the mechanisms involved (Garcia and Azambuja, 1991; Garcia et al., 1995; Garcia et al., 1999; Kollien and Schaub, 2000; Lopez et al., 2003; Azambuja et al., 2004, 2005; Borges et al., 2006).

Kollien and Schaub (1997) showed that diuresis rather than factors from the hemolymph or digestive products induced the development of metacyclic trypomastigotes of *T. cruzi*. Kollien and Schaub (2000) also showed that there are several aspects of competition of *T. cruzi* with the vector's nutrients, showing that feeding affects not only the parasite population density, but also changes the percentages of different evolutionary stages observed in the rectum.

In contrast with dipteran vectors that have trypsin for the digestion of blood proteins, triatomines use cathepsins that require acid pH in the intestinal contents. Borges et al. (2006) showed that in insects experimentally infected with *T. cruzi*, the level of cathepsin D activity increased 1 and 3 days after the blood meal.

Kollien et al. (2003) described and characterized cDNA encoding for a lysozyme from the gut of *Triatoma infestans* that was expressed differentially in the various regions of the digestive tract. Kollien et al. (2004) characterized two cysteine proteases in the digestive tract of *T. infestans* and Araújo et al. (2006) reported the sequence and expression patterns of defensins (def1 and def2), antibacterial peptides, and lysozyme (lys1) encoding genes from the gut of *T. brasiliensis*. The importance of these enzymes in the *T. cruzi*−insect interaction is unknown.

A hemolytic factor has been shown to be present in the stomach of *Rhodnius prolixus* (Azambuja et al., 1983) and its effect on trypanosomatids (Azambuja et al., 1989). These authors also verified that different *T. cruzi* strains present distinct susceptibility to the hemolytic factor, which suggests a selective advantage for the development of certain *T. cruzi* strains over others in the insect vector.

Parasite attachment in the gut of the insect vector is an important point to be considered in the *T. cruzi*−vector interaction. One process of *T. cruzi*−insect interaction involves attachment of the parasite to the gut's epithelial surfaces. On the anterior midgut surface, epimastigotes bound through the cell body or flagellum and on the posterior midgut occur only as flagellar attachment to perimicrovillar membranes (Gonzalez et al., 1999). *T. cruzi* epimastigotes adhere to the luminal surface of the triatomid vector's digestive tract by molecular mechanisms that are not completely understood yet. Nogueira et al. (2007) demonstrated that *T. cruzi* epimastigote glycoinositolphospholipids (GIPLs, formerly collectively known as lipopeptidophosphoglycan, LPPG) are the major cell surface glycoconjugates of the epimastigote forms of *T. cruzi* (Alves and Colli, 1975; Zingales et al., 1982; Lederkremer et al., 1991; Colli and Alves, 1999) involved in parasite attachment to the midgut and, somehow, are able to modulate the development of the parasite infection in *R. prolixus*, suggesting that glycoproteins from phosphomannomutase (PMM) and hydrophobic proteins from epimastigotes are important for parasite adhesion to the vector's posterior midgut cells.

It is known that not only the kinetics of *T. cruzi* epimastigote division, but also the metacyclogenesis process are dependent on the strains and clones of the infecting parasites (Lana et al., 1998; Pinto et al., 1998, 2000; Azambuja et al., 2005). *In vivo* and *in vitro* metacyclogenesis experiments with the Y and Berenice *T. cruzi* strains using different Triatominae species resulted in a higher percentage of metacyclics for both strains in *R. neglectus* gut than in *Triatoma maculata* (Carvalho-Moreira et al., 2003). Considering the high genetic variability of *T. cruzi*, some authors point out that an interaction and cooperation effect among the different parasite subpopulations in the insect gut should be considered (Lana et al., 1998; Pinto et al., 1998, 2000; Lima et al., 1999).

14.4 Biological Diversity of *T. cruzi*

14.4.1 In the Vertebrate Host

T. cruzi displays great polymorphism or **biological diversity** in several aspects, which may be correlated with the morphology of this parasite's bloodstream. Since the early studies of Chagas disease discovery (Chagas, 1909), **blood polymorphism** has been interpreted by some authors as indicative of sexual dimorphism of the parasite (Brumpt, 1912). Today, although the clonal theory of *T. cruzi* structure and evolution is accepted (Tibayrenc and Ayala, 1988), the hypothesis of *T. cruzi*'s sexuality remains an open question for some authors (Tibayrenc et al., 1986).

Some evidence suggests that the morphological variations of *T. cruzi* are related to the parasites' physiological variations. Studying different *T. cruzi* populations, Brener and Chiari (1963) and Brener (1965) verified that in some strains the slender forms (Figure 14.7A) are present throughout the course of infection, whereas in others the slender parasites are present only in the first days of infection and are gradually substituted by the broad forms (Figure 14.7C) that become predominant in the later days of acute infection.

Therefore, when blood trypomastigotes are inoculated via the intravenous route in normal mice, the slender forms quickly disappear from the circulation to reproduce within the cell. Moreover, the broad forms remain in the circulation without interacting and infecting the host cells (Brener, 1969). Additionally, it has been demonstrated that naturally surviving animals previously infected with *T. cruzi* and intravenously inoculated with slender trypomastigotes are rapidly destroyed while the broad ones are resistant, remain in the circulation for a long time. Afterward, Howells and Chiari (1975) demonstrated that broad trypomastigotes present greater ability to infect the triatomine vectors than the slender parasites, which degenerate without carrying out metacyclogenesis. Interestingly, blood trypomastigotes are antigenically different from trypomastigotes obtained in cellular culture (Da Silva et al., 1988). Moreover, blood trypomastigotes have a different ability to infect vertebrate and invertebrate hosts (MacHard and Neal, 1979; Bahia et al., 2002).

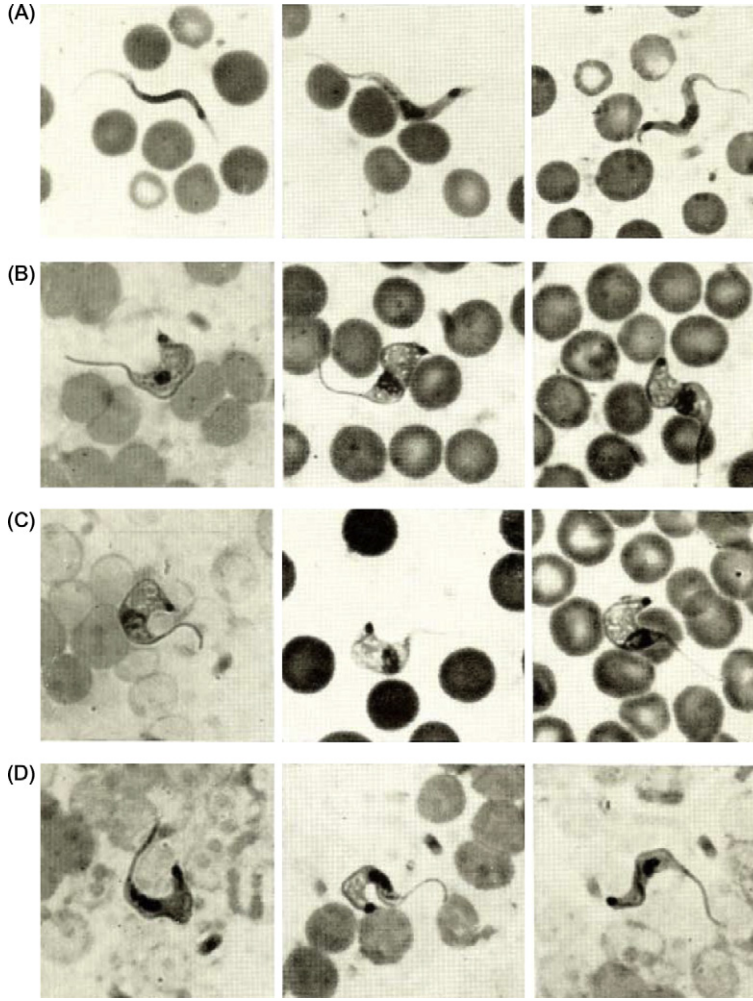


Figure 14.7 Morphological variation of blood trypomastigotes in *Trypanosoma cruzi*. (A) Slender; (B) intermediary; (C) broad; (D) stout.
Source: Photographs from Brener and Chiari (1963).

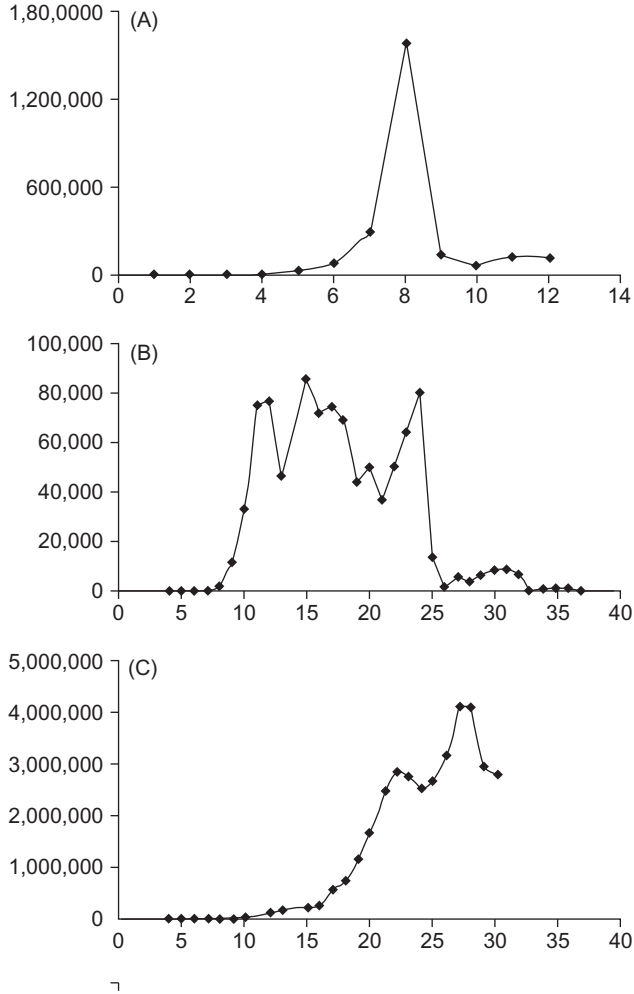
This dual behavior in strains with predominantly slender (Figure 14.7A) or intermediate (Figure 14.7B) versus large or stout trypomastigotes (Figure 14.7D) led Brener (1973) to propose the term "polar strains" for the Y and CL strains. Striking differences are observed in the parasitemia curves induced by the two strains in experimental hosts such as mice, dogs, and rabbits. The Y strain infects mice more efficiently, and higher parasitemia is observed early in the course of infection followed by early mortality, whereas in animals infected with the CL strain, infection and the increase of parasitemia and mortality are observed later. In addition,

the Y strain exhibits a tropism for cells from the mononuclear phagocytic system, as demonstrated by the peculiar parasitism of macrophages from the spleen, liver, and bone marrow; in contrast, the CL strain induces a negligible parasitism in these cells. As both strains infect muscle cells, the concept of macrophagotropic and non-macrophagotropic strains has been suggested to characterize these distinct tropisms. *In vitro* experiments with mouse peritoneal resident macrophages confirmed the *in vivo* findings. In addition, blood forms of the Y strain (but not the CL strain) collected from infected mice in the acute phase are readily lysed by complement via membrane-bound specific immunoglobulins. The resistance of the CL strain to the complement-mediated lyses strongly suggests that broad blood forms are equipped with evasion mechanisms that are lacking in the Y population.

The observation that different tissue tropism (cardiotropism or myotropism) of *T. cruzi* is verified in individuals infected with parasites with distinct genetic characteristics (Vago et al., 2000) led Macedo et al. (2004) to propose the clonal histiotropic model theory for this parasite since the correlation between *T. cruzi*'s genetic and tissue tropism could be associated with the different clinical manifestations of the disease (cardiopathy, megaesophagus, and megacolon), alone or in combination.

These variations in *T. cruzi* morphology are correlated with several other aspects of its infection in the murine model such as parasitemia curves, tissue tropism, mortality, and chemotherapy resistance or susceptibility. These associations led Andrade (1974) to propose the subdivision of *T. cruzi* populations into types I, II, and III. Type I includes the Y and Peruvian strains, which present a predominance of slender trypomastigotes, rapid multiplication in mice, a higher and early parasitemia peak (Figure 14.8A), higher and early mortality, and, particularly, tropism for macrophagic cells during the acute phase of infection and are susceptible or partially resistant to benznidazole and nifurtimox.

Type II includes the strains typical of Recôncavo baiano, Bahia state, Brazil, which presents a predominance of broad trypomastigotes but with slender forms at the beginning of infection, slow multiplication in mice (Figure 14.8B), irregular peaks of parasitemia between 12 and 20 days, null mortality in the acute phase, myocardial tropism, and is partially resistant or resistant to treatment. Type III includes, for example, the Colombian strain, which presents a predominance of broad trypomastigotes throughout infection, slow multiplication in mice, higher peaks of parasitemia between 20 and 30 days of infection (Figure 14.8C), lower rates of mortality occurring slowly throughout acute infection, tropism for the skeletal musculature, and resistance to treatment. Types I, II, and III correspond to biodemes I, II, and III, respectively (Andrade and Magalhães, 1996), since these associations were observed with the isoenzymatic profile of the parasites. Several other authors observed similar associations between the parasite genetics and its biological properties when comparatively studied clonal stocks in acellular culture (Laurent et al., 1997), cellular culture (Revollo et al., 1998), and mice (Toledo et al., 2002) of the three principal *T. cruzi* genotypes described by Tibayrenc and Brenière (1988) such as principal genotypes 19 and 20, or *T. cruzi* I, principal genotype 32, or *T. cruzi* II, and principal genotype 39, or *T. cruzi* hybrid genotype,



currently called *T. cruzi* I, *T. cruzi* II, and *T. cruzi* V groups, respectively, according to the recent genetic *T. cruzi* classification (Zingales et al., 2009). They are equivalent to biodemes I, II, and III, respectively, of Andrade and Magalhães (1996). Several authors demonstrated higher values for the growth and metacyclogenesis variables in acellular culture (Laurent et al., 1997), infectivity and differentiation in trypomastigotes in cellular culture (Revollo et al., 1998), virulence and parasitemia in mice (Toledo et al., 2002) for the *T. cruzi* I group in relation to *T. cruzi* II group, whereas *T. cruzi* V presented intermediate values for these same variables. With the same group of clonal stocks, Revollo et al. (1998) and Toledo

et al. (2003) also demonstrated that *T. cruzi* I was more resistant to benznidazole and nifurtimox (*in vitro*) and benznidazole and itraconazole (in mice), whereas *T. cruzi* II was the most susceptible, and the hybrid stocks (*T. cruzi* V) presented intermediate results. Moreover, all stocks belonging to the principal genotype 20 were 100% resistant to treatment with all the compounds assayed *in vitro* and *in vivo*. These results may explain the regional differences observed in human chemotherapy, which differs from region to region and explains why it is easier to cure patients from Argentina and Chile, where *T. cruzi* II is predominant, than in the north of the Americas where *T. cruzi* I is more widespread (Zingales et al., 1998; Briones et al., 1999).

The genetic polymorphism in *T. cruzi* is also related to trypomastigote small surface antigen (TSSA) sequences, a highly antigenic surface glycosylphosphatidyl inositol (GPI)-anchored mucin-like protein. Thus, Di Noia et al. (2002) described the first immunological marker in *T. cruzi* that discriminated between the two *T. cruzi* lineage groups (*T. cruzi* I and *T. cruzi* II).

14.4.2 In Invertebrate Vector

The genetic structure of *T. cruzi* is predominantly clonal, with restricted recombination. Various strains persist as stable genotypes that can spread through large geographic regions (Tibayrenc et al., 1986; Tibayrenc and Ayala, 1988). The clonal model does not totally exclude recombination, but is compatible with occasional genetic recombination (Tibayrenc et al., 1990; Machado and Ayala, 2001; Brisse et al., 2003) on the evolutionary scale to have an important impact on the adaptation of *T. cruzi* to new environments, including new vectors.

The role of triatomines in the selective transmission of *T. cruzi* has been studied by some authors, but several factors involved in the mechanisms and processes of parasite−vector interaction remain unknown. The number of parasites that develop into epimastigotes in the invertebrate host is apparently proportional to the number of broad forms in the ingested blood (Pereira da Silva, 1959; Brener, 1971). Pereira da Silva (1959) suggested that only broad forms of the parasite would be able to survive in the vector, while the slender forms would degenerate. On the other hand, there is no evidence that only broad forms evolve. The fact is that the same dual behavior observed between Y and CL strains in mice is also verified in vectors (Brener, 1971).

In experimental infections, it is known that not all *T. cruzi* strains developed in the same way in all species of triatomines (Perlowagora-Szumlewicz and Müller, 1982; Kollien et al., 1998; Cortez et al., 2002; Carvalho-Moreira et al., 2003). Few studies have been undertaken using genetically characterized *T. cruzi* clones in triatomine infections. Lana et al. (1998) and Pinto et al. (2000) confirmed that *T. infestans* does not always present the same efficiency in the transmission of different *T. cruzi* genotypes. In a recent investigation, the sylvatic species *Mepraia* (*Triatoma*) *spinolai* transmitted a greater number of subpopulations (TcI, TcIIb, TcIId, and TcIIe) than *T. infestans* (Campos et al., 2007).

14.5 Maintaining of *T. cruzi* in the Laboratory

T. cruzi can be cultivated and maintained in the laboratory in several conditions: acellular/axenic cultures, cellular cultures, successive passages in different laboratory animal models (especially mice), alternative passages through vertebrate (mice or other experimental models) and invertebrate hosts (triatomine vectors), and cryopreservation.

14.5.1 Acellular Culture

Acellular culture reproduces the biological cycle of *T. cruzi* developed in triatomine vectors. The first cultures of *T. cruzi* were in acellular biphasic or monophasic medium. The monophasic medium offers advantages because the parasite can be obtained with less contaminant and parasite growth can be assessed by cell counting using an electronic device. The most important and most widely used were liver infusion-tryptose (LIT) and Warren mediums. Camargo (1964) was the first to describe the cellular transformations of *T. cruzi* and growth in LIT medium, the most useful monophasic medium used in the laboratory. Several important studies on metacyclogenesis in LIT media have been described. Camargo (1964) was the pioneer followed by others (Chiari, 1974) who demonstrated that in the medium called M16, nutrient-poor with low pH, a higher percentage of metacyclogenesis is obtained, although the type of *T. cruzi* strain considered is also important.

The semidefined and defined media for trypanosomes were discovered by Yoshida (1975) and Roitman et al. (1972). Chemically defined TAUP (Contreras et al., 1985) or TAU3GAA (Goldenberg et al., 1987) media using components similar to vector urine were used for better differentiation *in vitro*. The existence of culture media free of macromolecules, especially the chemically defined medium, provides the large numbers and quantities of parasite cells necessary for antigen preparations, biochemical studies on nutritional requirements, metabolic pathways, and molecular characterization. Using the semidefined medium 4 as overlay and a monophasic medium using the blood of different animal species, high rates of *T. cruzi* and *Leishmania donovani* growth were obtained by Perlewitz and Koch (1985), which improved the chance of obtaining isolations of these parasites.

Bonaldo et al. (1988) verified that nutritionally poor medium promotes the metacyclogenesis of *T. cruzi*. The results of Duschak et al. (2006) indicate the presence of a novel cysteine proteinase secreted by metacyclic trypomastigotes and reinforces the important role played by these enzymes in *T. cruzi* metacyclogenesis. Interestingly, biological changes in the parasite's infectivity and metacyclogenesis have been observed after successive passages in acellular culture, which can be restored after maintenance by successive passages in triatomines (Contreras et al., 1994). De Lima et al. (2008) verified that cultivation of *T. cruzi* epimastigotes in low-glucose axenic media shifts its competence to differentiate at metacyclic trypomastigotes. Differential gene expression for different periods (6 and 24 h) of *T. cruzi* metacyclogenesis was observed by Krieger et al. (1999) and a proteomic

analysis (Parodi-Talice et al., 2007) identified relevant proteins involved in the metacyclogenesis process: their identification and molecular characterization is highly important to understanding the steps of parasite differentiation into the infective form.

14.5.2 Cellular Culture

In cellular media, the entire biological cycle of *T. cruzi* in the vertebrate host is reproduced. The first culture experiments were with tissue fragments absorbed with coagulated plasma or pendant drop (Kofoid et al., 1935; Meyer and Oliveira, 1948). Further monolayer culture of cell lineages regularly maintained in the laboratory was used. Cells of various tissues and organs such as heart, kidney, lung, skin, and skeletal musculature can be used. Metacyclic trypomastigotes originating from culture and vector, as well as bloodstream forms of the parasite, are able to infect cells in culture, although it has been demonstrated that vector trypomastigotes are more infective than the metacyclic forms of culture (Dvorak and Schmunis, 1972; Bertelli et al., 1977). Inside these cells, the parasite transforms into amastigotes, which after some time or several cell generations differentiate into new trypomastigotes that will be released in the extracellular medium and be able to invade new cells. With the cultivation of the cells in liquid medium containing mammal sera, several lineages of cells were adapted for *T. cruzi* culture. Today several different cell lineages are used for *T. cruzi* cultivation.

14.5.3 Cryopreservation

T. cruzi as well as other microorganisms can be preserved for long periods of time in liquid nitrogen (196°C) without changing its original characteristics. Filardi and Brener (1975) were the first to systematically study *T. cruzi* cryopreservation, verifying its effect on several biological characteristics of the parasite (infectivity to vertebrate and invertebrate hosts, morphology, parasitemia, and mortality in mice). These authors verified that initially blood trypomastigotes and epimastigotes obtained from artificial cultures need to be mixed with an equal volume of glycerin 10% and maintained at 73°C for 16−20 h before cryopreservation in nitrogen. Later the same authors (Filardi and Brener, 1976) verified that exemplars of triatomine vectors infected with *T. cruzi* can also be cryopreserved with preservation of parasite characteristics, including its infectivity to vectors. Dimethyl-sulfoxide (DMSO) 5−10% is also used as a cryoprotective agent for trypanosomes. However, Raether et al. (1988) demonstrated later that cryopreservation and thawing of *T. cruzi* may lead to severe damage of the mitochondrial apparatus and thus to severe disorders of metabolic function, exhaustion of the metabolic pool, and finally to death of the thus-damaged trypanosomes, despite the use of DMSO as a cryoprotective agent. Yaeger (1988) demonstrated that tissue homogenates containing *T. cruzi* amastigotes or *Leishmania* sp. were also rapidly frozen with 10% glycerol as cryoprotectant and the viability and pathogenicity of the parasites maintained for several years.

Cryopreservation made it possible to create banks of *T. cruzi* strains with parasites from around the world, which was very important to several studies on various aspects of this parasite and provided a better idea of its polymorphism. Moreover, cryopreservation avoids biological changes in its original characteristics induced by selection throughout long-term maintenance in the laboratory in different conditions such as successive passages in mice, the risk of accidental infections in the laboratory, and the comparative study of a large number of parasite populations of distinct origins.

14.5.4 Successive and Alternative Passages in Animals and Vectors

These alternatives are very frequently used in the laboratory to maintain the parasite for experimental studies. Mice models were the most widely used models for this purpose because they are very susceptible to infection and easy to reproduce and maintain in the laboratory. Parasite populations are maintained in this animal model by successive reproduction of the acute phase of the infection. Moreover, *T. cruzi* can also be maintained by successive passages in triatomines and alternative passages through triatomine vectors and laboratory animals. This type of maintenance has been considered to be very important to prevent the biological behavior of the parasite observed in the laboratory after long-term successive passages in animal models or culture.

Glossary

Blood polymorphism of *T. cruzi* the different morphological aspects observed in the blood trypomastigote stages of different *T. cruzi* stocks or strains or even during the acute phase of infection of the same *T. cruzi* strain. The morphological aspects can vary from slender, intermediate, broad, and stout forms.

Biological diversity of *T. cruzi* the polymorphism that *T. cruzi* presents in several parameters used for its biological characterization such as antigenicity, infectivity for vertebrate and invertebrate hosts, metacyclogenesis, virulence and pathogenicity for vertebrate hosts, susceptibility or resistance to drugs, and others.

Cytoskeleton a heavily microtubule based skeleton, containing many interesting protein structures building and sustaining the parasite shape. For details see De Souza (2002, 2009).

Flagellum an organelle usually attached to the cell body that emerges from an invagination called the flagellar pocket present in all trypanosomatids responsible for cell movement. It emerges at the anterior tip or somewhere along the side of the cell depending on the evolutive form or developmental stage. Its length also varies with their developmental stage. It has a basic structure similar to other flagella, showing a $9 + 2$ pattern of axonemal microtubules. For details see De Souza (2002, 2009).

Kinetoplast an important structure in the recognition of the order Kinetoplastida that includes the family Trypanosomatidae, comprising a mass of circular DNA inside the trypanosome's mitochondrion attached to the basal bodies of the flagellum. Division of the kinetoplast is a reliable marker of a certain point in the cell cycle, making it important in

cell cycle studies. Electron microscopy shows that the kinetoplast consists of a network of minicircle molecules associated with each other and with long linear molecules concatenated to the maxicircles. Restriction enzyme analysis has shown that the minicir cles are heterogeneous and their study has been used for the genetic characterization of the parasite. It is believed that the maxicircles contain genetic information. For details see De Souza (2002, 2009).

Lysosome an organelle present in the cytoplasm of the parasite rich in digestive substances and Ca^{++} ions that approximates from the phagocytic vacuoles during parasite penetra tion in the vertebrate cells.

Parasitophorous vacuole resultant of the transformations that the host mammalian mem brane cell and the *T. cruzi* membrane surface suffer after interaction, which results in the parasite offering temporary conditions of its survival before further transformation to amastigote stages. Inside this vacuole, the transformation of trypomastigote into amasti gotes occurs after passage by an intermediate epimastigote stage. For details see De Souza (2002, 2009).

Plasma membrane an organelle strongly associated with microtubules and microfilaments that cover the trypanosome surface with hundreds of important receptors. A large number of proteins are associated with the plasma membrane. Recent studies have characterized some details of the biosynthesis of the GPI anchor, which involves several important bio chemical steps for this parasite. The surface receptors are involved in the uptake of all the necessary host resources for the parasite. For details see De Souza (2002, 2009).

Spheromastigote the first *T. cruzi* evolutionary stage present prior to the intestine (midgut) of the invertebrate host (triatomine vectors) that initiates the epimastigote stage of the parasite in the vector described by Brack (1968).

Tc-Tox protein (Tc85 kDa) a complement 9 (C9) factor related molecule or hemolysin that, at low pH, destroys the parasitophorous vacuole membrane, releasing the parasite in the cytosol of the host cell (Andrews, 1990) where the biological cycle of *T. cruzi* continues.

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15 Biochemistry of *Trypanosoma cruzi*

Roberto Docampo[1,*] *and Silvia N.J. Moreno*[2]

[1]Department of Cellular Biology and Center for Tropical and Emerging Global Diseases, University of Georgia, Georgia, USA, [2]Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, Georgia, USA

15.1 Introduction

Completion of the *Trypanosoma cruzi* genome project (El-Sayed et al., 2005) and proteomic studies of the different stages of the parasite (Atwood et al., 2005; Ferella et al., 2008b; Cordero et al., 2009; Nakayasu et al., 2009a,b; Sant'Anna et al., 2009) have provided a wealth of information about their biochemistry and metabolic pathways. This is especially important because most metabolic studies were done before using the culture or epimastigote form of the parasite, and not the more clinically relevant mammalian stages. Until these studies are completed, the picture of *T. cruzi* biochemistry will be only fragmentary. The analysis and validation of these pathways will considerably increase our knowledge of the biology of *T. cruzi*. In addition, the rational development of new drugs against *T. cruzi* depends on the identification of differences between human metabolism and that of the parasite.

Developments in the study of the basic biochemistry of the parasite have allowed the identification of peculiar metabolic pathways in *T. cruzi* that provide or could provide novel targets for chemotherapy. Redox metabolism is involved in the mechanism of action of the drugs currently used against Chagas disease. The study of the isoprenoid pathway has resulted in drugs that are close to undergoing clinical trials against the disease, and other potent agents that are active *in vitro* and *in vivo* against *T. cruzi*, such as bisphosphonates and prenyltransferase inhibitors. Acidocalcisome metabolism has a number of characteristics that make these organelles potential targets for trypanocidal drugs. These metabolic pathways will be the subject of this chapter.

*E mail: rdocampo@uga.edu

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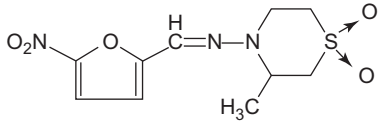
15.2 The Need for Chemotherapy of Chagas Disease

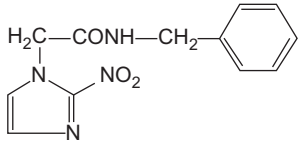
Chemotherapy against Chagas disease (Brener, 1979; Docampo and Moreno, 1984; Marr and Docampo, 1986; Docampo, 1990; Docampo and Schmunis, 1997; Urbina and Docampo, 2003; Urbina, 2009) depends on the use of two drugs, nifurtimox and benznidazole (Figure 15.1).

These drugs can cure at least 50% of recent infections as shown by the disappearance of symptoms and negativization of parasitemia and serology (Cerisola, 1969; Cerisola et al., 1969; Brener, 1979; Schmunis et al., 1980). However, results of treatment trials for acute infections have not been uniform in different countries (Brener, 1979; Schmunis et al., 1980), probably as a consequence of the different drug sensitivity of different *T. cruzi* strains. Both drugs have side effects that are more common in adults than in children, although they disappear when treatment is discontinued (Cerisola, 1969; Brener, 1979; Schmunis et al., 1980). Another drawback of these drugs is the need for an extensive treatment period. Nifurtimox is given for 30−120 days (Brener, 1979), whereas benznidazole is given for at least 30 days (Brener, 1979).

The use of these drugs in the indeterminate or chronic stage of the infection has been less frequent; after treatment, serology in most cases remains positive even when parasitemia is absent (Cerisola, 1969; Cançado, 1979; Schmunis et al., 1980). It has been shown that antiparasite treatment of chronic chagasic patients with benznidazole results in fewer electrocardiographic changes and a lower frequency of deterioration in their clinical condition (Viotti et al., 1994). Lack of progress in the myocardiopathy correlated well with negativization of serology (Viotti et al., 1994, 2006). Moreover, even when asymptomatic, some children aged 12 years or younger could be parasitologically cured when treated with benznidazole (de Andrade et al., 1996). Side effects were mild, and fewer children in the benznidazole-treated group showed myocardiopathy (de Andrade et al., 1996; Viotti et al., 2006). These findings match well with the fact that benznidazole treatment of *T. cruzi*-infected mice induces a late regression of lesions in the myocardium and skeletal muscle (Andrade et al., 1991), and that parasitization of heart tissue is both necessary and sufficient for the induction of tissue damage in *T. cruzi* infection (Tarleton et al., 1997;



(A) **Nifurtimox**



(B) **Benznidazole**

Figure 15.1 Structures of nifurtimox (A) and benznidazole (B), drugs currently used against Chagas disease.

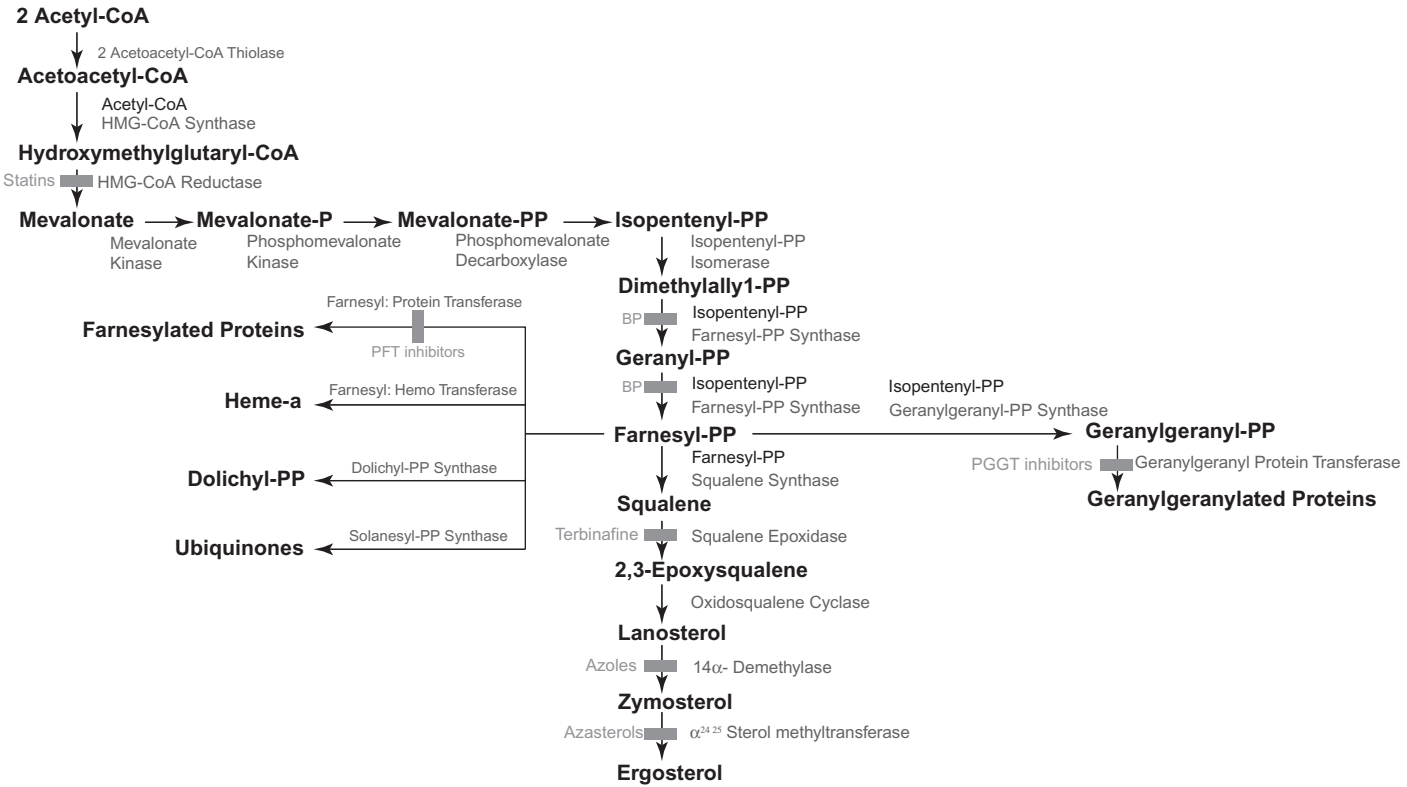


Figure 15.2 Isoprenoid pathway in *T. cruzi.* Enzyme names are in gray (blue in web version), products in black, and inhibitors in light gray (red in web version).

Tarleton and Zhang, 1999). These findings stress the need for chemotherapeutic agents that are effective against all strains of *T. cruzi*, and with fewer or no side effects than those currently available (Docampo and Moreno, 1985).

15.3 Metabolic Pathways in *T. cruzi* That Could Provide Targets for Drugs Against Chagas Disease

15.3.1 Isoprenoid Pathway

One pathway that has been particularly useful for the identification of new targets against *T. cruzi* is the isoprenoid pathway (Figure 15.2). Several enzymes of this pathway, involved in the synthesis of farnesyl diphosphate (FPP) (Docampo and Moreno, 2001) and sterols (Urbina, 2009), and in protein prenylation (Gelb et al., 2003) have been reported to be excellent drug targets against these parasites.

Isoprenoids are the most diverse and abundant compounds occurring in nature. Isoprenoids, such as steroids, cholesterol, retinoids, carotenoids, ubiquinones, and prenyl proteins are essential components of the cells of all organisms due to their roles in different biological processes. Despite their structural and functional variety, all isoprenoids derive from a common precursor: isopentenyl diphosphate (IPP), and its isomer, dimethylallyl diphosphate (DMAPP). In *T. cruzi*, IPP is synthesized exclusively via the so-called mevalonate pathway, which has the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase as the key regulatory enzyme (Banthorpe et al., 1972; Eberl et al., 2003) (Figure 15.2). Mevalonate is then converted to IPP with two continuous phosphorylation steps and one decarboxylation step. Isomerization of IPP by IPP isomerase yields DMAPP.

15.3.1.1 Polyprenyl Diphosphate Synthases

Once IPP is formed, polyprenyl diphosphate synthases are responsible for chain elongation and catalyze the sequential condensation of IPP with allylic prenyl diphosphates (Eberl et al., 2003). So far, only the genes encoding farnesyl diphosphate synthase (FPPS) (Montalvetti et al., 2001), and solanesyl diphosphate synthase (SPPS) (Ferella et al., 2006) have been cloned from *T. cruzi*. Both of these genes are single copy. Whereas the FPP synthase is localized in the cytosol (Ferella et al., 2008a), the SPP synthase is localized in the glycosomes (Ferella et al., 2006). Glycosomes are specialized peroxisomes that, like them, contain several enzymes in pathways of ether lipid synthesis, fatty acid β-oxidation, and peroxide metabolism, and, in addition, contain the Embden−Meyerhof segment of glycolysis (Parsons, 2004).

T. cruzi farnesyl diphosphate synthase (TcFPPS) catalyzes the consecutive condensation of IPP with DMAPP and with geranyl diphosphate (GPP) to form the 15-carbon isoprenoid compound, FPP (Figure 15.2). FPP is the substrate for enzymes catalyzing the first committed step for biosynthesis of sterols (which, in *T. cruzi*, is mainly ergosterol) (Docampo et al., 1981b), ubiquinones (mainly

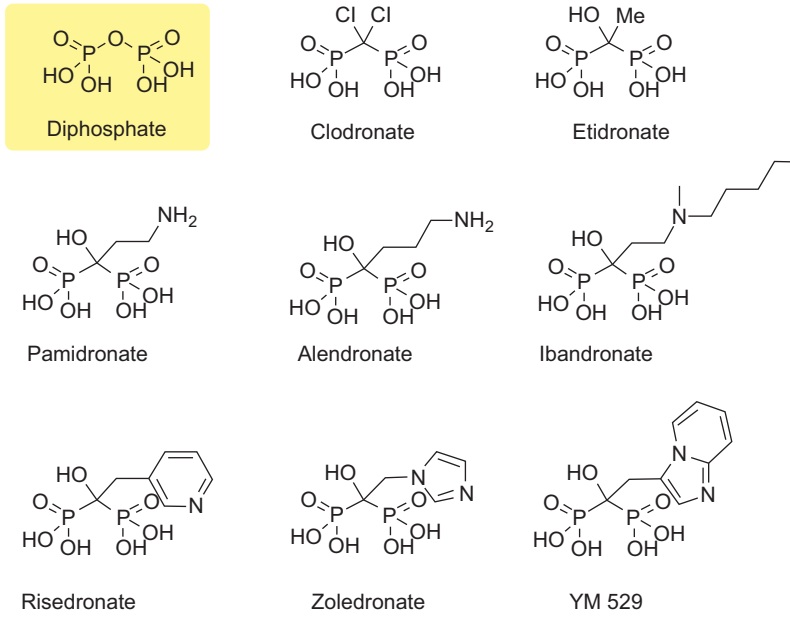


Figure 15.3 Structure of pyrophosphate (diphosphate) and selected bisphosphonates. First (clodronate and etidronate), second (pamidronate, alendronate, ibandronate), and third (risedronate, zolendronate, and YM 529) generation bisphosphonates are shown.

ubiquinone-9) (Ferella et al., 2006)), dolichols (which are required for the synthesis of complex carbohydrates and are present in *T. cruzi*) (Parodi and Quesada-Allue, 1982), heme-a (a component of the cytochrome oxidase, also present in *T. cruzi*) (Docampo et al., 1978b), and prenylated proteins (also present in *T. cruzi*) (Yokoyama et al., 1998; Nepomuceno-Silva et al., 2001; Cuevas et al., 2005). FPP could be condensed with an additional molecule of IPP by the geranylgeranyl diphosphate synthase (GGPPS) to form the 20-carbon isoprenoid GGPP (Figure 15.2), although this enzyme has not been studied in *T. cruzi*. The *FPPS* gene appears to be essential in all organisms (Blanchard and Karst, 1993, Song and Poulter, 1994). As most FPPSs that have been characterized, *T. cruzi* FPPS is an homodimeric enzyme, and requires divalent metal ions, such as Mg^{2+} or Mn^{2+} for activity (Montalvetti et al., 2001). The three-dimensional structure of TcFPPS has been solved in complex with different substrates and inhibitors (Gabelli et al., 2006; Huang et al., 2010).

TcFPPS is the main target of bisphosphonates in *T. cruzi* (Docampo and Moreno, 2001; Montalvetti et al., 2001). Bisphosphonates are pyrophosphate analogues in which a carbon atom replaces the oxygen atom bridge between the two phosphorus atoms of the pyrophosphate. The substitution of carbon with different side chains has generated a large family of compounds (Figure 15.3).

Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget's disease, hypercalcemia,

tumor bone metastases, and other bone diseases (Rodan, 1998). Selective action on bone is based on the binding of the bisphosphonate to the bone mineral (Rodan, 1998). It has been postulated that the acidocalcisomes, organelles rich in phosphorus and calcium in trypanosomes, are equivalent in composition to the bone mineral and that accumulation of bisphosphonates in these organelles, as they do in bone mineral, facilitates their antiparasitic action (Martin et al., 2001). Nitrogen-containing bisphosphonates were first found to be effective in the inhibition of *T. cruzi in vitro* and *in vivo* without toxicity to the host cell (Urbina et al., 1999; Garzoni et al., 2004a). *In vivo* testing of bisphosphonates against *T. cruzi* in mice has shown that risedronate can significantly increase the survival of mice infected by *T. cruzi* (Garzoni et al., 2004b; Bouzahzah et al., 2005). All these results indicate that bisphosphonates are promising candidate drugs to treat infections by *T. cruzi*.

T. cruzi solanesyl diphosphate synthase (TcSPPS) catalyzes the formation of the 45-carbon compound solanesyl diphosphate (SPP), which is an intermediate in the synthesis of ubiquinone-9. Ubiquinone is synthesized *de novo* in both prokaryotes and eukaryotes. The two parts of the molecule, the benzoquinone ring and the isoprene chain, are synthesized independently and assembled in a reaction catalyzed by a prenyl-4-hydroxybenzoate-transferase (Turunen et al., 2004). 4-Hydroxybenzoate originates from tyrosine or phenylalanine in eukaryotes (Ohnuma et al., 1991). In *T. cruzi*, it was shown that epimastigotes synthesize and keep mainly UQ-9 in their membranes (Ferella et al., 2006). Human tissues have mostly UQ-10 whereas rat tissues have UQ-9. Valuable functions have been adjudicated to this molecule, including acting as a component of the mitochondrial electron transfer system, as the only lipid-soluble antioxidant that is endogenously synthesized in both unicellular and multicellular organisms, and as an acceptor of electrons from sulfide (Kawamukai, 2002). Two genes with homology to the yeast (Ashby et al., 1992) and human (Forsgren et al., 2004) prenyl-4-hydroxybenzoate-transferases are present in the genome of *T. cruzi* differing in only two amino acids and possessing a mitochondrial targeting signal (Tc00.1047053510903.60 and Tc00.1047053505965.30). The protein has a putative polyprenyl diphosphate-binding domain similar to those found in other enzymes known to bind isoprenoid substrates. Bisphosphonates can also inhibit the activity of TcSPPS and amastigote growth in culture cells (Szajnman et al., 2008).

15.3.1.2 Protein Prenylation

The occurrence of protein prenylation in *T. cruzi* has been demonstrated (Yokoyama et al., 1998). Protein prenylation in mammals and yeast involves the attachment of 15-carbon farnesyl or 20-carbon geranylgeranyl groups to a conserved cysteine residue in a CaaX motif of a subset of cellular proteins (Figure 15.2). Many of these prenylated proteins are small GTPases, including Ras, Rac, Rab, and Rho, that have roles in cellular signal transduction and intracellular vesicle trafficking (Glomset et al., 1990; Glomset and Farnsworth, 1994). The known functions of prenyl groups attached to cellular proteins is to anchor proteins to membranes and to serve as molecular handles for mediating protein−protein

interactions (Yokoyama et al., 1992). Three enzymes have been identified in eukaryotic cells including those from mammals and plants and in yeast that attach prenyl groups to proteins: protein farnesyl transferase (PFT); protein geranylgeranyl transferase I (PGGT-I); and protein geranylgeranyl transferase II (PGGT-II) (Yokoyama et al., 1992; Casey and Seabra, 1996). Different studies have detected the presence of prenylated proteins and a farnesyl transferase activity in *T. cruzi* (Yokoyama et al., 1998; Nepomuceno-Silva et al., 2001; Cuevas et al., 2005). Over the past several years, hundreds of potent PFT inhibitors have been synthesized with the primary goal of developing anticancer drugs (Leonard, 1997). Some of these compounds have been shown to inhibit the growth of *T. cruzi* (Yokoyama et al., 1998) and are potential chemotherapeutic agents.

15.3.1.3 Ergosterol Synthesis

Squalene synthase (SQS) catalyzes the first step committed to the biosynthesis of sterols within the isoprenoid pathway, and several quinuclidine inhibitors of the enzyme were shown to have selective anti-*T. cruzi* activity both *in vitro* and *in vivo* (Sealey-Cardona et al., 2007). The enzyme is membrane-bound, and was expressed in truncated form in *Escherichia coli* and biochemically characterized (Sealey-Cardona et al., 2007). The following step in the synthesis of ergosterol is catalyzed by the squalene epoxidase, which is the target of terbinafine, a drug that is active *in vitro* and *in vivo* against *T. cruzi* (Urbina, 2009). Lanosterol is then synthesized by a reaction catalyzed by the lanosterol synthase or oxidosqualene cyclase (Figure 15.2). Several inhibitors of the enzyme showed activity *in vitro* against *T. cruzi* (Buckner et al., 2001). Interestingly, the antiarrhytmic bis-aryl-ketone amiodarone, which is used in chronic Chagas' patients with heart problems, also inhibits this enzyme and has activity *in vitro* and *in vivo* against *T. cruzi* (Benaim et al., 2006). Lanosterol is converted into zymosterol by a series of reactions including the sterol 14α-demethylase, a target of azole (imidazole and triazole) derivatives (Urbina, 2009). This enzyme is a member of the cytochrome P450 superfamily (CYP51), and catalyzes the oxidative removal of the 14α-methyl group from post-squalene sterol precursors. The gene encoding this enzyme was cloned and expressed, and its substrate preferences were studied (Lepesheva et al., 2006b). The *T. cruzi* enzyme prefers 24-methylenedihydrolanosterol as substrate and has functional similarities to the animal/fungal orthologues (Lepesheva et al., 2006a).

Azole compounds were first detected to have activity against *T. cruzi* in 1981 (Docampo et al., 1981b). Miconazole and econazole showed a potent growth inhibitory action parallel to a decrease in its 5,7-diene sterol content (Docampo et al., 1981b). Later studies showed that ketoconazole and other potent antimycotic azoles were also active in protecting mice against lethal infections with *T. cruzi* (McCabe et al., 1984; Raether and Seidenath, 1984), in inhibiting intracellular multiplication of the parasites (Beach et al., 1986; McCabe et al., 1986; Goad et al., 1989), and in blocking their biosynthesis of fungal-type sterols (Beach et al., 1986; Goad et al., 1989). More recent work on a number of inhibitors of this enzyme has been

reviewed (Urbina, 2009). Although the enzyme is present in mammalian cells, it is much less sensitive to the drugs than that present in fungi and trypanosomatids.

Ergosterol differs from cholesterol, the predominant mammalian sterol, by the presence of a 24-methyl group and Δ^7 and Δ^{22} double bonds. The enzymatic reactions that introduce the extra methyl group and the Δ^{22} double bond of ergosterol have no counterpart in mammalian sterol biosynthesis, and may be regarded as targets for new antiparasitic agents. In agreement with this hypothesis it has been shown that azasterols, which are $\Delta^{24(25)}$ sterol methyl transferase inhibitors, have a potent antiproliferative effect on *T. cruzi in vitro* and *in vivo* (Urbina et al., 1996).

15.3.2 Redox Metabolism

15.3.2.1 Deficient Metabolic Utilization of H_2O_2 in T. cruzi

More than three decades ago *T. cruzi* was reported to be deficient in enzyme systems necessary for the removal of hydrogen peroxide (H_2O_2) (Docampo et al., 1978a,c; Boveris et al., 1980; Docampo and Moreno, 1984, 1985, 1986; Docampo, 1990). Despite extensive studies on the antioxidant defenses of this and other trypanosomatids over subsequent years, this characterization appears to still be valid. *T. cruzi* lacks genes for catalase, selenocysteine-dependent glutathione peroxidases, glutathione reductase, and thioredoxin reductase (Irigoin et al., 2008; Krauth-Siegel and Comini, 2008). The two cysteine-dependent glutathione peroxidases that have been described are not able to hydrolyze H_2O_2 (Irigoin et al., 2008; Krauth-Siegel and Comini, 2008). One enzyme able to catalyze this reaction is the ascorbate peroxidase, first described in 1976 in *T. cruzi* (Docampo et al., 1976). The activity of this enzyme in homogenates was 6−15 nmol H_2O_2/min × mg protein (Boveris and Stoppani, 1978). The trypanothione-dependent peroxidase activity with H_2O_2 as substrate in extracts of epimastigotes (which include the activities of not only the tryparedoxin peroxidases but also of any other NADPH-dependent peroxidases (i.e., peroxidases) dependent on the reduction of $T(SH)_2$) was only 1.86 ± 0.54 nmol NADPH oxidized/min × mg protein (Wilkinson et al., 2000a).

It has been pointed out that these trypanothione-dependent peroxidase activities are quite low in comparison with approximately 150 nmol/min × mg protein found in lung mitochondria (Turrens, 1987). Assuming that 10^8 epimastigotes are approximately equivalent to 1 mg protein (Turrens, 1987) it seems that ascorbate peroxidases and trypanothione peroxidase are approximately 10 and 80 times less active, respectively, than the equivalent activities in mammalian tissues on a mg protein basis. In other words, trypanosomatids may be protected for dealing with a slow endogenous rate of H_2O_2 generation, but they are probably quite sensitive to an increased steady-state concentration of H_2O_2 (Turrens, 1987).

The reason for this deficiency is probably that there is little need for decomposing H_2O_2 in the conditions under which the parasite, a facultative aerobe, develops, either in the intestine of the insect vector in the case of epimastigotes, or in the cytosol of the host cell in the case of the intracellular amastigotes. In this regard, transformation of epimastigotes into metacyclic trypomastigotes is accompanied by

an increase in expression of antioxidant enzymes, such as ascorbate peroxidase, tryparedoxin peroxidase, tryparedoxin, trypanothione synthase, and iron superoxide dismutase (Atwood et al., 2005), a phenomenon that was proposed to indicate a preadaptation of metacyclic forms to withstand the potential respiratory burst of phagocytic cells in the mammalian host (Atwood et al., 2005). This deficiency in the metabolism of H_2O_2 also explains in part the susceptibility of *T. cruzi* to H_2O_2-generating drugs, such as napthoquinones (Boveris et al., 1977, 1978a,b; Cruz et al., 1978a,b; Docampo et al., 1977, 1978a,c; Lopes et al., 1978; Goncalves et al., 1980), and nifurtimox (Docampo and Stoppani, 1979, 1980; Docampo et al., 1981a), one of the drugs currently used against Chagas disease.

15.3.2.2 The Trypanothione System

In contrast to its mammalian hosts, which maintain their intracellular thiol homeostasis using glutathione/glutathione reductases, as well as thioredoxin/thioredoxin reductases, *T. cruzi* redox metabolism depends on the trypanothione/trypanothione reductase couple (Irigoin et al., 2008). Trypanothione (N^1, N^8-bis(glutathionyl)spermidine) is synthesized from glutathione (GSH) and spermidine (Figure 15.4).

The biosynthesis of GSH has not been studied in detail in *T. cruzi*, although it has been reported that 1-buthionine (S,R) sulfoximine (BSO), an inhibitor of gamma glutamylcysteine synthetase the first enzyme in the synthesis of GSH decreases GSH levels and increases the toxicity of nifurtimox and benznidazole in epimastigotes (Faundez et al., 2005). *T. cruzi* is auxotrophic for polyamines and unable to carry out *de novo* biosynthesis of putrescine (Algranati, 2009). Spermidine can be taken up by a transporter (Le Quesne and Fairlamb, 1996; Carrillo et al., 2006) or derived from putrescine through the reaction catalyzed by spermidine synthase. However, this enzyme has not been characterized in *T. cruzi*. An ATP-dependent enzyme then attaches GSH and spermidine covalently into a glutathionylspermidine conjugate, or monoglutathionylspermidine (GSH-SPD). Subsequently, a second molecule of GSH is added to yield dihydrotrypanothione ($T(SH)_2$). Both steps are catalyzed in *T. cruzi* by a trypanothione synthetase with

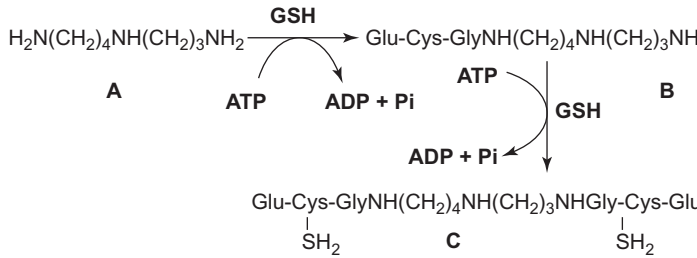


Figure 15.4 Trypanothione synthesis occurs by condensation of spermidine (A) with GSH to give glutathionyl spermidine (B). Addition of a second GSH leads to the formation of dihydrotrypanothione (C; $T(SH)_2$). Both reactions consume ATP and are catalyzed by trypanothione synthase.

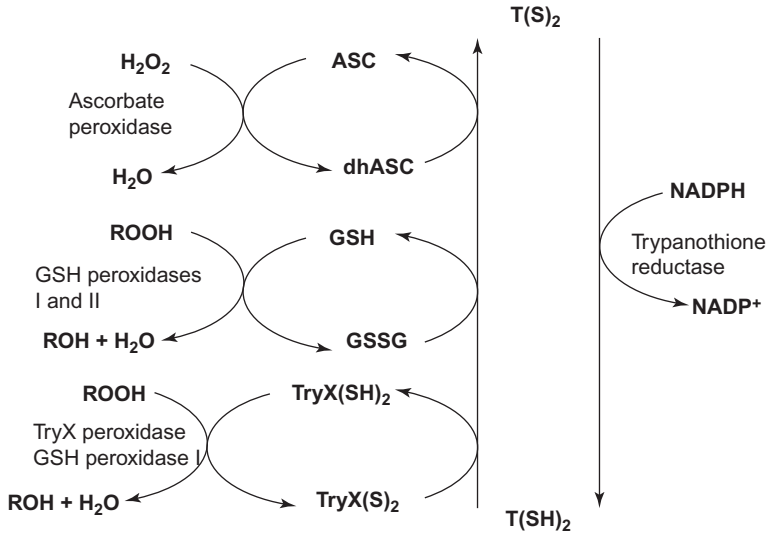


Figure 15.5 Redox metabolism of *T. cruzi*. Reduction of trypanothione ($T(S)_2$) to dihydrotrypanothione ($T(SH)_2$) is catalyzed by trypanothione reductase with conversion of NADPH into $NADP^+$. Oxidation of dihydrotrypanothione to trypanothione is coupled to the reduction of tryparedoxin ($TryX(S)_2$), GSSG, and dehydroascorbate (dhASC), and these compounds are regenerated by the action of peroxidases catalyzing the decomposition of H_2O_2 (ascorbate peroxidase) or hydroperoxides (ROOH; GSH peroxidases I and II, and tryparedoxin peroxidase).

the consumption of two ATPs (Oza et al., 2002b) (Figure 15.4). Oxidation of dihydrotrypanothione leads to the formation of trypanothione disulfide (TS_2), and trypanothione reductase (Jockers-Scherubl et al., 1989) catalyzes its reduction back to $T(SH)_2$ (Figure 15.5).

Apart from its synthetase activity, trypanothione synthetase has an opposite hydrolytic activity ($T(SH)_2$ amidase) located in its N-terminal region. These two activities were proposed to be relevant for the regulation of polyamine levels in response to their availability and growth phase (Oza et al., 2002a). Although *T. cruzi* has a gene homologous to the glutathionylspermidine synthase present in *Crihidia fasciculata* (Oza et al., 2002a), the function of this enzyme in the parasite has not been investigated. The trypanothione synthetase is a potential target for drugs, and high-throughput screenings of compounds against this enzyme are underway (Torrie et al., 2009).

The intracellular concentration of dihydrotrypanothione has been reported to be 0.12 ± 0.06 and 6.4 nmol/10^8 cells in epimastigotes grown in polyamine-deficient medium and in the presence of 100 μM extracellular putrescine, respectively, and 0.95 ± 0.34 nmol/10^8 cells in tissue culture-derived trypomastigotes (Ariyanayagam and Fairlamb, 2001). In another report, the concentration of $T(SH)_2$ was indicated as 0.18 ± 0.04, 0.25 ± 0.10, and 0.12 ± 0.04 nmol/10^8 cells in epimastigotes, trypomastigotes, and amastigotes, respectively (Ariyanayagam et al., 2003).

Interestingly, the intracellular concentration of GSH in epimastigotes (2.10 ± 0.53 nmol/10^8 cells) and of cysteine (0.40 ± 0.27 and 0.61 ± 0.12 nmol/10^8 cells for trypomastigotes and amastigotes, respectively), and GSH (0.29 ± 0.13 and 0.42 ± 0.04 nmol/10^8 cells in trypomastigotes and amastigotes, respectively) in the mammalian stages, are higher than those of $T(SH)_2$.

Taking into account the cell volumes of different stages of *T. cruzi* calculated by the inulin method (34.8 ± 3.3, 11.1 ± 1.9, and 14.6 ± 1.1 μl/10^9 cells for epimastigotes, trypomastigotes, and amastigotes, respectively (Rohloff et al., 2003)), the intracellular concentration of $T(SH)_2$ in epimastigotes (unsupplemented with exogenous polyamines), trypomastigotes and amastigotes would be: 35−52, 225−855, and 82 μM, respectively, and could reach 1.88 mM in epimastigotes supplemented with 100 μM putrescine. These values are important when considering the noncatalyzed reactions of $T(SH)_2$ with other molecules and reactions for which the Km for $T(SH)_2$ is in the micromolar level.

Trypanothione reductase was purified to homogeneity from *T. cruzi* (Krauth-Siegel et al., 1987) and the gene cloned and expressed to characterize the enzymatic properties of the recombinant enzyme (Borges et al., 1995), which were similar to those of the native enzyme (Krauth-Siegel et al., 1987). This enzyme catalyzes the NADPH-dependent reduction of trypanothione disulfide but not GSH. The enzyme is highly specific for trypanothione and has striking homology to glutathione reductase. Its crystal structure was solved alone or in complex with its substrate or inhibitors (Krauth-Siegel et al., 1993; Lantwin et al., 1994; Jacoby et al., 1996; Zhang et al., 1996; Bond et al., 1999; Saravanamuthu et al., 2004). A number of compounds, such as nitrofurans and naphthoquinones (Jockers-Scherubl et al., 1989; Aguirre et al., 2004), phenothiazines and related tricyclics (Benson et al., 1992; Chan et al., 1998; Khan et al., 2000; Gutierrez-Correa et al., 2001), crystal violet (Moreno et al., 1994), diphenylsulfide derivatives (Baillet et al., 1996), polyamine derivatives (O'Sullivan et al., 1996, 1997; Bonnet et al., 1997; Li et al., 2001), dibenzazepines (Garforth et al., 1997), bisbensylisoquinoline alkaloids (Fournet et al., 1998), ajoene (Gallwitz et al., 1999), acridines (Bonse et al., 1999), terpyridine platinum complexes (Bonse et al., 2000), Mannich bases (Lee et al., 2005), as well as some natural products (Cota et al., 2008) have been shown to inhibit *T. cruzi* trypanothione reductase and affect parasite growth *in vitro* or *in vivo*. However, most inhibitors have K_is in the micromolar range, and none has been curative against *T. cruzi* infection in mice. Because recombinant trypanothione reductase from *T. cruzi* was the first to be obtained, it has been used regularly in high-throughput screening campaigns to identify inhibitors (Holloway et al., 2007, 2009). The enzyme is predominantly cytosolic, although it cannot be ruled out that it could also be present in the mitochondria and glycosomes (Irigoin et al., 2008).

Oxidation of dihydrotrypanothione leads to the reduction of intermediates (oxidized glutathione (GSSG), dehydroascorbate, or the dithiol protein tryparedoxin), which are then used as a source of electrons for peroxidases: ascorbate peroxidase, cysteine-dependent glutathione peroxidases, and tryparedoxin peroxidases (Figure 15.5). Ascorbate peroxidase is reduced by ascorbate (which is regenerated by dihydrotrypanothione) and decomposes H_2O_2 but not organic hydroperoxides

(Docampo et al., 1976; Wilkinson et al., 2002b). The enzyme localizes in the endoplasmic reticulum (Wilkinson et al., 2002b), although early work found it in the glycosomes (Docampo et al., 1976), which is the site where ascorbate synthesis occurs (Logan et al., 2007). There are two cysteine-dependent glutathione peroxidases, which are characterized by the presence of cysteine instead of selenocysteine in their active site, in contrast to the mammalian homologues. Glutathione peroxidase I can be reduced by tryparedoxin or GSH and is localized in the glycosomes and cytosol (Wilkinson et al., 2000a, 2002a). Glutathione peroxidase II can be reduced only by GSH and is present in the endoplasmic reticulum (Wilkinson et al., 2002c). These peroxidases can decompose organic hydroperoxides but not H_2O_2 (Wilkinson et al., 2002c). Tryparedoxin peroxidases belong to the family of 2-cysteine peroxiredoxins, can decompose H_2O_2, and are reduced by tryparedoxin (Guerrero et al., 2000; Lopez et al., 2000; Wilkinson et al., 2000b). There are two isoforms, one cytosolic and the other mitochondrial (Wilkinson et al., 2000b). They can also decompose peroxinitrite (Thomson et al., 2003; Trujillo et al., 2004). The crystal structure of the cytosolic tryparedoxin peroxidase has been solved (Pineyro et al., 2005). In all cases, the activities of these peroxidases depend on the presence of trypanothione, which therefore has a central role in redox metabolism in *T. cruzi*.

15.3.2.3 Other Thiols

Several novel trypanothione analogues derived from spermine or other physiological polyamines have also been found in *T. cruzi* when supplemented with polyamines in the culture medium, among them homotrypanothione, N^1,N^{12}-bis(glutathionyl)spermine, N^1-glutathionyl-N^8-acetylspermidine, and N^1-glutathionyl-N^{12}-acetylspermine (Oza et al., 2002b; Ariyanayagam et al., 2003). These compounds result from the condensation of GSH with polyamines other than spermidine, such as cadaverin, spermine, *N*-acetylspermine, and N^1- and N^8-acetylspermine in reactions catalyzed by the trypanothione synthetase (Ariyanayagam et al., 2003). The physiological relevance of these thiols as well as ovothiol (N^1-methyl-4-mercaptohistidine) (Ariyanayagam and Fairlamb, 2001), which is also present in all three life cycle stages of this parasite is no known.

15.3.2.4 Superoxide Dismutases

Superoxide dismutases (SODs) catalyze the dismutation of superoxide anion ($O_2^{\cdot}$) to H_2O_2 and O_2. *T. cruzi* has four genes encoding for iron-dependent SODs, two of which have been cloned and the recombinant proteins characterized (Ismail et al., 1997; Temperton et al., 1998). Inhibitors of the iron-containing SODs of trypanosomatids have been found and proposed as possible trypanocidal agents (Meshnick et al., 1985). However, it is not self-evident that in an organism deficient in H_2O_2 detoxification, SOD inhibition will be toxic. This assumption takes for granted that superoxide is the ultimate toxic species. However, it is conceivable that the H_2O_2 formed by the action of SOD, and the hydroxyl radical eventually derived from it, are more cytotoxic than superoxide itself. If this is so, SOD produces the toxic

agent and SOD inhibition may be protective as long as H_2O_2 cannot adequately be detoxified. Interestingly, overexpression of cytosolic/glycosomal SOD (SODB1) in *T. cruzi* increases their susceptibility to benznidazole and crystal violet but has no effect on the action of nifurtimox (Temperton et al., 1998). The reason for these effects is unknown.

15.3.2.5 Chemotherapeutic Agents Used against Chagas Disease and Redox Metabolism

The enzymatic deficiencies of *T. cruzi* against oxygen toxicity were correlated with their sensitivity to both intracellularly generated and phagocyte-derived by-products of O_2 reduction (Docampo and Moreno, 1984; Docampo, 1990). The chemotherapeutic potential of these enzyme deficiencies was first recognized during work on the mode of action of the trypanocidal *o*-naphthoquinone β-lapachone and derivatives (Docampo and Moreno, 1984; Docampo, 1990). These studies showed that the metabolism of these compounds by *T. cruzi* involved, at least in part, the generation of superoxide anion and H_2O_2. H_2O_2 accumulated in the cells to cytotoxic levels and was also excreted (Docampo and Moreno, 1984; Docampo, 1990). The chemotherapeutic implications of these deficiencies were also apparent in the case of nifurtimox (Figure 15.1). Reduction of nifurtimox to a nitro anion radical followed by autoxidation of this radical with generation of superoxide anion and other oxygen reduction by-products such as H_2O_2, and hydroxyl radical have been implicated in the trypanocidal and toxic effect of this drug (Docampo, 1990). In contrast, the involvement of oxygen-reduction products in the trypanocidal action of benznidazole, which is a 2-nitroimidazole (Figure 15.1), could be ruled out (Docampo, 1990). As the rate of reduction of benznidazole is very low because of its lower reduction potential, redox cycling is considered a detoxification reaction that occurs by inhibition of the net reduction of the drug. The resultant low steady-state concentration of superoxide anion might be easily detoxified by the SODs present in *T. cruzi* (Moreno et al., 1982; Docampo, 1990).

Reactive oxygen species are also involved in the photodynamic action of crystal violet that has been described in *T. cruzi* (Docampo et al., 1983). Visible light causes photoreduction of crystal violet to a carbon-centered radical. Under aerobic conditions this free radical auto-oxidizes generating superoxide anion whose dismutation yields H_2O_2 (Docampo et al., 1983). Reducing agents known to enhance free radical formation from crystal violet in the presence of light enhance redox cycling of this dye (Reszka et al., 1986). In contrast to other photosensitizers, irradiation of crystal violet with visible light does not generate detectable amount of singlet oxygen (Reszka et al., 1986). The trypanocidal effect of crystal violet on *T. cruzi* epimastigotes and trypomastigotes is also enhanced by light (Docampo et al., 1983). The chemoprophylactic potential of the photodynamic action of crystal violet for the prevention of blood transmission of Chagas disease was also explored (Docampo et al., 1988). It was demonstrated that photoreduction with visible light in the presence of ascorbate reduces the effective dose and time of contact of the dye with *T. cruzi*-infected blood (Docampo et al., 1988). The scheme

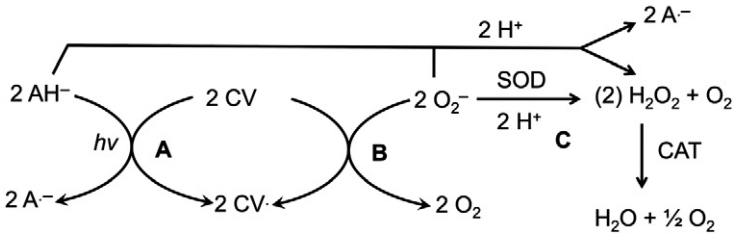


Figure 15.6 Mechanism of ascorbate enhancement of crystal violet toxicity. Light (*hv*) catalyzes the conversion of crystal violet into a carbon centered radical ($CV^{\cdot}$) in the presence of ascorbate (A^{-}), which generates ascorbyl radical ($A^{\cdot-}$). Autoxidation of the carbon centered radical generates superoxide anion ($O_2^{\cdot-}$) that dismutates to H_2O_2 in the presence of SOD or reacts with ascorbate to generate more ascorbyl radical. H_2O_2 can be decomposed by catalase.

shown in Figure 15.6 has been proposed to explain the enhancement of the cytotoxicity of crystal violet against *T. cruzi* by ascorbate (Docampo et al., 1988).

In reaction A, ascorbate anion reduces crystal violet under illumination. Under aerobic conditions, the crystal violet carbon-centered free radical then reduces O_2 to superoxide anion (reaction B); dismutation of superoxide anion produces H_2O_2 (reaction C). SOD increases the rate of H_2O_2 formation by catalyzing reaction C. The oxidation of ascorbate by superoxide anion contributes to the formation of H_2O_2 and is responsible for the generation of the ascorbyl radical that is detected in incubations of *T. cruzi*-infected blood upon illumination (Docampo et al., 1988). When catalase is present (as occurs in red and white blood cells but not in *T. cruzi*), H_2O_2 is detoxified. Formation of H_2O_2 may explain the photodynamic action of crystal violet/ascorbate on *T. cruzi* (Docampo et al., 1988) since the sensitivity of different *T. cruzi* stages to reagent H_2O_2, enzymatically generated H_2O_2, H_2O_2-generating drugs, and H_2O_2-generating phagocytic cells has been well documented (Docampo, 1990).

15.3.3 Acidocalcisome Biochemistry and Osmoregulation

15.3.3.1 The Role of Acidocalcisomes in T. cruzi Metabolism

The acidocalcisome is an acidic organelle rich in phosphorus, calcium, and other cations (Docampo et al., 2005; Docampo and Moreno, 2008; Moreno and Docampo, 2009). Phosphorus is present as pyrophosphate and polyphosphate (poly P) and is complexed with calcium and other cations. Poly P is a linear polymer of phosphate linked by high-energy phosphoanhidride bonds that could have from a few to several hundred phosphate units. The acidocalcisome membrane in *T. cruzi* contains a number of pumps (Ca^{2+}-ATPase, V-H^{+}-ATPase, H^{+}-PPase), and at least a channel (aquaporin), while its matrix contains enzymes related to pyrophosphate and poly P metabolism (Docampo et al., 2005; Docampo and Moreno, 2008; Moreno and Docampo, 2009) (Figure 15.7).

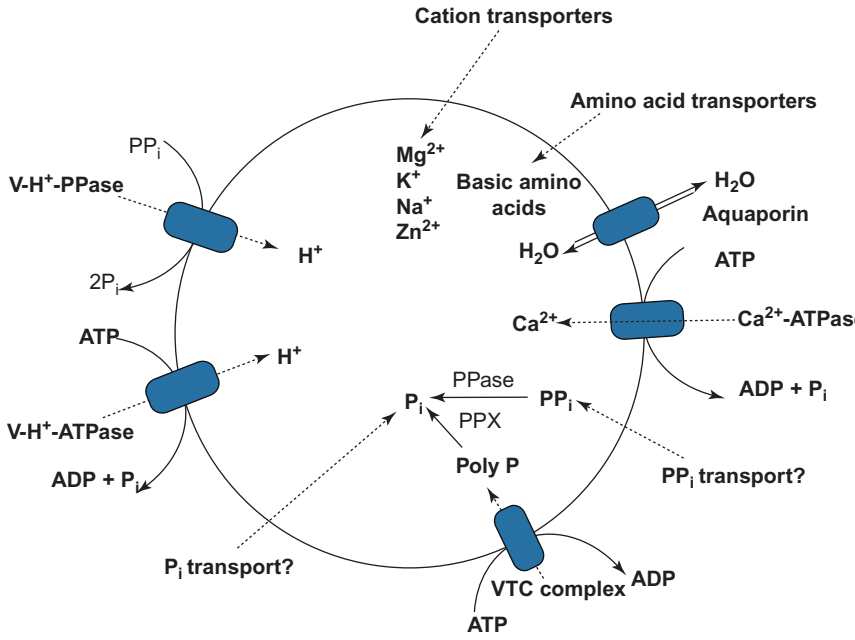


Figure 15.7 Scheme of a *T. cruzi* acidocalcisome. The matrix contains P_i, PP_i, and poly P combined with a variety of cations (Mg^{2+}, K^+, Na^+, Zn^{2+}) and basic amino acids, and also pyrophosphatase (PPase) and exopolyphosphatase (PPX) activities. The membrane possesses proton pumps (V H^+ ATPase, V H^+ PPase), a calcium pump (Ca^{2+} ATPase), a water channel (aquaporin), and a poly P synthase complex (VTC complex). Other transporters for cations, P_i, PP_i, and basic amino acids are also probably present (dashed lines).

Acidocalcisomes in *T. cruzi* are also rich in basic amino acids, such as arginine, ornithine, and lysine, probably also complexed with poly P (Rohloff et al., 2003). Some of the functions of acidocalcisomes in *T. cruzi* are the storage of cations, phosphorus, and amino acids, pyrophosphate and polyphosphate metabolism, and osmoregulation. The finding of novel enzymes in this organelle that are absent from mammalian cells has led to the discovery of novel targets for drug action (Docampo et al., 2005; Docampo and Moreno, 2008; Moreno and Docampo, 2009).

15.3.3.2 Acidocalcisomes and Osmoregulation in T. cruzi

T. cruzi encounters severe environmental stressors to which it must adapt as it progresses through its life cycle. One example is the parasite's ability to cope with fluctuations in osmolarity that occur within the gut of the vector (Kollien and Schaub, 2000; Kollien et al., 2001) and also as the parasite moves from the insect gut through the acidic phagolysosome to the cytosol of the host cell. The infective form of the parasite passes out of the vector in the highly concentrated excreta (600−700 mOsm) (Kollien et al., 2001) and rapidly encounters the interstitial fluid of the mammalian host with a much lower osmolarity (330 mOsm). Evidently, the

parasite has mechanisms that allow it to adapt to both hyperosmotic and hyposmotic stresses, although the response of the parasite to hyposmotic stress has been studied in more detail (Rohloff et al., 2003, 2004; Montalvetti et al., 2004; Rohloff and Docampo, 2008). Two organelles that have fundamental roles in these adaptations are the acidocalcisomes and the contractile vacuole complex. The contractile vacuole complex was described earlier in *T. cruzi* although its function was unknown for a long time (Rohloff and Docampo, 2008).

Upon exposure to a reduction in external osmolarity, cells initially swell but soon regain nearly normal cell volume by a process that has been known as the regulatory volume decrease (RVD; reviewed in Lang et al., 1998a,b), which is accomplished by the efflux of various inorganic ions (such as Na^+ and K^+) and organic osmolytes to the extracellular environment. The RVD mechanism is present in amastigotes, epimastigotes, and trypomastigotes of *T. cruzi* (Rohloff et al., 2003) and is due to the release of amino acids, K^+, and water (Rohloff and Docampo, 2008). This process is rapid and essentially complete in all *T. cruzi* stages after 5 min. Uncharged or acidic amino acids are mobilized during hyposmotic stress in all three stages and are probably released through an anion channel with properties similar to those previously described in other cells (Lang et al., 1998a,b).

Cell swelling causes a spike in intracellular cyclic AMP through activation of an adenyl cyclase, and results in a microtubule-dependent fusion of acidocalcisomes with the CV (Rohloff et al., 2004). A simultaneous rise in ammonia and its sequestration in acidocalcisomes as NH_4^+ (Rohloff and Docampo, 2006) increases their pH and probably activates an acidocalcisomal exopolyphosphatase, which cleaves poly P, releasing inorganic phosphate residues and also the various poly P-chelated osmolytes, such as basic amino acids and calcium (Rohloff and Docampo, 2008). The resulting osmotic gradient sequesters water (through the aid of an aquaporin), which is subsequently ejected into the flagellar pocket (Rohloff and Docampo, 2008). This pathway would terminate by hydrolysis of cyclic AMP by a phosphodiesterase, and inhibition of this phosphodiesterase could be a potential target against *T. cruzi*.

15.3.3.3 Acidocalcisomes as Drug Targets

A vacuolar proton translocating pyrophosphatase ($V\text{-}H^+$-PPase) is involved in the acidification of the organelle in *T. cruzi* (Scott et al., 1998). The enzyme uses pyrophosphate instead of ATP as an energy source, is K^+-stimulated (type I), and can be used as a marker for acidocalcisome purification (Scott et al., 1998). The gene encoding this pump has been functionally expressed in yeast (Hill et al., 2000). This enzyme is also found in the Golgi complex and in the plasma membrane of *T. cruzi* (Martinez et al., 2002) but is absent in mammalian cells.

Pyrophosphate analogs, bisphosphonates (containing a nonhydrolyzable P-C-P, rather that a P-O-P, backbone) as well as imidodiphosphate (containing a nonhydrolyzable P-N-P group) are inhibitors of the plant (mung bean, *Vigna radiata* L.) $V\text{-}H^+$-PPase (Kim et al., 1994). Imidodiphosphate and aminomethylenediphosphonate (AMDP), one of the best-known inhibitors of the $V\text{-}H^+$-PPase, inhibits the *T. cruzi* enzyme (Scott et al., 1998).

Acidocalcisomes possess another enzyme that is absent in mammalian cells: a poly P synthetase, also known as vacuolar transporter chaperone (VTC) complex (Fang et al., 2007). This complex is formed in yeast by four subunits: VTC1-4, being VTC4 the catalytic subunit (Hothorn et al., 2009). *T. cruzi* has genes homologous to those encoding VTC1 and VTC4 (Fang et al., 2007).

Acidocalcisomes are also known to accumulate drugs. Ormerod observed that these organelles, known at that time as volutin granules, become more visible under light microscopy when cells are treated with drugs (Ormerod, 1961). Further work showed that drugs like stilbamidine, quinapyramine, suramin, hydroxystilbaminine, and acriflavine accumulated in these organelles (Ormerod and Shaw, 1963; Macadam and Williamson, 1974). For this reason they were also called "chemotherapy granules" (Ormerod, 1951). Interestingly, some of these drugs are first concentrated in the kinetoplast and nucleus, then diffuse to the cytosol, and finally concentrate in acidocalcisomes. Such is also the case of diamidines like DB75 (furamidine) and DB820, which have been in phase III clinical trials against human African trypanosomiasis (Mathis et al., 2006, 2007). However, the impact that acidocalcisome accumulation has on the mechanism of action of these compounds is not known.

15.4 Conclusions

Studies on metabolic pathways in *T. cruzi* have in some cases revealed the reason for their susceptibility to drugs effective *in vivo* against this parasite. That is, for example, the case of nifurtimox and benznidazole (Figure 15.1), the drugs currently used in the treatment of Chagas disease. These studies have also helped us in understanding the effect of antifungal azoles, which have been tested or will be tested soon in clinical trials against Chagas disease. In other cases, studies on the metabolism of *T. cruzi* have shed light on potential targets for drug action (such as the acidocalcisomes) or have helped in the identification of compounds (such as bisphosphonates and prenyltransferase inhibitors) that could be of potential use against this disease. We should expect that the use of some of these compounds could result in an adequate treatment for the acute and chronic forms of Chagas disease. The advantage of some of these compounds is that many of them are under development for other uses by pharmaceutical companies and some are already FDA-approved.

The usefulness of specific anti-*T. cruzi* treatment of acute and chronic chagasic patients suggests that treatment is always beneficial. Even if the situation exists that cardiopathy or megas are not prevented in all those treated, the eradication of parasitemia will prevent transmission by blood transfusion and, in female children, congenital transmission years later.

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16 Ultrastructure of *Trypanosoma cruzi* and Its Interaction with Host Cells

Wanderley de Souza[1,2,3,*], *Tecia Maria Ulisses de Carvalho*[1,3] *and Emile Santos Barrias*[1,3]

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto Nacional de Metrologia, Normalização e Qualidade Industrial-Inmetro, Rio de Janeiro, Brazil, [3]Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Biomagens, Universidade Federal do Rio de Janeiro, Brazil

16.1 Structural Organization of *Trypanosoma cruzi*

Trypanosoma cruzi is protozoa of the Trypanosomatidae family that are agents of parasitic disease (Chagas disease, sleeping sickness, and leishmaniasis), which have a high incidence and negative economic impact in developing countries. Structural features of this family include structures/organelles such as the kinetoplast, the glycosome, the paraflagellar rod (PFR), a highly specialized flagellar pocket, and a layer of subpellicular microtubules. Another important feature of these parasites is their ability to change shape during their life cycle. In those species that switch from vertebrate to invertebrate hosts, the changes may be dramatic, involving the appearance of developmental stages that do not divide and stages that are highly infective. This process is generally described as protozoan differentiation or transformation (De Souza, 2008, 2009). Among the trypanosomatids, *T. cruzi* has one of the most complex life cycle. The parasite proceeds through several developmental stages in vertebrate and invertebrate hosts, living both in the bloodstream and inside the cells of the vertebrate host. Figure 16.1 shows a general view of the life cycle of this protozoan.

Let us consider that the cycle starts when insects from the Reduviidae family suck the blood of vertebrates infected with the parasite. The blood contains the trypomastigote form, which circulates in the bloodstream (known as bloodstream trypomastigotes). Once ingested with the blood, most of the trypomastigotes are lysed following interaction with bacteria in the insect's stomach (Castro et al., 2007). The surviving trypomastigotes transform a few days later either into

*E mail: wsouza@biof.ufrj.br

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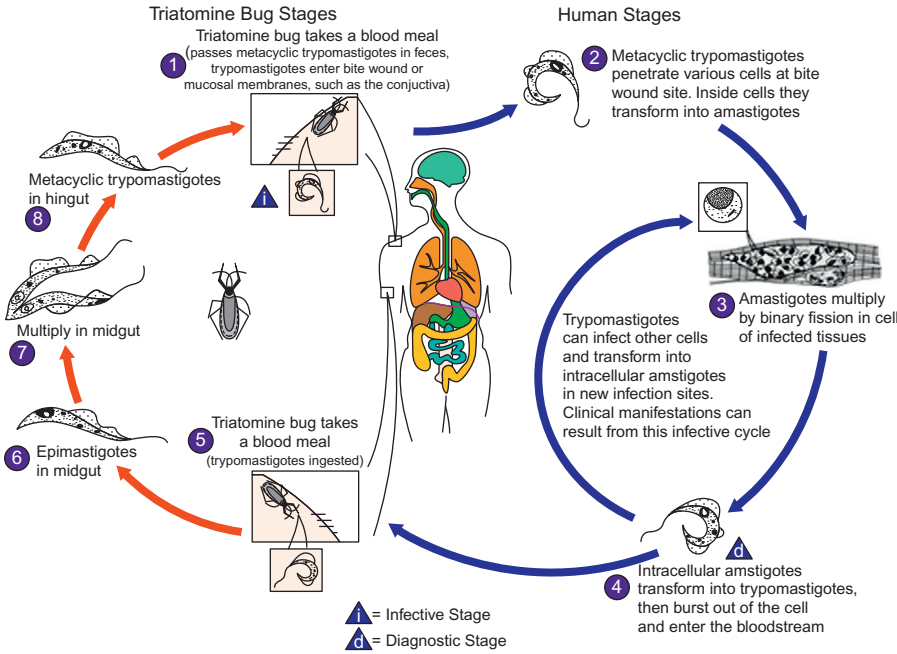


Figure 16.1 Life cycle of *T. cruzi* showing the different developmental stages of the protozoa in the invertebrate (triatomines) and vertebrate (mammals) hosts.
Source: Reproduced from the Center of Disease Control home page.

the spherical form, known as the spheromastigote, or into the epimastigote form. The latter migrates to the intestine, where it divides intensely and attaches. This attachment occurs via a specific interaction in which exposed glycosylphosphatidylinositol (GPI)-linked macromolecules on the parasite's surface are recognized by components of the intestinal cell surface and perimicrovillar membranes (Alves et al., 2007; Nogueira et al., 2007). At the most posterior regions of the intestine and at the rectum, many epimastigotes detach from the intestinal surface and transform into trypomastigotes, which are then released together with feces or urine (Garcia et al., 1991). These forms are also called metacyclic trypomastigotes and are highly infective to several mammal species, including man. The infection of mammals usually takes place following an insect bite, when the metacyclic trypomastigote penetrates directly through the ocular mucosa or skin lesion. Others infections routes are: 1) by ingestion of food contaminated with *T. cruzi*; 2) by blood transfusion; 3) organ transplantation; 4) congenital; 5) laboratory accident; 6) less usual sexual. (Yoshida, 2009). Once in the vertebrate host, the metacyclic trypomastigotes invade the cells found at the invasion site (fibroblasts, macrophages, and epithelial cells, for example) through several mechanisms (phagocytosis and induced endocytosis). After invasion, they appear within an endocytic vacuole known as the parasitophorous vacuole (PV). A few hours after cell infection, the long, thin trypomastigotes gradually became rounded with a short flagellum, characteristic of the amastigote form (also known as intracellular spheromastigotes). At the same time, the parasite secretes enzymes

that help in the digestion of the membrane lining the PV, allowing the amastigotes to enter into direct contact with host cell organelles. Since their initial description in 1909, the morphologies of the various developmental stages of *T. cruzi* have been the subject of intense investigation (Chagas, 1909). A color drawing found in the original paper describing *T. cruzi* (Figure 16.2) points to the variation in the general shape of the protozoan and the presence of several structures stained with Giemsa.

This chapter describes the main structures and organelles found in *T. cruzi*. Figure 16.3 presents a schematic view based mainly on images obtained with the use of transmission electron microscopy (TEM).

The most relevant structures and organelles will be described in detail.

16.1.1 The Nucleus

Initial observations showed a nucleus enveloped by typical membranes with pores, condensed chromatin dispersed throughout the nucleoplasm and a typical nucleolus found in epimastigotes, but not in amastigote or trypomastigote forms (De Souza and Meyer, 1974; Solari, 1995; Elias et al., 2001). It was also shown that the nuclear membrane remains intact throughout mitosis, which is characterized by the appearance of intranuclear microtubules, dispersion of the chromatin, and the appearance of dense plates whose number varies according to the trypanosomatid species (Solari, 1995). Still, there is no evidence that these plates correspond to chromosomes, which have been detected using a biochemical approach. The morphology of the nucleus changes significantly during the cell cycle. While it has a spherical shape in interphase cells, it elongates during cell division and then constricts in the middle. Metacyclic trypomastigotes have an elongated nucleus and a flagellum emerging from the posterior end of the cell. *T. cruzi* has 41 chromosomes (Weatherly et al., 2009).

16.1.2 The Kinetoplast–Mitochondrion Complex

Initial studies of trypanosomatids using electron microscopy (Meyer et al., 1958) identified a dark, electron-dense and slightly bent inclusion that corresponds to a structure known at that time as the kinetonucleus, which was identified based on observation of stained cells by light microscopy. This structure is now known as the kinetoplast. Its shape and structural organization vary according to the developmental stage of the protozoan. The trypanosomatids possess a unique and highly ramified mitochondrion. The kinetoplast appears as a dense structure and is made of a special type of DNA known as kinetoplast DNA (kDNA). The kDNA is found in a specialized portion of the mitochondrion (Figure 16.4) within the mitochondrial matrix, perpendicular to the axis of the flagellum.

The kinetoplast is always located close to the basal body. Filamentous structures connect the kinetoplast to the basal body (Figure 16.5) (Souto-Padron et al., 1984; Ogbadoiry et al., 2003).

Due to this connection, the position of the kinetoplast defines the cellular region in which the basal body is located and, consequently, the origin of the flagellum.

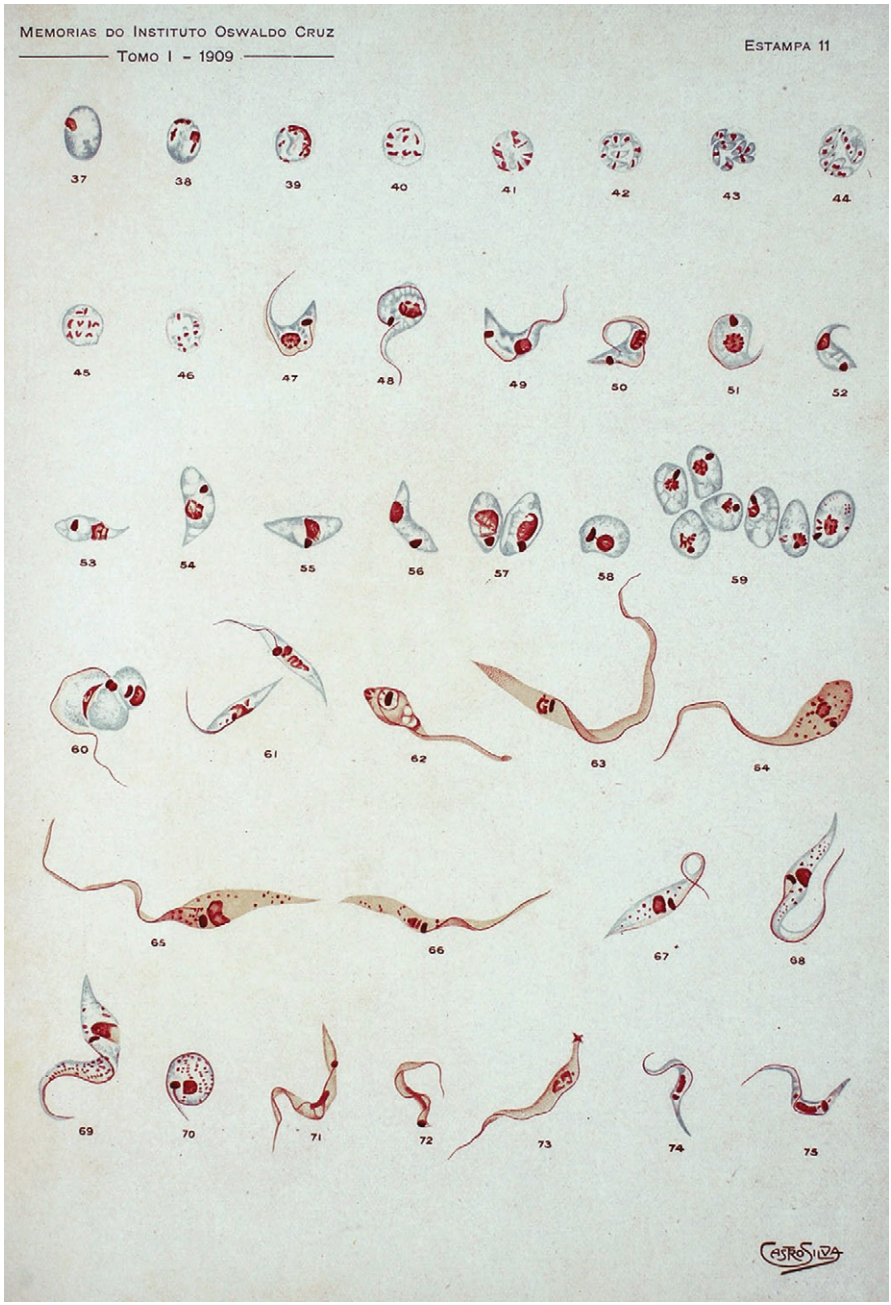


Figure 16.2 Reproduction of the original drawings of the first description of *T. cruzi* by Carlos Chagas in 1909, where developmental stages in both vertebrate and invertebrate hosts were seen.

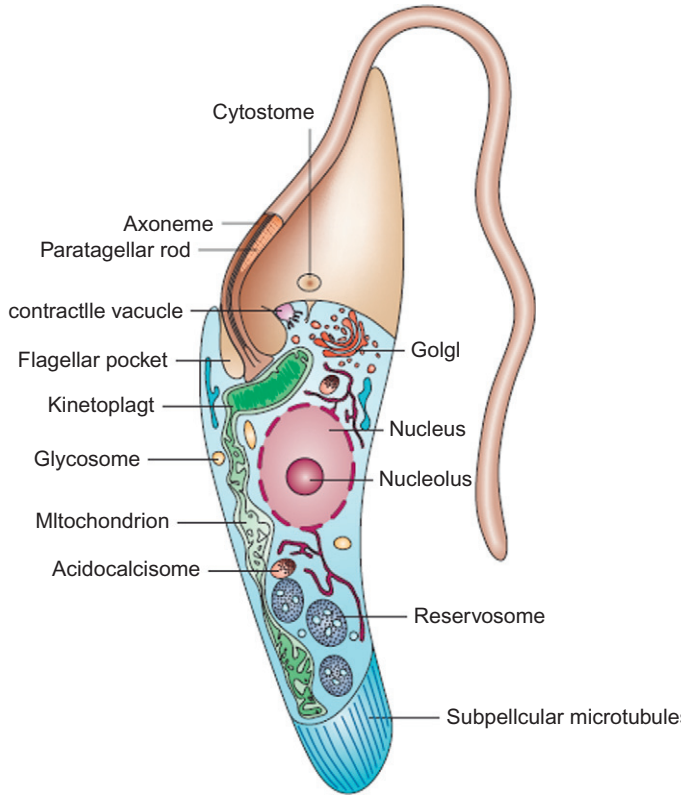


Figure 16.3 Schematic representation of longitudinal section of an epimastigote form of *T. cruzi* showing the main structures and organelles found in *T. cruzi*. The scheme is modified from a drawing by Flavia Moreira Leite, University of Oxford.
Source: After DoCampo et al. (2005).

A protein designated as p166 was identified and shown to be located in between the kDNA disk and the flagellar body (Zhao et al., 2008).

In addition to DNA, electron microscopy cytochemistry using the ethanolic phosphotungstic acid technique and ammoniacal silver (Souto-Padron and De Souza, 1978, 1979) showed the presence of basic proteins in the kinetoplast. It was suggested that these proteins could neutralize the negatively charged DNA molecules that are in close contact within the kinetoplast. Histone-H1-like proteins are known to be involved in the condensation of *T. cruzi* kDNA (review in De Souza and Cavalcanti, 2008).

Two types of circular DNA are present in the kinetoplast: minicircles and maxicircles. There are several thousand minicircles, which range in size from about 0.5 to 2.5 kb (depending on the species), and a few dozen maxicircles, which range from 20 to 40 kb (reviews in Shapiro and Englund, 1995). The minicircles encode guide RNAs that modify the maxicircle transcripts by extensive uridylate insertion

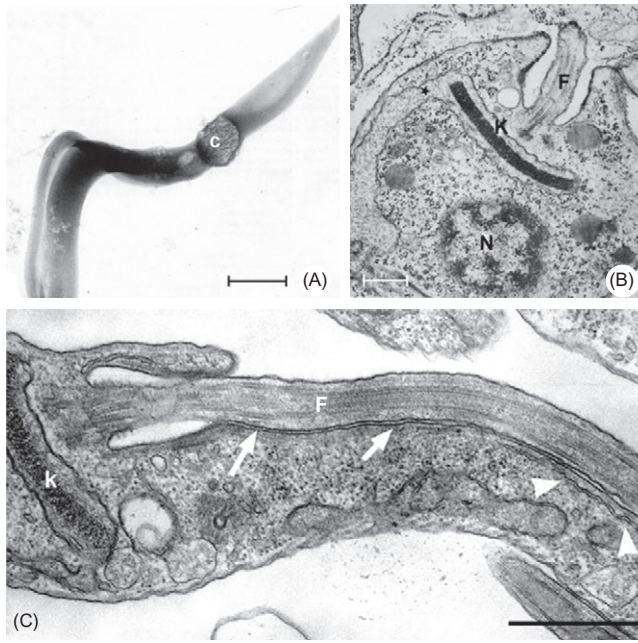


Figure 16.4 Different views of the kinetoplast and the general organization of the (A) trypomastigote, (B) amastigote, and (C) epimastigote forms of *T. cruzi*. F: flagellum; K: kinetoplast; N: nucleus. The white arrows point to the area of adhesion of the flagellum to the cell body. White arrowheads point to profiles of the ER. Bars = 1 μm.
Source: After De Souza et al. (2009) figures A and B; after Rocha et al., 2006 figure C.

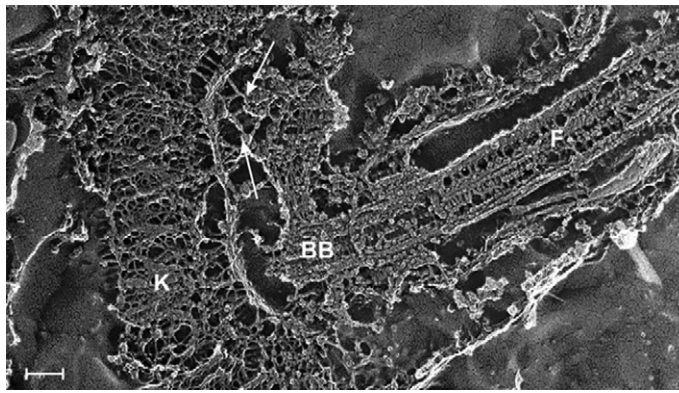


Figure 16.5 Anterior region of an epimastigote form of *T. cruzi* as seen in a replica of quick frozen, freeze fractured, deep etched, and rotary replicated cells. The network of DNA molecules that makes the kinetoplast (K) is shown as well as filamentous structures (arrow) connecting the basal bodies (BB) to each other and to the mitochondrial membrane. The axonemal microtubules which make the flagellum (F) are also seen. Bar = 100 nm.
Source: After Souto Padron et al. (1984).

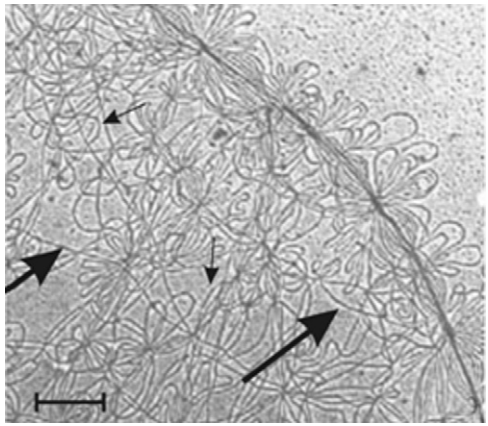


Figure 16.6 Organization of the kDNA fibers in maxicircles and minicircles. The kDNA network was dispersed in water, collected in a grid, and shadowed at low angle with platinum. Bar = 0.3 μm. *Source*: Courtesy of David Pérez Morga.

or deletion, a process known as RNA editing. The maxicircles are structurally and functionally analogous to the mitochondrial DNA that encodes rRNAs and subunits of the respiratory complexes in higher eukaryotes. *In situ* analysis of the kDNA structure showed that the network is formed not by circles, but by irregular polygonal structures (Figure 16.6).

Similar images of the whole kDNA network have been obtained using atomic force microscopy (AFM).

In light of the special characteristics of the kDNA and the observation that this structure is essential for life of the parasite, the kinetoplast has been considered an important target for the development of new antiparasitic drugs.

16.1.3 The Glycosome

All trypanosomatids contain spherical structures with a homogeneous matrix that are surrounded by a unit membrane and distributed throughout the cell. They were initially designated as microbodies because of their similarity to structures seen in mammalian cells. However, further studies in several trypanosomatids showed that these structures are a special type of peroxisome, designated as the glycosome due to the concentration of glycolytic pathway enzymes in this organelle (review in Opperdoes and Borst, 1977; Opperdoes, 1987). Usually, glycolysis takes place in the cytosol, and the organelle was thus designated as the glycosome. Since catalase is found in the glycosomes of monogenetic but not digenetic trypanosomatids, this organelle is now considered to be a special type of peroxisome. Electron microscopy cytochemistry aimed at the detection of basic proteins, as previously described in the kinetoplast section, showed intense labeling of the glycosomes (Souto-Padron and De Souza, 1978). These observations prompted biochemical studies demonstrating that the enzymes of the glycosome have higher isoelectric points than the cytosolic glycolytic enzymes of other eukaryotic cells. In addition to catalase, the peroxisomes of mammalian cells have more than 50 different enzymes involved in metabolic pathways such as peroxide metabolism, ß-oxidation of fatty acids, and ether phospholipid synthesis. Studies have found that, in addition to these pathways,

other metabolic pathways that occur in the cytosol of other cells also take place in the glycosomes of trypanosomatids, including carbon dioxide fixation (Opperdoes and Cotton, 1982), purine salvage and *de novo* pyrimidine biosynthesis, fatty acid elongation, isoprenoid biosynthesis, and sterol biosynthesis (review in Opperdoes, 1987). The glycosome does not possess a genome. Therefore, all of the proteins found in it are encoded by nuclear genes, translated on free ribosomes, and then posttranslationally imported into the organelle.

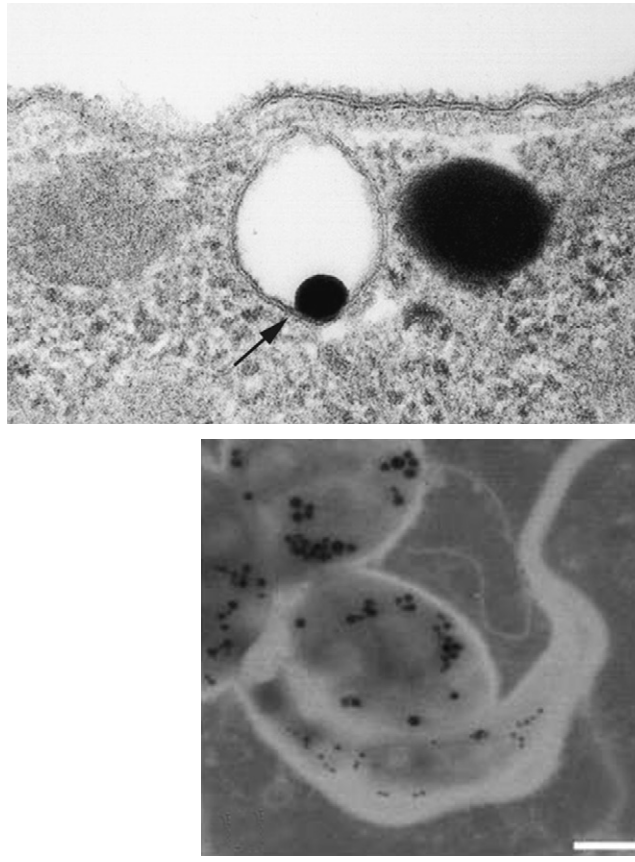
The biogenesis of the glycosome seems to be similar to that of peroxisome. Peroxins (PEX) have been described in human-pathogenic trypanosomatids and are homologous to PEX present in mammals, yeast, and plants, although with low levels of amino acid sequence similarity. Over 20 PEX proteins participate in protein import to glycosomes. Proteins targeted to glycosomes show a peroxisome targeting sequence (PTS) that binds to a soluble receptor (PEX 5 or PEX 7). This receptor/cargo (protein/PEX) complex binds to a PEX complex, allowing import into the glycosome (reviewed by Sommer et al., 1993). Studies with *Trypanosoma brucei* indicated that the only specific sequence for glycosomal 3-Phosphoglycerate kinase (gPGK) import into glycosomes is present in the C-terminal region and consists of three amino acids (serine-serine-leucine) (Sommer et al., 1993). By genetic manipulation, it was shown that decreasing their expression leads to growth arrest and death of the parasites. These proteins also show a low degree of sequence conservation. Those characteristics make PEX good drug targets.

16.1.4 The Acidocalcisome

Since the first morphological observations of *T. cruzi*, there have been descriptions of spherical structures distributed throughout the cell body. These structures are clearly depicted in the drawing published by Carlos Chagas (1909). Subsequently, they were named "polyphosphate granules" or "volutin granules," and electron microscopy observations revealed vacuolar structures containing electron-dense deposits. Only in 1994 was it shown that this organelle is capable of transporting protons and calcium, thus prompting its designation as the "acidocalcisome" (review in Docampo et al., 2005). Its appearance depends on the methodology used to process the samples for electron microscopy. With conventional methods, most of the dense content may disappear, leaving only a small electron-dense dot associated with the membrane lining the organelle (Figure 16.7).

When cryofixation was used, a much better preservation of the acidocalcisome content was achieved. Indeed, a homogenous electron-dense matrix was preserved when the cells were quick-frozen and freeze-substituted (Miranda et al., 2000). The organelles can also be visualized in whole cells dried on a grid using an electron microscope, especially if the microscope is equipped with an energy filter, as shown in Figure 16.8.

Electron microscopy microanalysis has played a key role in the determination of the ionic composition of the acidocalcisome. Using X-ray mapping, it was shown that the acidocalcisomes contain calcium, phosphorous, sodium, potassium, and zinc. In some trypanosomatids, iron has also been found (Miranda et al., 2004).



Figures 16.7 and 16.8 Acidocalcisome morphology seen in cells processed using routine methods for electron microscopy (Figure 16.7), and in whole cells examined using ESI (Figure 16.8). Bars = 200 nm (Figure 16.7), 2 μm (Figure 16.8).
Source: After De Souza et al. (2009).

The acidocalcisomes have many functions, including: (i) the storage of calcium, magnesium, sodium, potassium, zinc, iron, and phosphorous compounds, especially inorganic pyrophosphate and polyphosphate, as determined by biochemical analysis and X-ray microanalysis, (ii) pH homeostasis, and (iii) osmoregulation, a function that involves interaction of the acidocalcisome with the contractile vacuole (review in Docampo et al., 2005).

16.1.5 The Contractile Vacuole

Studies carried out with several protozoa, especially free-living ones, have shown that the contractile vacuole plays a fundamental role in the regulation of osmotic processes. There have been few reports on the presence of such a structure in trypanosomes. The structure was reported to consist of several tubules connected to a central vacuole located close to the flagellar pocket (Linder and Staehelin, 1977).

Aquaporin, a protein involved in water transport, was identified in *T. cruzi* epimastigotes and localized to both the acidocalcisomes and contractile vacuole (Montalvetti et al., 2004). These structures seem to be involved in the process of osmoregulation. It was shown that the fusion of acidocalcisomes to the contractile vacuole takes place in a process mediated by cyclic AMP (review in Rohloff and Docampo, 2008).

16.1.6 Lipid Inclusions

Spherical structures with variable diameters are observed in the cytoplasm of *T. cruzi*. They are surrounded not by a typical unit membrane, but by a phospholipid monolayer. Some are electron-transparent, while others present a medium density. Most of these structures are highly contrasted when the cells are fixed in an osmium tetroxide solution in immidazol buffer (Soares and De Souza, 1987). Very little is known about these structures.

16.1.7 The Cytoskeleton

TEM of thin sections of trypanosomatids showed the presence of subpellicular microtubules distributed throughout the cell body, except in the flagellar pocket region. Using conventional fixation techniques, it is possible to see that the microtubules are separated by a constant distance from each other (about 44 nm) and from the plasma membrane (about 12 nm) in all portions of the cell body. In the thinnest region of the cell, very few microtubules are seen. In the case of *T. cruzi* trypomastigotes, the largest number of microtubules is observed at the anterior region (Meyer and De Souza, 1976). In dividing amastigotes, up to 222 microtubules can be counted. In favorable sections, it is possible to see filamentous structures connecting the microtubules to the plasma membrane and to other microtubules. Profiles of the endoplasmic reticulum (ER) can be seen in between and below the subpellicular microtubules (Pimenta and De Souza, 1985). Each microtubule is made of 13 protofilaments, which can be better visualized when the cells are fixed in a glutaraldehyde solution containing tannic acid (Sauto Pardrón and De Souza, 1979).

One approach for observing the whole cytoskeleton of trypanosomatids is cell lysis on a water surface followed by critical point drying. Another approach is lysis during drying following adsorption of the cells onto a formvar carbon-coated grid and negative staining. With both techniques, it is possible to obtain images of the helicoidal array of microtubules. More recently, informative images of the organization of the subpellicular microtubules have been obtained using high-resolution scanning electron microscopy. The use of stable cold field-emission scanning electron microscopes produces images with excellent resolution (Sant'Anna et al., 2005; De Souza et al., 2008).

The quick-freeze, freeze-fracture, deep-etching, and rotary replication technique are ideal for revealing details of cytoskeleton organization (Souto-Padron et al., 1984). This approach has yielded new details about the organization of the

subpellicular microtubules and the flagellum of trypanosomatids. In regard to the subpellicular microtubules, it is possible to clearly see the filaments that connect the microtubules to each other, as well as those that connect the microtubules to the inner portion of the plasma membrane and to profiles of the ER (Figure 16.9).

Microfilaments have never been observed in the cytoplasm of *T. cruzi*. However, it has been shown (Correa et al., 2007, 2008) that cytochalasin B treatment leads to morphological alterations in the cytoskeletal elements associated with the cytostome−cytopharynx complex, which is responsible for transferrin uptake. Comparative genomic analysis identified a potential role for an actin−myosin system in *T. cruzi*, as this protozoan has, in addition to an actin gene, an expanded myosin family and a CapZ F−actin capping complex. It has been suggested that an actin−myosin system might function at the cytostome. Actin and actin-binding proteins were recently characterized in *T. cruzi* (De Melo et al., 2008). TcActin was observed in several patch-like cytoplasmic structures throughout the *T. cruzi* developmental stages. However, while the structure of *T. cruzi* actin is similar to that of higher eukaryote actin, homology modeling has revealed fundamental differences, predominantly in the loops responsible for oligomerization and interactions with

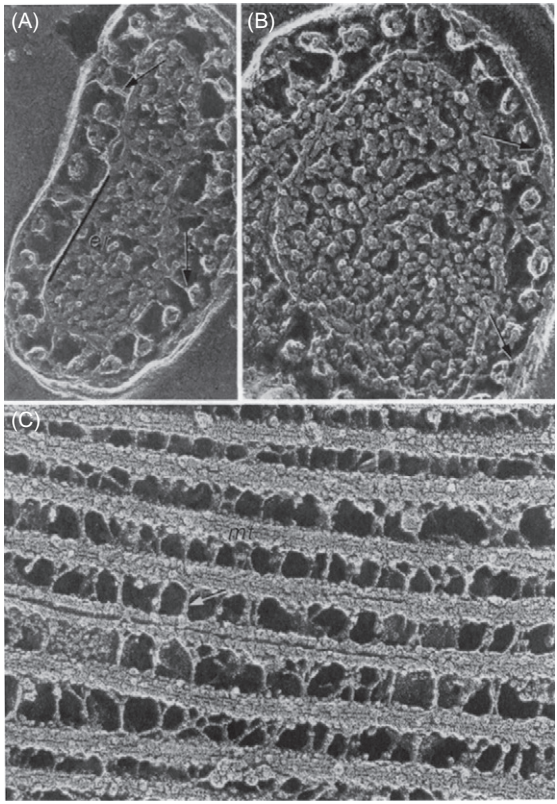


Figure 16.9 Deep etching view reveals subpellicular microtubules connecting with ER (A), with plasma membrane (B), and between them (arrows C). Bar = 100 nm.
Source: After Souto Padron et al. (1984).

actin-binding proteins. As a consequence, actin filaments have never been detected in *T. cruzi*.

16.1.8 The Flagellum

All trypanosomatids have one flagellar complex, with a canonical basal body at the base of the flagellum. Even in the so-called intracellular amastigote form, a short flagellum is observed. The flagellum consists of the typical array of nine pairs of peripheral microtubule doublets and one central pair (Figure 16.10B).

In addition, it contains an intriguing structure made up of a complex array of filaments that, due to its location, is called the paraxial or PFR. The use of a combination of tannic acid and glutaraldehyde significantly improves its preservation (Figure 16.10A–D). Using the quick-freezing, freeze-fracture, deep-etching, and rotary replication technique (Souto-Padron et al., 1984; Farina et al., 1986), several structures are visible that cannot be discerned in thin sections (Figure 16.10E). The PFR is made of a complex array of filaments linked to the axoneme. Two regions, designated as proximal (consisting of two plates) and distal (consisting of several plates), were identified in the PFR. The plates are formed by an association of

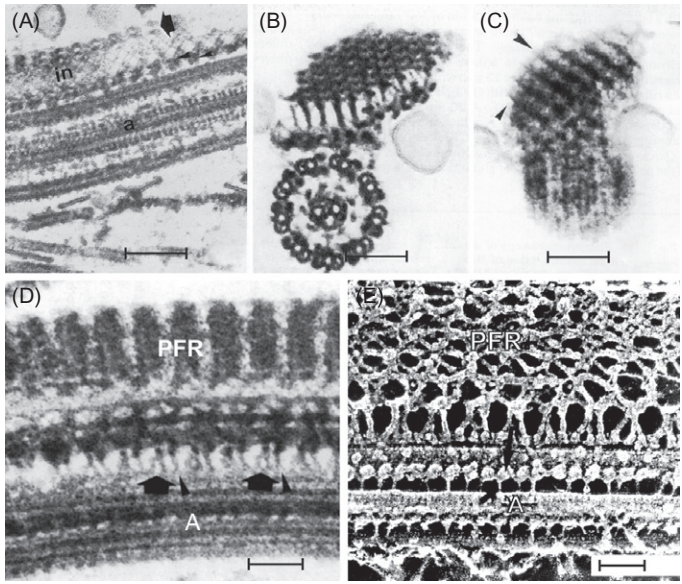


Figure 16.10 Different views of the flagellum of trypanosomatids as seen in longitudinal (A, D) and transversal (B, C) views of thin sections and in a replica of quick frozen, freeze fractured, deep etched, and rotary replicated samples (E). The axonemal (A) microtubules, as well as filaments which make the PFR structure, can be seen. Bridges connecting the axoneme to the PFR (arrows) and the plates which form the PFR are seen. Bar = 50 (A–C) and 100 nm (D, E).
Source: After Farina et al. (1986).

25-nm and 7-nm thick filaments that are oriented at a 50-degree angle in relation to the major axis of the axoneme (Farina et al., 1986). Biochemical analyses have shown that the PFR is composed of a large number of proteins, most of which have not yet been characterized. However, two major proteins have been characterized in some detail. These proteins, known as PFR 1 and 2, have molecular weights of 73 and 79 kDa, respectively. They are highly antigenic and thus are potential targets for vaccine and diagnostic kit development. The available evidence indicates that the PFR is an essential structure for parasite survival (review in Bastin and Gull, 1999).

AFM views of the flagellum revealed the presence of a furrow that separates the axoneme-containing portion of the flagellum from the PFR-containing portion (Rocha et al., 2007). Periodic structures associated with the furrow were also observed (Figure 16.11).

16.1.9 The Flagellar Pocket

In all trypanosomatids, the flagellum emerges from a deep invagination of the plasma membrane. At the emergence site, the flagellar membrane comes into contact with the membrane lining the cell body, such that the invagination becomes an almost closed pocket known as the flagellar pocket (review in Field and Carrington, 2009). The cell membrane that lines the flagellar pocket does not contain subpellicular microtubules. In addition, a large number of vesicles are seen in this region, which is where endocytosis and exocytosis take place. However, in the case of *T. cruzi* epimastigotes, it has been shown that the cytostome is the main site of endocytosis (Porto-Carreiro et al., 2000).

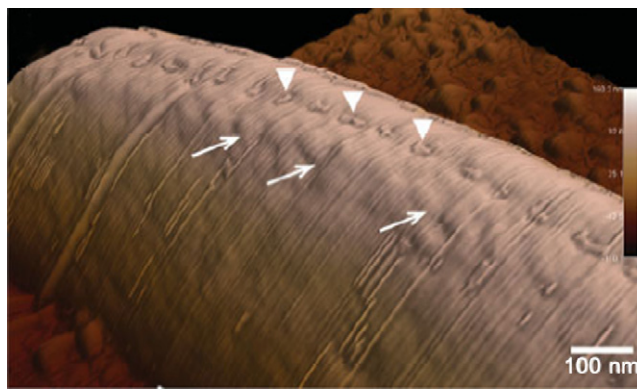


Figure 16.11 AFM image of the flagellum of a slightly detergent extracted epimastigote from *T. cruzi*. A furrow along the major axis of the flagellum is clearly seen. Periodically organized protrusions can be seen in the furrow (arrowheads).
Source: After Rocha et al. (2006).

16.1.10 The Secretory Pathway

The secretory pathway in *T. cruzi* involves the ER, the Golgi complex, and a system of vesicles that bud from the Golgi cisternae and migrate toward the flagellar pocket, where they fuse and discharge their contents into the flagellar pocket. ER cisternae are seen around the nucleus, and these radiate toward all regions of the cell, especially the peripheral microtubule-containing region. Both rough and smooth ER cisternae are present. The Golgi complex is always located close to the flagellar pocket and is essentially similar to that found in other cells. The Golgi complex can be labeled using gold-labeled lectins, which reveal sugar-containing molecules, indicating its involvement in protein glycosylation as reported for other eukaryotic cells. Rab7, a small GTPase involved in membrane trafficking, was also detected in the Golgi complex of trypanosomes (Araripe et al., 2005).

16.1.11 The Endocytic Pathway

Trypanosomatids are highly polarized cells, and their endocytic activity is restricted to the flagellar pocket and cytostome regions (review in De Souza et al., 2009). Studies of *T. cruzi* have shown that this protozoan exhibits certain peculiarities in its endocytic pathway that distinguish it from other cells. First, endocytosis only occurs at high levels in the epimastigote. Endocytosis is low or absent in metacyclic and bloodstream trypomastigotes and in intracellular amastigotes. Second, epimastigotes have two sites in which macromolecule uptake takes place: the flagellar pocket and a highly specialized structure known as the cytostome. Third, the cargo of the endocytic vesicles is delivered to unusual structures called reservosomes, which are located at the posterior end of the cell. As mentioned previously, *T. cruzi* epimastigotes use two specialized regions of the cell surface to ingest macromolecules from the environment: the flagellar pocket and the cytostome. The cytostome is a plasma membrane invagination coupled to a few special microtubules that penetrate the cell almost to the nucleus. The opening of this complex, which is known as the cytostome, has a diameter of up to 0.3 μm. This diameter is significantly smaller in the deeper portion, the cytopharynx, such that the structure resembles a funnel. There is a specialized region of the membrane lining the parasite that starts in the opening of the cytostome and projects toward the flagellar pocket. Quantitative analysis of the ingestion of gold-labeled macromolecules showed that in *T. cruzi* epimastigotes, about 85% of gold particles were associated with the cytostome (Porto-Carreiro et al., 2000). When epimastigotes are incubated with acridine orange, a weak base that accumulates in acidic compartments, the dye collects in the cytopharynx, suggesting that this compartment is acidic. Additionally, the presence of a P-type H^+-ATPase has been demonstrated, further supporting the acidic nature of the cytostome (Vieira et al., 2005). Following binding to the cytostome and flagellar pocket, macromolecules are rapidly internalized and appear in small endocytic vesicles, which bud from regions of these structures. Coated vesicles, suggestive of a clathrin coat, were first reported to bud from the Golgi complex. It was shown (Corrêa et al., 2007, 2008) that transferrin uptake is dependent

both on membrane cholesterol and on cytoskeletal elements that are associated with the cytostome. *In silico* analysis revealed the presence of clathrin, adaptin, and clathrin self-assembly genes.

Moreover, clathrin expression in *T. cruzi* has been demonstrated with western blots using polyclonal antibodies raised against bovine clathrin heavy chain. TcClathrin has been localized to the Golgi complex and flagellar pocket region. Curiously, agents that disturb receptor-mediated endocytosis do not impair transferrin uptake in epimastigotes (Correa et al., 2007, 2008). The fusion of endocytic vesicles with the tubule−vesicular network can be observed from the perinuclear region to the posterior tip of the cell. Using acridine orange to probe the pH of intracellular compartments, this tubular structure has been shown to be acidic. The cargo pathway kinetics and pH suggest that this compartment may correspond to the epimastigote early endosome (EE). The spatial distribution and morphology of the EE have been detailed with a 3D reconstruction of a sequence of ultrathin sections.

The major protease of *T. cruzi*, cruzipain, belongs to the cysteine protease family. It is very active in epimastigotes and is concentrated in reservosomes. The enzyme is a glycoprotein that is synthesized in the ER−Golgi system as a proenzyme and is then targeted to the endocytic pathway. The pro-peptide sequence is necessary and sufficient to drive cruzipain to reservosomes (Souto-Padron et al., 1990).

Macromolecules from the extracellular medium or from the ER−Golgi system are concentrated in structures known as reservosomes. These organelles are particularly interesting because they are found exclusively in members of the *Schizotrypanum* subgenus, such as *Trypanosoma vespertilionis*, *Trypanosoma dionisii*, and *T. cruzi*. Reservosomes are unique organelles that have a pivotal role in the life cycle of *T. cruzi*. They were named for their unusual capacity to accumulate all of the macromolecules that are ingested by the parasite via endocytosis (Soares et al., 1992). The main function of reservosomes in *T. cruzi* epimastigotes is to store macromolecules, although they also contain high concentrations of lysosomal hydrolases. In fact, reservosomes are also considered the main site of protein degradation and regulation. Each epimastigote has several reservosomes, primarily in the posterior region of the cell (Figure 16.12).

These organelles are surrounded by a unit membrane with a mean diameter of 0.6 μm. It is usually globular but may appear asymmetrical. Ultrastructural cytochemical studies have shown that the electron-dense portion of the reservosome matrix is mainly composed of proteins, while the electron-lucent inclusions are likely to be lipids. Proteins, especially basic proteins, can be localized using the ethanolic phosphotungstic acid technique in cells that have been previously fixed in glutaraldehyde but not post-fixed in osmium tetroxide. Recently, using different TEM approaches (Sant'Anna et al., 2008), the presence of internal vesicles both in isolated reservosomes and *in situ* was demonstrated. Long membrane profiles traversing the reservosome lumen were also observed, as was a noticeable rod-shaped electron-lucent structure bound by a membrane monolayer.

Using the DAMP technique to evaluate pH at the level of electron microscopy, reservosomes were found to be comparable to mammalian late endosomes

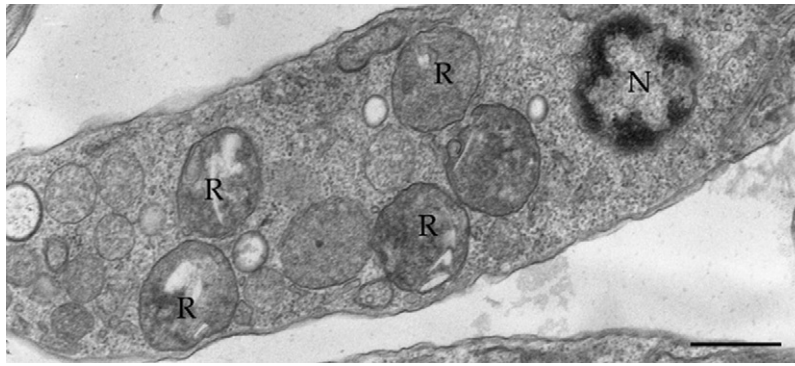


Figure 16.12 TEM of *T. cruzi* reservosomes. Ultrathin section of an epimastigote showing reservosomes (R) *in situ*, with their typical morphology and position, between nucleus (N) and posterior end of the cell. Bar = 1 μm.
Source: After Sant'Anna et al. (2009).

(prelysosomes). They have a pH of 6.0 and contain acid hydrolases but lack a lysosomal molecular marker (Soares et al., 1992).

Reservosomes have been described as a structure unique to epimastigotes. While lipid and protein uptake has never been demonstrated in either trypomastigotes or amastigotes, intracellular organelles that share many reservosomal features were recently described in the *T. cruzi* mammalian stages (Sant'Anna et al., 2008). Like reservosomes, these organelles are concentrated in the parasite's posterior region. Additionally, they accumulate cruzipain, as well as its natural inhibitor chagasin and serine carboxypetidase. The organelles are acidic and have a P-type H^+-ATPase. Interestingly, rod-shaped electron-lucent lipid bodies, similar to those that were recently characterized in the reservosome lumen, were also found in trypomastigote and amastigote hydrolase-rich compartments. Collectively, these results indicate that these two types of compartments are closely related. Nonetheless, the hydrolase-rich compartments differ from reservosomes in the ability to store external macromolecules. Because of their low internal pH and accumulation of lysosomal hydrolases, we have proposed that epimastigote reservosomes and trypomastigote and amastigote organelles can both be considered lysosomal-related organelles (LROs) since they share fundamental properties with mammalian lysosomes.

It has been shown that typical reservosomes disappear during the transformation of epimastigotes into metacyclic trypomastigotes *in vitro*. Morphometrical analysis showed that the reservosomes may account for 6% of the total cell volume in epimastigotes. During the transformation into trypomastigotes, the lipid-like structures disappear first, followed by the contents of the reservosome matrix.

Reservosomes are thought to be the final destination for macromolecules captured from the extracellular medium and are also a major site of accumulation for parasite proteases. This organelle probably has lysosomal functions, especially because classical lysosomes have never been identified in *T. cruzi*. Nevertheless, aryl sulfatase

activity, which is characteristic of lysosomes, has been detected inside small vesicles distributed all over the cell body of epimastigotes and trypomastigotes (Adade et al., 2007). The digestive function of these compartments has not been addressed.

16.1.12 The Cell Surface

Scanning electron microscopy (SEM) is the most commonly used electron microscopy approach to analyze cell surfaces because it allows for the visualization of the whole surface of the cell. Recently, more detailed information has been obtained with the use of a high-resolution field-emission scanning electron microscope (Sant'Anna et al., 2005). Using such an instrument, it is possible to visualize specific areas of the cell surface, such as the cytostome of epimastigotes, a structure involved in the uptake of macromolecules from the medium through endocytosis. SEM has also been used with gold-labeled lectins, which can be imaged using electron backscattering, to visualize the surface domains of the protozoan.

Conventional TEM of thin sections is certainly the most common approach for analyzing the structural organization of cells. However, it only gives a general idea of the thickness and density of the glycocalyx. It has been shown that the trypomastigote form of *T. cruzi* has a surface coat that is thinner than the thick coat that is characteristic of the bloodstream form of *T. brucei* (Vickerman, 1969; De Souza and Meyer, 1975). The surface coat can be better visualized using cytochemical methods that detect carbohydrate-containing macromolecules (De Souza and Meyer, 1975). Cationic dyes, which bind to components of the coat and are intrinsically dense (e.g., cationized ferritin particles and colloidal iron hydroxide particles) or osmiophilic (e.g., ruthenium red), can also be used (De Souza et al., 1977).

The quick-freezing, freeze-fracture, deep-etching technique allows for simultaneous observation of the protoplasmic face of the plasma membrane and a small area of the actual cell surface. Another possibility for visualization of large areas of the actual cell surface is the use of the fracture-flip technique. Figure 16.13 shows the surface of trypomastigote forms of *T. cruzi* that were prepared with this technique (Pimenta et al., 1989).

It is clear that the surface of the trypomastigote is very rugose. The rugosity probably corresponds to proteins exposed on the cell surface. In the case of epimastigotes, the lining of the cytostome region is more rugose than that covering the cell body and flagellum.

The conventional freeze-fracture technique allows for the examination of the inner portion of the plasma membrane. Figure 16.14 shows an image of a *T. cruzi* epimastigote that was prepared using the freeze-fracture technique (Martinez-Palomo et al., 1976).

The use of this technique makes it evident that the plasma membrane is not homogeneous in terms of density and distribution of intramembranous particles (Figure 16.14). Indeed, this technique made it possible to identify at least three macrodomains of the membrane (Martinez-Palomo et al., 1976; De Souza et al., 1978; review in De Souza, 2007): the cell body, the flagellum, and the flagellar

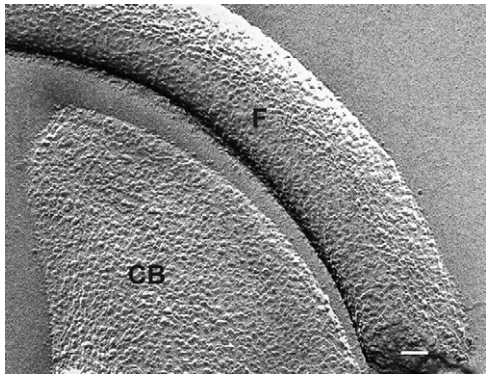


Figure 16.13 Fracture flip of the surface of the cell body (CB) and flagellum (F) of the trypomastigote form of *T. cruzi*. The surface appears with many rugosities. Bar = 100 nm.
Source: After Pimenta et al. (1989).

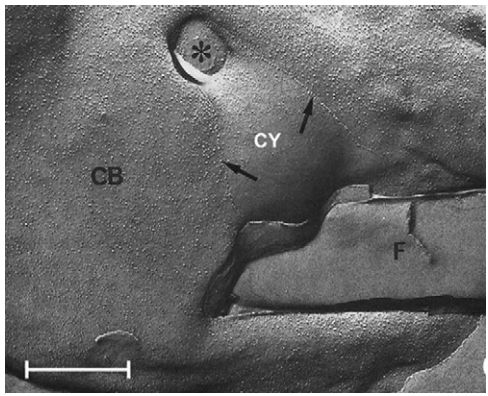


Figure 16.14 Freeze fracture view of *T. cruzi* epimastigotes where it is possible to identify three macrodomains of the membrane: the cell body (CB), the flagellum (F), the aperture of cytostome (CY).
Source: After Martinez Palomo et al. (1976).

pocket. Each of these macrodomains possesses specific microdomains, such as (i) the flagellar necklace, localized at the basal portion of the flagellum; (ii) the zone of attachment of the flagellum to the cell body, where a linear array of intramembranous particles exists on both fracture faces of the flagellar membrane lining the adhesion region; and (iii) the cytostome region, observed in epimastigote and amastigote forms of *T. cruzi*. The cytostome is an invagination of the plasma membrane accompanied by a few specialized microtubules that penetrate almost to the nucleus of the cell. The opening of the cytostome may reach a diameter of 0.3 μm, but it is significantly smaller in the deeper portion, the cytopharynx, which resembles a funnel. Freeze-fracture studies have shown that the membrane lining the cytostome is delimited by a palisade-like array of closely associated particles, corresponding to transmembrane proteins that remain unidentified 30 years after their initial description. The delimited area is almost devoid of transmembrane proteins, appearing smooth in freeze-fracture replicas. However, when the replicas were flipped, exposing the actual surface, the membrane lining the cytostome appeared very rugose. As described earlier, this area contains a fibrillar material exposed on the surface. The combination of freeze-fracture and cytochemical data indicates that the membrane lining the cytostome is rich in glycoconjugates that are not inserted in the membrane.

Figure 16.15 Freeze fracture image revealing a special organization of intramembranous particles in the portion of the flagellar membrane involved in the adhesion of the flagellum to the cell body. Bar = 100 nm.
Source: After Martinez Palomo et al. (1976).

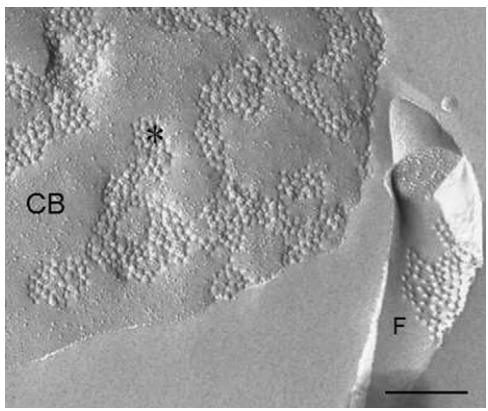


Figure 16.16 Freeze fracture image of an epimastigote form of *T. cruzi* incubated in the presence of filipin and then fixed. Filipin−sterol complexes (asterisks) can be seen both in the flagellar membrane (F) and in the membrane lining the cell body (CB). Bar = 0.5 μm.
Source: After Souto Padrón et al. (1983).

Freeze-fracture also revealed details of the area of adhesion of the flagellum to the cell body. A unique array of intramembranous particles could be seen in the fracture faces of the membrane lining the flagellum and cell body at the attachment regions (Figure 16.15).

The Flagellum Adhesion Zone (FAZ) region of the epimastigote shows a row of Intra Membrane Particle (IMP) clusters and a linear array of IMPs on both the Protoplasmic Face (PF) and Extracellular Face (EF) faces that could be involved with the FAZ region. The IMPs were longitudinally oriented in relation to the main axis of the flagellum. The cytostome was observed as a region with no IMPs that was delimited by a linear array of IMPs (Rocha et al., 2006).

Freeze-fracture cytochemistry allows for the identification of components of the plasma membrane. The most successful example of this was the use of digitonin and filipin to localize cholesterol and other ß-hydroxy sterols in *T. cruzi* epimastigotes. These compounds establish complexes with sterols and induce the appearance of either cylindrical (digitonin) or protruding (filipin) structures, which can be easily recognized in freeze-fracture replicas. Figure 16.16 shows the distribution of filipin−sterol complexes in *T. cruzi* epimastigotes (Souto-Padron and De Souza, 1983).

16.2 Fine Structure of the Interaction of *T. cruzi* with Host Cells

The first steps of the *T. cruzi*−mammalian host cell interaction process can be divided into three stages: (i) adhesion and recognition, (ii) signaling, and (iii) invasion. The adhesion step involves the recognition of molecules present on the surface of both the parasite and the host cell. Adhesion is a process that depends on receptors restricted to membrane domains.

The adhesion process is seen more clearly with the use of cytoskeleton inhibitors, like cytochalasins, that inhibits the invasion process (Meirelles et al., 1982; Rosestolato et al., 2002). It is well-known that lectins or lectin-like molecules are involved in the adhesion process (Meirelles et al., 1999). The sugar residues involved vary according to the parasite developmental form and host cell. One example is the participation of sialic acid. In macrophages, the depletion of parasite surface sialic acid by different treatments enhances both adhesion and infection. Here, the sugar residue recognized by macrophages could be galactose/*N*-acetyl galactosamine (Araújo Jorge and De Souza, 1984).

Different strains of *T. cruzi* as well as different forms of the parasite (tissue culture-derived trypomastigotes, metacyclic trypomastigotes, and amastigotes) express different molecules on their surface. These surface molecules interact with host components to help the parasite invade mammalian cells. In the case of trypomastigotes, several surface-exposed glycoproteins have been described that play roles in the interaction process, including the following: (i) gp90, which seems to have glycosidase activity, downregulates host cell invasion probably due to its lack of Ca^{2+} signal-inducing activity (Dorta et al., 1995; Yoshida, 2006); (ii) mucins, which are the major *T. cruzi* surface glycoproteins, have sugar residues that interact with mammalian cells (Villalta et al., 1984; Yoshida et al., 1989). Many mucins have been implicated in the host cell infection process (Yoshida et al., 1989; Di Noia et al., 1995; Buscaglia et al., 2006); (iii) the Tc85 molecule, which is abundant in trypomastigotes, is part of the gp85/trans-sialidase family, which includes other proteins such as gp85, gp82, TSA-1, and trans-sialidases. This superfamily shares common motifs with bacterial neuraminidases; however, all members of this superfamily contain a conserved sequence (VTVXNVFLYNR) that is absent in bacterial neuraminidases (Cross and Takle, 1993). Tc85 forms a population of heterogeneous GPI-glycoproteins with similar molecular masses but different electric charges (Katzin and Colli, 1983; Andrews et al., 1984; Abuin et al., 1989; Giordano et al., 1994). Because the Tc85 family is composed of multiadhesive glycoproteins, its members are capable of binding to different receptor molecules either located on the cell surface, like host cell cytokeratin 18 (Giordano et al., 1999), or belonging to components of the extracellular matrix, like fibronectin (Ouaissi et al., 1986) and laminin (Giordano et al., 1999); (iv) two groups of glycoproteins, gp82 and gp35/50, are also involved in parasite invasion. Both proteins are expressed on the surface of metacyclic trypomastigotes (Teixeira and Yoshida, 1986). These glycoproteins constitute the main surface molecules of the metacyclic form in different

T. cruzi strains and appear to be highly immunogenic. Mice immunized with heat-killed metacyclic trypomastigotes produce antibodies that predominantly recognize these antigens and lyse the metacyclic forms in a complement-dependent reaction (Teixeira and Yoshida, 1986). It is capable of activating Ca^{2+} signaling in this host cell. In 1998, Favoretto and colleagues demonstrated that gp82 is the signaling receptor that mediates protein tyrosine phosphorylation, which is necessary for host cell invasion. Phospholipase C and IP3 are also involved in this signaling cascade, which is initiated at the parasite cell surface by gp82 and leads to the $Ca2^{+}$ mobilization required to target cell invasion (Favoretto et al., 1998, reviewed by Yoshida and Cortez, 2008). These mucin-like gp35/50 molecules, which are abundant on the surface and resistant to protease digestion, are responsible for protecting the metacyclic trypomastigotes from destruction during infection by the oral route (Cortez et al., 2006); (v) another group of molecules present on the surface of culture trypomastigotes are the trans-sialidases. Trans-sialidase is an enzyme that is unique to *T. cruzi*. It is a surface-bound protein that is shed by the parasite into the external milieu. This trans-sialidase is also a modified sialidase that, instead of releasing sialic acid, can transfer it from sialoglycoconjugates in the host to terminal β-galactoses in the glycoconjugates of the parasite, which are unable to synthesize these molecules (Previato et al., 1985). This enzymatic activity is different from the known eukaryotic sialyltransferases present in the Golgi complex that exclusively use CMP-sialic acid as the donor substrate. The trans-sialidase gene family comprises at least 140 members, which can be classified into three groups according to the structure and function of the protein product (Acosta-Serrano et al., 2001). Trans-sialidases are expressed by trypomastigotes and are anchored by GPI to the parasite surface membrane. They have two main regions: an N-terminal catalytic region and a C-terminal extension with repeats of 12 amino acids (SAPA repeats) in tandem. Sialic acid is incorporated by trans-sialidases mainly into mucins (Schenkman et al., 1991). Sialylation of *T. cruzi* was shown to confer resistance to the human complement, which is a prerequisite for infection (Tomlinson et al., 1994); (vi) present in all strains of *T. cruzi*, Gp83 is a ligand employed by the parasite to attach and enter both professional and nonprofessional phagocytic cells (Lima and Villalta, 1988; Villalta et al., 2008). It is expressed only in infective trypomastigotes (Villalta et al., 1992); (vii) Penetrin, a 60 kDa protein that has an affinity to extracellular matrix elements, selectively binds to heparin, heparan sulfate, and collagen and promotes fibroblast adhesion and penetration (Ortega-Barria and Pereira, 1991); (viii) some *T. cruzi* proteases, including cruzipain, oligopeptidase B, and Tc80, have been implicated in the process of host cell infection. Cazullo and colleagues (1990) isolated and characterized a lysosomal cysteine proteinase from epimastigotes of *T. cruzi* that was given the trivial name "cruzipain." Souto-Padron and colleagues (1990) demonstrated that this cysteine proteinase is found in the endosomal−lysosomal (reservosome) system of epimastigotes but is also expressed on the surface of epimastigotes and amastigote−trypomastigote transitional forms. Furthermore, addition of antiproteinase antibodies to the interaction medium significantly inhibited the ingestion of parasites by macrophages. This cysteine protease is secreted through the flagellar pocket of

T. cruzi and has been described to cleave host high-molecular-weight kininogen to generate short-lived kinins, which bind to the bradykinin receptor to stimulate IP3-mediated Ca^{2+} release (Murta et al., 1990).

Oligopeptidase B, a serine endopeptidase, is secreted by *T. cruzi* trypomastigotes (Burleigh and Andrews, 1995; Burleigh et al., 1997). This soluble factor is generated by the action of a 120-kDa alkaline peptidase on precursors present only in infective trypomastigotes. This 80-kDa cytosolic serine peptidase indirectly induces $[Ca^{2+}]_i$-transients during *T. cruzi* invasion (Burleigh et al., 1997; Burleigh and Woolsey, 2002).

Tc80 is a prolyl oligopeptidase and a member of the serine protease family that hydrolyses human collagens type I and IV at neutral pH. Fibronectin is important for the parasite's transit through the extracellular matrix (Santana et al., 1997), and Grellier and colleagues (2001) showed that the parasite's entry into the host cell was blocked by treatment with selective inhibitors of Tc80. Thus, this molecule has been indicated as potential target for Chagas disease chemotherapy.

The amastigote surface components that are involved in the attachment and internalization of the parasite into host cells have not yet been well described. Evidence indicates that a carbohydrate epitope recognized by a monoclonal antibody 1D9 and abundant in lineage 1 *T. cruzi* (Verbisk et al., 1998; da Silva et al., 2009) could be implicated in this process, because Mab 1D9 specifically inhibits parasite invasion. Recently, da Silva and colleagues (2009) described a 21-kDa protein that is secreted at high levels by extracellular amastigotes. Pretreatment of host cells with P21-His_6 inhibited the cell invasion of extracellular amastigotes. On the other hand, when the protein was added to host cells at the same time as amastigotes, an increase in cell invasion was observed, suggesting that p21 may be involved in *T. cruzi* cell invasion.

Several surface-exposed molecules on the host cell have been shown to be involved in the process of *T. cruzi*–host cell interaction. These include proteins/glycoproteins released by trypsin and/or neuraminidase, lectin-binding sites, and lectin-like molecules (Scharfstein and Morrot, 1999; Magdesian et al., 2001).

Since *T. cruzi* entry into the host cell is a multifactorial process, many molecules that are present in the membrane of the host cell are potential partners for recognition. These factors can vary depending upon the cell type involved. One class of receptors present in mammalian cells consists of lectin-like molecules. Galectin-3 (Vray et al., 2004), a β-galactosyl-binding lectin, is a type of lectin involved in *T. cruzi* attachment, and its binding has been suggested to mediate parasite attachment and entry into both dendritic cells and smooth muscle cells (Kleshchenko et al., 2004). Using electron spectroscopic imaging (ESI) and lectins like WGA, RCAI, and Con-A, Barbosa and Meirelles (1992) detected galactosyl, mannosyl, and sialyl residues in regions of host cell plasma membrane that are internalized along with the parasite. Glycosylation mutants of Chinese hamster ovary (CHO) cells showed that adhesion and invasion of *T. cruzi* was impaired in cells that express very few sialic acid residues. If the deficient cell line was incubated in the presence of exogenous fetuin and *T. cruzi* trans-sialidase, the infection process was similar to that observed in the parental cells (Pereira et al., 1991). Integrins,

receptors that mediate attachment between two cells or between a cell and the extracellular matrix, are involved in the invasion processes. Tc85, which is present in the *T. cruzi* membrane and has been associated with parasite invasion, contains fibronectin-like binding sequences (Claser et al., 2008) and a laminin-binding domain (Giordano et al., 1999). Besides functioning as a cellular link to laminin or fibronectin, integrins also function as receptors that can activate PI3 kinase signaling pathways. Tc85 can bind to cytokeratin 18, a cytoskeletal protein that was suggested to function as a *T. cruzi* receptor (Magdesian et al., 2001). However, when cytokeratin 18 expression was silenced by RNAi, the binding of trypomastigotes to host cells was not affected (Claser et al., 2008). Another molecule present on the host cell surface and involved in trypomastigote entry is the TGFβ receptor. Signal transduction through TGFβ receptors facilitates *T. cruzi* entry into epithelial cells (Scharfstein and Morrot, 1999; Hall and Pereira, 2000). The trypomastigote molecule that is capable of binding to the TGFβ receptor has not yet been identified. Ming and colleagues (1995) proposed that infective stages of *T. cruzi* secrete a TGFβ-like molecule or a factor capable of activating latent host TGFβ. The exposure of phosphatidylserine on the surface of *T. cruzi* trypomastigotes (DaMatta et al., 2007) and its deactivating effect on macrophages by the induction of TGFβ suggests that phosphatidylserine is a possible activator of host cell TGFβ receptor.

The bradykinin receptors are another class of receptors used by trypomastigotes to penetrate mammalian cells. These receptors are coupled to a heterotrimeric G-protein, and the class consists of two subtypes: the bradykinin 2 receptor, which is constitutively expressed by cardiovascular cells, and the bradykinin 1 receptor, whose expression is upregulated in injured tissues (Grellier et al., 2001; Leeb-Lundberg, 2004). *T. cruzi* trypomastigotes are thought to use bradykinin B2 receptors, because infection of B2R-null mice results in increased parasitemia, mortality, and myocardial parasitism. To activate the bradykinin 2 receptor during invasion, *T. cruzi* relies on cruzipain to enzymatically generate bradykinin 2 (Leeb-Lundberg, 2004).

TrkA, a nerve growth factor receptor present in neuronal and dendritic cells, leads to *T. cruzi* invasion by binding to a trypomastigote trans-sialidase (Weinkauf and Pereira-Perrin, 2009). Moreover, many types of neurons and glial cells express a neurotrophic receptor called tyrosine kinase C (TrkC). *T. cruzi* binds to TrkC to maximize host−parasite equilibrium in the nervous system. The *T. cruzi*−TrkC interaction is mediated by trans-sialidase/parasite-derived neurotrophic factor (PDNF), which was previously identified as a TrkA ligand. Thus, TrkC is a new neurotrophic receptor that *T. cruzi* engages to promote the survival of neuronal and glial cells (Weinkauf and Pereira-Perrin, 2009).

Campos et al. (2001) reported that *T. cruzi*-derived GPI anchors linked to mucin-like glycoproteins and glycoinositolphospholipids (GIPLs) are recognized by TLR2/CD14 of host cells. Toll-like receptors activate nuclear factor NFκB and the interferon regulatory factor-dependent pathway (Tarleton, 2007). *T. cruzi*-derived GPI anchors can also phosphorylate mitogen-activated protein kinases (MAPKs) and IkB in macrophages (Ropert et al., 2004). Maganto-Garcia and colleagues (2008) demonstrated that Toll-like receptor 2 induces the phagocytosis of

trypomastigotes by activating Rab5. Tissue culture-derived trypomastigotes initiate an inflammatory process by triggering Toll-like receptor 2 in macrophages (Schmitz et al., 2009). The other Toll-like receptors that recognize *T. cruzi* are Toll-like receptor 4 and Toll-like receptor 9. A GPl ceramide isolated *T. cruzi* from epimastigotes has been suggested to interact with Toll-like receptor 4. Toll-like receptor 9 is known to be activated by GPI-anchors and the CpG-rich DNA of *T. cruzi* (Tarleton, 2007).

16.2.1 Triggering of Endocytosis

Following binding to and recognition of the parasite by the host cell surface, a series of cell signaling processes take place, which culminate in the invasion of the host cell. The available evidence indicates that the *T. cruzi* trypomastigotes use several mechanisms of signaling and invasion, including the following: (i) phagocytosis/macropinocytosis, an actin-mediated process in which the cells emit pseudopods. In professional phagocytes such as macrophages, the activation of tyrosine kinase proteins occurs, followed by the recruitment of PI-3 kinase and actin filaments (a process that has been associated with the mechanism of phagocytosis) at the trypomastigote entry site (Vieira et al., 2002). Thus, phagocytosis is the main mechanism of *T. cruzi* entry into macrophages (Figure 16.17); (ii) endocytosis, without the emission of pseudopods but with the participation of actin filaments; and (iii) invagination of the membrane, without the participation of actin filaments. This latter process has been regarded as an active mechanism for parasite entry that wastes energy (Schenkman and Mortara, 1992).

In the initial moments of the recognition between *T. cruzi* and the host cell, a transient increase of cytoplasmic levels of calcium occurs in both the parasite and

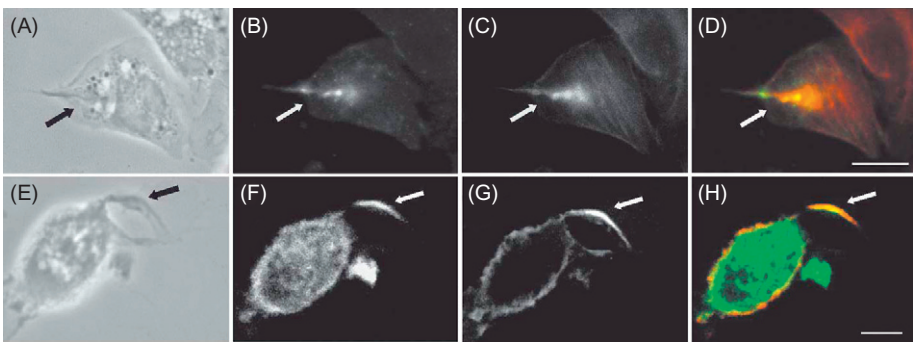


Figure 16.17 Immunofluorescence microscopy colocalization (arrows) of phosphorylated proteins (B, G), detected using antiphosphotyrosine antibody, actin filaments (C), detected using phalloidin, and PI3 kinase (F) detected using anti PI3 kinase antibody, during penetration of *T. cruzi* into macrophages. The overlay images (D, H) show the areas of colocalization demonstrating that phosphorylated proteins and microfilaments participate in internalization of *T. cruzi* trypomastigotes by macrophages. Bars = 5 μm.
Source: After Vieira et al. (2002).

the host cell (Wilkowsky et al., 1996; Garzoni et al., 2003; Yoshida et al., 2008). If this transient increase in cytoplasmic calcium is blocked (by treatment with thapsigargin, for example), there is a reduction in parasite invasion (Rodríguez et al., 1995). Additionally, in nonprofessional phagocytic cells, there is a recruitment of lysosomes to the place of parasite invasion, although this phenomenon occurs in only about 20% of parasites that enter. The lysosome-dependent pathway is initiated by targeted Ca^{2+}-regulated exocytosis of lysosomes at the plasma membrane. Another pathway used for parasite internalization in nonphagocytic cells is the lysosome-independent pathway. In this model, parasites enter cells through plasma membrane invaginations that accumulate PIP3, the major product of class I PI3K activation. As a result of this mode of entry, around 50% of total internalized parasites are contained in vacuoles enriched in plasma membrane markers, and about 20% are in early endosomes (EEA1 labeled) at 10 min postinfection. In this case, the immature vacuole becomes filled with lysosomes within an hour (Burleigh, 2005). Transient increases of calcium in the host cell cytoplasm after interaction with *T. cruzi* trypomastigotes have been shown to cause a reorganization of the actin cytoskeleton (Low et al., 1992; Rodríguez et al., 1995). Several studies using host cells treated with cytochalasins D or B (agents that depolymerize actin filaments) before the process of interaction with trypomastigotes produced controversial data. Some authors say that the treatment inhibits the entry of trypomastigote forms (Meirelles et al., 1982; Barbosa and Meirelles, 1995; Rosestolato et al., 2002), while others describe a sharp increase in entry (Tardieux et al., 1994) and still others report almost no effect. However, the time of treatment, trypomastigote interaction times, host cell nature, and strains of *T. cruzi* used varied among the experiments. In addition, we cannot exclude the possibility that attached parasites were considered as ingested.

Recently, it was shown that the actin cytoskeleton is important in the retention of trypomastigotes in the cytoplasm of the host cell (Woolsey and Burleigh, 2004). However, this paper also described that cytochalasin-treated host cells showed a reduction in the number of parasites inside them, confirming earlier data (Rosestolato et al., 2002) that showed a decrease in infection of cytochalasin pretreated host cells after 40 min of interaction with trypomastigotes (Shenkman and Mortara, 1992; Tardieux et al., 1994). The entry of the trypomastigote activates signaling processes in the host cell and parasite that lead to parasite invasion. In professional phagocytes, tyrosine kinase activation and recruitment of PI3 kinase and actin to the site of parasite entry also occur (Tardieux et al., 1994; Vieira et al., 1994).

Activation of tyrosine kinases does not occur in nonprofessional phagocytic cells, as shown in studies in which inhibitors of these enzymes were shown to produce no reduction in the invasion process. However, the activation of PI3 kinase does occur (Woolsey et al., 2003), and this activation seems to be a regulator of the phagocytosis that involves host cell lysosomes (Woolsey et al., 2003). The involvement of tyrosine phosphatase in this process has also been shown.

Host cell plasma membrane microdomains were also shown to be involved in *T. cruzi* entry both in nonphagocytic and phagocytic cells (Barrias et al., 2007;

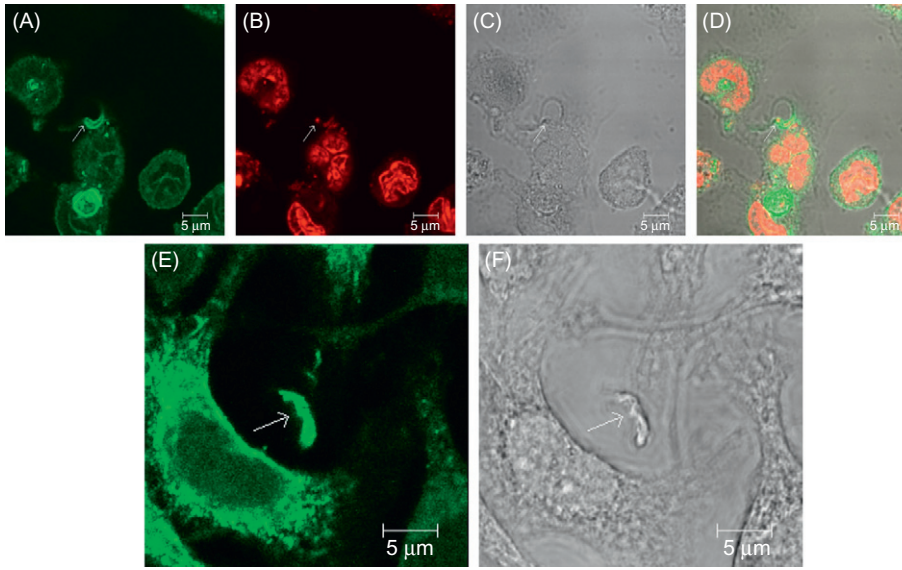


Figure 16.18 Imunofluorescence microscopy localization of GM1 (A−D) and flotillin 1 (E, F) during internalization of *T. cruzi* trypomastigotes by macrophages suggests the participation of membrane microdomains in this process. A−D: Colocalization of GM1, using cholera toxin subunit B (A), and an intracellular parasite (C arrow). B shows labeling of the nucleus and kinetoplast labeled with propidium iodide. C corresponds to a DIC image; D is a merge image. E, F: Colocalization of flotillin 1 (A), detected using a specific antibody, and trypomastigotes (B arrows). Bars = 5 μm.
Source: After Barrias et al. (2007).

Fernandes et al., 2007). Both groups demonstrated that cholesterol, major membrane raft components, and microdomain molecular markers like flotillin-1 colocalize with trypomastigote and amastigote entry sites, suggesting the participation of microdomains in *T. cruzi* invasion (Figure 16.18).

In the case of phagocytic cells, once the parasite attaches to the cell surface, there is activation of NAD(P)H oxidase. Enzymatic activity can be detected at the parasite−host cell interface, as well as within the PV.

16.2.2 PV Assembly

After the recognition process between the *T. cruzi* trypomastigote and a host cell, the parasite may or may not invade that cell. The participation of sugar residues in adhesion and internalization was described earlier. With the use of labeled Concanavalin A (fluorescein- or ferritin-labeled), it was possible to show that Con-A binding sites on the macrophage surface are internalized together with the trypomastigote, as Con-A was observed to be associated with the PV membrane (Meirelles et al., 1983). The interaction of trypomastigotes with cationized ferritin

prelabeled macrophages (at 4°C) showed that the PV containing the parasite was negative for cationized ferritin, although this marker could be observed inside some cytoplasmic vesicles. Macrophages incubated with cationized ferritin and horseradish peroxidase at 37°C ingested both makers and concentrated them in macrophage endocytic vacuoles. When these prelabeled macrophages were allowed to interact with trypomastigotes, the parasites were observed in vacuoles labeled with horseradish peroxidase but not in those labeled with cationized ferritin (Meirelles et al., 1984).

Carvalho and De Souza (1987) showed that opsonized trypomastigote and epimastigote forms interacting with macrophages activate NAD(P)H oxidase at the host cell membrane, and this enzyme is kept activated inside the PV. The first study describing the PV membrane composition used a macrophage cell line and showed (Hall et al., 1991) the presence of Fc receptors if trypomastigotes were opsonized with anti-*T. cruzi* antibodies, β1 integrin, and lysosomal membrane glycoproteins (lgp). If epimastigotes were opsonized with anti-*T. cruzi* antibodies, the presence of complement receptors (CR3), Fc receptors, β1 integrin, and lgp could be detected. The authors concluded that the host cell plasma membrane glycoproteins incorporated into the surrounding PV membrane differ depending upon the stage of parasite being internalized. These differences reflect, at least in part, selective ligation of cell surface receptors that mediate uptake. In addition, the nonopsonized trypomastigotes did not incorporate FcR or CR3 to be taken up by host cells, but they did not escape to fuse with lysosomes. Using muscle cells, Barbosa and Meirelles showed with Thiéry staining that glycoconjugates were present in the PV membrane. They also used ferritin-labeled RCAI to detect galactosyl residues and showed that in muscle cells, galactosyl residues accumulate in the parasite adhesion region, and these residues are internalized during parasite invasion. Tardieux and colleagues (1994) and Rodríguez et al. (1996) showed that lysosomes migrate early to the parasite entry site in nonphagocytic host cells, contributing membrane during the formation of the PV. They also described, as a requirement for parasite entry, the participation of microtubules and kinesin in the migration of lysosomes migration from the perinuclear region to the cell periphery (Rodriguez et al., 1994). Additionally, they showed that lysosome-membrane fusion is dependent on calcium (Rodríguez et al., 1999). Using macrophages as host cells, Ochatt and colleagues (1993) showed that GTP-regulated factors, but not calcium-regulated elements, are involved in the early inhibition of phagosome−lysosome fusion in *T. cruzi* infected macrophages. Carvalho and colleagues (1999) used fluorescent markers to show that host cell membrane lipids, proteins, and sialoglycoconjugates contribute to the membrane lining the PV, which contains the epimastigotes and trypomastigotes ingested by the macrophage. Lysosome fusion at the parasite entry site during early infection of macrophages by trypomastigotes has not been clearly shown. Using GFP-Rab5 and confocal microscopy, Wilkowsky and colleagues (2002) demonstrated the presence of Rab5 in early PVs containing *T. cruzi*, indicating that some PV fuses first with endosome vacuoles. Woolsey and colleagues (2003), using briefly infected nonprofessional phagocytic cells, showed that 50% or more of invading

T. cruzi trypomastigotes use host cell plasma membrane during PV formation. The authors suggested that this process is facilitated by the depolymerization of host cell actin microfilaments. They also showed that this vacuole is enriched in products from PI3 kinase and negative for lysosomal markers. Also, approximately 20% of *T. cruzi*-containing vacuoles were positive for EEA1 and Rab5, as well as Lamp-1. While investigating the early residence of *T. cruzi* in a phagolysosome, Andrade and Andrews (2004) blocked *T. cruzi* lysosome-mediated invasion and showed that the parasites were not retained inside the host cell. They concluded that the phagolysosome fusion is essential for parasite retention and development. Concerning the lysosome fusion at the parasite entry site, Tyler and colleagues (2005) showed that host cell microtubules polymerized at this site act as a secondary microtubule-organizing center. More recently, Romano and colleagues (2009) showed that the PV containing *T. cruzi* is decorated by the autophagic protein LC3. This paper also observed that: (i) Host cell starvation or pharmacological induction of autophagy before the infection with *T. cruzi* significantly enhances the number of infected cells, while inhibitors of this process prevent parasite invasion; (ii) the absence or reduction of two proteins required in the initial step of autophagosome formation (Atg5 and Beclin 1) reduces both parasite entry and Lamp-1 association with the PV; (iii) autolysosomes are recruited to the site of parasite entry. Fernandes et al. (2007) and Barrias et al. (2007) observed in the PV containing *T. cruzi* the presence of GM1, flotillin, and caveolin 1 shortly after infection, thus suggesting the presence of microdomains in the membrane lining the Tc PV. More recently, Barrias and colleagues (2010) demonstrated that dynamin is essential for PV formation, because the blockage of its GTPase activity by dynasore (a dynamin inhibitor) impairs parasite internalization (Figure 16.19).

16.2.3 Lysis of the PV Membrane

T. cruzi trypomastigotes use different receptors/linkers to get into host cells. Regardless of the mechanism used (fusion of lysosomes at the site of entry, participation of components of the plasma membrane, or initial fusion with endosome compartments at the site of invasion), the parasite will be located in a vacuole. Even after complete formation of the PV, fusion of endosomes and lysosomes with the vacuole takes place (Figure 16.20).

A few hours after internalization, the trypomastigote gradually transforms into an amastigote via an epimastigote-like intermediate stage. In the PV, trypomastigotes release trans-sialidase/neuraminidase, which removes sialic acid residues from the PV membrane, making it sensitive to the action of TcTox (a peptide that shares homology with human complement factor 9) (Andrews et al., 1990). At the acidic pH of the PV, this molecule begins to destroy the PV membrane, possibly by pore formation (Carvalho and De Souza, 1989; Andrews et al., 1990) (Figure 16.21).

We hypothesize that the formation of these small pores, which are associated with the action of secreted enzymes like trans-sialidase/neuraminidase and proteases, by the parasite leads to the fragmentation of the PV membrane. Treatment

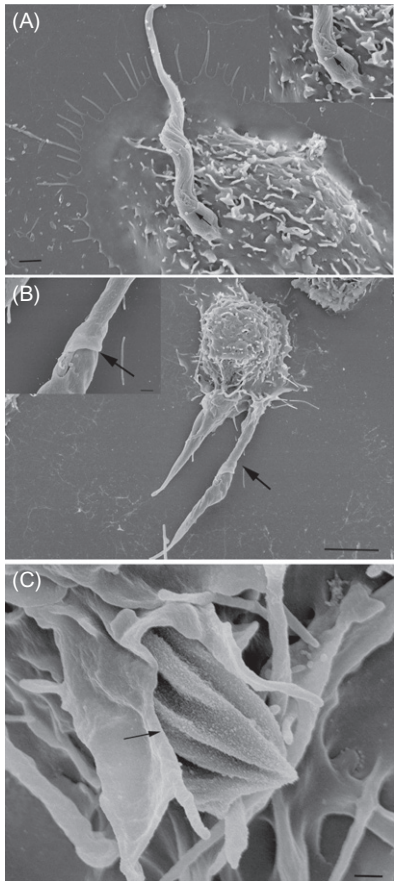


Figure 16.19 Field emission scanning electron microscopy of the interaction between peritoneal macrophages treated with 60 μM dynasore (for 20 min) and allowed to interact with *T. cruzi* trypomastigotes (A), epimastigotes (B), and amastigotes (C). All parasite evolutive forms were partially recovered by the macrophage plasma membrane indicating that the blockage of GTPasic dynamin activity did not impair the pseudopod extension, impairing only the complete vacuole formation. The interaction time was enough to complete the parasite entry into control macrophages. Bars = 1 μm.
Source: After Barrias et al. (2010).

of the host cell with drugs that raise the intracellular pH delays the fragmentation of the PV membrane (Ley et al., 1990). On the other hand, observations using CHO cells that are deficient in sialylation showed that the absence of sialic acid makes the PV membrane more sensitive to lysis (Hall et al., 1991). The presence of sialic acid residues seems to protect the lysosome membrane from lysis.

Some authors refer to this process as "escape" of the parasite from the PV. We prefer to call it "disruption" of the PV membrane. Following this disruption, the amastigote enters into direct contact with the host cell cytoplasm and starts a process of binary division. Further studies are necessary to better characterize the mechanism involved in this important step of the *T. cruzi* life cycle.

The amastigote form was also shown to be infective to both phagocytic and non-phagocytic cells (Carvalho and De Souza, 1983; Nogueira and Cohn, 1976). Such an infective ability is important for an organism that cycles through different hosts (Mortara et al., 2008). Observing blood smears of mice infected with *T. cruzi*, we could detect the presence of amastigotes (or an amastigote-like form) (Figure 16.22).

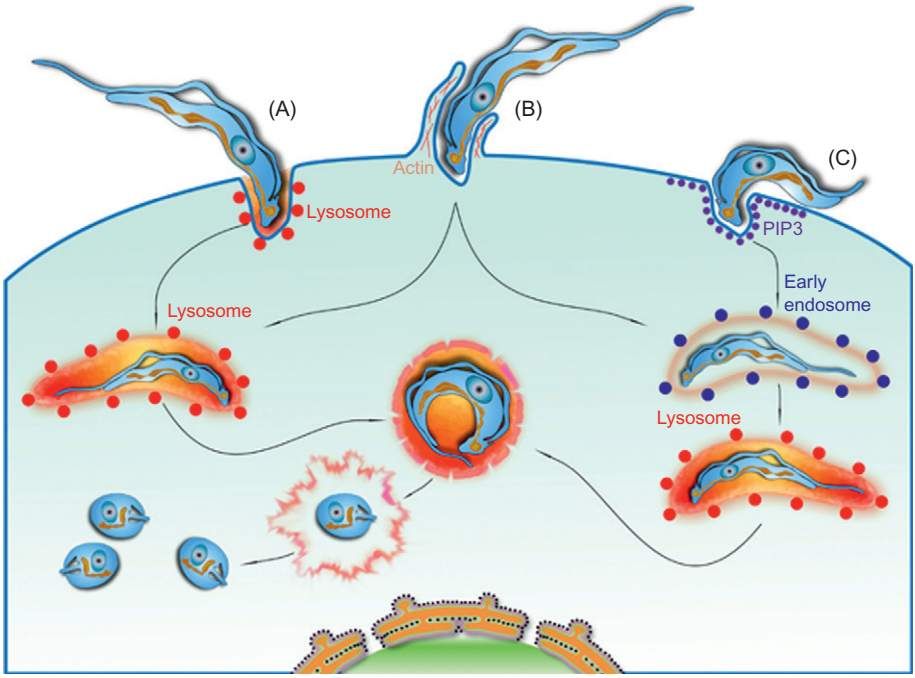


Figure 16.20 Model of *T. cruzi* invasion. The model indicates three distinct mechanisms of *T. cruzi* entry into host cell. (A) The lysosome dependent pathway is initiated by targeted Ca^{2+} regulated exocytosis of lysosomes in the plasma membrane. (B) In the actin dependent pathway trypomastigotes penetrate into a host cell through a plasma membrane expansion that culminates in assembly of a PV. Either early endosomes or lysosomes can fuse with the PV. (C) In the lysosome independent pathway, parasites enter cells through plasma membrane invaginations that accumulate PIP3 (product of class I PI3K activation). Subsequently, internalized parasites are contained in vacuoles formed from the plasma membrane and it maturates with the acquisition of early endosome markers (Rab5 and EEA1) and subsequently with the acquisition of lysosome markers. Later on, the trypomastigote form gradually transforms into a amastigote form with simultaneous lysis of the PV membrane. Then, amastigotes in direct contact with the cytoplasm start to divide.

Andreoli and colleagues (2006) showed that amastigotes were recognized by a monoclonal antibody against C9 complement protein, suggesting that TcTox is also present and active in the intracellular amastigote. Amastigotes may be able to use trans-sialidases, proteases, and TcTox to lyse the PV membrane more quickly than the trypomastigote can. However, Stecconi-Silva and colleagues (2003) showed negligible trans-sialidase and hemolytic activity (TcTox activity) by amastigotes after 12 h of incubation with red blood cells. They also showed that when the pH of the host cell cytoplasm is raised using chloroquine, metacyclic trypomastigotes remain in the PV almost twice as long, although the treatment had no effect on the

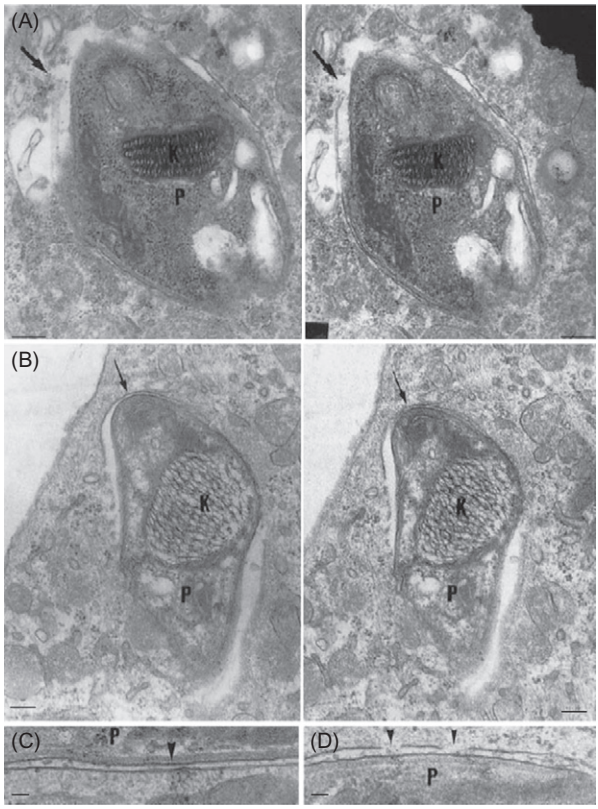


Figure 16.21 TEM of thin sections of macrophages infected with trypomastigote forms of *T. cruzi*. Micrographs taken at different inclination angles of the section. Focal disruption of the membrane lining the vacuole is observed (arrows in A and B), especially in C and D; K = kinetoplast, P = parasite. Bars = 1 μm. *Source*: After Carvalho and De Souza (1983).

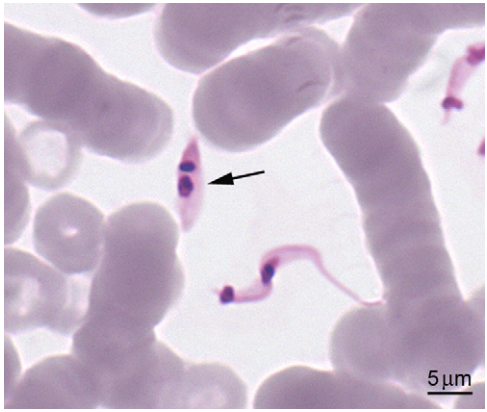


Figure 16.22 Amastigote like form (arrow) observed in a blood smear of mice experimentally infected with *T. cruzi.* A typical bloodstream trypomastigote is also seen. Bar = 5 μm. *Source*: Tecia Maria Ulisses de Carvolho.

release of amastigotes. The kinetics of the escape of metacyclics and amastigotes from the vacuole were not affected.

After several cycles of intracellular division, the amastigotes start the process of transforming into trypomastigotes, which are subsequently released into the

intercellular space. These trypomastigotes eventually reach the bloodstream and infect other cells.

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17 Genetics of *Trypanosoma cruzi*

17.1 Nuclear Genome

Daniella C. Bartholomeu[1], Gregory A. Buck[2], Santuza M.R. Teixeira[3] and Najib M.A. El-Sayed[4,*]

[1]Department of Parasitology, University of Minas Gerais, Minas Gerais, Brazil, [2]Center for the Study of Biological Complexity, Virginia Commonwealth University, Virginia, USA, [3]Department of Biochemistry and Immunology, Federal University of Minas Gerais, Minas Gerais, Brazil, [4]Department of Cell Biology and Molecular Genetics, Center for Bioinformatics and Computational Biology, Maryland Pathogen Research Institute, University of Maryland, Maryland, USA

17.1.1 Sequencing of the CL Brener Reference Strain: A Historical Perspective

In April 1994, an international group of parasitologists along with representatives of the World Health Organization met in Rio de Janeiro, Brazil, to discuss the initiation of efforts toward the sequencing of the genome of several human parasites, among them *Trypanosoma cruzi*. The clone CL Brener was selected as the reference strain for sequencing since it is well-characterized biologically. This clone, isolated by Professors Brener and Pereira (Centro de Pesquisa René Rachou, Fiocruz, Belo Horizonte, Brazil) presents important *T. cruzi* characteristics: (i) it was isolated from the domiciliary vector *Triatoma infestans*; (ii) its pattern of infectivity in mice is very well-known; (iii) it has preferential tropism for heart and muscle cells; (iv) it shows a clear acute phase in accidentally infected humans; and (v) it is susceptible to drugs used clinically in Chagas disease (revised by Zingales et al., 1997). Because the funds were limited at the time, initial priorities were set to generate karyotype data, physical maps, and **EST** sequences (Cano et al., 1995; Henriksson et al., 1995; Brandão et al., 1997; Verdun et al., 1998; Porcel et al., 2000). These activities were distributed among approximately 20 laboratories from 15 countries. In 1999, additional funds were obtained from the National

*E mail: elsayed@umd.edu

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Institutes of Allergy and Infectious Diseases/National Institutes of Health (NIAID/NIH) by a consortium formed by three genome centers, the Institute for Genomic Research (Rockville, USA), the Seattle Biomedical Research Institute (Seattle, USA), and Karolinska Institute (Stockholm, Sweden), which enabled the deciphering the nuclear genome of the parasite. Approximately 80 researchers from 14 countries contributed to the data analysis that was published in 2005 (El-Sayed et al., 2005a) along with the *Trypanosoma brucei* (Berriman et al., 2005) and *Leishmania major* (Ivens et al., 2005) genomes and a comparative analysis of the genome architecture of the three parasites (El-Sayed et al., 2005b). The genome information is central for a better understanding of the biology of these parasites and will hopefully aid in the translation of basic research into clinical therapies.

17.1.2 Sequencing Strategy, Genome Organization, and Content

The CL Brener strain is a representative of the *T. cruzi* hybrid lineage VI (Zingales et al., 2009). The parasite genome is diploid but displays a high degree of difference between **homologous chromosomes**. Also, **heterologous chromosomes** may have similar sizes and triploidy has been reported (Henriksson et al., 1995; Obado et al., 2005; Branche et al., 2006). In addition to its hybrid nature (Brisse et al., 1998; Machado and Ayala, 2001; Westenberger et al., 2005), CL Brener is one of the most repetitive genomes sequenced to date, with a repeat content close to 50%. The genome consortium's choice of sequencing strategy was constrained.

The high complexity of the *T. cruzi* molecular karyotype precluded the use of a whole chromosome shotgun (WCS) approach, and the high repeat content complicated the bacterial artificial chromosome (BAC) "map-as-you-go" clone-by-clone strategy in the early stages of the project. A whole genome shotgun (WGS) strategy was finally adopted. As expected, the high level of allelic variation between the CL Brener **haplotypes** and the overall repeat content made the genome assembly quite challenging. Long repeats are problematic because genome assemblers cannot differentiate well true reads overlaps from those induced by repeats. In addition, typical assemblers are not able to handle highly polymorphic genomes. Ambiguities derived from allelic variations need to be discriminated from sequencing errors. This is possible because base-calling errors are frequently associated with low quality values and they do not tend to be confirmed by other reads. Highly polymorphic genomes, however, require a much higher level of sequence **coverage** to ensure these inferences are reliable. In the case of *T. cruzi*, 14× coverage was achieved. The assembly of this polymorphic genome generated a redundant dataset. This is because homologous regions displaying a high level of polymorphism were assembled separately, generating two **contigs**, one corresponding to each haplotype.

Based on sequence coverage on CL Brener genomic regions represented by the two haplotypes, it was estimated that the diploid genome is around 106–110 Mb

(El-Sayed et al., 2005a). The genome assembly, however, resulted in 67 Mb, represented by 5489 **scaffolds** that were built from 8740 contigs. Of these, 60.4 Mb were used as substrate for gene finding and the downstream analysis such as annotation; this is because contigs in scaffolds smaller than 5 kb or contigs not incorporated into scaffolds and smaller than 5 kb were excluded. The rationale behind excluding this dataset was that its fragmented nature would hamper the gene prediction. Nevertheless, a large proportion of nonannotated sequences correspond to long, near-identical segmental duplications, including noncoding sequences and members of nonpolymorphic multigene families organized in tandem, which were collapsed or misassembled (El-Sayed et al., 2005a; Arner et al., 2007). For instance, the *T. cruzi* 195-bp satellite DNA is the most abundant repetitive DNA sequence of the parasite (Gonzalez et al., 1984). Copies of this repetitive element can be duplicated as tandem arrays of approximately 30 kb (Elias et al., 2003). By analyzing CL Brener individual reads, it was estimated that this repeat corresponds to approximately 5% of the CL Brener genome, but it represents only 0.09% of the annotated dataset, indicating that a large number of satellite copies were not incorporated into the assembled data (Martins et al., 2008). It is important to emphasize that the nonannotated dataset and the individual reads are also available at the Genbank and should always be analyzed before strong statements regarding gene content are made.

To be able to distinguish the two haplotypes, postassembly sequence comparisons with sequences from a representative of the parental subgroup Tcll (formerly named llb lineage) were performed (El-Sayed et al., 2005a). Approximately 120 Mb of Esmeraldo sequences (2.5 × genome coverage) were generated and compared with CL Brener annotated contigs, which were then classified into the following categories: (i) similar to the Esmeraldo haplotype (Tcll, formerly lineage IIb), (ii) dissimilar to Esmeraldo haplotype, (iii) homozygous or haploid regions, (iv) repetitive regions, and (v) merged regions. When an Esmeraldo read matched exactly two contigs, the contig region that displayed a higher identity match with the Esmeraldo read was classified as Esmeraldo-like haplotype, and the corresponding region of the other contig was classified as non-Esmeraldo haplotype. Regions where the Esmeraldo reads matched only a single contig were considered to represent haploid regions corresponding to the Tcll parent, if the coverage and/or single nucleotide polymorphism (SNP) density was low. On the other hand, if the coverage and/or SNP density was high, these regions were classified as homozygous or heterozygous regions with very similar sequence that had merged during assembly. Conversely, contigs showing no match to Esmeraldo reads (or matches covering less than 90% of the Esmeraldo read) were presumed to represent haploid regions from the non-Esmeraldo-like haplotype, although they may represent unsampled regions of the Esmeraldo because of low sequence coverage. When the Esmeraldo reads matched three or more contigs, the corresponding regions were classified as repetitive. Around 50.5% of the annotated dataset corresponds to heterozygous regions (Esmeraldo-like or non-Esmeraldo-like haplotype), 37.2% to repeats not merged, 9% to merged sequences (repeats and homozygous regions), and 3.3% to haploid regions.

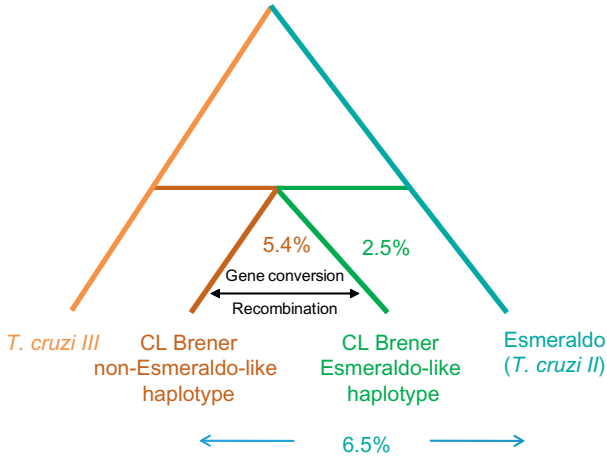

Figure 17.1.1 Sequence divergence among CL Brener Esmeraldo like and non Esmeraldo like haplotypes and Esmeraldo reads. The pairs of corresponding regions among Esmeraldo reads and the two CL Brener haplotypes were identified by nucmer (Delcher et al., 2002) and the average percentage divergence computed.

The two haplotypes display high levels of gene **synteny**, with most differences due to insertions/deletions in intergenic and subtelomeric regions and/or amplification of repetitive sequences. The average sequence divergence between the two CL Brener haplotypes is 5.4%. As expected, this value is smaller when comparing the Esmeraldo-like haplotype and Esmeraldo reads (2.5%) and larger when comparing the non-Esmeraldo-like haplotype and Esmeraldo reads (6.5%) (Figure 17.1.1).

Pairs of alleles were identified for approximately half of the CL Brener genes. The average divergence between the coding regions of CL Brener genes is 2.2% (versus 5.4% overall difference), indicating that the large difference between the two haplotypes is due to polymorphism in the intercoding regions. The haplotype classification for each single CL Brener gene based on the type of match with Esmeraldo reads is available through Genbank and Tritryp database (http://www.tritryp.org).

The annotated genome dataset corresponds to 838 scaffolds, built from 4008 contigs and totaling 60.4 Mb. The diploid genome contains approximately 23,000 genes, with more than 50% of the genome consisting of repeated sequences. Those include retrotransposons and members of large multigene families encoding surface proteins such as the trans-sialidases (TS) superfamily, mucins, the surface glycoprotein 63 proteases (gp63), and a novel family of about 1400 mucin-associated surface protein (MASP) genes (El-Sayed et al., 2005a; Bartholomeu et al., 2009) These gene families are mostly *T. cruzi*-specific, account for one-fifth of the total protein-coding genes, and occur in dispersed clusters of tandem and interspersed repeats. Putative functions were assigned to approximately 50.8% of the annotated protein coding genes, based on significant sequence similarities with previously characterized proteins and the presence of functional domains. All datasets and genome annotations are available through GeneDB (http://www.genedb.org) and Tritryp database (http://www.tritryp.org).

17.1.3 Comparative Genome Sequencing and Analyses

17.1.3.1 Comparative Genome Analysis with Other Trypanosomatids

The simultaneous availability of the complete genome sequences of two other trypanosomatids, *T. brucei* (Berriman et al., 2005) and *L. major* (Ivens et al., 2005), allowed comparisons of the gene content and genome architecture of the three parasites and a better understanding of the genetic and evolutionary bases of the shared and distinct parasitic modes and lifestyles of these pathogens. These analyses revealed that the three genomes display striking synteny (Figure 17.1.2) and share a conserved core of approximately 6200 genes, 94% of which are arranged in syntenic directional gene clusters (El-Sayed et al., 2005b).

An amino acid alignment of a large subset of the three-way clusters of **orthologous genes** (COGs) reveals an average 57% identity between *T. cruzi* and *T. brucei*, and 44% identity between *T. cruzi* and *L. major*, reflecting the expected phylogenetic relationships (Lukes et al., 1997; Haag et al., 1998; Stevens et al., 1999; Wright et al., 1999). Most species-specific genes, of which *T. cruzi* (32%)

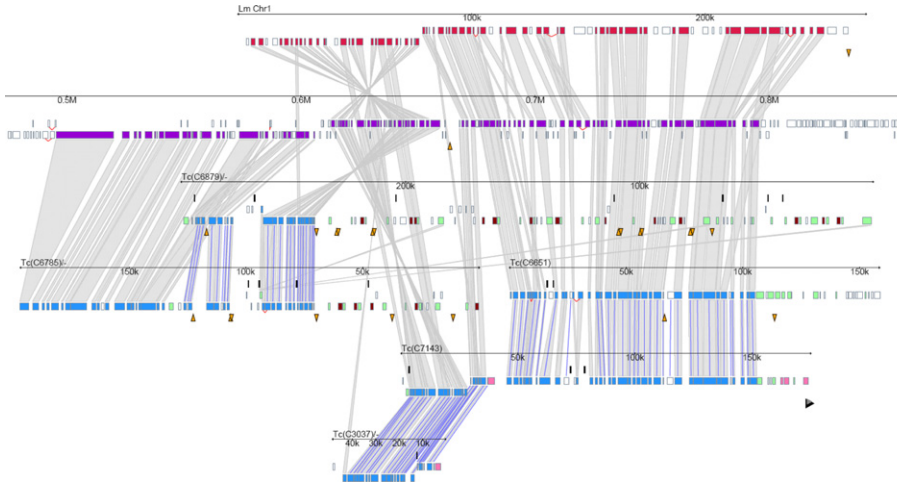

Figure 17.1.2 Comparative architecture of *T. cruzi* CL Brener haplotypes (Tc), *T. brucei* (Tb), and *L. major* (Lm) genomes. Lm chromosome 1 was selected as reference to illustrate the organization of the three genomes and their striking degree of synteny. Lm, Tb, and Tc genes are colored in red, purple, and blue, respectively. Gray lines link genes that belong to the same COGs. Genes colored in white represent singletons, while those in gray belong to COGs that are not shown. Yellow and black rectangles represent retrotransposons and telomeric repeats, respectively. Blue lines link pairs of alleles of each CL Brener haplotype. Tc colored genes represent surface protein coding genes. Tc arrays of surface protein genes are boxed in red. Tc scaffolds are labeled with the last digits of a unique identifier where Cxxxx refers to Scaffold 104705351xxxx. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this book).

and *T. brucei* (26%), have a much greater proportion than *L. major* (12%) are found to occur at non-syntenic internal and subtelomeric regions and consist of members of large surface antigen families. Retroelements, structural RNAs, and gene family expansion are often associated with breaks in conservation of gene synteny, which along with gene divergence, acquisition, and loss, and rearrangements within syntenic regions, have shaped the genomes of each parasite (El-Sayed et al., 2005b).

Compared with the other two trypanosomatids, a remarkable feature of the *T. cruzi* genome is the extensive expansion of species-specific genes, the large majority encoding surface proteins, such as TS superfamily, MASPs, mucins TcMUC, GP63, among others, all of them involved in important host−parasite interactions (El-Sayed et al., 2005b). The *T. cruzi* surface protein-encoding genes are often clustered into large arrays that can be as large as 600 kb, preferentially associated with large chromosomes (Di Noia et al., 1995; Vargas et al., 2004; Baida et al., 2006; Bartholomeu et al., 2009). The available assembled data clearly demonstrates that the clusters of surface proteins are internal in the chromosomes at regions of synteny breaks with *T. brucei* and *L. major* (Figure 17.1.2). The synteny breaks of the hybrid CL Brener at the arrays of surface protein genes (Figure 17.1.2) suggest that these regions are or were subject to intense rearrangements during the parasite's evolution (El-Sayed et al., 2005a; Bartholomeu et al., 2009). It is likely therefore that much of the striking polymorphism among the *T. cruzi* isolates that is reflected in several epidemiological and pathological aspects of Chagas disease may be in part due to variability within these regions. Whole genome comparisons of distinct *T. cruzi* lineages would allow further investigation.

17.1.3.2 *Pathogenomics of* T. cruzi*: Planned Sequencing of Additional Strains*

As described in Chapter 19, a wide range of pathologies is found within the *T. cruzi* lineage. Thus, there is a wide range of evolutionary and pathological space yet to be explored through additional comparative sequencing. With the advent of massively parallel sequencing technologies, sequencing of additional trypanosomatid strains can be performed at a fraction of the cost of the sequencing of the reference genomes. In late 2006, two institutes within the National Institutes of Health (NIAID and National Human Genome Research Institute (NHGRI)) initiated a collaboration aimed at coordinating a sequencing effort to provide publicly available genomic data for the most significant eukaryotic pathogens and disease vectors. A target selection process (http://www3.niaid.nih.gov/LabsAndResources/resources/gsc/pathogen/selection.htm) was put in place and a world community of several hundred investigators were queried as to the value of sequencing additional isolates from the three main groups of trypanosomatid pathogens (including *T. cruzi*) and for advice as to which isolates are the best candidates for future sequencing. The consensus led to the identification of six first-priority isolates/strains of *T. cruzi* and is published online at http://www.genome.gov/Pages/Research/DER/PathogensandVectors/PathogensofTrypanosomatid.pdf. The American trypanosome strains prioritized for sequencing include *T. cruzi* Silvio X10 (*T. cruzi* I),

Esmeraldo (*T. cruzi* II), 3869 (*T. cruzi* III), Can III (*T. cruzi* IV), NRcl3 (*T. cruzi* V), and Tula cl2 (*T. cruzi* VI). These strains were strategically selected according to two main principles: coverage of the major subgroups within *T. cruzi* (Zingales et al., 2009) and coverage of closely related strains/isolates with clearly different pathogenesis.

Outlined below are two of the most outstanding questions in the pathogenesis of *T. cruzi* that we can begin investigating by sequencing.

1. *What is/are the genotypes associated with the ability of different strains or isolates to cause widely varied clinical manifestations? Are certain metabolic, regulatory, or genetic networks required for or associated with disease?* Chagas disease presents with a wide variety of clinical outcomes, including chronic chagasic cardiomyopathy, the "mega" syn dromes, or even totally asymptomatic carriers, and many patients do not manifest disease until years after the infection. The genetic bases of the diversity of clinical outcomes in these parasites are largely unknown. Genomic dissection of a select group of these para sites will provide a window into the genetic basis of these pathogenic characteristics. For example, it has been widely shown that the major lineages *of T. cruzi* exhibit significant differences in pathogenic potential. *T. cruzi* I, generally less pathogenic for humans, has a lower acute infectious profile and progression, a more extensive chronic profile, and invades and causes pathology in different organs. Comparison of at least one *T. cruzi* I isolate (e.g., Silvio X10) with the other isolates will provide an opportunity to discover the genetic basis of these phenomena.
2. *What are the genetic bases of the phenotypic (cell cycle, host range, vector selection, pathogenic and clinical manifestations, etc.) characteristics of the major groups of patho genic trypanosomatids?* Isolates of the six lineages of *T. cruzi* are quite divergent in many respects. Although superficially similar, their preferred hosts and vectors, method of invasion, effects on the invaded cells, levels of parasitemia, mechanisms of pathogene sis, and clinical outcomes are quite different. Whereas it is quite well established that the differences among *T. cruzi* isolates are genetically programmed, it is not yet established which genes or gene networks confer these different phenotypes. Thus, obtaining a good draft sequence, with the subsequent gene annotation and metabolic and other network reconstructions of these isolates (*T. cruzi* I Silvio X10, *T. cruzi* II Esmeraldo, *T. cruzi* III 3869, *T. cruzi* IV Can III, *T. cruzi* V NRcl3, and *T. cruzi* VITula cl2) will provide a basis for a comparison that will identify the genetic roots of these differences. A detailed description of the *T. cruzi* strains targeted for sequencing is described at http://www .genome.gov/Pages/Research/DER/PathogensandVectors/PathogensofTrypanosomatid.pdf.

A comprehensive genomic analysis of selected members of *T. cruzi* will provide an excellent step toward a better understanding of their biology and pathogenesis.

17.1.4 Transcription Mechanisms and Genetic Expression in *T. cruzi*

17.1.4.1 Unique Mechanisms of Control of Gene Expression and Gene Expression Profiling

There are marked differences in the way prokaryotes and eukaryotes regulate their gene expression. Being part of a group of early branching eukaryotes,

trypanosomatids have attracted the attention of parasitologists, not only for their medical relevance but also because they present distinctive features in their mechanisms controlling gene expression. Distinct promoter sequences controlling the expression of individual protein coding genes, which are recognized by RNA polymerase II and the requirement of pre-mRNA cis-splicing as a RNA-processing event are major characteristics of eukaryotic gene transcription (Licatalosi and Darnell, 2010). In trypanosomatids, transcription is polycistronic (i.e., several genes are transcribed in one large pre-mRNA) and because of the lack of introns, with only four exceptions (Ivens et al., 2005), cis-splicing does not occur in these organisms. However, since primary transcripts are polycistronic, cleavage of the pre-mRNA has to occur in the nucleus in order to produce monocistronic mRNAs that are capped and polyadenylated. In trypanosomes, cleavage of the pre-mRNA is linked to the addition of the 39 nucleotide miniexon (or spliced leader, SL) containing a methylated cap at the 5′ end and the poly (A) tail at the 3′ end of each mRNA (Matthews et al., 1994). Biosynthesis of mRNA in these organisms is also notable because of the fact that some protein coding genes can be transcribed by RNA polymerase I (Günzl et al., 2003) and most mitochondrial mRNAs have to undergo extensive RNA editing before mitochondrial proteins can be produced (Stuart and Panigrahi, 2002).

Adaptation of trypanosomes to distinct environments in the vertebrate and invertebrate hosts, as well as differentiation in distinct parasite forms, calls for major changes in morphology, surface composition, biochemical pathways, and thus, complex mechanisms to control gene expression. In most eukaryotes, transcriptional regulation is a major step of gene expression control. Recent studies, however, together with the completion of the Tritryp genomes, not only revealed a total lack of evidence for differential regulation of RNA polymerase II transcription but also no identifiable RNA polymerase II promoter consensus sequence in the genomes of trypanosomes. Thus, the lack of transcription initiation control implies that the knowledge of elements involved in posttranscriptional processes, such as trans-splicing, mRNA stabilization, and translation, is crucial for the understanding of gene expression in these organisms.

As previously indicated, gene organization in trypanosome chromosomes is also very peculiar. Large polycistronic transcription units encoding 20 or more proteins in one strand separated by strand switch regions (i.e., changes of the coding strand) were found initially in the *L. major* genome (Myler et al., 1999) and later in the Tritryp genomes (El-Sayed et al., 2005b). Before transcription is completed, the long pre-mRNA is processed in the nuclei by cleavage reactions that are coupled to two cotranscriptional RNA-processing events: trans-splicing of a small capped RNA of 39−41 nucleotides, the spliced leader RNA (SL-RNA) which is added to the 5′-terminus of all known protein-encoding RNAs, and 3′-end polyadenylation (El-Sayed et al., 2005b). Both events are dependent on polypyrimidine motifs (polyPY) located within the intergenic regions (Matthews et al., 1994). Again, in contrast to most eukaryotes, no canonical polyA addition signal has been identified, and only AG dinucleotides situated downstream from a polyPY motif are used as an SL acceptor site (El-Sayed et al., 2005b; Campos et al., 2008). Since

mRNAs derived from the same polycistronic mRNA precursor can present vast differences in their steady-state levels, gene expression modulation must depend heavily on regulatory pathways acting at the control of mRNA half-life. By employing this type of regulation, trypanosomes can ensure that rapid changes associated with transmission between insect vector and mammalian host are followed by an instant reprogramming of genetic expression.

Most of the early studies on gene expression in trypanosomatids were focused on the process of antigenic variation, the powerful survival strategy devised by African trypanosomes and allowing *T. brucei* bloodstream forms to escape the immunological attack from the mammalian host. Variant surface glycoproteins (VSGs) (Boothroyd et al., 1980) are the main surface molecules present at the surface of *T. brucei* bloodstream forms. While the genome of this parasite contains about 1000 VSG genes, only one VSG, present in telomeric locations called VSG bloodstream expression site (BES), is active at a time (Donelson, 2003). Understanding the mechanisms controlling VSG expression, particularly the *in situ* switch (i.e., the mechanism responsible for the activation of one telomeric BES concomitantly with the inactivation of all another BES) (there are about 15 VSG BESs), has been a difficult task, but has allowed the discovery of a large body of information about gene expression in this group of organisms (for a recent review, see Stockdale et al., 2008). Compared with *T. brucei*, studies on gene expression in *T. cruzi* had a late start. Whereas the first *T. cruzi* gene was cloned in 1986 (Peterson et al., 1986), characterization of some of the key players involved in gene expression control in this parasite has only recently begun.

Initial studies on stage-specific gene expression in *T. cruzi* indicated that, similar to what had already been described for *T. brucei* and *Leishmania*, the majority of *T. cruzi* genes are constitutively transcribed in epimastigotes, trypomastigotes, and amastigotes (Teixeira et al., 1995; Bartholomeu et al., 2003; Gentil et al., 2009). Further studies on a number of gene models showed that change in mRNA stability is a main mechanism employed by *T. cruzi* to control stage-specific gene expression of protein coding genes (Teixeira et al., 1995; Abuin et al., 1999; D'Orso and Frasch, 2001; Bartholomeu et al., 2003; Gentil et al., 2009). From these studies, the 3′UTR has emerged as a main regulatory site involved in controlling mRNA stability. Using transient transfections with CAT or luciferase reporter genes, various groups have demonstrated the presence of elements in the 3′UTR of several mRNAs that confer developmental regulation of the reporter genes (Lu and Buck, 1991; Nozaki and Cross, 1995; Teixeira et al., 1995). The two examples below illustrate some of these studies. In the *T. cruzi* genome a tandem array of alternating genes encoding amastin, a surface glycoprotein and tuzin, a G-like protein, is polycistronically transcribed in all three forms of the parasites' life cycle. In spite of the constitutive transcription, steady-state levels of amastin genes are 60-fold higher in amastigotes compared to epimastigote forms, whereas tuzin mRNA levels do not change significantly. It has been shown that the half-life of amastin mRNAs is sevenfold longer in amastigotes than in epimastigotes and that a 180-nt sequence present in the 3′UTR is responsible for amastin up-regulation (Coughlin et al., 2000). This positive effect is likely mediated by a sequence that binds to an RNA

stabilizing factor present in amastigotes (Teixeira et al., 1995; Coughlin et al., 2000). Mucin genes are part of an even larger family of cell surface proteins of *T. cruzi* with hypervariable regions (HVR) and with members of distinct subfamilies expressed in various stages of the parasite life cycle (Buscaglia et al., 2006). Di Noia et al. (2000) have shown that mRNAs for one group of mucins, SMUG mucin mRNAs, are more abundant in the insect stage and that the mRNA turnover is controlled by an AU-rich element (ARE) located in their 3′UTR. These authors have also demonstrated that an RNA-binding protein named TcUBP-1 is involved in mRNA destabilization *in vivo* through binding to the ARE of SMUG mucin mRNAs (D'Orso and Frasch, 2002). They have gone further in characterizing this trans-acting factor, showing that TcUBP-1 is part of a approximately 450 kDa ribonucleoprotein complex with a poly(A)-binding protein and a novel 18 kDa RNA-binding protein, named TcUBP-2 (De Gaudenzi et al., 2003). These two examples show that both positive and negative regulatory elements controlling mRNA stability are found in the genome of *T. cruzi* and that these sequences are recognized by trans-acting factors. Trypanosomatid genomes encode for numerous proteins containing an RNA recognition motif (RRM) (De Gaudenzi et al., 2005). It is thus likely that a large number of these proteins are key players in processes controlling pre-mRNA trans-splicing, transport, and mRNA decay, but so far, only a few of them have been characterized in *T. cruzi* (Pérez-Díaz et al., 2007; Noé et al., 2008; Alves et al., 2010).

Transitions in gene expression that occur during differentiation of *T. cruzi* have also been analyzed using high throughput strategies that recently became available with the genome sequencing data. Several reports on microarray analyses confirm that it is a valuable screening tool for identifying stage-regulated genes in *T. cruzi* (Minning et al., 2003, 2009; Baptista et al., 2004). From these studies, we can infer that a total of almost 5000 transcripts (approximately 50% of *T. cruzi* genes) are regulated during the parasite life cycle, supporting the conclusion that transcript abundance is one of the main levels of gene expression regulation in *T. cruzi*. Together with more recent studies using next-generation cDNA sequencing technologies, these analyses allow researchers to identify groups of genes that are part of what has been called "posttranscriptional regulons," consisting of mRNAs that show almost identical patterns of regulation (Queiroz et al., 2009). In contrast to the earlier studies described earlier, where each gene was individually analyzed, the identification of these "posttranscriptional regulons" may allow researchers to find common regulatory motifs in *T. cruzi* mRNAs. When identified, these motifs will be useful as powerful "baits" for the screening of key trans-acting regulatory factors.

17.1.4.2 Genetic Manipulation of T. cruzi

Most of our current knowledge about the mechanisms controlling gene expression in trypanosomatids resulted from the development of transfection protocols, which allowed manipulation of genes, generation of knockout mutants, and introduction of reporter genes and genetic markers in the parasite genome. In contrast to *T. brucei*, in which homologous recombination of the foreign sequences with the

parasite genome is the main strategy that allows the generation of stable transfection lineages, two types of transfection vectors are used in *T. cruzi* and in various *Leishmania* species. Vectors containing the foreign gene flanked by *T. cruzi* sequences allow the integration of the foreign DNA, by homologous recombination in the parasite genome. Episomal vectors have also been used to obtain high levels of expression of foreign genes, if they contain SL/polyadenylation addition sites present both upstream (for trans-splicing) and downstream (for polyadenylation) from the exogenous gene (for a review see Teixeira and DaRocha, 2003). Work from our laboratory has identified sequences derived from various genes that can be used to provide efficient trans-splicing and polyadenylation (DaRocha et al., 2004a,b). With regard to the choice of promoters that can be used in *T. cruzi* expression vectors, we are quite limited: while VSG and Procyclin promoters (both of them recognized by RNA polymerase I) work well in *T. brucei* expression vectors, the only option currently available in *T. cruzi* is the rRNA promoter. However, similar to what has been observed in various species of *Leishmania*, it is also possible to obtain relatively high levels of expression of foreign genes using episomal vectors that do not contain promoter sequences at all (Laban and Wirth, 1989; Teixeira et al., 1995).

An important breakthrough allowing a better control of genetic manipulation in trypanosomatids was achieved with the development of inducible expression of gene products under the control of tetracycline repressor. In this system, which has been initially developed for *T. brucei*, transgenic parasites expressing the tetracycline repressor of *E. coli* exhibit inducer (tetracycline)-dependent expression of a reporter gene cloned downstream from a trypanosome promoter bearing one or more copies of the Tet operator (Wirtz and Clayton, 1995). Such an inducible expression system has been successfully introduced in *T. cruzi* (Wen et al., 2001; DaRocha et al., 2004a), although its efficiency in controlling transcription in response to tetracycline does not seem to be as high as in *T. brucei*. The availability of such a repressor/operator system is an excellent tool for the dissecting function of essential genes and for the expression of toxic gene products in the parasite.

A second major advance that provided a powerful tool for genetic manipulation in trypanosomes was described by Ngô et al. (1998), who were able to generate "knockdown" mutants by targeting an mRNA through the mechanisms of RNA interference (RNAi). RNAi is a very specific gene silencing mechanism guided by double-strand RNA (dsRNA) bearing sequences derived from a target gene. Briefly, exogenously synthesized or internally expressed dsRNAs homologous to the coding sequence of a target gene are processed into 20−24-nt long RNAs, which work as active guides for mRNA degradation (Filipowicz, 2005). RNAi is particularly convenient as a methodology to study trypanosomatid genes where antisense RNA has failed and conventional gene knockout is hindered by the fact that several genes are encoded by multicopy gene families (Ullu et al., 2004). Besides *T. brucei*, reports of successful RNAi "knockdown" have been described for *Trypanosoma congolense* (Inoue et al., 2002) and evidence for a functional RNAi machinery has been found in *Leishmania brasiliensis* (Peacock et al., 2007). Unfortunately, although several reports now show that this approach has changed

the way to do genetic manipulation in *T. brucei*, RNAi methodology seems to be more difficult to apply to *T. cruzi* (DaRocha et al., 2004b) and most *Leishmania* species (Zhang and Matlashewski, 2000; Ullu et al., 2004) which lack the underlying RNA silencing pathway. Genome database mining shows that *T. cruzi* and *Leishmania* lack DICER and AGO1 homologs, two key components of the RNAi pathway. However, as recently described in *Saccharomyces cerevisiae*, another RNAi-negative eukaryote, introducing Dicer and Argonaute from *Saccharomyces castellii* restores RNAi in the budding yeast and the reconstituted pathway is able to silence exogenous GFP as well as endogenous yeast retrotransposons (Drinnenberg et al., 2009). Testing whether a similar strategy introducing Dicer and Argonaute from *T. brucei* will work in *T. cruzi* is a top priority for future investigation.

Glossary

Contigs contiguous sequence generated by overlapping series of sequence reads.

Coverage The average number of times a genomic segment is represented in a collection of clones or sequence reads.

EST expressed sequence tag single pass sequence derived from the 5′ or 3′ end of a clone selected randomly from a cDNA library.

Haplotype combination of alleles at multiple loci on one chromosome that tend to be inher ited together.

Homologous chromosomes pair of chromosomes with the same biological features and which contain the same genes at the same loci, although the alleles can differ.

Heterologous chromosomes chromosomes that do not belong to the same pair.

Orthologous genes genes in different species that originated from a single genetic locus in the last common ancestor.

Scaffolds a set of contigs that are ordered, oriented, and positioned with respect to each other by mate pair reads. Mate pair reads are a pair of sequences derived from the 5′ and 3′ ends of a single clone.

Synteny conservation of gene content, order, and orientation between chromosomes of dif ferent species.

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17.2 Kinetoplast Genome

Jenny Telleria[1]* and Michal Svoboda[2]

[1]Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS), Montpellier, France, [2]Laboratoire de Chimie Biologique, Faculté de Médecine, Université Libre de Bruxelles (ULB), Belgique

17.2.1 Introduction

The protozoan parasite *Trypanasoma cruzi* belongs to the kinetoplastida order.

Like other kinetoplastid flagellates, *T. cruzi* possesses a unique mitochondrion containing an unusually complex assembly of mitochondrial DNA known as kinetoplastid DNA (kDNA).

17.2.2 kDNA Organization

The morphology of the kinetoplast varies depending on the species. Either it has the structure of a disk (polykDNA), or its form is less well-defined and is pankinetoplastid. The kinetoplast in *T. cruzi* has the polykDNA structure and contains 4.9×10^7 nucleotide pairs (5.4×10^{14} g) (Lanar et al., 1981). It represents a nonnegligible part of the total DNA, approximately 15% of the total DNA cells (Baptista et al., 2006).

The DNA of the kinetoplast presents a particular structure. Its size and shape are variable in the different developmental stages of the protozoan. In trypomastigotes, the kinetoplast appears like a basket due to a particular arrangement of the DNA loops in several layers, whereas in epimastigotes and amastigotes the kDNA presents a rod-like aspect.

The kinetoplast appears as a dense structure. It forms a giant network composed of interlocked DNA rings: maxicircles and minicircles concatenated between them.

The maxicircles are present in a few dozen copies and represent the equivalent of classical mitochondrial DNA; apparently, they are identical copies varying between 20 and 38 kb, which have slipped into the concatenated single layer of minicircles (Guilbride and Englund, 1998).

Each *T. cruzi* kinetoplast contains approximately 20,000−30,000 DNA minicircles. The size of *T. cruzi* minicircles is relatively constant, approximately 1.4 kb, but in heterogeneous sequences.

Despite the high heterogeneity of minicircle sequences, similar sequence features are present in all *T. cruzi* minicircles. Each minicircle contains four regions of

*E mail: Jenny.Telleria@ird.fr

approximately 100 bp with a nucleotide sequence which is almost identical for all minicircles of all *T. cruzi* lineages. These constant domains are equidistant, located on 3, 6, 9, and 12 o'clock of the minicircle. Segments separating conserved domains contain 280−320 bp and are called HVR (Degrave et al., 1988; Liu et al., 2005).

17.2.3 Replication Model of kDNA

The structure of the kDNA network has an unusual replication mechanism.

Replication of kDNA minicircles was first characterized in *Crithidia fasciculata* (for review see Shapiro and Englund, 1995). Nevertheless, the replication mechanism is similar to that of *T. cruzi* (Guilbride and Englund, 1998). The minicircles are not linked to the network when they replicate themselves.

Their replication is initiated at dodecamer 5′-GGGGTTGGTGTA-3′ which is the universal minicircle sequence (UMS). During the early stages of replication, this dodecamer binds specifically to a zing finger protein known as UMS-binding protein. This UMS sequence is identical for trypanosomid minicircles and the UMS-binding protein sequence presents a high similarity between various *Trypanosoma* species (Coelho et al., 2003).

The minicircles may be freed by the enzyme topoisomerase II, enabling them to replicate freely. They are in a natural state closed covalently in their replication. The first step is replication in θ-like structures. Then, in the second step, Okazaki fragments are synthesized and their descendants, which contain gaps, are subsequently reconnected to the periphery of the network (Guilbride and Englund, 1998). This mechanism is fundamentally different from that of all other cells. In either prokaryotes or eukaryotes, Okazaki fragments are ligated immediately after their synthesis (Kunkel and Burgers, 2008).

When the network is being replicated, the central region (which is not involved) diminishes in size, and the peripheral region containing the new replicates of the minicircles with gaps grows bigger. Once the minicircles have replicated (minicircles containing the gaps), the number has doubled.

Whereas the connection sites are opposed, the new minicircles containing gaps are rapidly uniformly distributed around the periphery of the network in a sequential manner. This uniform distribution is considered to be a movement relative to the kinetoplast disk as well as to the protein complexes. It has been suggested that the kinetoplast disk rotates between the two protein complexes. Due to the distribution of recently replicated minicircles in the network, it has been called the "annular" replication mechanism (Guilbride and Englund, 1998).

In order for this mechanism to function, it is necessary for the gaps between the Okazaki fragments to be repaired, and then the network divides into two. The latter process is possibly mediated by the enzymes contained in the antipodal site such as endonuclease I (SSE-1), responsible for primer removing and which colocalizes with the kinetoplast topoisomerase II and DNA polymerase β during replication

(Engel and Ray, 1999). These enzymes then untie neighboring minicircles along the cleavage line of the network (Guilbride and Englund, 1998).

Following primer removal, the gaps between fragments are repaired by DNA polymerase β and DNA ligase kβ as well as mitochondrial DNA helicase (Liu et al., 2009). The new replicates of the minicircles are connected, in opposite positions, to the periphery of the network. Several authors have suggested that these positions are adjacent to two protein complexes, known to contain topoisomerase II and a DNA polymerase β (Abu-Elneel et al., 2001).

17.2.4 Maxicircles and Minicircles: kDNA Coding

Maxicircles contain mitochondrial rRNA genes and genes, which encode hydrophobic mitochondrial proteins. These proteins are predominantly involved in the process of oxidative phosphorylation. *T. cruzi* uses this pathway in its transformation to the epimastigote stage in the vector. Other proteins take part in the glycolitic pathway, used by the parasite in its mammal host.

For *T. cruzi*, the genome of maxicircles has been mounted and annotated for the CL Brener and Esmeraldo strains by the TIGR-SBRI-KI *T. cruzi* Sequencing Consortium (TSK-TSC). This maxicircle genome is schematized in Figure 17.2.1 (reprinted from the original paper of Westenberger et al., 2006).

The order of the rRNAs and protein genes on the *T. cruzi* maxicircle is identical with both the *T. brucei* and *L. tarentolae* maxicircles.

The selective pressure requirement for active gene production is noticeable in the sequence comparison of the maxicircle coding domain. Comparison of maxicircle encoded genes of *T. cruzi* with *T. brucei* and *Trypanosoma tarantolae* demonstrated that whereas nonedited genes (*ND5*, *ND4*, *COI*, *COII*, *ND1*, *MURF1*, *MURF2*, *Cyb*) have a similarity of more that 75%, extensively edited genes (*COIII*, *ATPase6*, *ND7*, *ND8*, *ND9*, *CR4*, *CR5*, *RPS12*) have a similarity of less than 50% only at the DNA level. However, the similarity of translated edited genes rose to 75% for the majority of comparisons.

The absence of selective pressure is obvious in the noncoding domain of maxicircles: no similarity was found between *T. cruzi* and *T. brucei* and *T. tarantolae*. Furthermore, almost no homology was evidenced between two sequenced maxicircles of two different lineages of *T. cruzi*. Variable sequences of maxicircles could also be potentially used for the determination of *T. cruzi* lineages. However, the variable sequences of maxicircles of each lineage have not yet been published, and it has been considered that the number of maxicircles is 100 times lower than the number of minicircles.

In 1986, Benne et al. described the presence of four nonencoded uridylate (U) residues in the mRNA of the maxicircle gene encoding subunit 2 of cytochrome c oxidase (cox) of two kinetoplastids, *T. brucei* and *Crithidia fasciculate* (Benne, 1994, Benne et al., 1986).

We now know that the DNA of maxicircles encodes 20 genes, corresponding to edited and nonedited genes. However, the majority of primary transcriptions of the

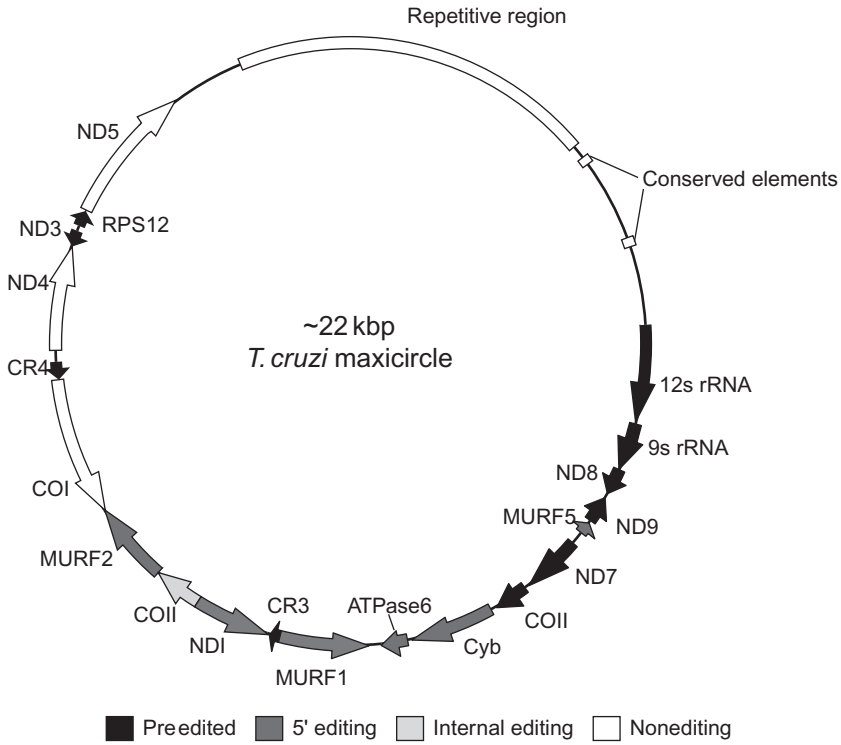

Figure 17.2.1 Maxicircle of the *T. cruzi* CL Brener and Esmeraldo reference strains (from Westenberger et al., 2006). All annotated genes are shown as arrows indicating coding direction. The noncoding regions of both genomes are distinct from one another, with the exception of a duplicated conserved element lying between the repetitive region and the *12S rRNA*.

maxicircles cannot be directly translated because they often contain many errors relating to the open reading frame (ORF) and should be "published" before the translation. That is why, at first sight, the genome seems to be lacking several genes characteristic of mitochondrial genomes, whereas other genes lack key elements for the translation, such as initiation codons or contiguous ORFs.

Whereas the information contained in a genomic sequence is, in most cases, accurately reproduced in the RNA, in the case of *T. cruzi*, the transcription undergoes a genuine correction that modifies its sequence thanks to the process refereed to as "RNA edition." It regroups addition mechanisms, the suppression, and more rarely, the conversion of nucleotides in precise positions of the coding region of maxicircle primary transcriptions (for review see Stuart et al., 2005). This process may be defined as a programmed alteration of RNA primary structure, enabling the production of a functional sequence. Thus, initiation codons are created, or the correction of internal reading is realized, if not, the transcriptions are unrecognizable for the creation of the ORF (Westenberger et al., 2006).

Since the discovery of mitochondrial RNA editing, we know that this process is the result of a perfect collaboration between genes contained in the maxicircles and the minicircles. The maxicircles provide pre-edited RNA and the minicircles contribute RNA guides (gRNA). The existence of these gRNA in *T. cruzi* and their role in the edition of the transcribed RNA maxicircle has been demonstrated by Avila and Simpson (1995) and Thomas et al. (2007). This RNA edition must be extremely accurate in order to avoid the insertion or the suppression of a wrong number of uridines, which would falsify the DNA edition.

The consequence of such errors could lead to the synthesis of an untranslatable reading frame, or modify a senseless sequence giving rise to a full reading frame. The key to this precision lies with the RNA guides (Blanc and Davidson, 2003). They possess a complementary sequence of the edited region, which determines the precise number of uridines to add, suppress, or convert.

Nevertheless, the level of the variability in the gRNA sequence, without loss of functional information, is impressive, due to the fact that any link with G or residues of U in the mRNA is not affected by the transition mutations in the gRNA (Westenberger et al., 2006). But the genes in the maxicircle must maintain a certain degree of fidelity to the gRNA genes in order for the correction to be made (Baptista et al., 2006).

The gRNA, in their 5′ region, present a so-called "linking sequence," which pairs with pre-edited transcriptions, and their 3′ region, particular to RNAg, is a poly-tail (U) that could be involved in the stabilization of the mRNA−gRNA complex.

The formation of the first mRNA−gRNA complex is crucial to the activation of the edition process. The gRNA associated with the transcription serves as a matrix for the insertion or suppression of uridines. In some cases, the edition creates a new linking site for a second gRNA. The consecutive action of the gRNA means that the edition is a 3′ to 5′ oriented process, which repeats itself until the mRNA is completely edited.

A series of enzymatic reactions triggered by the pairing of the gRNA enables the endonuclease to cleave the messenger RNA at the level of the first wrongly paired base. The 5′ fragment thus formed is maintained close to the 3′ fragment via RNA−RNA interactions, bringing into play the poly-tail (U) of the gRNA (Blum and Simpson, 1990) and proteins. This group is called "editosome."

Secondly, the addition, suppression, or the conversion of uridines takes place in the 3′ of 5′ fragment thanks to 3′ terminal uridylyl transferases (TUTases). The newly added uridines pair with the gRNA. The two RNA fragments (5′ and 3′), which remain together due to complementarity with gRNA, are finally linked by an RNA ligase, giving rise to the mature transcription (Blanc and Davidson, 2003).

The edition process of maxicircle transcriptions contributes to the evolution of the stages of cellular life and the unusual energetic metabolism of *T. cruzi* in certain stages of life, passing through rich glucose sanguine trypomastigotes to intracellular amastigotes, and to the poorest epimastigotes living in the energy environment of insects' intestines.

17.2.5 Determination of *T. cruzi* Lineages Analyzing Minicircles DNA Sequences

T. cruzi undergoes essentially clonal evolution with only very rare sexual recombination (Tibayrenc et al., 1986). The occurrence of hybridization in natural populations of *T. cruzi* has been unequivocally demonstrated (Machado and Ayala, 2001; Westenberger et al., 2005; de Freitas et al., 2006). Nevertheless, the rarity of such sexual recombination allows propagation of clonal genotypes over long periods of time (de Freitas et al., 2006). Consequently, lineages of this parasite are identifiable on the basis of their genotype and phenotype. Recently, the nomenclature of *T. cruzi* intraspecific variability has been revised, taking into account the six principal genotypes (Zingales et al., 2009).

The identification of the lineage could be based either on genomic DNA sequences, on the polymorphism of isoenzymes (i.e., products of genomic DNA), or on the presence of specific sequences in hypervariable kDNA region. It is largely accepted that at least two hybridization events occurred in *T. cruzi* natural populations in which a fusion between ancestral TC I and II (former DTU I and DTU IIb, respectively) genotypes gave rise to a heterozygous hybrid that homogenized its genome to become the homozygous progenitor of TC III and TC IV (former DTU IIa and DTU IIc, respectively). The second hybridization was between TC II and TC III (former DTU IIb and DTU IIc, respectively) strains that generated TC V and TC VI (former DTUs IId and DTU IIe, respectively). It is noteworthy that the reference strain CL Brener, whose genome was first sequenced, pertains to a hybrid TC VI (former DTU IIe) lineage.

For various reasons developed later, the genetic pressure to maintain the constant gRNA encoding domain of minicircles appears to be low. It is therefore not surprising that the long and separate evolution of parasites led to the evolution of the sequence diversity of hypervariable domain minicircles. The relationships between nuclear genotypes specific to given lineages and sets of minicircle sequences is, however, maintained. Consequently, each lineage may be characterized by a set of minicircle sequences roughly specific to this lineage. The sequence diversity of minicircles is the foundation of various typing methods determining parasite lineage.

A first approach to *T. cruzi* kDNA characterization, described by Mattei et al. (1977), was based on the variability of restriction fragment length polymorphism (RFLP) of the minicircles. The kDNA should be purified, which requires extraction of kDNA from a large number of cultured parasites. Later, Morel et al. (1980) used this method for genotyping *T. cruzi* strains and proposed the term "schizodeme" to refer to groups of parasites presenting the same pattern of kDNA.

Schizodeme typing is based on the separation of fragments of the digested kDNA by electrophoresis. Most of the digestions for schizodeme analysis were performed in polyacrylamide gels. When agarose gels were used, the resolution was not sufficient, and hybridization (southern blot) was necessary. These methods are time consuming.

PCR amplification of hypervariable domain of minicircles was already described in 1989 (Sturm et al., 1989). Hypervariable domains are amplified using primers based on the constant region. An amplicon of approximately 320 bp was obtained. This amplicon contains the hypervariable domain but also about 100 bp of the constant domain (primers plus 60 bp of relatively constant sequence).

This PCR approach yielded good results with relatively pure parasites isolated from bugs, but did not allow direct identification of parasites in blood samples. In order to increase sensitivity and specificity, hybridization of amplicons was proposed (Brenière et al., 1992). This approach was developed by Veas et al. (1990), who amplified the kDNA hypervariable domain using modified primer containing restriction sites, allowing subsequent elimination of constant domains from amplicons (HVRm).

Probes prepared by Veas et al. (1990) were used for strain typing. Brenière et al. demonstrated that probes obtained by amplification of kDNA from clonet 39, hybridized only with DNA from clonet 39, probes obtained from clonet 43, hybridized only with DNA of clonet 43, and probes from clonet 20, hybridized with clonet 19 and 20, respectively (Brenière et al., 1992, 1998). Subsequently, the method was validated for epidemiological purposes, using total kDNA as probes (Brenière et al., 2002).

Systematic sequencing of large numbers of hypervariable sequences originating from different lineages confirmed sequence specificity of each group and demonstrated that if some hypervariable sequences are rare or unique, other sequences are frequently repeated in one lineage but, in proportion, varying from one strain to another. This was observed mainly in lineage TC V (former DTU IId) (Telleria et al., 2006).

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18 Experimental and Natural Recombination in *Trypanosoma cruzi*

*Michael D. Lewis, Martin S. Llewellyn, Matthew Yeo and Michael A. Miles**

Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

18.1 Introduction

It has been more than 100 years since the remarkable discovery of *Trypanosoma cruzi* by Carlos Chagas. The centenary commemorations resulted in a profusion of reviews on Chagas disease. There has also been a spate of recent original publications relevant to the topic of this chapter. Accordingly, here we will be relatively brief and when appropriate will refer the reader to primary sources and other reviews.

The discovery of Chagas disease is scientifically and historically interesting because such rapid progress was made in a short period of time from the unusual starting point of finding *T. cruzi* in the triatomine vector, prior to confirmation of its presence in any patients. It is also politically interesting because of circumstances surrounding Chagas' nominations and consideration for a Nobel prize (Miles, 2004).

Despite progress with vector control programs, notably through a series of international cooperative initiatives, Chagas disease remains a major public health problem in Latin America; approximately 8 million people are thought to be infected (Rassi Jr et al., 2010). Furthermore, sporadic cases may arise beyond the traditional endemic regions through transfer of contaminated blood or organs and congenital transmission. The present chemotherapy is far from ideal and improved low-cost drugs are desperately needed to allow routine and reliable treatment of all those infected, including adults, who are considered to be more susceptible to side effects from the two available drugs, benznidazole and nifurtimox. Potential new drugs under trial for treating Chagas disease are posaconazole and ravuconazole, although their cost at present is far beyond reach for routine use in endemic areas.

* E mail: Michael.Miles@1shtm.ac.uk

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The disease manifestations of *T. cruzi* infection in individual patients and in different geographical regions of Latin America are diverse, as is the response to chemotherapy. This is thought to be partially dependent on the very high degree of intraspecific genetic diversity in *T. cruzi*. Genetic diversity is shaped by several evolutionary forces, including mutations, migration of individuals, genetic drift, and natural selection. Recombination can also have a dramatic impact because it generates new combinations of alleles. Recombination occurs during meiosis as part of the process of sexual reproduction in most eukaryotes. Meiosis is a specialized form of cell division involving a halving of the number of chromosomes to generate gametes (gametogenesis). Gametes subsequently fuse to form progeny (fertilization) with a full complement of chromosomes restored.

While sexual reproduction involving meiosis is obligatory in almost all multicellular eukaryotes, this is not necessarily true of protozoa. These unicellular eukaryotes can typically reproduce clonally by repeated rounds of binary fission (mitosis) without recombination, and while most are now thought to be capable of meiotic sexual reproduction it is not obligatory, and evidence indicates that clonality often predominates (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2002). The population structure of disease-causing microorganisms, such as *T. cruzi*, is therefore determined by the relative rates of recombination and clonality. Understanding these processes is important because they influence the spread of epidemiologically relevant traits such as virulence, transmission potential, or resistance to therapeutic drugs. Accurate knowledge of such factors can thus be important in designing disease control strategies. In this chapter we will examine our understanding of recombination in *T. cruzi*. Research in this area is important not only for the reasons laid out above with respect to Chagas disease epidemiology, but also for the intrinsic biological interest due to its potential evolutionary novelty.

18.2 Genetic Diversity of *T. cruzi*

T. cruzi is still considered to be a single species. Early comparisons between strains suggested a diverse range of phenotypic characteristics. The application of biochemical methods, especially multilocus enzyme electrophoresis (MLEE) to study intraspecific diversity, led to the description of distinct genetic lineages (or discrete typing units, DTUs). Initially three main groups were described and they were called called principal zymodemes. Further application of MLEE revealed six discrete genetic lineages. A plethora of molecular biological methods was subsequently applied, largely confirming the existence of six major genetic lineages. These corresponded with those originally defined by MLEE but also uncovered appreciable diversity within some of the lineages, dependent on the degree of resolution of the molecular method applied. From a recent consensus review of the subspecific nomenclature of *T. cruzi*, these six lineages or DTUs were designated as TcI−TcVI (Zingales et al., 2009). It is anticipated that as sampling coverage of the endemic areas improves, novel *T. cruzi* lineages will be described. For example, a somewhat diverse group, provisionally named TcBat, presenting distinctive genotypic

characteristics has recently been described from bats and appears most closely related to TcI (Marcili et al., 2009). In addition a subspecies of *T. cruzi* that is restricted to bats is named *T. cruzi marinkellei*.

TcI–TcVI have distinctive, yet partially overlapping, geographical distributions and ecological associations (Miles et al., 2009). TcI is relatively diverse and widespread; it has a primarily arboreal transmission cycle with secondary cycles in terrestrial rodents and in humans; it is the main agent of Chagas disease in endemic regions north of the Amazon, for example, in Venezuela and Colombia. TcII, TcV, and TcVI are less genetically diverse than TcI and are almost exclusively found in domestic transmission cycles in the Southern Cone region of South America, where they are the main agents of Chagas disease; they are presumed to have sylvatic cycles but these appear scarce, and remain poorly understood. TcIII is geographically widespread and has a dispersed terrestrial sylvatic cycle that involves primarily the armadillo, *Dasypus novemcinctus*. TcIV is little studied but has divergent genotypes in South and North America. Both TcIII and TcIV are generally associated with sylvatic hosts and vectors and although both can cause human infection they as yet only sporadically invade domestic transmission cycles. This present understanding of the genetic diversity of *T. cruzi* is a valid framework but needs to remain dynamic as more extensive sampling and research continue.

In the following sections we will briefly review research on experimental recombination in *T. cruzi* and the evidence for recombination in natural populations. We will also suggest future methodological approaches to understanding genetic recombination in *T. cruzi* and its epidemiological importance. A further account of experimental recombination in *T. cruzi* and its relevance for formal genetics and epidemiology is given in a companion volume (Tibayrenc, M. (Ed.), Genetics and Evolution of Infectious Diseases, Elsevier, in press).

18.3 Experimental Recombination

Experimental attempts to produce recombinants between TcI and TcII, then referred to as Z1 and Z2, were first undertaken in the 1970s. The experimental approach was either simply to passage together mixed populations of the two lineages in blood agar cultures or sequentially from mouse to triatomine to mouse and back into culture, then recharacterizing the population mixtures. No recombinants were detected and either a mixture of TcI and TcII or TcII alone was recovered from these experiments. In retrospect, it was ambitious to attempt an experimental cross between these genetically divergent lineages. Furthermore, there were no genetic markers available for the selection of recombinants, which would have been undetectable if they had been present as minor populations in the output of such experiments.

Experimental genetics for *T. cruzi* became feasible with the development of recombinant DNA technology that allowed genetic transformation of parasite populations to confer resistance to different drugs. Trypanosomes can now be marked to carry a wide range of reporter genes, including fluorescent markers of various

colors: it is possible to direct such markers to intracellular organelles of special interest or to tag genes of interest that are expressed during different life cycle stages. Without this technology the study of genetic exchange in kinetoplastid parasites, which remains ongoing, would have been severely hampered.

In 1996 putative parental homozygous and recombinant heterozygous genotypes of TcI were described on the basis of phosphoglucomutase (PGM) isoenzyme phenotypes in isolates from a single locality in the Amazon basin of Brazil, potentially compatible with active intralineage recombination (Carrasco et al., 1996). It was suggested that genetic exchange was most likely to be achieved experimentally by crossing strains from within a single *T. cruzi* lineage. Accordingly, two putative parental strains from the Amazon locality were transformed with episomal recombinant DNA plasmids bearing drug-resistance genes conferring resistance to specific antibiotics, either hygromycin B or neomycin. Experimental crosses were attempted *in vitro* in mammalian cell cultures, and *in vivo* in mice and triatomine bugs by coinfection with both transgenic parental strains (Gaunt et al., 2003). Parasite populations subsequently derived from the coinfections were subjected to selection with both drugs simultaneously. Six double drug-resistant *T. cruzi* recombinant hybrid clones were obtained from mixed infections in the mammalian cell cultures and were shown to contain both drug-resistance marker genes indicating that they were the products of genetic exchange between the coinfecting strains. The six clones were characterized by MLEE, karyotypes, microsatellites, and sequencing of some housekeeping genes. This analysis demonstrated that parental genetic markers had not been inherited in typical Mendelian ratios. Rather than inheriting one allele per locus from each of the parent strains, as expected in typical meiotic F1 heterozygous progeny, the hybrid clones contained all alleles from both parents at most loci. However, at a small minority of loci some parental alleles were not present. Each of the hybrid clones had one of the parental kDNA maxicircle genotypes but not both. It was concluded that fusion of the diploid parental strains had occurred to produce a tetraploid hybrid, with limited subsequent genome erosion and an unclear level of concomitant genetic recombination between parental sequences.

In terms of virulence, pathogenesis, and epidemiological relevance it was of considerable interest to see how these hybrid *T. cruzi* clones behaved in experimental mice. The hybrid clones proved to be at least as virulent as the parental strains in immunocompromised mice infected with metacyclic trypomastigotes from stationary phase cultures (Lewis et al., 2009a). They produced abundant pseudocysts in heart and skeletal muscle, with some detectable infection of smooth muscle of the alimentary tract. This showed that these *in vitro*−generated hybrids were capable of all the morphogenic transitions required to complete a full life cycle, and were able to survive in a mammalian host. Whether the hybrid clones display increased virulence or "vigor" in comparison with their parents, or would compete with the parents in coinfections, is a topic that requires further study. This is true for these and future experimental hybrids, for natural hybrid lineages (see following section) and for any intralineage recombinants that may be found among natural populations.

Flow cytometric analysis of DNA content provided further insight into the genomic composition and ploidy of the experimental hybrid clones and the process of genome erosion. This approach was originally applied to *T. cruzi* by James Dvorak in the 1980s and revealed substantial variation in the DNA content of natural isolates (Dvorak et al., 1982). The DNA content analysis demonstrated that all six clones had, on average, 69% more DNA than the parental strains. This was compatible with an aneuploid chromosome complement intermediate between 3*n* and 4*n*, and so the hybrids were considered to be subtetraploid (Lewis et al., 2009a). There was no dramatic decline in DNA content when the clones were passaged through immunocompromised mice or in response to stressful growth conditions, such as heat shock, indicating that they were relatively stable. The DNA content analysis thus further supported the hypothesis that the hybrids underwent limited genome erosion from a tetraploid fusion product as previously suggested by the genetic marker analyses.

The ploidy level, however, is not absolutely stable. Following prolonged passage in axenic cultures, a gradual, progressive decline in DNA content has been observed (Lewis et al., unpublished data), with a pattern that is not compatible with any true meiotic reductive division that would result in rapid, ordered reduction of ploidy. Further comparisons of the parental and experimental hybrid karyotypes and genotypes, particularly using heterozygous parental allelic markers, are required to understand the mechanism of genome erosion in *T. cruzi*. Nevertheless, it is clear these *in vitro*−generated intralineage TcI hybrids are not the result of the typical eukaryotic program of genetic exchange, since neither the parents nor the hybrids underwent a meiotic reductive division. However, the process of fusion of diploids followed by genome erosion is reminiscent of the parasexual reproductive cycle of the pathogenic yeast *Candida albicans*, which is characterized by cellular and nuclear fusion of diploid cells producing tetraploid intermediates, followed by random, concerted chromosome losses giving rise to recombinant progeny with an approximately diploid chromosome complement (Bennett and Johnson, 2003). Whether the diploid fusion/genome erosion model of genetic exchange applies to field populations of *T. cruzi* or whether sexual reproduction involving normal meiosis could occur under different conditions or between different strains remains an open question.

Performing experimental crosses in *T. brucei* using transgenic strains expressing different fluorescent proteins (red or green variants) has proven to be a powerful tool for studying recombination under laboratory conditions since hybrid organisms coexpressing both markers appear as having yellow fluorescence and can be identified microscopically. This has helped to pinpoint the developmental stage of the parasite that is involved in genetic exchange (Gibson et al., 2008). The same approach is likely to prove fruitful for experimental crosses in *T. cruzi*. Red and green fluorescent strains of *T. cruzi* as well as the closely related species *T. rangeli* have been described, and the potential for exploiting these reporters to track coinfections *in vitro* and in mice and triatomine bugs *in vivo* has now been demonstrated (Guevara et al., 2005, Pires et al., 2008). As yet no hybrid parasites have been recovered from such experiments, although relatively few conditions or strains appear to have been tested.

In *T. cruzi* the experimental hybrid clones described above were derived from mammalian cell cultures, it was not proven that hybridization was an intracellular event. In *T. brucei* and *Leishmania*, recombination occurs in their invertebrate vectors, the tsetse fly, and sand fly, respectively (Jenni et al., 1986; Akopyants et al., 2009). In the *T. cruzi* cross the mammalian cells were infected with a mixture of metacyclic trypomastigotes and epimastigotes from stationary phase cultures. It is therefore possible that hybridization occurred between epimastigotes prior to invasion and establishment of intracellular forms. Alternatively, hybridization may have taken place between trypomastigotes emerging from the mammalian cells during the prolonged infection, which could have encompassed up to four rounds of invasion and intracellular multiplication. This implies that hybridization between extracellular forms that would be found in natural infections of triatomine bug vectors should not be ruled out. This possibility requires further investigation, ideally with transgenic *T. cruzi* strains carrying both drug-resistance and fluorescent markers to allow visualization of interactions between coinfecting strains. This will require careful experimental design because there are around 140 known species of triatomines, and the six known lineages of *T. cruzi* each encompasses considerable genetic diversity. Triatomine species appear to differ in their susceptibilities to infection with different *T. cruzi* lineages and so the behavior of *T. cruzi* strains in one combination of triatomine species and *T. cruzi* lineage will not necessarily be typical.

The experimental demonstration of hybridization was a milestone in the research on *T. cruzi*. It proved that *T. cruzi* has an extant capacity for genetic exchange. It also revealed an unusual, nonmeiotic mechanism involving fusion of diploids followed by genome erosion, for which the precise details remain to be understood. This mechanism may be operating among natural populations of *T. cruzi*, but the occurrence of other genetic mechanisms among natural populations cannot be excluded.

18.4 Recombination in Natural Populations

18.4.1 Interlineage (Inter-DTU) Recombination: TcV and TcVI

The clonal theory of parasitic protozoa implied that genetic exchange was either absent or a rare event in *T. cruzi* and of little or no epidemiological significance (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2002). This theory was supported by several features of the genetic diversity of *T. cruzi*, including strong linkage disequilibrium, identical genotypes spread over vast geographical distances, and phylogenetic correlation between independent sets of genetic markers. Nevertheless, early MLEE studies of isolates from Bolivia and Paraguay, now incorporated within lineage TcV and TcVI, respectively, revealed highly distinctive, heterozygous MLEE profiles, with at least one corresponding homozygous profile seen among other isolates from the same locality (Tibayrenc and Miles, 1983; Chapman et al., 1984). For example, the dimeric enzyme glucose phosphate isomerase (GPI) had the profile of three equidistantly separated bands, with a central band that was more intense, as would be expected for recombinant strains. Furthermore, this

profile was sustained in *T. cruzi* clones and could not be due to a mixture of populations. The TcV and TcVI profiles were similar in that both showed a high level of heterozygosity but they also had some distinguishing isoenzyme bands.

TcV and TcVI have an unusual geographical distribution. They were found to be particularly common in the foothills of the Andes and the greater Gran Chaco regions of Bolivia, Chile, Paraguay, northern Argentina, and in the extreme south of Brazil, where there were wider fluctuations in environmental temperature than in the Amazon and Atlantic forests. This suggested that the heterozygous MLEE profiles might be adaptive, giving enhanced fitness in triatomine bugs through metabolic flexibility over a range of environmental temperatures. Such an adaptive advantage was proven to occur in other systems, for example, in fish moving between warm and cold climatic conditions. The metabolic significance of the predominantly heterozygous profiles of TcV and TcVI was explored by purifying the GPI isoenzymes and testing their catalytic rates at different temperatures. Although the isoenzymes certainly differed in temperature stability, as was readily seen directly by their differing persistence on incubated MLEE gels, there was no proof of differences in catalytic efficiency at diverse temperatures for the purified isoenzymes (Widmer et al., 1987). Nevertheless, the maintenance of high levels of heterozygosity in TcV and TcVI suggests that such experiments to test for associations with fitness remain worthwhile.

The hybrid nature of TcV and TcVI was confirmed by other molecular methods, notably by sequencing of housekeeping genes (Machado and Ayala, 2001; Brisse et al., 2003). Comparison of nucleotide sequences showed that TcV and TcVI must have arisen through hybridization between genetically distinct parents because they were found to possess fully intact alleles from two other DTUs (TcII and TcIII) that are so distinct that they could not possibly have arisen independently. As in the experimental hybrids described in the previous section, sequencing of kDNA maxicircle genes showed they were inherited uniparentally by TcV and TcVI, with the TcIII parent the donor in each case. Analysis of existing natural hybrid isolates is highly relevant since it could illuminate the circumstances under which they arose and in which other interlineage hybrids might arise in the future.

The close genetic similarity between TcV and TcVI has made it difficult to determine whether they are the products of a single hybridization event followed by limited clonal diversification or of two independent events involving genetically similar TcII and TcIII parental strains. Limited microsatellite data based on five loci have indicated that more than one event is the most likely explanation (de Freitas et al., 2006).

It is clear that while high-resolution markers can reveal some differences within TcV and TcVI, as well as between them, both these DTUs are genetically relatively homogeneous. Furthermore, scrutiny and comparison of sequence-based data can identify the closest putative parents from within TcII and TcIII and give insight into the geographical location of the hybridization events. For example, analysis of 5S rDNA sequences has shown that the TcII-like allele for these sequences found in TcV and VI hybrid strains is more similar to TcII isolates from Bolivia and Chile than to others from Brazil (Westenberger et al., 2006). Additional sequence and

microsatellite data of both TcII and TcII isolates also support a geographical origin for both parents in the Southwest of the continent (Lewis et al., unpublished data).

Intuitively, the more recent the natural hybridization event(s) that gave rise to TcV and TcVI, the more likely it is that new natural hybrid lineages could be expected to emerge. Moreover, given the abundance of TcV and TcVI in human infections across large endemic areas, there may be a significant risk associated with such potential new hybrids. However, the resolution afforded by the genetic markers used so far has not been sufficient to resolve the evolutionary timeframe for the origin of TcV and TcVI. The use of phylogenetic molecular clocks may permit progress to be made in this area; for example, analysis of nucleotide sequences has indicated that all six *T. cruzi* lineages last shared a common ancestor within the last 3−10 million years (Machado and Ayala, 2001). Nevertheless, observation of genetic exchange in the laboratory suggests that at least some natural isolates are likely to be capable of hybridization.

The ecological circumstances of the origin of TcV and TcVI are not known. Hybridization must have occurred in either a mammalian host or a triatomine bug coinfected with parasites from both TcII and TcIII lineages. It is not clear where this might have occurred because TcII and TcIII have little overlap in their geographical distribution or ecological associations (Miles et al., 2009). TcIII has been documented occasionally from domestic or peridomestic cycles in parts of the Southern Cone where it might have been sympatric with TcII. Sylvatic records of TcII are currently too scarce to suggest a scenario where it might have come into contact with TcIII in a sylvatic cycle. Isolates of TcV and TcVI have so far rarely been found in sylvatic transmission cycles, and are normally associated with humans, domestic animals, and domiciliated triatomine species, particularly *Triatoma infestans*. The apparent lack of genetic diversity within both hybrid DTUs from isolates covering a vast geographic area is most plausibly a result of a recent spread of these lineages in association with the spread of domiciliated *Triatoma infestans*, which is itself due to human activities and population movements? (Bargues et al., 2006). Overall, the genetic evidence, host records, and geographical distributions of the parental and hybrid lineages suggest that TcV and TcVI originated in a sylvatic setting and subsequently invaded the domestic ecological niches, or alternatively they actually originated in a domestic setting.

If TcV and TcVI were, like the experimental hybrids, products of genome fusion of diploids to yield aneuploid progeny, the redundant extra copies of genes might confer versatility and evolutionary advantage, in that those genes would potentially be free to evolve rapidly and independently and to acquire alternative independent functions. Accordingly, flow cytometric analysis of DNA content and multilocus genotyping has been applied to natural isolates of *T. cruzi* representing the known *T. cruzi* lineages (Lewis et al., 2009a). Unlike the experimental TcI hybrids, the natural hybrids of TcV and TcVI were found to have DNA contents consistent with diploidy, and equivalent to the average DNA contents of isolates representative of their TcII and TcIII parents. Where sufficient resolution has been possible, TcV and TcVI appear to possess one TcII allele and one TcIII allele, although only a limited number of chromosomes have been covered so far. There are, therefore,

fundamental differences between the naturally occurring hybrid strains and the experimental hybrids both in terms of allelic inheritance patterns and overall DNA content. It is not clear whether these differences reflect the operation of fundamentally different mechanisms of genetic exchange. It is possible that developmental cues that would cause the experimental hybrids to return to diploidy were absent under laboratory conditions. Alternatively, if TcV and TcVI are indeed derived from fusion of diploids, genome erosion may have progressed sufficiently to result in reversion to a diploid state. The mechanism of genetic exchange in natural populations is thus yet to be fully understood and potentially differs from that documented so far by experimental observations.

While the precise biological circumstances that governed the origin and spread of TcV and TcVI remain to be fully understood, the epidemiological impact of these hybrids is striking. They are highly abundant in domestic settings and may even predominate among human infections in Bolivia, Chile, Paraguay, northern Argentina, and the extreme south of Brazil. Chagas disease manifestations are often severe in these regions and chagasic cardiomyopathy, megaesophagus, megacolon, and congenital transmission are all common. The epidemiological importance of genetic exchange in *T. cruzi* is therefore no longer in doubt: it has been and may still be profoundly important.

18.4.2 Interlineage (Inter-DTU) Recombination: Other Lineages

It has also been proposed that TcIII and TcIV are themselves the products of a more ancient interlineage hybridization event between TcI and TcII (Sturm et al., 2003; Westenberger et al., 2005). The evolutionary relationships between TcI and TcIV had been unclear for some time. In phylogenetic analysis of various nuclear loci, TcI and TcII were consistently found to be the most genetically distant lineages. The positions of TcIII and TcIV, however, could not be satisfactorily resolved since some markers indicated close relationships with TcI while others indicated a stronger affinity with TcII. The reasons for this became clear in a multilocus sequence typing (MLST) analysis of nine nuclear genes (Westenberger et al., 2005), which showed that the copies of each gene carried by TcIII and TcIV strains contained single nucleotide polymorphisms (SNPs) that were otherwise only found in TcI or TcII strains. Importantly, the TcIII and TcIV sequences contained both TcI-like and TcII-like SNPs in mosaic patterns, with the ratio of TcI- to TcII-like SNPs varying between genes. This explained the incongruent phylogenetic trees that were observed for different genes. The number of SNPs contributing to these mosaic patterns was too high to be a result of the same mutations arising in each lineage independently (homoplasy) and so must have been a result of genetic exchange between TcI and TcII ancestral populations. TcIII and TcIV also had their own unique SNPs interspersed between the TcI- and TcII-like SNPs, reflecting independent evolution since their divergence. Analysis of mitochondrial sequences again showed that uniparental inheritance of kDNA maxicircles occurred, with TcI identified as the donor.

Accordingly, an evolutionary scenario for TcI–IV was proposed in which two diploid homozygous lineages (TcI and TcII) diverged from a universal *T. cruzi* common ancestor and subsequently representatives of these two lineages underwent hybridization via a mechanism of genetic fusion, producing an aneuploid or polyploid heterozygous hybrid. After this initial hybridization event, several evolutionary processes are thought to have led to the current composition of the TcIII and TcIV genomes. Homologous recombination between the TcI- and TcII-like alleles produced mosaic sequences; extensive genome homogenization fixed these mosaic alleles in a homozygous state; chromosomal loss restored diploidy, and novel mutations accumulated over time during clonal diversification, resulting in the current TcIII and TcIV lineages.

Analysis of the current diversity within TcIII and TcIV shows that although ancient recombination events contributed to their origin, their multilocus genotypes are consistent with long-term evolution in isolation from the other major lineages and they should be treated as distinct in any applied studies. TcIII also has a characteristic DNA content that is significantly greater than the other DTUs (Lewis et al., 2009a). Furthermore, as mentioned above, TcIII has distinct ecological associations, being commonly isolated from *Dasypus* spp. over a wide geographical range (Yeo et al., 2005). TcIV is not a well-sampled *T. cruzi* lineage, yet analysis of the limited isolates that are available has pointed toward abundant intralineage genetic diversity with a highly distinct subgroup present in North America.

18.4.3 Mitochondrial Introgression as a Signature of Genetic Exchange

As described in the previous section, the current view of *T. cruzi* evolution involves two ancestral lineages (TcI and TcII), two ancient highly homozygous hybrids that have diverged extensively (TcIII and TcIV), and two more recent highly heterozygous hybrids that have diverged minimally (TcV and TcVI). Thus, there are four distinct *T. cruzi* clades based on nuclear haplotypes (TcI–TcIV), with TcV and TcVI containing one haplotype derived from TcII and one from TcIII. In contrast, there are only three principal mitochondrial maxicircle sequence clades (Machado and Ayala, 2001; de Freitas et al., 2006). The TcI and TcII DTUs carry highly distinct maxicircles, whereas TcIII, TcIV, TcV, and TcVI maxicircle sequences are all quite similar. It is presumed that the hybrids TcV and TcVI retained only the TcIII parental maxicircle genotype and that the TcII maxicircle genotype was lost.

The maxicircle sequences of TcIII and TcIV are highly distinct compared to TcI and TcII, further evidence of their long-term independent evolution from the parental types since hybridization. However, when TcIII and TcIV maxicircles are compared with each other, they show far less divergence than is seen for their nuclear sequences. This is unexpected because mitochondrial sequences are normally expected to evolve more rapidly, not slower, than nuclear ones. This suggests a process of relatively recent mitochondrial introgression between TcIII and TcIV (Machado and Ayala, 2001). Incongruence in the phylogenetic relationships inferred by nuclear and mitochondrial sequences also identified putative interlineage genetic exchange via mitochondrial introgression between TcIV and TcI

populations in North America in this case one strain possessing TcI-like nuclear genes was found to have virtually identical maxicircle sequences to specific TcIV strains. Other North American TcI and TcIV strains have been shown to have unexpectedly high DNA contents, potentially linking mitochondrial introgression in this region to hybridization events (Lewis et al., 2009a). There is some additional evidence that TcIV may be involved in recombination events more frequently than previously suspected: TcIV strains can have at least three alternative miniexon genotypes, including the TcI type, three alternative 24Sα rDNA genotypes, including the TcII type, and they contain multiple microsatellite alleles that are otherwise specific to other DTUs (Lewis et al., 2009a,b).

The key message from these observations is that although interlineage genetic exchange is a relatively rare occurrence, it is a more common phenomenon than previously thought. It is not clear to what extent these genetic exchange events might involve obscured or partial genetic exchange between the nuclear genomes of the strains involved. The dynamics of kDNA maxicircle and minicircle evolution are poorly understood, and strains exhibiting mitochondrial introgression would therefore be of interest for further comparative genomics analysis and a source of new insight into recombination mechanisms in natural populations of *T. cruzi*.

18.4.4 Intralineage (Intra-DTU) Recombination

The existence of natural hybrid lineages shows that while interlineage genetic exchange may well be rare in *T. cruzi*, it has had a major impact on its evolution and current diversity. The current genetic lineages, including hybrids, appear to be stable and independently propagating with little gene flow between them. However, the frequency of recombination within these lineages remains relatively unexplored. Molecular epidemiological studies on *T. cruzi* have not generally permitted meaningful analysis of intralineage recombination rates due to a lack of sufficiently diverse markers or insufficient sampling. The landmark publication of the *T. cruzi* genome sequence in 2005 (El-Sayed et al., 2005) means that genetic markers covering a wide range of evolutionary rates and, hence, levels of resolution can now be selected and exploited for sequence comparisons between strains. Previously the repetitive nature of the *T. cruzi* genome has hampered the search for suitable targets; at least 50% of the *T. cruzi* genome consists of tandemly repeated genes (El-Sayed et al., 2005). Now MLST, multilocus microsatellite typing (MLMT) and, ultimately, comparative genomics have the capacity to improve dramatically the understanding of *T. cruzi* population genetics and disease epidemiology.

MLST of nuclear or mitochondrial genes is more broadly applicable than MLMT for formal genetic, phylogenetic, and taxonomic studies. It has the advantage that the functional roles of many genes are known, lower mutation rates means they are less prone to homoplasy, selective pressures can be inferred, and the mutational mechanism is well characterized. This means that selection of appropriate targets permits both recent and potentially ancient recombination events to be deduced. A basic MLST approach for *T. cruzi*, comparing incongruence between individual phylogenetic trees, was employed by Machado and Ayala (2001) in their

study of genetic recombination in natural populations that confirmed TcV and TcVI were interlineage hybrids. A standardized panel of suitable MLST loci for *T. cruzi* is under development. MLST based on nine genetic loci (Yeo et al., unpublished data) has demonstrated accurate lineage assignment, incongruent phylogenetic topologies, potential gene mosaics within DTUs, and putative intralineage hybrid and parental isolates based on patterns of SNPs.

Microsatellites display a far higher degree of intralineage polymorphism than protein-coding nucleotide sequences. MLMT thus has the advantage of being a high-resolution approach to investigating recent evolutionary history, population structure, multiclonality, and evidence of recombination among closely related isolates, and is particularly applicable to intralineage recombination. This approach provides a powerful means of unraveling the microdynamics of *T. cruzi* in different epidemiological situations. Until recently only a small number of loci had been applied to relatively few strains (Oliveira et al., 1998).

Analysis by MLMT involves genotyping isolates at a large number of microsatellite loci distributed on many different chromosomes. Microsatellites are composed of short tandem repeats of di-, tri- or tetra-nucleotide motifs (e.g., TCTC TCTC, GATGATGAT, TTTATTTATTTA). Because of the repetitive nature of these sequences, they are prone to replication errors that result in frequent expansion or contraction of the tandem repeat; for example, from 10 repeat units to 12. Over time these mutations accumulate at a far higher rate than nucleotide substitutions and so microsatellite loci are typically highly polymorphic, enabling resolution at an intralineage level. At each locus the number of repeats present in any strain can be determined using PCR and used to build up multilocus genotypes that can then be compared between different strains. Improved population sampling combined with the use of these high-resolution markers is now beginning to provide a better understanding of intralineage population structures.

MLMT has been applied to analyze the population substructures in South America of both TcI and TcIII, revealing extensive intralineage diversity that had previously been hidden (Llewellyn et al., 2009a,b). Patterns of diversity among isolates that form genetic subpopulations can be analyzed to detect signatures of recombination at a local scale. Population genetic theory provides us with numerous tools to do this, which are useful so long as we are careful to interpret their output in the light of the assumptions on which they are based. Fundamental among these tools are statistics that measure the statistical likelihood for co-occurrence of alleles (linkage) at different loci among samples (e.g., Maynard Smith et al., 1993), although allele frequencies at individual loci and the frequency of repeated genotypes can also be informative. In general, recombination should disrupt such associations between alleles whereas clonal reproduction will maintain them. MLMT analysis has shown that at the intralineage level, the co-occurrence of alleles at different loci among samples is commonplace in those *T. cruzi* populations so far examined, suggesting that widespread clonality prevails at this level, without sufficient amounts of recombination to break up associations between microsatellite alleles (Llewellyn et al., 2009a,b). However, not all observations of *T. cruzi* intralineage population structures are consistent with a total lack of recombination.

Strictly asexual (diploid) populations are expected to be characterized by fixed heterozygosity due to alleles at the same locus independently acquiring different mutations over time, a process often referred to as the "Meselson effect" (Welch and Meselson, 2000). However, this phenomenon is not observed for TcI or TcIII; natural sylvatic populations actually show unexpectedly low levels of heterozygosity. This also appears to also be true of TcII and TcIV, though sampling of these lineages has been far less comprehensive (Machado and Ayala, 2001; Westenberger et al., 2005). The hybrid lineages TcV and TcVI are highly heterozygous, but this is a consequence of relatively recent hybridization rather than long-term clonal reproduction. Explanations for the high levels of homozygosity in TcI−IV may include frequent loss of heterozygosity (LOH) due to high rates of gene conversion or mitotic recombination. Alternatively, sexual recombination, even at relatively low frequencies, could prevent allelic divergence; this could occur between individuals that are genetically identical (selfing), closely related (inbreeding), or genetically distinct (outcrossing) (Machado and Ayala, 2001).

The factor that most limits the usefulness of MLMT in *T. cruzi* is not the availability of highly variable markers, as they are ubiquitous throughout the genome. It is appropriately sampled populations that are extremely rare. To draw accurate conclusions regarding the frequency of recombination from population genetic data, samples must be collected at high density from restricted foci across a limited time interval, and ideally involve analysis of multiple parasite clones taken from the same host or vector (Prugnolle and De Meeus, 2010). This stems from the basic expectation that genetic exchange is likely to occur among strains that are frequently in physical contact with one another and remains an important consideration for future research directions. Application of such an approach to *T. cruzi* will be required to determine the true relative rates of recombination and clonality in natural populations.

18.5 Conclusions and Future Research

In conclusion, the concept that genetic exchange is of little importance to *T. cruzi* is no longer tenable. Experimental work has demonstrated that *T. cruzi* has an extant capacity for genetic exchange, albeit by a somewhat unusual mechanism of fusion of diploids and genome erosion. Interlineage genetic exchange, though infrequent, has clearly shaped the evolution of the species, giving rise to at least two of the principal DTUs, TcV, and TcVI. Furthermore, this has had a profound epidemiological impact: TcV and TcVI are widespread and probably recently dispersed agents of severe Chagas disease. Mitochondrial introgression suggests additional interlineage recombination events. Mechanisms of genetic exchange in natural populations may be more varied and distinct from fusion of diploids and genome erosion discovered in the laboratory. The extent of intralineage genetic exchange is still not clear and demands improved, intensive multiclonal sampling and genetic analysis of populations from single hosts, vectors, and communities. Ideally, further experimental crosses combined with genome-scale sequence analysis of parents and resulting hybrids, as well as higher resolution analysis of natural hybrids and

their putative parents, would give rapid and incisive insight into mechanisms of genetic exchange in *T. cruzi.*

In terms of control of Chagas disease, it is important to re-examine the association of *T. cruzi* genotypes with drug susceptibility, virulence, and pathogenesis, all of which are traits that may be rapidly spread by genetic exchange within and between lineages. Given the high level of genetic diversity within *T. cruzi* and the complexity of its population structure, new drugs should be tested against representatives of all the lineages, including hybrid strains.

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19 Reticulate Evolution in *Trypanosoma cruzi*: Medical and Epidemiological Implications

Michel Tibayrenc, Christian Barnabé and Jenny Telleria*

Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS), Montpellier, France

19.1 Introduction

The title of this chapter may sound strange to those who are not familiar with the concepts of evolutionary genetics. It refers to the evolutionary pattern in which discrete genetic lines undergo preponderant separate evolution, partly countered by occasional bouts of hybridization events. This situation is reached in many higher plants. It appears to be the best way to summarize the evolutionary strategy of *Trypanosoma cruzi*.

19.2 An Indispensable Recall of Evolutionary Genetics

19.2.1 Preponderant Clonal Evolution—What Does It Mean?

This is the basic evolutionary model proposed for *T. cruzi* (Tibayrenc et al., 1986). Many misleading interpretations have been made, willingly or unwillingly, of the model designated by this term. Clarification is hence necessary. It cannot be emphasized enough that clonality, according to this model, refers to all cases where offspring have **multilocus genotypes** that are identical or extremely similar to parental lines, whatever the cytological mechanism of reproduction may be. Preponderant clonal evolution, in this genetical meaning, is synonymous with lack or rarity of *genetic recombination* (reassortment of **genotypes** occurring at different loci). This situation can originate from: (i) mitotic propagation; (ii) several cases of parthenogenesis; (iii) gynogenesis, hybridogenesis; (iv) self-fertilization in the homozygous state; and (v) extreme cases of homogamy.

* E mail: Michel.Tibayrenc@ird.fr

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Mitotic propagation (i) is the usual case observed in many bacterial species and does occur in *T. cruzi*. However, clonal evolution, again, has a much broader meaning. Cases (ii)−(v) are able to generate genetic clones as well. Parthenogenesis (ii) is observed in many insects, reptiles, and even mammals. Specific cases of parthenogenesis are recorded in some fish and salamander species. Interestingly, gynogenetic and hybridogenetic females mate and therefore mimic the behavior of sexuality. Only the genetic analysis of their offspring can evidence that they actually generate clonal lines. Cases (iv) and (v) have been presented as an alternative hypothesis to clonal evolution in the case of *Leishmania* parasites (Rougeron et al., 2009), while on the contrary, they obviously constitute only a specific case of it (Tibayrenc and Ayala, 1991, 2002). Another recurrent source of misunderstanding comes from the fact that the clonal model does not by any means rule out occasional bouts of genetic exchange (Tibayrenc et al., 1990). It only stipulates that such events are rare and interfere only at an evolutionary scale. The clonal model therefore does not amount to absolute clonality, as sometimes claimed (Sturm and Campbell, 2009).

Lack of, or severe restrictions to, *genetic recombination* is the only, necessary and, sufficient criterion to settle the working hypothesis of broad-sense clonality (genetic clonality). The relevant **population genetics** statistics is *linkage disequilibrium* (LD), or nonrandom association of genotypes occurring at different loci. By definition, LD evaluates the obstacles to recombination and is the only statistical approach able to do this. Unilocus segregation analysis based on *Fis*, *Fst*, Hardy−Weinberg equilibrium analysis (De Meeûs et al., 2007), although very useful to explore mating strategies in depth, by its very nature cannot estimate the strength of recombination inhibition.

LD analysis is based on the very simple principle that the expected frequency of multilocus genotypes is the product of the observed frequencies of the unilocus genotypes they are composed of. For example, if two loci A and B are surveyed, and the observed frequencies of genotypes A1 and B2 are 0.3 and 0.5, respectively, the expected frequency of the bilocus genotype A1 + B2 is $0.3 \times 0.5 = 0.15$. When a large number of loci are surveyed, this analysis becomes very powerful, because the mere fact that a multilocus genotype is recorded more than once could become highly improbable. Calculating this by hand and simple chi-square analysis is possible but rapidly becomes cumbersome. Various indices have been published (Tibayrenc et al., 1990; Maynard Smith et al., 1993). Biases due to time or geographical separation (Wahlund effect) have been previously discussed (Tibayrenc, 1995).

LD tests evidence severe obstacles to recombination. However, in some cases, they could be positive in situations where the genetic clones in a given species are ephemeral and soon disappear in the common gene pool of the species (epidemic clonality; Maynard Smith et al., 1993). From an epidemiological and medical point of view, the important parameter to evaluate is the stability of the genetic clones in space and time. For this, LD analysis is usefully completed by two approaches: (1) direct observation and (2) **phylogenetic** analysis.

1. Direct observation is done at a limited time scale. *T. cruzi* strains have now been charac terized for long enough to score recurrent observations of multilocus genotypes that have been repeatedly sampled for more than 30 years over vast geographical areas. This is in

itself a strong indication of stable clonal propagation. Such observations cannot be made in highly recombinant pathogens such as *Helicobacter pylori* (Go et al., 1997) or *Neisseria gonorrhoeae* (O'Rourke and Stevens,1993).

2. Phylogenetic analysis addresses a much longer time scale than population genetics and aims at reconstructing the evolutionary past of a given species over thousands or millions of years. This chapter is not the place to provide a comprehensive presentation of phylo genetic analysis many valuable textbooks have detailed the matter. Instead, I will pres ent a few general principles that are specifically relevant for surveying the subspecific phylogenetic diversity of *T. cruzi*.

19.2.2 T. cruzi *Is Undergoing Some Genetic Exchange*

In an organism such as *T. cruzi*, in which some recombination is occurring (see introduction and further), phylogenetic analysis should be understood in a specific manner. Indeed, by definition, phylogenetic analysis surveys the evolution of discrete lines that are genetically strictly separate from each other (**clades**). When some genetic exchange occurs between these lines, it clouds the phylogenetic picture of the species under study (Tibayrenc and Ayala, 2002). The observed departures from an ideal phylogenetic reconstruction are in themselves useful information on how and how much occasional recombination is operating. On the other hand, the cleaner the phylogenetic reconstruction is, the stronger the evidence is that recombination is exceptional or absent.

19.2.3 *"Gene Trees and Species Trees Are Not the Same" (Nichols, 2001)*

The evolutionary history of a given gene often does not reflect the general evolution of the species under study. The gene could undergo strong selective pressures or have a specific evolutionary rapidity (**molecular clock**). The general, most welcome, tendency is now to base phylogenetic reconstruction of species on a broad range of genes. Although gene sequencing conveys a great deal of information, the use of classical markers such as multilocus enzyme electrophoresis (MLEE) or random amplified polymorphic DNA (RAPD), if it is based on a broad range of loci, could be more reliable than the analysis of the sequence of only one gene or a few genes to reconstruct the evolution of a species. Many studies on *T. cruzi* have relied on the analysis of only one gene or a few genes (O'Connor et al., 2007; Spotorno et al., 2008; Marcili et al., 2009). These studies convey useful information on the precise genes they investigate. However, extrapolation to the entire species should be done with caution. The ideal approach is of course to combine the power of gene sequencing and multilocus analysis. This is done by multilocus sequence typing (MLST) (Maiden et al., 1998).

19.2.4 *Comparing the Information of Different Markers*

Different genetic markers explore different regions of the genome that have specific evolutionary patterns. For example, MLEE explores only genes that code for enzyme variants, while RAPD also surveys noncoding sequences and is able to

evidence copy-number polymorphism (insertions/deletions/inversions), contrary to both MLEE and MLST. When the **phylogenies** designed after different markers corroborate each other, this is particularly strong evidence that these phylogenies are valid, according to the congruence principle (Avise, 2004) and a specific strong case of LD (correlation between independent sets of genetic markers: criterion g Tibayrenc et al., 1990).

19.2.5 A Wrong Statement: Microsatellites Cannot Be Used for Phylogenetic Reconstruction

It has been claimed that microsatellites exhibit too much **homoplasy** to be useable for phylogenetic reconstruction. Actually the same can be said for the other uses of microsatellites, such as population genetics and strain typing. For example, in population genetics, homoplasy could mimic genetic recombination and lower the observed rate of LD. In strain typing, due to homoplasy, two strains could appear more closely related than they actually are. Microsatellites can be used for phylogenetic reconstruction, but must be used with caution. One has to address microevolutionary levels only, in accordance with the fast molecular clock of this marker. Moreover, the risk of homoplasy should be kept in mind.

19.3 The Results: How Does *T. cruzi* Evolve?

Having performed evolutionary analysis based on the principles stated here, the following results have emerged.

19.3.1 *Is* T. cruzi *a "Good" Species?*

This is the first question to arise from a medical and epidemiological point of view, especially when studying molecular epidemiology (strain typing). Defining what a "good" species is refers to the very definition of what a species is. Briefly, species are generally defined as: (i) a community of sexual reproduction (the biological species concept); (ii) a clade (a monophyletic lineage with only one ancestor; the phylogenetic species concept); or (iii) a set of organisms that share specific **phenotypic** traits (the phenotypic species concept). The biological species concept is not easy to handle and inadequate in those organisms in which sexual reproduction is not a constant and mandatory process, as for the agent of Chagas disease. On the other hand, there is no doubt that *T. cruzi* meets the criteria for (ii) and (iii) (phylogenetic and phenotypic species concepts). Phylogenetic studies have brought all *T. cruzi* strains into a single clade that is distinguishable from closely related taxa (*T. cruzi marenkellei*, a close relative of *T. cruzi*, a bat parasite, is the best choice of such an **outgroup**). Moreover, all *T. cruzi* strains share a set of specific phenotypic characteristics (morphological aspect, vectorial transmission by triatomine bugs, potential host range extended to all mammal species but restricted to them, geographical distribution limited to the New World, and potential pathogenic power all point to Chagas disease). Consequently, according to the phylogenetic and phenotypic concepts, *T. cruzi* is a so-called "good" species. The fact that *T. cruzi* is a

unique clade makes it possible to design various molecular markers that will be specifically shared by all strains of the taxon and only by them (in the **cladistic** jargon, synapomorphic characters; see also the concepts of DTU and tags below).

19.3.2 T. cruzi *Is Undergoing Predominantly Clonal Evolution*

It can actually be said that the agent of Chagas disease is a paradigmatic case of this evolutionary model. Recurrent observations have been made of multilocus genotypes that have persisted unchanged for more than 30 years over vast geographical areas. Moreover, the LD in *T. cruzi* is considerable and has been verified for a large set of genetic markers, including MLEE (Tibayrenc et al., 1986; Barnabé et al., 2000), restriction-fragment length polymorphism of kinetoplast DNA (Tibayrenc and Ayala, 1987), RAPD (Tibayrenc et al., 1993; Brisse et al., 2000a), and microsatellites (Oliveira et al., 1998). LD is so strong in *T. cruzi* that it also involves the polymorphism of expressed genes surveyed by random amplified differentially expressed sequences (RADES) (Telleria et al., 2004).

Again, clonality and clonal evolution are taken here in the genetic sense and the precise cytological mechanism of reproduction continues to be debated. Homogamy and self-fertilization (Rougeron et al., 2009) could play a role in some cycles, as suggested by results obtained with microsatellite markers (Llewellyn et al., 2009b). Nevertheless, the important result remains: genetically homogeneous lines isolated from each other (genetic clones by definition) persist over long periods of time and vast geographical areas in natural populations of *T. cruzi*.

19.3.3 *Genetic Variants of* T. cruzi *Represent Clonets, Not Clones*

Once predominant clonal evolution has been ascertained, it comes to mind that the genotypes identified by MLEE, RAPD, microsatellites, etc., represent clones of this parasite. Now let us identify genotypes with 15 MLEE loci. For example, 43 different genotypes can be observed (Tibayrenc et al., 1986). If we take a broader range of loci, say 22 of them, many of the previously identified presumably homogeneous genotypes split into several additional ones (Barnabé et al., 2000). This is a never-ending story. The only way to exhaust the complete clonal diversity of a given species would be to sequence all of its strains, an unfeasible task. Moreover, this would not take into account genomic rearrangements, which also play a role in clonal diversity and could be involved in gene expression.

To overcome this difficulty, the term "clonet" has been coined (Tibayrenc and Ayala, 1991) to refer to those sets of stocks that appear to be identical with a given set of genetic markers in a basically clonal species. *T. cruzi* zymodemes, schizodemes, RAPDdemes, etc. should therefore be considered clonets rather than true clones. From an evolutionary point of view, clonets represent sets of closely related clones.

19.3.4 *Recombination Operates at the Evolutionary Scale: Reticulate Evolution*

The presence of hybrid genotypes in *T. cruzi* natural populations has long been hypothesized (Bogliolo et al., 1996; Carrasco et al., 1996; Macado and Ayala,

2001; Brisse et al., 2003). The potentiality of genetic recombination in this species has been experimentally demonstrated by Gaunt et al. (2003). These experimental hybrids have been only obtained from parental genotypes that are genetically closely related. They seem to be generated by fusion of diploid parents followed by genomic erosion, slowly returning to a diploid state. Natural hybrids seem to be of a different nature. They are predominantly diploid and can be the result of hybridization between distantly related parental genotypes (Lewis et al., 2009a). Different scenarios and genealogies have been proposed to explain the generation of the currently observed natural hybrids in *T. cruzi* (Sturm and Campbell, 2009). This will be detailed further below. At this step, a number of important points about recombination in *T. cruzi* should be underlined: (i) these events are exceptional in this parasite's natural populations. By definition, frequent genetic exchange would be incompatible with the strong inhibition of genetic recombination evidenced by LD. (ii) Once generated, the hybrid genotypes are stabilized by clonal propagation and behave like genetic clones. They have been recurrently observed unchanged over long periods of time and large geographical areas. (iii) These natural hybrid clones are widespread in domestic chagasic cycles and could represent a specific adaptation to these transmission cycles, in which they behave like successful genotypes.

19.3.5 T. cruzi *Is a Structured Species*

Natural populations of *T. cruzi* are divided into discrete and stable subdivisions between which the **genetic distances** convey reliable evolutionary information (Tibayrenc, 1995). The existence of such clusters was first postulated by the pioneering studies of Miles et al. (1977, 1978). It is interesting to note that, in spite of a lack of genetic interpretation of these data, the MLEE variants identified at that time (zymodemes I, II, and III) are still there, although the picture has of course been refined by further studies (Table 19.1). Again, the permanency of the multilocus genotypes to which these zymodemes correspond is in itself a strong indication of stable clonal propagation.

Table 19.1 Correspondence between the Most Recent Nomenclature of *Trypanosoma cruzi* Genetic Variants and Previously Published Nomenclatures

1	2	3	4	5
DTU I	DTU 1	1–25	Z I	X10 cl1, Cuica cl 1
DTU II	DTU 2b	26–29	Z III	CAN III cl 1
DTU III	DTU 2c	30–34	Z II	TU 18 cl2
DTU IV	DTU 2a	35–37	Z II	M5631, M6241
DTU V	DTU 2d	38, 39	Z II	MN cl2, SC43 cl1
DTU VI	DTU 2e	40, 43	Z II	CL Brenner, Tulahuen cl2

1 = Zingales et al. (2009); 2 = Brisse et al. (2000a); 3 = Tibayrenc et al. (1986); 4 = Miles et al. (1977); 5 = Representative stocks.

19.3.6 The Six Discrete Typing Units

The term "discrete typing units" (DTUs) was coined to overcome the difficulty that occasional hybridization events make it impossible to consider *T. cruzi*'s genetic subdivisions as true clades. Indeed, these subdivisions are not strictly separated from each other, as true clades should be, and hybrid clones have two parental genotypes while true clades should originate from one ancestral parent only. The concept of DTUs has therefore been proposed (Tibayrenc, 1998). These are sets of stocks that are genetically closer to each other than to any other stock and are identifiable by common molecular, genetic, biochemical, or immunological markers called tags. The genetic subdivisions identified within *T. cruzi* fully match this definition, whereas they very imperfectly fit the definition of clades.

Detailed analysis by MLEE (Barnabé et al., 2000), RAPD (Brisse et al., 2000a), and gene sequencing (Brisse et al., 2000b; Brisse et al., 2001) have congruently evidenced the existence of six DTUs within *T. cruzi* (see Figure 19.1). These DTUs have also been uncovered by RADES (Telleria et al., 2004), which shows that this structuring persists when surveying coding expressed genes. PCR-RFLP typing (Rozas et al., 2007) has also corroborated this clustering into six DTUs. In addition, multilocus gene sequencing is consistent with DTU structuring, although gene phylogenies uncover less reliable subdivisions than classical markers (Subileau et al., 2009).

The partition of *T. cruzi* into two major subdivisions (Tibayrenc, 1995; Souto et al., 1996), DTU 1 and 2 (Barnabé et al., 2000; Brisse et al., 2000a), named TC I and TC II (TC = *Trypanosoma cruzi*; Anonymous, 1999) is presently questioned, although not necessarily ruled out. In a recent meeting, Chagas disease experts decided to retain the subdivision of *T. cruzi* into six DTUs as the official reference model for this parasite's genetic variability. However, it was decided to renumber the DTUs into TC I−VI instead of DTUs 1, 2b, 2c, 2a, 2d, and 2e, respectively (see Table 19.1) to take into account the present debate on the presence of two major subdivisions (Zingales et al., 2009).

It has proven to be impossible to reliably identify each DTU with unique markers. Instead, a convenient set of three markers has been proposed (Lewis et al., 2009b). Thus far, the six DTUs have been upheld. The studies that have evidenced them have sometimes relied on impressively extensive samples, the most exhaustive of which is the MLEE study by Barnabé et al. (2000), which involved no less than 434 different stocks. It is possible that this classification will be slightly modulated by further samplings involving more sylvatic cycles. However, to date these, studies have evidenced further variability within the DTUs and have revealed some very interesting new epidemiological features, but have corroborated the classification (Llewellyn et al., 2009a,b).

19.3.7 Evolutionary Origin of the DTUs

Some DTUs have a clear hybrid origin. The number of hybrid events and the precise sequence that have generated the present DTUs continues to be debated (Sturm and Campbell, 2009). It is generally recognized that the evolutionary origin

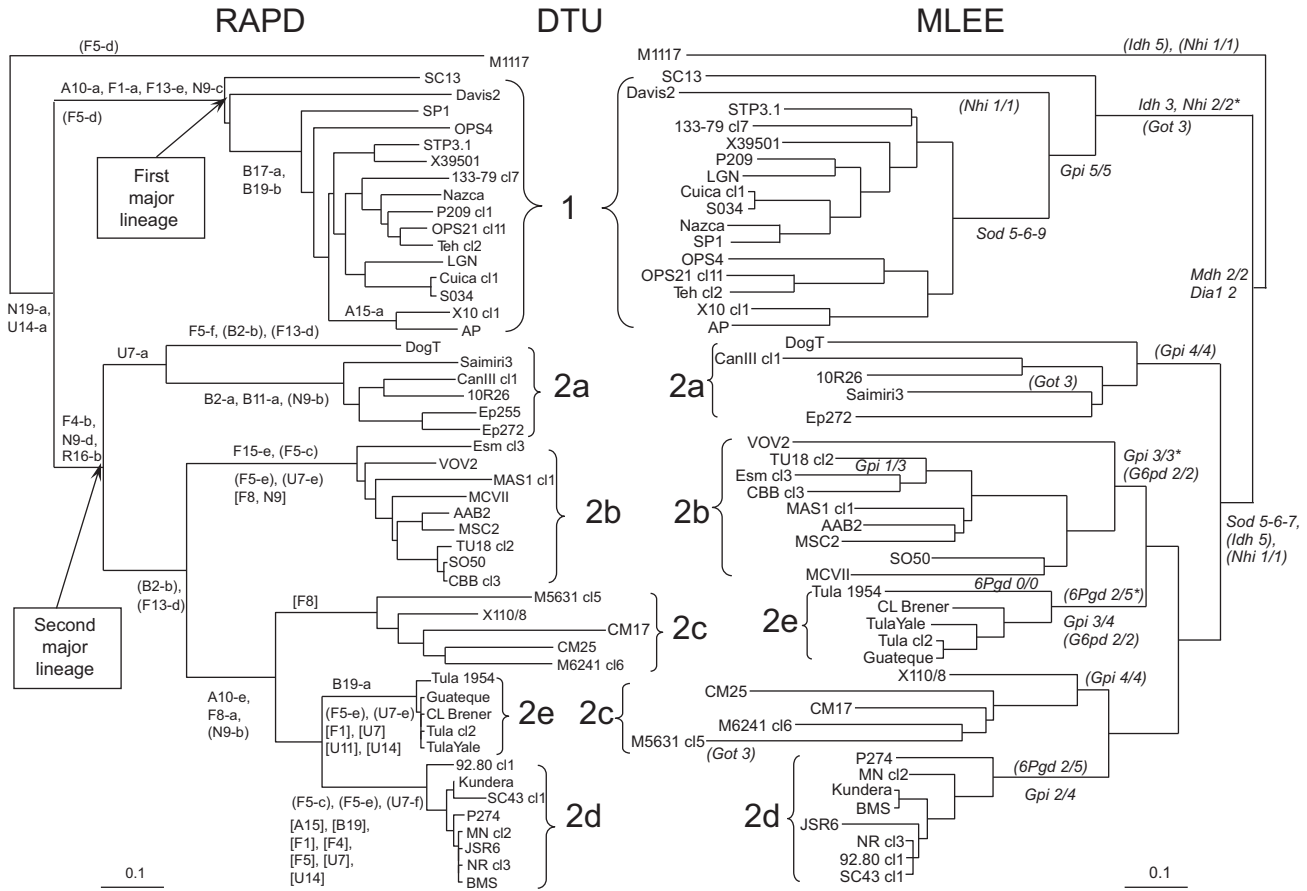

Figure 19.1 Two phylogenetic trees depicting the evolutionary relationships among *Trypanosoma cruzi* genotypes: isoenzymes (left) and RAPD (right). The strong agreement between the two trees is a clear manifestation of LD.
Source: From Brisse et al. (2000a).

of some DTUs results from the following recombination events: (i) DTU III and IV probably originated from a hybridization event between ancestral DTU I and II genotypes (Westenberger et al., 2005) and (ii) DTUs V and VI probably resulted from the hybridization of genotypes similar to DTU II with DTU III (Brisse et al., 2003; Westenberger et al., 2005).

19.3.8 Nomenclature Considerations

The name of the DTU has thus far been limited to the scientific literature on the agent of Chagas disease, although it could be valuably applied to many pathogenic microorganisms.

We have already explained why the term "clade" is inappropriate to designate *T. cruzi* genetic subdivisions. "Clusters" and "groups" are also inadequate, because, contrary to DTUs, they have no precise definition.

A meeting of experts on *T. cruzi* nomenclature suggested replacing the DTUs with six Linnaean species with binomial Latin names, but the proposal was not accepted. The group has agreed that new species of pathogenic microorganisms should be based on the congruence of clear phylogenetic and medicophenotypic characteristics only.

19.3.9 Medical and Epidemiological Characteristics of the DTUs

These specificities have often been exaggerated. For example, it is incorrect to consider that DTU I is specifically linked to sylvatic cycles (Sturm and Campbell, 2009). It has been recorded many times in classical domestic cycles involving chronic chagasic patients and the vector *Triatoma infestans*. DTU IV (zymodeme III), classically considered to be linked to sylvatic Amazonian cycles, has been also recorded in chronic chagasic patients in Ecuador (Garzón et al., 2002).

Now the null hypothesis that DTU subdividing is neutral compared to Chagas disease epidemiology and geographical distribution can be reliably rejected.

The geographical distribution of the DTUs is not uniform. Most of them are present in most places of Chagas transmission, including the USA, which shows that *T. cruzi* pertains to the native fauna of that country (Barnabé et al., 2001). However, the hybrid DTUs V and VI are predominant in the southern part of the area of Chagas transmission and are specifically related to domestic cycles, for example. DTU I is largely predominant in Mexico and Venezuela. Still, exhaustive mapping of DTU geographical distribution remains to be done.

The question of a clinical specificity of *T. cruzi* genetic variants has long been open (Miles et al., 1981) and has not yet received a final answer. The DTUs show clear pathogenic specificities in animal experiments (De Lana et al., 2000) but this cannot be extrapolated to human disease.

More generally, *T. cruzi* DTUs often show strong statistical associations between various experimental parameters such as growth speed in *in vitro* and *in vivo* culture, pathogenicity for laboratory animals, transmissibility through insect vectors, and *in vitro* and *in vivo* sensitivity to antichagasic drugs (see e.g., Revollo

et al., 1998). However, these various studies are complex and therefore have investigated a limited number of stocks (approximately 20).

Lastly, there are clear links between DTU classification and gene expression surveyed by proteomic analysis (Telleria et al., in preparation).

19.3.10 *Relevance of* T. cruzi *Genetic Variability in Applied Research*

The concepts of clonet and DTU are robust, and it is likely that they will not be called into question by further studies. Clonets and DTUs are convenient units of analysis for molecular epidemiology (epidemiological tracking, strain typing). However, clonet identification should be based on a broad range of markers. A set of three markers has been proposed for reliable, routine characterization of the DTUs (Lewis et al., 2009b).

Apart from epidemiological tracking, DTUs should be taken into account for all applied studies investigating *T. cruzi*: clinical studies, drug development, diagnostic testing, and vaccine design. It is crucial, for example, to verify that a new drug is effective in all genetic variants of *T. cruzi*. A set of stocks representative of the DTU classification should therefore be used for all these types of study.

Lastly, clonets and DTUs are ideal units of analysis for experimental evolution, a domain that has thus far been insufficiently exploited in Chagas disease (Tibayrenc, 2009).

19.4 Conclusion: *T. cruzi* Is a Star in the Field of Pathogen Population Genetics

This is unexpected, since the community of scientists working on Chagas disease is very limited by comparison with those working on AIDS, tuberculosis, and malaria. Nevertheless, it can be said that *T. cruzi* is by far the pathogenic microorganism that population genetics knows best and can compete in this domain with the bacterium *Escherichia coli*. In spite of the obstacle that it is a pathogenic agent, it is our hope now to "sell" *T. cruzi* as a pet model for basic evolutionary research, together with *E. coli*, *Drosophila melanogaster*, *Mus musculus*, and *Caenorhabditis elegans* (Tibayrenc, 2009).

As far as applied research is concerned, it is our cherished hope that *T. cruzi*'s genetic variability is far from having revealed all its secrets and will continue to make great contributions to the survey, control, and cure of Chagas disease.

Glossary

Clade evolutionary lineage defined by cladistic analysis. A clade is monophyletic (it has only one ancestor) and is genetically isolated (which means that it evolves independently) from other clades.

Cladistic analysis a specific method of phylogenetic analysis based on the polarization of characters that are divided into ancestral (plesiomorphic) and derived (apomorphic) characters.

Genetic distance various statistical measures inferred from genetic data, estimating the genetic dissimilarities among individuals or populations. Genetic distances can be based on the percentage of band mismatches on gels (as for markers such as MLEE or RAPD) or allelic frequency differences or the percentage of sequence divergence.

Genotype genetic constitution of a given organism; see also **Phenotype**.

Homoplasy possession shared by distinct phylogenetic lines of identical character that do not come from a common ancestry. The origin of homoplasic character can be: (*a*) con vergence (possession of identical character derived from different ancestral character, due to convergent evolutionary pressure; f), (*b*) parallelism (possession of identical character derived from a single ancestral character, and generated independently in different phylo genetic lines), and (*c*) reversion (restoration of an ancestral character from a derived character).

Molecular clock in its strict, original sense (more appropriately called the DNA clock hypothesis), the concept that the rate of nucleotide substitutions in DNA is constant. In a broader sense, it is simply the evolutionary speed of the part of the genome that encodes the variability of a given marker. This speed is commended by the rate of substitution/ mutation. It may be regular or irregular.

Multilocus genotype the combined genotype of a given strain or a given individual estab lished with several genetic loci.

Outgroup in cladistics or phylogenetics, an outgroup is a (monophyletic) group of organ isms that is used as a reference group for determining the phylogenetic relationship in a set of monophyletic groups of organisms. The outgroup is selected to be closely related to the groups under study, but less closely than any single one of the groups under study is to another.

Phenotype all observable properties of a given individual or a given population apart from the genotype. The phenotype is not limited to morphological characteristics and can include, for example, physiological or biochemical parameters. The pathogenicity of a microorganism is a phenotypic property. The phenotype is produced by the interaction between genotype and the environment.

Phylogenetics a branch of genetics that aims at reconstructing the evolutionary past and genetic relationships of taxa, species, strains, or of separate evolutionary lines.

Phylogeny evolutionary relationships between taxa, species, organisms, genes, or molecules.

Population genetics analysis of allele and genotype frequency distribution and modifica tions under the influence of genetic drift, natural selection, mutation, and gene flow. It also takes into account the factors of population subdivision and population structure.

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20 Implications of *Trypanosoma cruzi* Intraspecific Diversity in the Pathogenesis of Chagas Disease

Andréa M. Macedo and Marcela Segatto*

Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Minas Gerais, Brazil

20.1 Introduction

As will be detailed in Chapter 27, Chagas disease is characterized by a variable clinical course. Following parasite infection, there is a short acute phase characterized by abundant parasitemia but frequently mild and nonspecific symptoms make the recognition of the contagion difficult. In less than 5% of the cases, fatal neurological and/or cardiac complications are observed during the acute phase. The infection then proceeds to a chronic phase, with scarce parasitemia and an unpredictable clinical course that ranges from the absence of symptoms to severe disease with cardiovascular and/or gastrointestinal involvement (Prata, 2001; Pimenta, 2002).

Geographical variations in the prevalence of clinical forms and morbidity of Chagas disease have been reported. In Brazil, the asymptomatic or indeterminate form is the most common (60−70%) followed by the cardiac and digestive forms (20−30% and 8−10%, respectively). The cardio-digestive form is the rarest. However, in Central Brazil and Chile, the digestive form of Chagas disease predominates but is practically nonexistent in Venezuela and Central America (Luquetti et al., 1986; Dias, 1992). The reason for this geographical heterogeneity and why different patients develop different clinical forms remains unexplained, but certainly, both host and parasite factors are involved.

It is interesting to observe that the relative importance attributed to parasite versus human-related variables has changed in the evolution of pathogenetic explanations for Chagas disease. Historically, it is possible to distinguish three main phases of our pathogenetic knowledge of Chagas disease. The first, which we refer to as the "parasite phase," corresponds to the period immediately following the

* E mail: andrea@icb.ufmg.br

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discovery of the disease when there was no questioning of the primary pathogenetic role of *T. cruzi*. In this period, there was an intense, although largely fruitless, search for biological and biochemical methods and markers capable of establishing a link between the variability of the parasite and the clinical form of the disease. The failure of these endeavors, associated with the scarcity of parasites in the blood and tissues of chronic symptomatic patients, led to the "autoimmune phase." In this period, the pathogenetic importance of the parasite was relegated to a lower tier, except as a provider of cross-reacting epitopes capable of unleashing autoimmune reactions (Schmuñis, 1987; Kalil and Cunha-Neto, 1996). More recently, the primary role of *T. cruzi* in the pathogenesis of Chagas disease has been reestablished, and we are now experiencing the third wave, which we call the "genomic phase." Infirmity is seen as the product of the interaction of two highly variable genomes: the parasite's and the human's. The main purpose of this chapter is to review the parasite counterparts that have led to this genomic paradigm.

20.2 The Genomic Paradigm

In 1909, Carlos Chagas first described the disease that bears his name in a 2-year-old child from the state of Minas Gerais, Brazil (Chagas, 1909). Indeed, in 1909, Chagas had already noted a peculiar dimorphism (so-called slender and stout forms) in the bloodstream trypomastigotes of *T. cruzi*, which was later amply confirmed by others (Da Silva, 1959; Brener, 1965). Today, these morphological forms are believed to emerge from epigenetic phenomena, and their pathological relevance is obscure (Howells and Chiari, 1975). At an early stage, the idea of differential tissue tropism in the origin of the pathogenesis of Chagas disease was also proposed (Vianna, 1911; Campos, 1927) and persisted despite only tenuous evidence based mostly on the parasite distribution in different tissues during the acute phase of experimentally infected animals (Taliaferro and Pizzi, 1955; Hanson and Roberson, 1974; Melo and Brener, 1978; Andrade et al., 1983; Andrade and Magalhaes, 1996).

In chronic Chagas disease, parasites are rarely found in tissues examined by routine staining techniques (Koberle, 1968; Palacios-Pru et al., 1989). However, their presence has been unveiled by application of powerful new immunohistochemical techniques developed over the past two decades (Higuchi Mde et al., 1993). Moreover, application of the exquisitely sensitive polymerase chain reaction (PCR) to the study of Chagas disease provides reliable molecular evidence that cogently ties tissue lesions to the presence of the parasite (Jones et al., 1993; Brandariz et al., 1995; Vago et al., 1996a). These new studies highlight the primary role of *T. cruzi* in the pathogenesis of Chagas disease and set the stage to establish the notion that genomic variation of *T. cruzi* might influence the course of the disease.

20.3 The Genetic Variability of *T. cruzi*

Different studies, including biological, biochemical, and molecular ones, have demonstrated that *T. cruzi* is a heterogeneous **species** (Macedo and Pena, 1998; Macedo et al., 2001; Devera et al., 2003). In this chapter, we will recapitulate some

of these studies, with emphasis on those that have contributed to a better understanding of the *T. cruzi* population structure and its role in the pathogenesis of Chagas disease.

The creative nomenclature assigned to the various groups of *T. cruzi* reflects the unique biology of the organism, and in the literature, subgroups of *T. cruzi* are partitioned into **biodemes, zymodemes, schizodemes, strains, stock, clones, clonets, lineages, clades**, and, most recently, **discrete typing or taxonomic units (DTUs), haplotypes**, and **haplogroups**. Definitions of these important concepts can be found in the glossary at the end of this chapter.

20.3.1 Zymodemes or Multilocus Enzyme Electrophoresis

The first experimental method demonstrating the extent of genetic diversity of *T. cruzi* was the analysis of electrophoretic variants of cellular enzymes (isoenzymes). This procedure involves electrophoresis of parasite extracts on starch gels or cellulose acetate plates and biochemical staining with colorimetric or fluorescent substrates for different enzymes. On the basis of the variability of six loci, Miles and others proposed the existence of two isoenzymatic clusters: zymodeme 1 (Z1), circulating mostly in the sylvan environment, and zymodeme 2 (Z2), present in the domestic cycle of the protozoan and encompassing the vast majority of the infected human hosts in Southern Cone countries (Miles et al., 1977). A third isoenzyme group of *T. cruzi* was further characterized as zymodeme 3 (Z3) (Miles et al., 1978). This enzymatic variant, originally described in the Amazon region, was also associated with the *T. cruzi* sylvatic transmission cycle and was rarely isolated from humans (Barrett et al., 1980; Miles et al., 1981b; Povoa et al., 1984). Subsequent phylogenetic analyses among Z3 isolates from the Brazilian Amazon revealed a clear dichotomy in the Z3 group, defining two groups that were named Z3A and Z3B. These findings were confirmed by three independent genetic markers (Mendonca et al., 2002). During *T. cruzi* diversity studies in Bolivia, Chile, and Paraguay, two additional and apparently hybrid zymodemes were identified and named Bolivian Z2 and Paraguayan Z2 due to their close relationship to the Brazilian Z2 zymodeme (Tibayrenc and Miles, 1983). Subsequent studies using 15 isoenzyme loci demonstrated much higher genetic diversity, and 43 genotypes or clonets were proposed for *T. cruzi* isolated from different hosts from other regions of South America (Tibayrenc et al., 1986a; Tibayrenc and Ayala, 1988). Despite the extraordinary intraspecific diversity identified in *T. cruzi* numerical taxonomic analyses, these data demonstrated the existence of two major, highly heterogeneous, phylogenetic lineages of *T. cruzi* differing in several biological properties (Tibayrenc, 1995) that were subsequently partitioned into six DTUs or major lineages: I, IIa, IIb, IIc, IId, and IIe (Brisse et al., 2000a, 2001).

20.3.2 Schizodemes or kDNA Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphism (RFLP) analysis is a genotyping technique based on the existence of single nucleotide polymorphisms (SNPs) within recognition sites for specific restriction enzymes. Therefore, DNA samples from different organisms (digested by the same restriction enzyme) frequently present fragments of

different lengths that can be resolved by gel electrophoresis. RFLPs are suitable for intraspecific variability studies among closely related taxa and have been largely used for *T. cruzi* genotyping approaches using both the mitochondrial and nuclear genomes.

The first studies on DNA polymorphism of *T. cruzi* were published in 1980 by Morel et al., who reported on RFLPs of kinetoplast DNA (kDNA) minicircles (Morel et al., 1980). All members of the order Kinetoplastidae are characterized by the presence of a kDNA network within a single large mitochondrion. The kDNA constitutes 10−30% of the total cell DNA, and in *T. cruzi* comprises $5-20 \times 10^3$ minicircles of 1.42 kb and 20−50 maxicircles of 36 kb (Simpson, 1987). Each minicircle is organized into four 120-bp, highly conserved regions separated by four highly variable regions (Degrave et al., 1988). Parasite populations displaying identical or similar kDNA minicircle restriction patterns are called schizodemes (Morel et al., 1980).

kDNA RFLP analyses unraveled an unexpectedly high amount of genetic diversity in *T. cruzi*. Together with cloning experiments, kDNA restriction analysis demonstrated for the first time that single strains of *T. cruzi* could contain two or more distinct clonal genotypes (Morel et al., 1980). The existence of these multiclonal *T. cruzi* strains was later confirmed by several groups using different techniques (Deane et al., 1984; De Araujo and Chiari, 1988; Carneiro et al., 1991; Macedo et al., 1992a; Oliveira et al., 1998, 1999). Schizodemes presented some correlation with the isoenzyme classification, demonstrating that linkage disequilibrium can even occur between different (nuclear and mitochondrial) genome compartments (Tibayrenc and Ayala, 1987).

The RFLP technique, however, has some drawbacks. It requires a large amount of DNA (1×10^8 parasites), and the process and analysis are labor intensive. This technique was later simplified with a previous amplification of the hypervariable regions of kDNA minicircles by PCR using primers designed from flanking conserved regions, followed by digestion of the products, a technique known as PCR-RFLP, which requires many fewer parasites (Sturm et al., 1989).

20.3.3 Random Amplified Polymorphic DNA Analysis

The PCR-based random amplified polymorphic DNA (RAPD) technique and the less-used but closely related simple sequence repeat anchored primer PCR (SSR-PCR) have provided a sensitive approach for *T. cruzi* profiling and constitute useful tools for establishing genetic relationship among isolates (Dias Neto et al., 1993; Steindel et al., 1993; Tibayrenc et al., 1993; Oliveira et al., 1997; Brisse et al., 2000b). Complex and strain-specific banding patterns are evident for different *T. cruzi* populations, although no correlation with clinical aspects has been uncovered (Oliveira et al., 1997). However, when we compare the RAPD profiles of strains isolated from patients in the chronic phase of the disease with isolates from patients in the acute phase and with *T. cruzi* populations obtained from sylvatic vectors and mammals, a clear increasing gradient in variability is observed (Table 20.1). These results are compatible with the idea that not all *T. cruzi* strains are capable of establishing effective infections in *Homo sapiens* and that humans thus function as a filter in selecting more adapted subpopulations (Macedo and Pena, 1998; Macedo et al., 2002, 2004).

Table 20.1 Reduction of the *T. cruzi* Population Complexity During the Course of Human Infection as Revealed by the RAPD and MLMT Techniques

Strain Origin	Average Proportion of Shared Bands between Two Strains (RAPD Data Analysis)	Proportion of Strains with Two or More Peaks Per Locus (MLMT Data Analysis)
Strains isolated from nonhuman sources	22%	58%
Strains isolated from patients with acute Chagas disease	39%	ND
Strains isolated from patients with asymptomatic chronic Chagas disease	51%	ND
Strains isolated from patients with symptomatic chronic Chagas disease	53%	10%

MLMT: multilocus microsatellite typing; ND: not determined; RAPD: random amplified polymorphic DNA.
Source: Adapted from Macedo et al. (2004) with modifications.

The RAPD technique is based on the amplification of genomic DNA with single, and usually short, oligonucleotide primers of arbitrary sequence, which generate complex banding profiles (Welsh and McClelland, 1990; Williams et al., 1990). Essentially, any primer can be used for any organism if sufficiently low-stringency conditions are used and small amounts of biological material are required. However, this lack of specificity means that RAPD cannot be used for analyzing *T. cruzi* directly in infected tissue. Different authors have also pointed out that RAPD presents problems of reproducibility because the profile may vary according to the quality and quantity of DNA in the reaction, the composition of the buffer, and the brand of *Taq* polymerase used for PCR (Macedo et al., 1992b; Riedy et al., 1992; Oliveira et al., 1997). However, the RAPD profiles proved to be stable even after prolonged parasite cultivation (up to 100 generations of a *T. cruzi* clone), indicating that under rigidly controlled conditions, RAPD can have good reproducibility (Zingales et al., 1997).

RAPD has been largely used for *T. cruzi* numerical taxonomy studies, and strong correlations between the MLEE and the RAPD patterns have been identified (Steindel et al., 1993; Tibayrenc et al., 1993; Revollo et al., 1998). Moreover, RAPD profiling was also utilized for corroborating the *T. cruzi* taxon division into major lineages (Tibayrenc, 1995; Souto et al., 1996).

20.3.4 Low-Stringency Single Specific Primer PCR

A major drawback of RAPD (discussed earlier) is that the primers used cannot distinguish between host and parasite DNA. Thus, their use is limited to cultured

parasites, and it is not possible to examine organisms isolated directly from patients. One solution to this problem is to use a two-phased PCR protocol called low-stringency single specific primer PCR (LSSP-PCR). In LSSP-PCR, a purified DNA fragment is subjected to PCR using a single specific primer under low-stringency conditions. The primer hybridizes specifically to its complementary region and nonspecifically to multiple sites within the fragment, in a sequence-dependent manner, producing a highly complex set of reaction products that can be resolved by electrophoresis to generate "gene signatures" (Pena et al., 1994). The rationale of the approach is the use of specific primers to amplify a variable region of the parasites' DNA directly from blood samples or biopsies and then to probe the sequence variations in the amplified parasite-specific DNA fragment.

LSSP-PCR has been successfully applied to the approximately 330-bp variable DNA fragment of *T. cruzi* kDNA minicircles to produce "kDNA signatures," allowing profiling of parasites present in the tissues of chronically infected patients for the first time (Vago et al., 1996b). The initial idea of the differential tissue tropism playing fundamental importance in determining the pathogenesis of Chagas disease, proposed as early as 1911 (Vianna, 1911), was revitalized by the demonstration that parasites with different genetic profiles can be found in distinct tissues (esophagus and heart) of the same patient (Vago et al., 2000).

20.3.5 Multilocus Microsatellite Typing

An alternative approach to characterize *T. cruzi* directly in biological samples emerged with the discovery of polymorphic microsatellites in the *T. cruzi* genome (Oliveira et al., 1998). Microsatellites are polymorphic tandem repeats of 2- to 6-bp-long simple motifs that can exhibit great levels of polymorphism provided by variable numbers of repetitive units (Levinson and Gutman, 1987; Litt and Luty, 1989; Henderson and Petes, 1992). These markers evolve rapidly due to a higher mutation rate compared to that found in other regions of the genome. Polymorphic microsatellites represent approximately 1% of the *T. cruzi* genome and are distributed throughout the nuclear chromosomes. In addition, microsatellite loci are generally located in nonexpressed regions of the parasite nuclear genome, which makes them insensitive to selective pressures that can occur with other classical markers (e.g., isoenzymes or rDNA genes), and contrasting to other polymorphic DNA markers or techniques (e.g., RAPD and LSSP-PCR), the microsatellites are unilocal markers and present only one copy per haploid genome. Therefore, microsatellites provide a powerful tool to perform more refined population studies because the alleles present in each *T. cruzi* strain can be exactly determined (Oliveira et al., 1998, 1999; Macedo et al., 2001; Llewellyn et al., 2009a,b).

Presently, more than 50 polymorphic microsatellite loci, composed of di-, tri-, and tetranucleotide motifs, have been described for different *T. cruzi* genome analyses (Oliveira et al., 1998; Macedo et al., 2001, 2010; Freitas et al., 2006; Valadares et al., 2008; Llewellyn et al., 2009a). However, one of the most interesting applications of polymorphic microsatellites is to determine whether a specific parasite population is mono- or polyclonal. Monoclonal populations always present

only one or two peak patterns, indicating homo- or heterozygosity at the respective microsatellite locus. However, strains consistently presenting amplification patterns of three or more fragments of different sizes correspond to multiclonal populations (Oliveira et al., 1998; Macedo et al., 2001; Llewellyn et al., 2009b). Interestingly, microsatellite analyses indicate that the percentage of multiclonal populations decreases progressively as we compare strains isolated from the sylvatic cycle with those isolated from man, confirming the previous idea of a human filter selecting more adapted strains (Macedo and Pena, 1998). Usually less than 10% of strains isolated from humans in the chronic phase of infection are polyclonal, compared up to 58% of multiclonal populations isolated from sylvatic vectors in the Amazon (Table 20.1) (Macedo et al., 2004).

20.3.6 Miniexon and rDNA Polymorphisms

The mRNA of trypanosomes is unique in that most, if not all, mature transcripts bear an identical 35-bp splicer leader (SL) at their 5′-termini (Borst, 1986). This SL is encoded in blocks of tandemly repeated units, the miniexon genes (MEs). Comparative alignment of MEs from different *T. cruzi* strains shows the presence of highly conserved sequence regions of 39 bp (the exons), similar regions of 73 bp with more than 98% identity (corresponding to the introns), and divergent intergenic regions or ITS (less than 59% similarity) (Murthy et al., 1992; Souto et al., 1996) (Figure 20.1A).

By using a multilocal PCR strategy, it was possible to cluster *T. cruzi* strains into two groups according to the amplification products. Strains that yielded 300-bp products were designated as belonging to group 1, and those that yielded 350-bp products were designated as belonging to group 2 (Souto et al., 1996). Subsequent analysis of ME sequences of Z3 strains indicated the presence of a third group (2′) characterized by an approximately 50-bp insertion/deletion in the nontranscribed spacer region of the MEs (Fernandes et al., 1998). Thus, new sets of primers were designed to amplify the Z3 strains (Fernandes et al., 2001; Burgos et al., 2007).

Another singular characteristic of the trypanosomatids is their rRNA cistron organization. Both the small subunit (SSU) and the large subunit (LSU) are considerably larger than the typical eukaryotic rRNA species. Particularly interesting is the LSU, which contains two-high-molecular weight RNA (24Sα/24Sβ) and six low-molecular-weight RNA (S_1-S_6) components (Leon et al., 1978; de Arruda et al., 1990; Hernandez et al., 1990). The rDNA cistron is composed of repeated sequences in which the coding regions of the SSU and the LSU are separated by two internally transcribed spacers (ITSs). ITS1 separates the coding region of the 18S subunit and the 5.8S rDNA, and ITS2 separates the 5.8S rDNA sequence from the 24Sα rDNA (Figure 20.1B).

Comparison between the 24Sα rRNA gene of *T. cruzi* and other trypanosomatids showed high homology, except for discrete regions. The most divergent domain (D7) is a segment of about 100 bp located at the 3′ end of the *T. cruzi* gene (de Arruda et al., 1990; Souto and Zingales, 1993). Initially, PCR amplification of a sequence

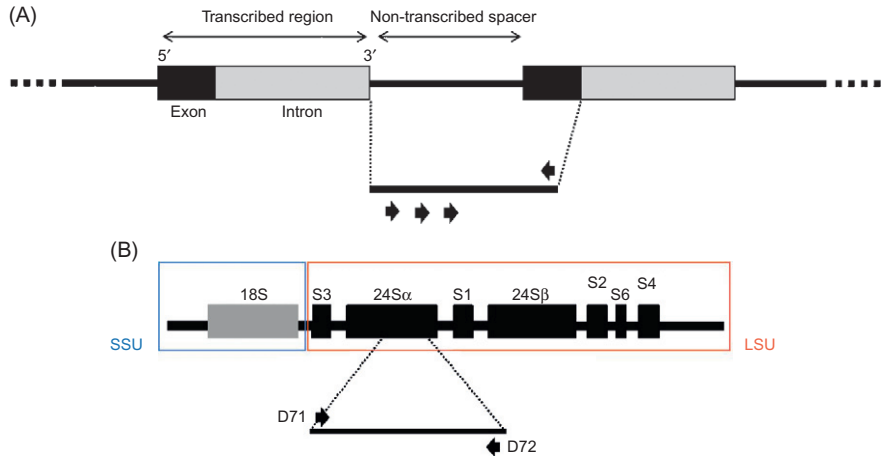

Figure 20.1 (A) Schematic representation of the *T. cruzi* ME locus. The ME locus is organized as tandem repeated units in which the exon region is conserved throughout the kinetoplastida (black boxes). Intron regions are indicated by the gray boxes, and the nontranscribed spacer is represented by the black connecting line. ME typing is based on multiplex PCR using an exon specific primer (right arrow) and lineage specific primers located in the spacer region. (B) Schematic organization of *T. cruzi* rDNA cistron. The SSU is indicated by a gray box, and the LSU is indicated by black boxes. The rDNA cistron is composed of repeated sequences in which the coding regions of the SSU and the LSU are separated by two ITSs. ITS1 separates the coding region of the 18S subunit and the 5.8S rDNA, and ITS2 separates the 5.8S rDNA sequences from the 24S rDNA. rDNA typing is performed using primers D71 and D72, which amplify a dimorphic region at the 3′ end of the 24Sα rRNA gene. This figure is adapted and modified from Macedo et al. (2004).

from the D7 domain revealed a size dimorphism, and three different groups or lineages of *T. cruzi* strains were detected (Souto and Zingales, 1993; Souto et al., 1996). Strains from lineage 1 gave 125-bp products, and strains from lineage 2 gave 110-bp products. A third group of strains presented both PCR products and was denoted group 1/2. Later, by using more sensitive analysis methodology, additional groups of strains presenting D7 amplicons of 117/119 bp (Pimenta, 2002; Augusto-Pinto et al., 2003) or 120/130 bp (Brisse et al., 2001) were also identified.

20.3.7 Other Nuclear and Mitochondrial Gene Polymorphisms

Using two different nuclear genes (trypanothione reductase (TR) and dihydrofolate reductase-thymidylate synthase (DHFR-TS)) and a region of the mitochondrial DNA partially encompassing the maxicircle-encoded genes (cytochrome oxidase subunit II (COII) and NADH dehydrogenase subunit 1 (ND1)), Machado and Ayala (2001) proposed the division of *T. cruzi* strains into four different clades named A, B, C, and D. Clade A included all DNA sequences from strains belonging to zymodeme 1 (Z1). However, the DNA sequences obtained from strains of

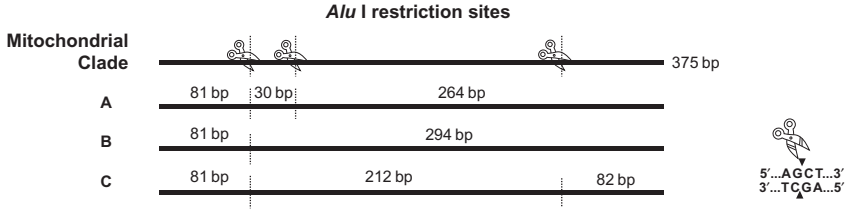

Figure 20.2 *Alu*I restriction sites and RFLP patterns corresponding to the three mitochondrial haplogroups.

Z2 and Z3 were not monophyletic and were clustered into two (mitochondrial data) or three (nuclear data) distinct clades (i.e., B, C, and D).

Based on these previous results, Freitas and coworkers developed a simple PCR-RFLP method for typing the mitochondrial genome of *T. cruzi* populations. Following the PCR amplification of the 3′ end of the COII regions, an *Alu*I restriction reaction produces three different RFLP patterns corresponding to three mitochondrial haplogroups (Figure 20.2).

The haplogroup A encompasses the zymodeme 1, D7 rDNA 2, miniexon 2 strains; haplogroup C encompasses the zymodeme 2, D7 rDNA 1, miniexon 1 strains; and haplogroup B encompasses the hybrids and the zymodeme 3 strains (Freitas et al., 2006).

Similarly, Augusto-Pinto et al. (2003) cloned and sequenced the mismatch repair mutS homologue 2 (MSH2) of *T. cruzi,* which proved to be polymorphic. Examination of 13 different strains identified three clusters of sequences that could be used for dividing the taxon into three distinct clades (A, B, and C). It was shown that strains belonging to clade A correspond to those presenting D7 rDNA 2, miniexon 2, and zymodeme Z1. Similarly, strains belonging to clade C correspond to those strains sharing D7 rDNA 1, miniexon 1, and zymodeme Z2. Strains in clade B include the hybrid strains, such as those presenting D7 rDNA 1/2 and CL Brener or zymodemes Z3A and Z3B.

20.4 Major Lineages in *T. cruzi*

As highlighted previously, *T. cruzi* is a polymorphic species, and the overall population structure is far from being completely understood. Until the end of 1980s, the prevalent idea was that *T. cruzi* strains could not be grouped into discrete groups that represent natural taxa (Morel et al., 1980; Tibayrenc et al., 1986b). Instead, a multiclonal population structure was proposed, with the different clones evolving from an ancient ancestor, essentially by clonal reproduction (Tibayrenc et al., 1986a; Zhang et al., 1988). However, the identification of a strong correlation between different DNA markers (e.g., zymodemes, miniexon, D7-24S rDNA, RAPD, microsatellites, and mitochondrial genes) generated a consensus about the existence of at least two major phylogenetic lineages within the taxon (Tibayrenc,

1995; Souto et al., 1996; Nunes et al., 1997; Zingales et al., 1998). The basic dichotomy of the species was further correlated with a plethora of epidemiological, biochemical, biological, and molecular markers (Fernandes et al., 1998; Oliveira et al., 1998; Momen, 1999; Zingales et al., 1999; Macedo et al., 2001; Henriksson et al., 2002; Buscaglia and Di Noia, 2003), but the nomenclature of the major groups became confusing.

In April 1999, during the International Symposium commemorating the 90th anniversary of the discovery of Chagas disease in Rio de Janeiro, Brazil, the subdivision of *T. cruzi* into two ancestral lineages (*T. cruzi* I and *T. cruzi* II) was recognized (Anonymous, 1999). *T. cruzi* I includes the strains presenting common characteristics (e.g., zymodeme Z1, rDNA and miniexon group 2, and mitochondrial clade A), whereas *T. cruzi* II includes strains presenting zymodeme Z2, rDNA and miniexon group 1, and mitochondrial clade C (Momen, 1999; Machado and Ayala, 2001). However, some parasite strains could not be properly grouped into either of these two major lineages. Among these unclassified strains were those identified as belonging to zymodeme Z3 (Miles et al., 1980) and other hybrid strains, such as those characterized as 24Sα rDNA group 1/2 (Souto et al., 1996; Stolf et al., 2003). Subsequently, other subdivisions were proposed. Based on MLEE and RAPD markers, Brisse et al. (2000a, 2001) proposed the subdivision of *T. cruzi* into two major lineages of DTUs, or DTUs I and II. The latter DTU was later subdivided into five sublineages named DTU IIa−e. DTU I and DTU IIb correspond, respectively, to the ancestral lineages *T. cruzi* I and *T. cruzi* II, whereas DTU IId and DTU IIe encompass hybrid strains, such as the strains from rDNA group 1/2 and CL Brener (Brisse et al., 2001, 2003). Later, based on microsatellite and mitochondrial DNA analyses, Freitas et al. (2006) proposed the existence of a third ancestral lineage, named *T. cruzi* III, which corresponds to DTU IIc.

Recently, during the XIII International Congress of Protistology, XXV Annual Meeting of the Brazilian Society of Protozoology, and the XXXVI Annual Meeting on Basic Research in Chagas Disease held in Armação dos Búzios, Rio de Janeiro, Brazil (August 2009), a second consensus was reached for *T. cruzi* intraspecific nomenclature. The *T. cruzi* strains are now referred to by six DTUs named *T. cruzi* I to *T. cruzi* VI. *T. cruzi* I and *T. cruzi* II correspond, basically, to the major lineages originally defined 10 years ago during the first Satellite Meeting (Anonymous, 1999). Two additional DTUs (*T. cruzi* III and *T. cruzi* IV) include the strains belonging to zymodeme Z3A and Z3B, respectively (Mendonca et al., 2002). The other two DTUs (*T. cruzi* V and *T. cruzi* VI) include hybrid strains corresponding to zymodeme Bolivian Z2 and zymodeme Paraguayan Z2, respectively (Zingales et al., 1999). The designation of these six DTUs and their equivalence to previously published nomenclature are detailed in the previous chapter, but summarized information is also presented in Table 20.2.

20.5 The Origin of the *T. cruzi* Hybrid Strains

The occurrence of genetic exchange in *T. cruzi* by homologous recombination has been controversial over the years, ranging from absolute clonality (Tibayrenc et al.,

Table 20.2 The New *T. cruzi* Nomenclature[a] and its Correspondence to Previous Nomenclature Defined by Different Molecular and Biochemical Markers

Zingales et al. (2009)	Anonymous (1999)	Miles et al. (1978)	Tibayrenc et al. (1986a)	Souto et al. (1996)	Fernandes et al. (1998)	Brisse et al. (2001)	Freitas et al. (2006)	Reference Strains
T. cruzi I	*T. cruzi* I	Z1	1–25	Lineage 2	Lineage 2	Lineage I	*T. cruzi* I	Silvio X10, Colombiana
T. cruzi II	*T. cruzi* II	Z2	30–34	Lineage 1	Lineage 1	Lineage IIb	*T. cruzi* II	Esmeraldo, JG
T. cruzi III	ND	Z3/Z1 ASAT	35–37	ND	Sublineage 2'	Lineage IIc	*T. cruzi* III	M5631, M6241
T. cruzi IV	ND	Z3	26–29	ND	Sublineage 2'	Lineage IIa	ND	CanIII
T. cruzi V	ND	Z2b	38–39	Group 1/2	Lineage 1	Lineage IId	Hybrid	MN cl2, SC43
T. cruzi VI	ND	ND	40–43	Lineage 1	Lineage 1	Lineage IIe	Hybrid	CL Brener

[a]Zingales et al. (2009).
Source: Adapted from Brisse et al. (2001).

1986b) to rare and discrete hybridization events (Westenberger et al., 2005; Freitas et al., 2006; Tomazi et al., 2009; Venegas et al., 2009) to a high-frequency, cryptic event (Carranza et al., 2009). Most of the evidence was based on the identification of putative natural hybrid strains by studies of individual genes (Bogliolo et al., 1996; Carrasco et al., 1996; Souto et al., 1996; Machado and Ayala, 2001; Brisse et al., 2003; Sturm et al., 2003). Gaunt et al. (2003), however, clearly demonstrated the formation of hybrids of *T. cruzi* in experimental infections by a nuclear fusion mechanism followed by nonmeiotic hybridization events. In this process, the two parental nuclei fuse in their diploid forms, resulting in polyploid progeny that can undergo recombination between alleles and, through subsequent chromosomal loss, eventually return to the diploid state. The length of time required for the return to diploid is unknown. This so-called parasexual pathway resembles the mechanism of genetic exchange observed in certain fungi (Heitman, 2006). Experimental hybrids of *T. cruzi* appear to be aneuploid, containing 1.65−1.72 times more DNA than the parental cells (Gaunt et al., 2003; Lewis et al., 2009a).

Gaunt et al. (2003) also showed that hybridizations in *T. cruzi* strains also occur without mitochondrial fusion. Thus, natural hybrid strains derived from the same evolutionary lineages will have the same type of mitochondrial DNA. In agreement with this premise, all identified hybrid strains share the mitochondrial DNA clade or haplotype B (Machado and Ayala, 2001; Freitas et al., 2006). Nonetheless, the phylogeny of the whole *T. cruzi* taxon is far from being completely understood. The current prevailing view is that *T. cruzi* I and *T. cruzi* II are ancient lineages, and the *T. cruzi* V and *T. cruzi* VI strains are the products of a minimum of two hybridization events occurring in the past between *T. cruzi* II and *T. cruzi* III strains (Freitas et al., 2006; Westenberger et al., 2005; Tomazi et al., 2009). Curiously, both parental strains have contributed to the constitution of the nuclear genome of the hybrids, but only *T. cruzi* III was the mitochondrial genome donor. The origins of *T. cruzi* III and *T. cruzi* IV, however, remain controversial. A possible explanation is that *T. cruzi* III and *T. cruzi* IV are ancient and closely related DTUs from which both *T. cruzi* I and *T. cruzi* II originated by long-term divergence. An equally possible alternative scenario is that *T. cruzi* III and *T. cruzi* IV are hybrid strains that originated from an early hybridization event between the *T. cruzi* I and *T. cruzi* II strains (Freitas et al., 2006; Westenberger et al., 2005; Tomazi et al., 2009). Further studies will be necessary to clarify this point.

20.6 Genotyping the Six Major Lineages of *T. cruzi*

The standardized nomenclature recently recommended for the *T. cruzi* DTUs will certainly improve communication within the scientific community and contribute to clarify the epidemiological and pathogenic aspects specifically associated with each of the six *T. cruzi* major lineages. To achieve this, however, it is also necessary to standardize a simple and reproductive genotyping strategy applicable in any basic laboratory working on *T. cruzi*.

The biological, biochemical, and genetic diversity of *T. cruzi* strains have long been recognized, and over the years, numerous approaches have been used to characterize the parasites. However, none of the individual markers described earlier allows complete DTU resolution, and, in any case, reliance on a single marker is inadvisable because of the consequent loss of resolution and the potential influence of genetic exchange on some lineages (Lewis et al., 2009b). Thus, to effectively standardize *T. cruzi* classification, a simple and reproducible schema for typing isolates into their respective DTUs is required.

Brisse and coworkers were the first to propose a multiple assay system based on a combination of miniexon, 24Sα rRNA (Souto et al., 1996), and 18S rRNA (Clark and Pung, 1994) PCR product size polymorphism assays suitable for *T. cruzi* assignment into each of the six DTUs (Brisse et al., 2001). However, several of the assignments were based on the absence, rather than the presence, of bands, which is not recommended for a gold standard typing method.

Rozas et al. (2007) described a multilocus PCR-RFLP (MLP) analysis of genetic polymorphism of 12 loci, most of which are involved in host−parasite relationships. Several of these PCR-RFLP assays demonstrated inter-DTU differences, and a single assay permits the typing of several DTUs in a "single shot." In the case of ambiguities, the combination of a second assay (or, if needed, a third one) allows complete typing (Rozas et al., 2007).

Recently, Miles' group proposed a simpler typing strategy using currently available markers in the form of a triple assay that employed rDNA PCR (Souto et al., 1996) and PCR-RFLP of the HSP60 (heat shock protein 60) and GPI (glucose-6-phosphate isomerase) loci (Westenberger et al., 2005). The combined application of these three PCR-RFLP markers (Figure 20.3A) is enough to discriminate all six DTUs, with only three exceptions among 48 analyzed strains (Lewis et al., 2009b).

An alternative tool for simple DTU assignment, devised by Macedo and coworkers (D'Avila et al., 2009), is schematized in Figure 20.3B.

Based on this strategy, a triple step assay comprising PCR-RFLP-COII (Freitas et al., 2006), amplification of ITS leader (Burgos et al., 2007), and 24Sα rRNA (Souto et al., 1996) genes, applied in that order, represents a reliable methodology for *T. cruzi* strain typing.

The utility of all these assays in practice, however, is subject to complications arising from the existence of appreciable heterogeneity within each *T. cruzi* DTU and the occurrence of mixed infections, which are already well documented in both vectors and mammal hosts, including humans (Bosseno et al., 1996; Yeo et al., 2007; Cardinal et al., 2008). Depending on the parasite compositions and the typing strategy chosen, mixed genotype profiles can be clearly recognized, resulting in unequivocal DTU identification. In contrast, observation of unexpected multilocus genotypes can indicate undiscovered lineages or recombinant strains that warrant further study. Certainly, a multicentric study would be necessary to standardize and validate the different protocols for genotyping *T. cruzi* strains. To achieve this goal, comparative typing protocols should include the same representative set of reference and laboratory strains as well as a great number of field isolates.

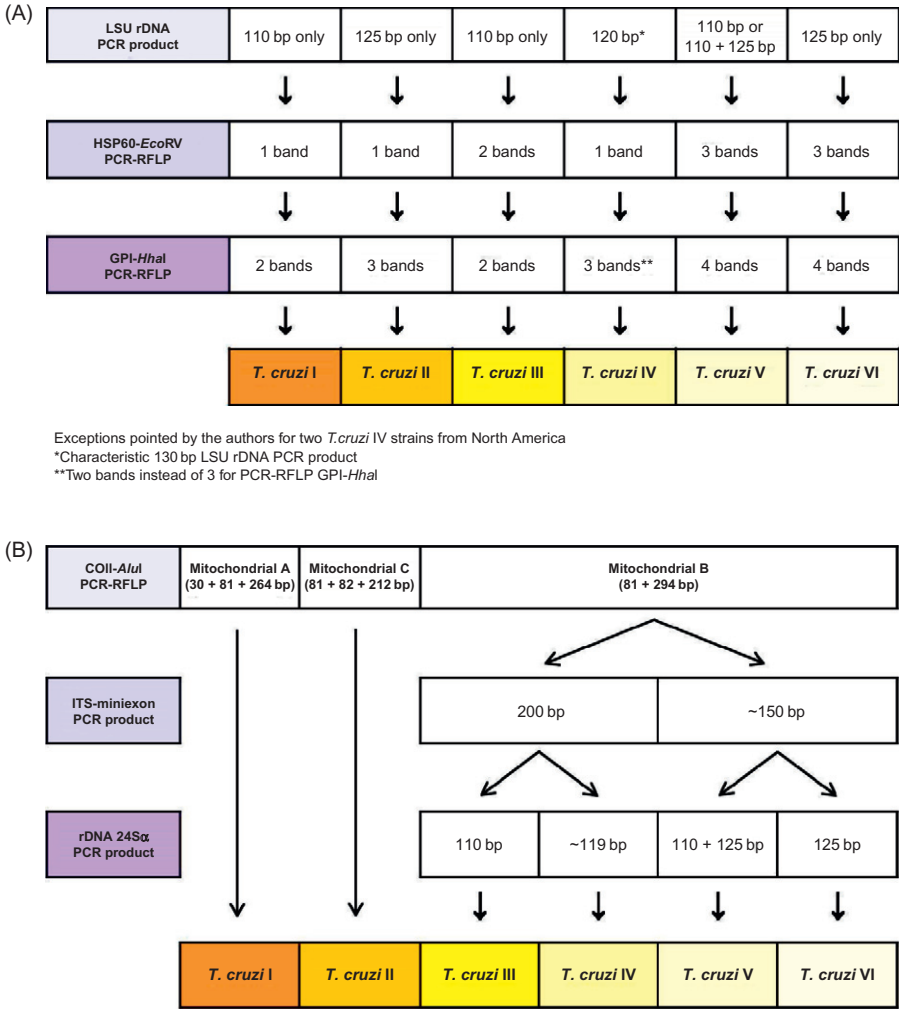

Figure 20.3 (A) Triple assay as proposed by Miles and colleagues (Lewis et al., 2009b) for *T. cruzi* DTU assignments. The strategy includes a PCR of LSU rDNA (Souto et al., 1996), followed by PCR RFLP of the HSP60 (heat shock protein 60) gene using the restriction enzyme *Eco*RV and, finally, another PCR RFLP using GPI (glucose 6 phosphate isomerase) as the targeted gene and *Hha*I as the restriction enzyme (Westenberg et al., 2005). (B) Triple assay as recommended by Macedo and coworkers (D'Avila et al., 2009) for discriminating the *T. cruzi* DTUs. Initially, strains should be analyzed by PCR RFLP of the COII gene followed by digestion with *Alu*I (Freitas et al., 2006), which allows the discrimination of *T. cruzi* I and *T. cruzi* II from the others (*T. cruzi* III−VI). A second step comprising ITS leader gene PCR (Burgos et al., 2007) is applied to these unclassified strains, resulting in two distinct clusters, one formed by *T. cruzi* III and *T. cruzi* IV and another by *T. cruzi* V and *T. cruzi* VI. The final step consists of rDNA 24Sα PCR (Souto et al., 1996).

20.7 Epidemiological and Clinical Implications of the Major *T. cruzi* Lineages

Under natural conditions, the flagellate *T. cruzi* infects over 100 mammalian species from different orders, to which it is transmitted by dozens of infected hematophagous hemiptera from the family Reduviidae. In addition to triatomines, parasites can be transmitted by blood transfusion, laboratory accidents, organ transplantation, and contaminated foods and drinks (Coura et al., 1994; Prata, 2001; Dias, 2006). Transmission from mothers with Chagas disease to their fetuses has also been reported, with the incidence varying from less than 1% in Brazil to 4−12% in endemic areas of Argentina, Bolivia, Chile, and Paraguay (Dias, 1992; Prata, 2001).

The geographic distribution of triatomines and vertebrate hosts associated with the hematophagous vector's preferences for specific blood sources define two different transmission cycles: one related to sylvatic hemiptera and generally involving wild nonhuman vertebrate hosts (the "sylvatic" cycle) and another dependent on home-dwelling hemiptera and primarily involving humans and household animals (the "domestic/peridomiciliary" cycle). The connection between the two cycles is made by infected rats, mice, bats, opossums, and hemiptera that migrate to the human environment, forced by the destruction of forests and adult insects attracted to dwellings, especially to artificial light (Coura et al., 2002).

The prevalence of the *T. cruzi* major lineages in the enzootic cycle has not been fully established yet. Pioneering studies led to the identification of *T. cruzi* I as the most relevant DTU in the sylvatic transmission cycle, at least in French Guiana (Lewicka et al., 1995). In contrast, subsequent analysis of several sylvatic isolates from the Brazilian Atlantic rainforest demonstrated that both *T. cruzi* I and *T. cruzi* II are equally represented (Fernandes et al., 1999). In both cases, however, the preferential association of each major *T. cruzi* lineages with different hosts (*T. cruzi* I with marsupials and *T. cruzi* II with raccoons, monkeys, and other placental mammals) was evident (Clark and Pung, 1994; Lewicka et al., 1995; Fernandes et al., 1999; Jansen et al., 1999).

Understanding *T. cruzi* population structure and phylogeny is critical because they are connected to both transmission cycles and disease aspects. Efforts to comprehend the genetic composition and population structure of *T. cruzi* are justified by their correlations with biological properties of the parasite, including geographical distribution, host specificity, and the clinical outcome of infection. Although no global correlations have been clearly demonstrated, there are many instances of local associations between parasite genetics and biology (Revollo et al., 1998; Campbell et al., 2004; Miles et al., 2009).

Chagas disease is distributed in a range from southern North America to southern South America. Generally, this parasite is considerate, more benign at the northern end of its geographical range in Mexico, Central America, and northern South America and to cause more severe heart and digestive disease at the southern end of its range in Bolivia, Brazil, Argentina, and Chile (Dias, 1992; Sturm and Campbell, 2010). It is reasonably accepted that this geographical heterogeneity is caused primarily by genetic variation of *T. cruzi* because there is no clear

correlation with any pattern of ethnic, human genetic, or environmental variation. In this chapter, a modest compilation of the major literature findings in relation to *T. cruzi* intraspecific variability and the possible correlation to epidemiological and clinical aspects are presented (Table 20.3). However, because *T. cruzi* genotyping

Table 20.3 Clinical Aspects, Transmission Cycle, and Geographical Distribution of Chagas Disease Associated with the Major *T. cruzi* DTUs

DTU	Transmission Cycle	Geographic Distribution of Chagas Disease	Major Clinical Aspects
TcI	Predominantly in sylvatic cycle	Amazon region	Usually associated with milder chronic Chagas disease Possibly associated with severe Chagas disease in Venezuela and Colombia Associated with oral transmission and severe acute cases in Brazil Possibly associated with neuro encephalitis in immunocom promised patients
TcII	Predominantly in domestic cycle; rare in sylvatic cycles	Main agent in the Southern Cone region of South America	Primary cause of severe acute and chronic Chagas disease Megaesophagus and megacolon in Brazil
TcIII	Predominantly in sylvatic cycle; rare in domestic cycles	?	Rarely causes human Chagas disease
TcIV	Predominantly in sylvatic cycle; rare in domestic cycles	Venezuela and Amazon region	Poorly understood DTU Few strains have been isolated from humans Possibly associated with oral transmission and severe acute cases in Brazil
TcV	Predominantly in domestic cycle; rare in sylvatic cycles	South America	Severe acute and chronic Chagas disease Megaesophagus and megacolon in Bolivia Possibly linked to congenital transmission in Bolivia, Argentina, and southern region of Brazil
TcVI	Predominantly in domestic cycle; rare in sylvatic cycles	South America	Possibly associated with severe acute and chronic Chagas disease in the Southern Cone region

Source: Adapted from Miles et al. (2009).

methods and the major lineage nomenclature in the literature were not standardized, some uncertainties are expected for these compiled data.

It is interesting that the geographical distribution of the *T. cruzi* major lineages tracks quite well with the severity gradient of Chagas disease across this range. *T. cruzi* I predominates in the USA, Mexico, Central America, and the countries in northern South America. *T. cruzi* II and the hybrids *T. cruzi* V and *T. cruzi* VI are relatively more abundant in the Southern Cone countries. *T. cruzi* IV is found predominantly from the southern USA to the Brazilian Amazon (Coura et al., 2002; Martins et al., 2008), whereas *T. cruzi* III has been isolated in many South American countries (Lewis et al., 2009b). The coincidence between both gradients has led to the general but not absolute association of *T. cruzi* I with milder disease and transmission via the sylvatic cycle, and *T. cruzi* II, V, and possibly VI with more severe disease and the domestic transmission cycle (Miles et al., 1978, 2003; Souto et al., 1996; Fernandes et al., 1999). Exceptions, however, are common.

20.7.1 T. cruzi *I*

T. cruzi I is the predominant agent of Chagas disease in countries in the Amazon region. The rarity of megasyndromes (megaesophagus and megacolon) in these regions has been attributed to the local predominant endemicity of *T. cruzi* I (Miles et al., 1981a). Moreover, cases of chronic human infection by *T. cruzi* I strains are rare and usually asymptomatic in Southern Cone countries (Miles et al., 1981a; Zingales et al., 1999; Coura et al., 2002; Buscaglia and Di Noia, 2003). Nonetheless, disease and death due to *T. cruzi* I in regions of Venezuela, Colombia, and part of Brazil where *T. cruzi* II is rare or absent (Miles et al., 1981a; Anez et al., 2004; Teixeira et al., 2006; Zafra et al., 2008; Llewellyn et al., 2009b) indicate that pathogenesis is also an inherent property of *T. cruzi* I strains (Miles et al., 2009; Sturm and Campbell, 2010).

T. cruzi I is associated with severe acute cases resulting from oral infection outbreaks (Shikanai-Yasuda et al., 1991; Coura, 2006; Dias et al., 2008; Steindel et al., 2008) and incursion of sylvatic rodents into houses (Luquetti et al., 1986). *T. cruzi* I is also related to reacutized cases of Chagas disease in individuals seriously immunocompromised due to immunosuppressant treatment after heart transplantations or coinfection with human immunodeficiency virus (HIV). Curiously, in these cases, *T. cruzi* I is frequently associated with central nervous system complications and meningoencephalitis (Anez et al., 2004; Burgos et al., 2008).

T. cruzi I shows substantial genetic heterogeneity (Barnabé et al., 2000; Herrera et al., 2007; O'Connor et al., 2007; Spotorno et al., 2008) that is evident in localized studies from Mexico (Lopez-Olmos et al., 1998; Bosseno et al., 2002), Colombia (Herrera et al., 2007), Venezuela, and Bolivia (Llewellyn et al., 2009b). Current studies suggest four subdivisions (haplotypes Ia−d) within *T. cruzi* I (Herrera et al., 2007; Falla et al., 2009), although these have not been integrated into the nomenclature revision proposed in the Second Satellite Meeting (Zingales et al., 2009). Interesting findings indicate that haplotypes Ia and Ib (sometimes referred as domestic strains of *T. cruzi* I) are particularly associated with the

domestic/peridomestic transmission cycle and human infections in Colombia (Falla et al., 2009) and Venezuela (Llewellyn et al., 2009b).

Although a predominance greater than 90% of *T. cruzi* I was initially described in Colombia (Widmer et al., 1985; Montilla et al., 2002; Zafra et al., 2008), increasing detection of *T. cruzi* II, III, and V has occurred in this country, especially by using parasite genotyping methods directly in infected human tissues (Cuervo et al., 2002; Zafra et al., 2008; Mantilla et al., 2010). These findings indicate that the epidemiology of Chagas disease is much more complex in Colombia than initially foreseen, with possible consequences on the pathology and morbidity of the disease.

20.7.2 T. cruzi *II,* T. cruzi *V, and* T. cruzi *VI*

T. cruzi II is the main agent of Chagas disease in the Southern Cone region of South America, where *Triatoma infestans* is or was the principal domestic vector. *T. cruzi* V and *T. cruzi* VI are hybrid lineages derived from hybridization events between *T. cruzi* II and *T. cruzi* III, and similar to *T. cruzi* II, they are extremely rare in the sylvatic transmission cycle (Zingales et al., 2009).

T. cruzi II is largely associated with severe cases of Chagas disease in South America, especially in central and southern Brazil, where cardiac, megacolon, and megaesophagus forms of Chagas disease are almost exclusively associated with this DTU (Freitas et al., 2005; Yeo et al., 2005; Lages-Silva et al., 2006). Nonetheless, increasing data also implicate *T. cruzi* V and possibly *T. cruzi* VI as the major lineages involved in human infections in the Gran Chaco region and neighboring countries, including Bolivia, Chile, northern Argentina, and southern Brazil (Diosque et al., 2003; Virreira et al., 2006b; Cardinal et al., 2008). For instance, megacolon in Bolivia is associated predominantly with *T. cruzi* V and to a lesser extent with *T. cruzi* II (Virreira et al., 2006b).

Remarkably, *T. cruzi* V is associated with congenital transmission of Chagas disease in Argentina, Bolivia, and the southern region of Brazil (Virreira et al., 2006a, 2007; Valadares, 2007; Corrales et al., 2009). The frequency of congenital infection is much higher (4−12%) in areas where *T. cruzi* V predominates and relatively rare (below 1%) in *T. cruzi* II-circulating areas (Valadares et al., unpublished data). Thus far, no case of congenital infection involving any of the other four major *T. cruzi* lineages has been reported. Whether these correlations result from the intrinsic biological characteristics of these major lineages or only reflect the local abundance of the lineages remains to be demonstrated.

Aside from *T. cruzi* II and *T. cruzi* V, different authors have indicated *T. cruzi* VI as one of the major lineages associated with severe Chagas disease in southern South America (Miles et al., 2009). However, due to their close genetic relationship and depending on the method chosen for parasite genotyping, *T. cruzi* VI cannot always be distinguished from *T. cruzi* II or *T. cruzi* V in the current literature (Campbell et al., 2004; Burgos et al., 2008; Lewis et al., 2009a). Thus, the specific contribution of *T. cruzi* VI in human infections is difficult to evaluate properly.

20.7.3 T. cruzi *III and* T. cruzi *IV*

T. cruzi III and *T. cruzi* VI are primarily associated with the sylvatic transmission cycle. They are distributed throughout a vast geographical range from the southern USA to Argentina, being relatively frequent in the Amazon region (Clark and Pung, 1994; Coura et al., 2002; Roellig et al., 2008). However, understanding the specific distribution and phylogeography of *T. cruzi* III and *T. cruzi* IV is complicated by the fact that several genotyping methods used in the literature fail to distinguish these DTUs.

T. cruzi III rarely, if ever, causes human infections, but it has been recorded in domestic dogs and peridomiciliar triatomines in northern Brazil (Chapman et al., 1984; Cardinal et al., 2008; Marcili et al., 2009; Câmara et al., 2010), threatening to be an emergent disease agent as transmission cycles evolve (Miles et al., 2009).

T. cruzi IV is the least characterized major *T. cruzi* lineage. Although *T. cruzi* IV is the secondary cause of Chagas disease in Venezuela (Miles et al., 2009), few strains have been isolated from humans. The reference strain CANIII is the best characterized among them. Nonetheless, *T. cruzi* IV was responsible for the first recorded outbreak of presumed orally transmitted simultaneous acute cases of Chagas disease in Canudos, Belém, Pará State, Brazil (Miles et al., 1978). Subsequently, several cases of oral transmission were confirmed in the Amazonia basin, which is currently considered an endemic area for this mode of transmission of Chagas disease (Coura et al., 2002). The coincidence of the high incidence of oral transmission and the high prevalence of *T. cruzi* IV overlapping with *T. cruzi* I in the Amazon region has given rise to the hypothesis that these major lineages are particularly associated with severe acute cases of Chagas disease caused by contaminated foods and drinks. Accordingly, *T. cruzi* I and Z3 population (likely *T. cruzi* IV) were recently implicated in an outbreak of acute Chagas disease supposedly transmitted by contaminate açaí juice in Amapá State, North Brazil (Valente et al., 2009).

20.8 Clonal–Histotropic Model of Chagas Disease

Humans are considered a recent accident in the evolutionary history of *T. cruzi*. It is estimated that *T. cruzi* emerged as a species over 150 million years ago, and the first contact with human was much more recent (i.e., in the late Pleistocene, approximately 15,000 years ago) when human first settled in the Americas (Briones et al., 1999; Macedo et al., 2001). Thus, it is natural to assume that not all *T. cruzi* strains can infect humans and cause Chagas disease.

Despite the identification of a differential distribution of lineages in the wild and domiciliary cycles of *T. cruzi* transmission associated with specific aspects of Chagas disease pathology, it is still not possible to establish a precise association between the parasite's lineages or clones and the clinical form of the disease based on the current studies of the parasite's genetic diversity. For instance, as addressed earlier, it is evident that in Brazil the severe human disease is much more associated with *T. cruzi* II strains, although some *T. cruzi* I, *T. cruzi* IV, *T. cruzi* V, and probably *T. cruzi* VI could be eventually involved. However, it is not yet clear what

factors determine the different clinical forms of the disease even in close endemic areas, such as a small villa or even a single dwelling. Although, we cannot discard the influence of patient-associated factors, an important role of the parasite genetics is currently recognized (Vago et al., 1996a,b). Conversely, many studies have been unsuccessful in correlating specific elements of the variability of the parasites with the clinical characteristics of the disease (Morel et al., 1980; Macedo et al., 1992a). One possible explanation for this is that strains of *T. cruzi* represent swarms of clones that may present symbiotic relationships but also compete fiercely for available resources. In addition, due to biological polymorphism, different clones from a strain may present tropism for different tissues (e.g., cardiac muscle, myenteric plexuses in the esophagus and rectum, etc.), and thus an important factor determining the clinical course of disease might be the specific "constellation" of infecting clones in the swarm and their specific tropisms. Because most genetic profiling techniques used for the characterization of *T. cruzi* require parasite isolation from patient blood and growth in laboratory animals or cultures, there are many opportunities for clonal selection, and consequently, the trypanosome populations available for analysis may differ from those actually causing the tissue lesion. This scenario constitutes the core of what we call the "clonal−histotropic model" of Chagas disease (Figure 20.4) (Macedo and Pena, 1998; Macedo et al., 2001, 2002).

The demonstration that it is possible to achieve genetic typing of *T. cruzi* directly from clinical tissues by LSSP-PCR (Vago et al., 1996a,b, 2000; Lages-Silva et al., 2006), rDNA (Burgos et al., 2005, 2007; Freitas et al., 2005), and microsatellite analysis (Burgos et al., 2008; Valadares et al., 2008) provides invaluable tools to study the molecular epidemiology of Chagas disease. For instance, when applied to experimentally double-infected mice with JG and Col1.7G2 populations, these techniques reveal a perfect concordance between the *T. cruzi* DNA profile obtained from the hearts (JG strain pattern) and from the recta (clone Col1.7G2 signature) of the same animals and the type of lesion observed in these respective tissues (Andrade et al., 1999). Similarly, Vago et al. (2000) clearly demonstrated that different *T. cruzi* populations are associated with the heart or esophagus of single patients. Even more notable, parasites from distinct major lineages (a mixture of *T. cruzi* II/V and *T. cruzi* I in peripheral blood and *T. cruzi* I in cerebrospinal fluid) were found in different tissues of the same patient (Burgos et al., 2008). Similarly, Mantilla et al. (2010) clearly demonstrated the presence of *T. cruzi* II in the heart and *T. cruzi* I in the esophagus of the same patient in Colombia, an endemic area where *T. cruzi* I was supposedly the main agent of Chagas disease. In fact, *T. cruzi* II was detected in 25% of the hearts of patients presenting chagasic cardiomyopathy, contrasting to 9% blood samples from the same patients (Mantilla et al., 2010). Taken together, these results undoubtedly demonstrate the necessity of genotyping the parasites directly in the infected tissues for a better understanding of the pathogenesis of Chagas disease.

20.9 The Role of Host Genetics

Studies of the differential tissue distribution of the Col1.7G2 and JG *T. cruzi* populations in BALB/c mice, among others, clearly demonstrate the importance of the

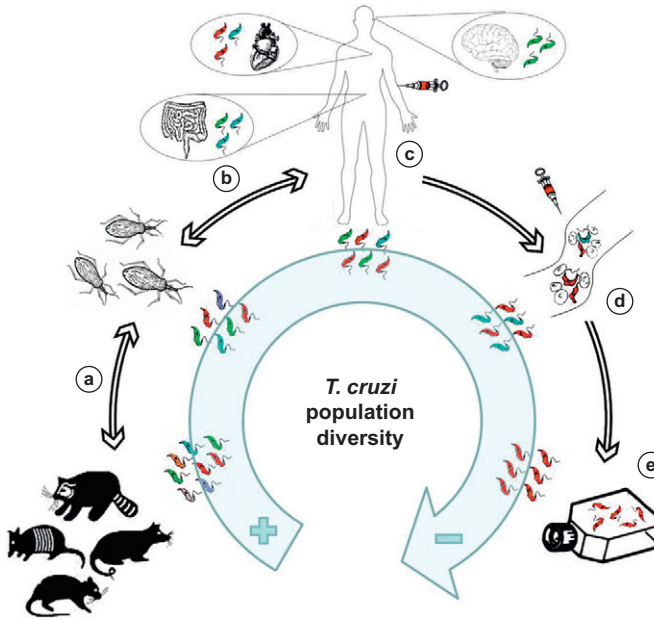

Figure 20.4 Diagram of the clonal−histotropic model of Chagas disease. Most of the natural strains of *T. cruzi* are multiclonal, and the number of clones may change drastically in different environments. (a) In the sylvatic cycle, many different animals are known to be reservoirs, and some triatomines are vectors. Thus, the same hosts may be simultaneously infected by different *T. cruzi* strains due to frequent exposures to insects and to the heterogeneous composition of the *T. cruzi* populations. (b) The passage of the multiclonal population from the invertebrate vector to a human is a particularly stressful situation (e.g., owing to host defense mechanisms). This will select against some parasite clones, thereby decreasing the complexity of the infecting strain in the acute phase. This reduction is even greater in the chronic phase of the infection owing to further elimination of clones, likely as a result of immunological factors or parasite−strain−intrinsic factors (e.g., the multiplication capability, nutritional requirements). Aside from this, different clones from a strain may present (c) tropism for different tissues (cardiac muscle, myenteric plexuses in the esophagus and rectum, etc.), and thus an important factor determining the clinical course of the disease might be the specific "constellation" of infecting clones in the swarm and their specific tropisms. Clonal selection might also occur during (d) parasite isolation and (e) maintenance *in vitro*. Thus, parasites usually submitted to molecular characterization might be different from those directly present in the infected tissues and likely more associated to the different clinical forms of the disease.

parasite genetic background in tissue tropism and lesion formation in Chagas disease (Andrade et al., 1999). Intriguingly, however, the Col1.7G2 clone (isolated from a cardiac patient) showed tropism for smooth muscles in BALB/c mice, whereas the JG strain (isolated from a patient with the digestive form of the disease) showed unequivocal cardiotropism. This could be explained as a result of parasite subpopulation selection during blood collection from the patients (which

may conceivably be different from those actually involved in the host tissue injury) and laboratory culture, as described earlier. However, it is also important to consider host genetic aspects; especially taking into account that parasitism is a result of the interaction of two varying genomes. Therefore, the behavior of parasites in distinct host may be different from each other.

Studies on experimental infection of different isogenic and outbred mice lineages with the Col1.7G2 clone and the JG strain clearly demonstrate a similar tissue distribution in BALB/c and DBA-2 mice but a different distribution in C57BL/6 and Swiss mice. Because BALB/c and DBA-2 lineages share the major histocompatibility complex (MHC) haplotype (H-2d), and the C57BL/6 lineage has a different haplotype (H-2b), it has been hypothesized that the host MHC gene region might influence the differential tissue tropism of *T. cruzi* strains in these mice (Andrade et al., 2002). This hypothesis was later confirmed using different congenic mice lineages (Freitas et al., 2009). Congenic mice are animals generated in the laboratory that present the same genetic backgrounds (BALB/c or C57BL/6 for instance) but differ from each other by a single selected gene region (MHC in this case). By using this strategy, it was irrevocably shown that the predominance of the JG or Col1.7G2 population in the hearts of the mice was dependent on the MHC genes rather than the mice's genetic background. Thus, the hearts of animals presenting the H-2b haplotype always selected for Col1.7G2, whereas the H-2d haplotype continually selected for the JG population. These observations strongly suggest that there is a significant role of host MHC and/or closely associated genes in the differential tissue tropism of *T. cruzi* strains, which in turn may also be an important factor in determining the clinical forms of the Chagas disease in human infections (Freitas et al., 2009).

Certainly, the phenomenon of differential tissue tropism is an effect of interactions between macromolecules on the surface of both parasite and host cells. Many parasite surface antigens have been described as important surface molecules involved in invasion (Burleigh and Andrews, 1995), although little is known about this parasite−host interaction. It is likely that more than one molecule is implicated in this process, and the genetic markers that are most appropriate to ascertain the major host genes determining parasite tropism and virulence remain to be discovered.

Individual variation in immune response efficiency has also been implicated in the genesis of the variability of Chagas disease in humans. Efficient immune responses control the parasite level, limiting the tissue damage, whereas inefficient responses fail to adequately control the parasite burden, thus leading to persistent inflammatory reactions and a more severe clinical condition (Tarleton, 2001).

Different authors have already suggested an influence of the genetic variability of mice in the resistance or susceptibility to *T. cruzi* infection and disease severity (Trischmann et al., 1978; Trischmann and Bloom, 1982; Wrightsman et al., 1982; Rowland et al., 1992). More recently, heterozygosity for the S180L variant of MAL/TIRAP, a gene expressing an adaptor protein in the Toll-like receptor pathway, was associated with a lower risk of humans developing chronic Chagas cardiomyopathy (Ramasawmy et al., 2009). Although no specific loci have been firmly identified yet, the MHC locus (Tarleton et al., 1996; Garcia Borras et al., 2009), production of the transcription factor NF-kβ (Hall et al., 2000), cytokine

levels, and polymorphisms (Bahia-Oliveira et al., 1998; Powell et al., 1998; Umekita and Mota, 2000; Cruz-Robles et al., 2009) have all been implicated in host modulation of *T. cruzi* infection.

Glossary

Biodemes, Biological types, Types classification of *T. cruzi* strains proposed by Andrade (1974) based on the biological parameters of the mouse−parasite interaction, such as parasitaemia, morphology, tissue tropism, and histopathologic lesions (Andrade, 1974; Andrade and Magalhães, 1996).

Clade group of strains that share a common ancestor and thus genetic similarities unraveled by analyses with different genetic markers. For example, Machado and Ayala (2001) based on sequence analyses of different mitochondrial genes defined three clades in *T. cruzi* taxon.

Clone clone is each individual of a whole population that reproduces by binary division and remains unaltered for a great number of generations until mutations occur.

Clonet all the individuals of a clonal species that share a similar profile for a particular set of genetic markers (Tibayrenc and Ayala, 1991).

Discrete typing or taxonomic units (DTUs) defined as sets of stocks that are genetically more related to each other than to any other stock and that are identifiable by common genetic, molecular, or immunological markers (Tibayrenc, 1998, Zingales et al., 2009).

Haplogroup a set of strains that share similar haplotypes.

Haplotype a group of genes within an organism that was inherited together from a single parent and is transmitted stably from generation to generation. In addition, the term "hap lotype" can also refer to the inheritance of a cluster of single nucleotide polymorphisms (SNPs), which are variations at single positions in the DNA sequence among individuals.

Lineages group of strains sharing common biological and molecular characteristics that are derived from a single ancestor.

Schizodemes parasite populations displaying identical or similar kDNA minicircle restric tion patterns (Morel et al., 1980).

Species the modern concept of a species is that of a population that is simultaneously a reproductive community, an ecological unit, and a genetic unit. Obviously, basically clonal asexual species such as *T. cruzi* cannot be defined in reproductive terms and thus the concept of genetic unity, in the sense of a shared genome, becomes critical.

Stock a microbial line kept in a laboratory, taken originally from a vector or host (often called isolate).

Strains isolates obtained from vectors or hosts which have already been characterized by genetic, molecular, or immunological markers.

Zymodeme a set of stocks that share an identical isoenzyme profile for a given set of enzyme systems (Godfrey and Kilgour, 1976). Zymodeme is the corresponding term for electrophoretic type in the parasitic protozoa literature.

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21 Vector Transmission

Simone Frédérique Brenière[*,1], *Christine Aznar*[2] *and Mireille Hontebeyrie*[3]

[1]Institut de Recherche pour le Développement (IRD), Montpellier, France, [2]Université des Antilles et de la Guyane, Cayenne, French Guyana, [3]Institut Pasteur, Paris, France

21.1 How Does the Transmission Work?

Chagas disease is commonly transmitted by bloodsucking triatomine vectors living in residential dwellings (domestic vectors) in close contact with humans. Indeed, the main interventions to decrease transmission focus on eliminating vectors that live in dwellings. Generally, transmission occurs during the night when the insects are most active, at which time they take their blood meal on sleeping humans. The parasite is transmitted during this contact, but the free-infection form (flagellated) is in the feces of the insect rather than the salivary glands as in most other arthropod vectors. The parasites are concentrated in the rectal bulb in the terminal part of the digestive tube of the insect, and during the blood meal or soon after, the bugs defecate and deposit the infected feces on the skin or near mucosa. The bite causes a skin abrasion that allows the parasite to enter underneath the skin. This transmission is complex, and all the steps are not well understood. Its load depends on many factors including those that promote the human−vector contact and those that allow the parasite to enter its host (Figure 21.1).

The first step is the contact between the vector and the mammal. What attracts the bugs to their prey? Heat, carbon dioxide, and odors could be cues and lures that direct the bugs. *Triatoma dimidiata* and *Rhodnius prolixus* are major vectors of Chagas disease in Central and South America and in experimental tests, these vectors were more attracted by heat or CO_2 alone than by selected chemicals (Milne et al., 2009). In experiments where the breathing of a host was mimicked by pulses of CO_2 and where a continuous flow of CO_2 was provided, *Triatoma infestans*, the main vector in the Southern Cone, was attracted (Barrozo and Lazzari, 2006). In 2002, it was speculated that *T. infestans* possess thermoreceptors as in other animals and insects, which aids in hunting and feeding (Campbell et al., 2002). Furthermore, moisture could increase the attraction of a hot spot, but this effect would be limited to short distances (Barrozo et al., 2003). Recently, a double-choice

*E mail: Frederique.Breniere@ird.fr

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Figure 21.1 *Rhodnius prolixus* taking a meal on human skin.
Source: Electronic publication from "Centro de Análisis de Imágenes Biomédicas Computarizadas," CAIBCO, Instituto de Medicina, Tropical, Facultad de Medicina, Universidad Central de Venezuela.

olfactometer was used to test volatile substances and showed that *R. prolixus* is also attracted by the odors of human skin and even microflora compounds present mainly on the face, which would explain why the bites frequently occur on the human face (Ortiz and Molina, 2009).

The duration of the blood meal of triatomines is long because the insect must obtain a large quantity of blood. It is important that the host does not react in a hostile manner because of the pain caused by the bite. A number of biological properties of triatomine saliva are similar to that of other arthropods and help the vector obtain its meal while also facilitating the transmission of the pathogenic agents. Thus, saliva contains anesthetic factors, anticoagulants, and vasodilators that facilitate the intake of blood (Santos et al., 2007; Assumpção et al., 2008). Although the anesthetic effect has been studied little, parasitological xenodiagnosis was used with different species of triatomine. The patient was exposed to the bite of 30−40 nymphs, and the bites were rather painless (WHO, 2002). Interestingly, Voigt et al. (2006) proposed the use of triatomines to obtain blood from small animals with cryptic veins as an alternative to conventional blood sampling; the authors report that the animals may be less stressed and possibly did not notice the bloodsucking insect. However, it should be noted that the inhabitants of hyperendemic areas testified frequently that the bug density was so high in their house (*T. infestans*) that they would be awakened at night by the attack of bugs, and they were forced to sleep elsewhere ("nos sacaban de la casa").

The parasites contained in feces can penetrate skin cells through the abrasion made by the bite. In fact, intact skin is an effective barrier against *Trypanosoma cruzi*, but very small abrasions of the skin (e.g., triggered by scraping) could allow

the parasite to enter. In experimental trials on mice of the natural route of infection via skin, the actual invasion of cells happened very quickly (Schuster and Schaub, 2000). The results showed that the parasite spread rapidly (less than 15 min) from the site of inoculation. At least a few parasitic forms can be immediately transported, possibly through the lymphatic or blood system, and then disseminated to other tissues of the host. Of course, if the parasites are transmitted via the mucosa, cells are easier to infect. In the invasion process, the saliva of *Triatoma* still plays a role. The saliva has an inhibitory effect on the activation of the classical complement pathway that acts in the lysis of foreign agents (Cavalcante et al., 2003). The role of the complex salivary secretion of triatomines is not well known, but immunomodulating proteins exist in the saliva of several hematophagous arthropods, and it has been widely observed that saliva can enhance the infectivity of pathogens (Titus et al., 2006). The saliva particularly affects the activation of T- and B-lymphocytes, macrophages, and dendritic cells. Thus, saliva is able to create a local environment conducive to cell invasion by the parasite.

Human infection requires the multiplication of the parasite, which occurs only in the host cells. In fact, *T. cruzi* invades a wide variety of vertebrate cells by endocytosis, including phagocytic cells, using a mechanism distinct from phagocytosis (De Araujo-Jorge et al., 1992). There are many mechanisms of recognition between the parasite and host cells, and *T. cruzi* has adapted to invade a wide variety of specialized cells (Snary, 1985; Andrews, 1993; Stafford et al., 2002).

21.2 Who Transmits the Parasite?

One hundred and thirty species of the subfamily Triatominae have been described. These are grouped into 16 genera. Six genera include species that are important vectors of Chagas disease or live in the human environment and therefore constitute a potential danger: *Dipetalogaster* (Usinger, 1939); *Eratyrus* (Stål, 1859); *Meccus* (Stål, 1859), *Panstrongylus* (Berg, 1879); *Rhodnius* (Stål, 1859); *Triatoma* (Laporte, 1832). The vector capacity of each species is difficult to determine. However, several indicators are usually used for laboratory colonies, and most often, several species are compared under the same experimental conditions (WHO, 1991). However, the vector capacity appears to be shaped by environmental conditions mainly related to food availability.

21.2.1 Vector Capacity

The vector capacity of a species will depend on the ability of *T. cruzi* to complete its life cycle along the gut of the insect and to produce infective forms at the rectal gland (metacyclogenesis process) that are then deposited near skin abrasions or on mucous membranes. Metacyclogenesis is the transformation of epimastigote forms (noninfective) to trypomastigotes, which are metacyclic forms that are capable of infecting mammalian cells when released into the feces. The transitions to metacyclic forms seem to occur primarily *in situ* in the rectal gland, where both epimastigotes and trypomastigotes are attached by the flagellum (Schaub, 1988; Zeledón

et al., 1984). In the small intestine, transitional forms occur but are scarce. Metacyclogenesis appears to be vector dependent (Perlowagora-Szumlewicz and Moreira, 1994; Carvalho-Moreira et al., 2003). For example, during experimental comparisons of several species (*R. prolixus*, *R. neglectus*, *P. megistus*, *T. sordida*, *T. infestans*, *T. brasiliensis*, *T. rubrovaria*, *T. speudomaculata*, and *T. dimidiata*) reared in the laboratory, the authors observed a significant difference in the rate of metacyclic forms between species at the 120th day of infection, where metacyclic forms comprised 50% of individuals in *R. neglectus* and 37% in its congener *R. prolixus*, but were dramatically lower in the majority of *Triatoma* species (5% in *T. sordida*, 3% in *T. brasiliensis*, and 0% in *T. pseudomaculata*) (Perlowagora-Szumlewicz and Moreira, 1994). Several studies have also shown that the rate of metacyclogenesis varies from one strain of *T. cruzi* to another. In *T. infestans*, a strain belonging to the Discrete Typing Unit (DTU) *T. cruzi* I nearly always reached higher trypomastigote densities in the rectum than another strain belonging to the *T. cruzi* II-IV DTUs (Schaub, 1989). Several studies have examined the metacyclogenesis process *in vitro*. The incubation of epimastigote forms in an appropriate medium induced metacyclogenesis, and different strains exhibited highly heterogeneous differentiation rates, and in particular, *T. cruzi* I strains exhibited the highest level of differentiation (Sanchez et al., 1990; Laurent et al., 1997). Another factor that influences metacyclogenesis is the meal of the triatomine. Starvation reduces the total number of parasites and the number and percentage of trypomastigotes; moreover, feeding the vector after 40 days of starvation induces the appearance of pure populations of trypomastigotes (Kollien and Schaub, 1998). These observations indicate that vector capacity depends on the availability of food sources.

Another important factor determining the vector capacity of triatomine species is their feeding behavior and defecation reflex such that the feces are deposited near the bite. Several indices were measured in laboratory colonies using main vector species (*T. infestans* and *R. prolixus*) as references. The voracity (i.e., the delay before initiating a meal), the meal period, and especially the postfeed defecation delay have been analyzed. It is impossible to formulate a comprehensive description of the feeding behavior of triatomines, but it is important to note that the main vector species are not the only species that exhibit eating and defecation behaviors that favor transmission of the parasite. The species *T. protracta* and *T. rubida* are common triatomines in southwestern North America and are considered poor vectors; a large proportion of defecations occurred after the bugs left the vicinity of the host (Klotz et al., 2009). However, some species that are not currently recognized as vectors may be adapting to the human environment. For example, 69% and 58% of the nymphs of *T. patagonica* and *T. infestans*, respectively, produced their first defecation within 5 min after being fed, and the nymphs of *T. patagonica* were capable of defecating during or immediately after feeding (Rodríguez et al., 2008). Similarly, several Mexican species exhibit a short delay in defecation (less than 10 min for *T. lecticularia*, *T. protracta*, and the youngest nymphs of *T. gerstaeckeri*), which suggests that these three species may be important potential vectors of *T. cruzi* for human populations in areas of Mexico where these species are currently present (Martínez-Ibarra et al., 2007).

21.2.2 Eclectic Species

Some species of triatomines are adapted to specialist niches, but many others are generalists more eclectic and may be a danger to humans by transmitting Chagas disease. These species, classified as secondary, are a concern because the strictly domesticated species of vectors have been targeted for extermination through various regional initiatives fighting Chagas disease in most endemic countries. In more general terms, in many areas the problem is made worse by wild species of triatomines now playing a role in transmission. This transmission relates to sylvatic zoonotic foci and generally results in sporadic occurrences of human cases. However, these regions must be under entomological supervision because an increased risk of contact between humans and infected vectors might emerge from uncontrolled environmental factors. *T. brasiliensis* is now considered the most important Chagas disease vector in the semiarid zones of northeastern Brazil. In a study on domiciliary infestation from 1993 to 1999, 21 triatomine species were captured within the geographic range of *T. brasiliensis* in Brazil, but the highest domiciliary infestation rates was for *T. brasiliensis* (Costa et al., 2003). In the "Brazilian Caatinga" region, reinfestation by *T. braziliensis* occurred after treatments of habitats. Based on genetic markers and population genetics, there appeared to be a triatomine flow (*T. brasiliensis*) between the neighboring sylvatic and artificial environments, such that the peridomestic area was a main interface function (Borges et al., 2005) and the elimination of *T. braziliensis* was more complex. Lastly, for the first time, a study reported the capture of *T. brasiliensis* in sylvatic environment, using light traps indicating that *T. brasiliensis* is attracted by light and that the colonization of houses might also be due to artificial light sources (Carbajal de la Fuente et al., 2007).

Among the unexpected species, *T. tibiamaculata*, which is often associated with *P. megistus* and *T. sordida*, was found inside houses in São Paulo State, Brazil. The triatomines captured within the domiciles were mostly adults, but about half of them had fed upon humans (Carvalho et al., 2002, 2003). Further south on the west coast of Brazil in the state of Santa Catalina (municipality of Navegantes), an outbreak of acute human cases occurred in 2005 because humans had ingested sugar cane juice contaminated by the feces of triatomines. This was the first report involving *T. tibiamaculata* in peridomestic areas (Steindel et al., 2008). Further north by 1630 km, in the state of San Salvador, the main habitat of *T. tibiamaculata* is marsupial and rodent nests in bromeliads; in this area, it was suspected that *T. tibiamaculata* was transmitting Chagas disease (Dias-Lima and Sherlock, 2000). Similarly, in a periurban zone in Santiago, Chile, a study on the prevalence of blood sources in *Mapraia spinolai* (regarded as a sylvatic species) identified several human feeding sources; this suggests that the wild insect might become a vector of greater epidemiological importance (Canals et al., 2000). Various entomological studies highlight complex situations where several triatomine species coexist in environments neighboring developed areas, and many of these species are capable of entering the residential dwellings. In communities in the northeastern province of Corrientes, Argentina, several species were identified

in domestic ecotopes. *T. infestans* was the most dominant species. The second most dominant species was *T. sordida*, which is widely distributed in South America and most frequently found in extra-domiciliary ecotopes. Although it is scarcely domiciliated, it could be considered capable of colonizing human dwellings (Damborsky et al., 2001). In the same province, a study showed a remarkable rate of infestation by *T. sordida* of palm trees on a farm (96−100% of *Butia yatay* and *Acrocomia aculeate*, respectively). The use of fronds in walls and roofs can favor the passive transport of wild triatomines to the domestic environment, as has been reported for *Rhodnius* spp. in tropical forests (Bar and Wisnivesky-Colli, 2001). Although *T. sordida* has not been considered dangerous, in the Velasco province, Bolivia, in 1995, it was the only species that colonized houses, and some autochthonous cases of infection were detected (Noireau et al., 1997). In the USA, a country considered to be nonendemic, the transmission of Chagas disease may occur at a very low level, as six autochthonous human cases have been detected. Interestingly, a recent study used geographical information system (GIS) and survey analyses of Chagas infection patterns to predict the impact of increase in temperature in the year 2030 on the geographical distribution of three triatomine species (*Triatoma sanguisuga*, *T. lecticularia*, and *T. protracta*) in the southern USA. The results indicated that there is a risk of Chagas disease emerging in the USA (Lambert et al., 2008) (Figure 21.2).

21.2.3 Special Attention for Amazonian Region

Special attention should be devoted to the Amazonian region. Indeed, this region has become a focus of scientific investigation, and Chagas disease is now a public health priority in the region. In 2004, concerned countries launched "The Initiative for the Prevention and Control of Chagas Disease" (Pan American Health Organization; AMCHA: Initiative of the Amazon Countries for Surveillance and Control of Chagas Disease; http://www.paho.org/english/ad/dpc/cd/dch-amcha.htm). Since the 1970s, the number of autochthonous cases has increased, where 205 cases had been identified as of 2000 (Coura et al., 2002). This region covers nine countries and 44% of the area of South America. At least 24 species of triatomines have been found infected, and 14 of these were recently recorded exclusively in French Guiana (Bérenger et al., 2009). An extremely broad range of mammals (e.g., marsupials, bats, rodents, and toothless and carnivorous primates) have been infected, indicating the complexity of this enzootic disease. Environmental and social changes in the last 30 years have led to the transmission of *T. cruzi* to humans. This transformation in the disease's epidemiological pattern in the Amazon can be explained by new human settlements near forested areas (especially with palm trees), seasonal and permanent rural−urban migrations, deforestation, cattle raising, mining, sedentary living, and an increase in the presence of domestic animals (Aguilar et al., 2007; Briceño-León, 2009). Among the reported acute cases, oral infections comprise a large portion, but vector transmission is also involved; thus, continuous entomological surveillance is necessary.

Figure 21.2 New patterns of vector transmission linked to wild populations of triatomines. (A) A typical suburban house in Quillacollo city (Department of Cochabamba, Bolivia) at risk of invasion by a population of *Triatoma infestans* living in rocky outcrops near the home. (B) A typical village in the Ameca valley (Jalisco State, Mexico). Wild populations of *Triatoma longipennis* are widely distributed in the surrounding countryside and are concentrated especially in the dry stone walls separating fields; the dwellings are at risk of invasion. (C) A typical house in the Sierra Nevada, Colombia, at risk of invasion by wild populations of *Rhodnius* sp. and *Triatoma dimidiata* that are living in the forest where palm trees provide habitat.
Sources: Part A photographs by S.F. Brenière. Part B photographs by S.F. Brenière and E. Magallón. Part C photographs by J. Dib.

21.3 Where Does the Transmission Occur?

21.3.1 In Houses

Unlike other vector-borne diseases, the vector transmission of Chagas disease occurs almost exclusively in the home, as it relates to vectors that live in the home or vectors that occasionally enter the house. Historically, areas in which Chagas disease was found were rapidly associated with the presence of domiciliated triatomines. Several studies have demonstrated this association. For example, in the Northern Goias state

(Brazil), the presence of triatomines in dwellings or evidence of triatomine colonization was found to be statistically correlated with seropositivity in children (De Andrade et al., 1995). Similarly, in an area in Argentina where *T. infestans* was endemic, there was a high correlation between an indicator of entomological risk (the number of risky bites per human) and the seroprevalence in children (Catalá et al., 2004).

21.3.2 In Rural, Urban, and Periurban Areas

Furthermore, the traditional idea of vector transmission is that transmission takes place in rural areas where the habitat is more favorable to infestation by bugs, but in fact, urban areas are not spared. Chagas disease should also be considered an urban health problem. In Cochabamba, Bolivia, a recent study found that vector transmission persists in periurban areas (Medrano-Mercado et al., 2008). In the city of Arequipa, Peru, scientists are concerned about the possible emergence of transmission in periurban areas due to the infestation of houses by *T. infestans* (Levy et al., 2006). Additionally, *T. dimidiata* has been found in several cities and towns in Costa Rica (Zeledón et al., 2005) as well as in Mérida, Mexico, where infestation was more related to environmental factors (e.g., location of houses on the outskirts of the city, near abandoned land) than to the quality of the house (Guzman-Tapia et al., 2007). More surprising, a huge colony of *T. infestans* was discovered in a hen building for egg production in an urban neighborhood of Greater Buenos Aires (Gajate et al., 2001). *T. infestans* was found in apartments in an urban neighborhood of the capital city of the province of San Juan, Argentina. Its occurrence was related to the proximity of poor housing areas, and dispersal by flight from this area was assumed to be a main mechanism of infestation (Vallvé et al., 1996).

21.3.3 Risk Factors of Domiciliary Infestation by Triatomines

Because the disease is transmitted within human dwellings, many studies have examined the types of building that facilitate the development of colonies of triatomines. However, an approach based on identifying risk factors for intradomiciliary infestation revealed the human activities that play a role in the disease epidemiological cycle. The results of these studies clearly showed that infestation is multifactorial and differs by region. Increasingly, it appears that the manner in which society and individuals position themselves and act against the disease and the vectors is crucial (Walter, 2003). Several studies found a strong association of disease prevalence with the type of construction, the materials used for walls and roofs and the conditions of the materials. These factors facilitate triatomine colonizations inside a house, but other factors play a large role, including limited knowledge about the disease, socioeconomic status, crowded rooms, cleanliness (household hygiene), conviviality, and household pets (Gürtler et al., 1998; Sanmartino and Crocco, 2000; Catalá et al., 2004; Campbell-Lendrum et al., 2007; Feliciangeli et al., 2007; Rojas et al., 2008; Bustamante et al., 2009). For example, intervention by the inhabitants themselves through better household hygiene and housing construction would have the advantage of reducing the risk of infestation by triatomines

for a longer duration than the standard intervention by insecticide use (Monroy et al., 2009).

In addition, peridomestic space is often a source of domestic infestation. The vectors may colonize several structures primarily related to domestic animals, but different vector species may colonize different structures. In rural localities in the state of Ceara, Brazil, *T. brasiliensis* primarily colonized brick piles and roofing tiles; *T. pseudomaculata* preferred wood poles and woodpiles; and *R. nasutus* was mainly found in roofing straw (Sarquis et al., 2006). In a rural community in the western part of Mexico, peridomestic infestation risks by *T. longipennis* and *T. barberi* (evaluated with multivariate logistic regression analysis) were related to the density of permanent and temporal structures but not with domestic animals because the main food source of triatomines was *Rattus rattus* (Bosseno et al., 2006; Walter et al., 2007). In the home, residents must rely on management changes to limit the infestation of triatomines in peridomestic areas. In other cases, attention must be paid to the immediate vegetation. For example, *T. guasayana* is a semisylvatic species that invades peridomestic sites in rural northwestern Argentina, and its spatial distribution is related to the local abundance of goats, but also with the density of vegetation habitats near houses including bromeliads, dry cacti, and firewood (Vazquez-Prokopec et al., 2008). The importance of the immediate environment in neotropical regions is even more striking because most species of *Rhodnius* are primarily associated with palms.

21.3.4 Does Vector Transmission Occur Outside of Human Dwellings?

The existence of vector-borne transmission outside dwellings is virtually undocumented; however, it may exist. Only one report suggests this kind of transmission. In the northern part of the state of Amazonas, three cases of chronic chagasic cardiopathy have been reported in patients who were bitten several times by triatomine bugs in their camping huts while gathering pia ava fibers (Viñas Albajar et al., 2003). Moreover, some species are aggressive, and the authors have personally experienced severe attacks by *T. guasayana* and *T. sordida* outside at the dusk in the Gran Chaco region, Bolivia. For the Indians of the Sierra Nevada in Colombia, the economy is based on agriculture located in different climatic zones. They travel into the mountains to reach their crops and commonly stay in camps. In addition to the classical transmission related to colonization of dwellings by *R. prolixus* and *T. dimidiata*, the practice of camping results in the exposure of people to triatomine bites independent of human dwellings (Dib, personal communication). Moreover, a Yucatan farmer reported to us that he had collected wild *T. dimidiata* specimens during a 1 month camping trip in the forest where he practiced hunting; every day at dusk he was attacked by about five triatomines when he was resting in his hammock.

21.4 The Perception of Vectors and a Need for Education

Unfortunately, even now, humans in the affected regions do not associate the presence of triatomines in their home with the transmission of any illnesses. The

ignorance surrounding Chagas disease works against efforts to control it. To fight this ignorance, people should become aware that living with triatomines could be dangerous to their health. It is urgent that people exposed to vector transmission be made aware of this danger so that they can become involved in the fight against Chagas disease; health care needs to be facilitated, particularly at diagnosis, and heart and digestive health need to be monitored. Knowledge about Chagas disease should be disseminated throughout the at-risk population to prevent incidents like that of José.

> *"Montero is a town 100 km from Santa Cruz de la Sierra in Bolivia. A large family of 10 children are living in a house near the sawmill on the outskirts of the city. The nuisance is so great that the mother regularly struggles against the triatomines and cockroaches invading the house. She periodically removes the mattresses and places them in the sun. José remembers: 'Frequently at night, we'd catch* Triatoma infestans*, and then Mom would send us to sleep with our uncle as she treated the house with insecticide overnight. But it is impossible to get rid of these disgusting insects. When did I know that the* Triatoma infestans *was transmitting the disease? When my sister, Juana, began her nursing studies, she discovered that she was chagasic and began treatment but soon discontinued her treatment because she had adverse reactions. No one in the family realized that Chagas disease could be a problem for the other members of the family, and everyone needed a checkup. A few years later in 2000, my brother suffered a heart attack during a football game and died. He was 46 years old. Necropsy shows infection by* Trypanosoma cruzi. *Then, we were all concerned about our health, and the siblings are all chagasic. Juana is in Italy and I am in France, the countries to which we immigrated a few years ago. During recent years, two other siblings died, both suddenly. We did not know about the bugs, the insects that transmit Chagas disease. And then later, we heard only that Chagas disease could not be treated, and that we should live with it without worrying about it'. I talked with José, and I explained several things. I told him that parasites are in the feces and that the parasite is not in the salivary glands; he did not know. He remembered that when he was a young boy, he and his siblings frequently found* Triatoma infestans *in the bedroom, usually engorged with blood, and they would remove the head, but they had not taken any precau tions, and they would get blood and feces on their hands."*

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22 Maternal–Fetal Transmission of *Trypanosoma cruzi*

Yves Carlier and Carine Truyens*

Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles (ULB), Bruxelles, Belgium

22.1 From Maternal–Fetal Transmission of *Trypanosoma cruzi* to Congenital Chagas Disease: Definitions and Limits

Maternal–fetal transmission of *T. cruzi* induces congenital infection (from its Latin etymology "cum" (with), and "genitus" (engendered). This implies a "prenatal" (*in utero*) or a "perinatal" transmission (at the time of delivery) (see Section 22.5) of live parasites that persist after birth. It excludes the "postnatal" transmission of parasites (mainly through maternal milk by breast-feeding) and the transmission of dead parasites, parasite DNA, or other molecules released from parasites in the mother and likely to be found in fetal blood.

The terms "mother-to-child transmission" or "**vertical transmission**" have a broader connotation corresponding to transmission from one generation to the next, including prenatal, perinatal, as well as postnatal transmission of live parasites. The term "congenital infection with *T. cruzi*" refers to asymptomatic as well as symptomatic cases of infection, whereas the term "congenital Chagas disease" should be used only for symptomatic cases (Carlier and Torrico, 2003), though, often, both terms are confounded.

The main previous reviews on congenital Chagas disease have been written by Howard and Rubio (Howard and Rubio, 1968), Schmunis and Szarfman (Schmunis and Szarfman, 1977), Bittencourt (Bittencourt, 1976, 1988, 2000), Freilij and Altcheh (Freilij et al., 1994; Freilij and Altcheh, 1995), Moya and Moretti (Moya and Moretti, 1997), and, more recently, Schijman (Schijman, 2007).

* E mail: ycarlier@ulb.ac.be

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22.2 Epidemiological Aspects of Congenital Infection with *T. cruzi*

22.2.1 Past and Present Situation in Latin American Countries

Historically, Carlos Chagas himself, in 1911, referred to the possibility of congenital transmission of *T. cruzi* infection when he found trypomastigotes in the blood smear of a 2-month-old child whose mother was infected (Chagas, 1911). Dao (1949) was the first in reporting cases of congenital Chagas disease from Venezuela.

The main countries affected by American trypanosomiasis and where congenital transmission has been studied are **Argentina** (Saleme et al., 1971; Moya et al., 1985, 1989, 2005; Arcavi et al., 1993; Zaidenberg and Segovia, 1993; Streiger et al., 1995; Freilij and Altcheh, 1995; Zaidenberg, 1999; Contreras et al., 1999; Blanco et al., 2000; Gurtler et al., 2003; Altcheh et al., 2005; Mora et al., 2005; Sanchez et al., 2005; Burgos et al., 2007, 2009; Sosa-Estani et al., 2008, 2009b; Mallimaci et al., 2010), **Bolivia** (Azogue et al., 1985; Azogue and Darras, 1991; Carlier and Torrico, 2003; Torrico et al., 2004, 2007; Virreira et al., 2006a; Brutus et al., 2007, 2008; Salas et al., 2007; Sosa-Estani et al., 2008; Bern et al., 2009b), **Brazil** (Bittencourt et al., 1975, 1985b; Bittencourt, 1976; Hoff et al., 1978; Nisida et al., 1999; Rassi et al., 2004; Neto et al., 2004; Luquetti et al., 2005b), **Chile** (Apt and Niedmann, 1964; Howard and Rubio, 1968; Munoz et al., 1982; Tello et al., 1982; Mendoza et al., 1983; Thiermann et al., 1985; Aguilera et al., 1988; Schenone et al., 1991; Munoz et al., 1992; Munoz et al., 1994; Apt et al., 2010), **Paraguay** (Russomando et al., 1998, 2005), and more recently **Mexico** (Guzman-Bracho et al., 1998; Olivera et al., 2006; Sosa-Estani et al., 2008), and **Peru** (Mendoza Ticona et al., 2005). Data on infection in pregnant women are available for **Honduras** (Sosa-Estani et al., 2008), whereas no information is available for the other Latin American countries.

Table 22.1 compiles information from the main published reports on infection prevalence in pregnant women and rates of congenital transmission in such women, according to the studied time periods and areas of corresponding countries. The transmission rate, defined as the number of congenital cases/number of *T. cruzi*−infected mothers, is more frequently observed between 1% and 12% (Carlier and Torrico, 2003). Table 22.2 resumes the WHO/PAHO estimations on the numbers of infected women of fertile age and the numbers and incidences of congenital cases for each Latin American country for the year 2005 (WHO, 2006). A pool of approximately 1,809,000 currently infected women of fertile age is considered at risk of congenital transmission, and 14,400 neonates as being infected per year, most of them being encountered in Argentina, Bolivia, and Brazil (Table 22.2).

National averages are probably underestimated for some countries. For example, studies in 2003−2004 indicated a prevalence of infection in pregnant women up to 40% in the Bolivian department of Tarija (Table 22.1; Salas et al., 2007), whereas there is a mean prevalence of 6.7% for all Bolivia. A similar remark can be made for Mexico, currently starting extensive studies on Chagas disease, where recent

Table 22.1 Main Epidemiological Published Reports on Pregnant Women and Congenital Cases Infected with *T. cruzi* in Latin American Countries

Country	Area	Study Year	Seropositive Pregnant Women		Congenital Cases					References
			n/N	%	*n/N*	%	Diagnostic Methods	Familial Clustering	*T. cruzi* Genotype	
Argentina	Buenos Aires	87–93	ND	ND	71/1118	6.3	1,3,5	+(10)	ND	Freilij and Altcheh (1995)
		90–91	62/729	8.5	2/38	5.3	1	ND	ND	Arcavi et al. (1993)
		95–04	ND	ND	4/159	2.5	1,5,6	+(10)	ND	Altcheh et al. (2005)
		00–06	ND	ND	47	ND	1,5	ND	V,VI	Burgos et al. (2007)
	Cordoba	89–97	ND	ND	37/721	5.1	1,3,4	ND	ND	Moya et al. (1985, 1989); Moya and Moretti (1997)
	Salta	80–97	ND	ND	102	ND	1,2	ND	ND	Zaidenberg (1999)
		<93	149/937	15.9	6/149	4.0	1,2,4	ND	ND	Zaidenberg and Segovia (1993)
		<99	34/276	12.3	3/34	8.8	1	ND	ND	Contreras et al. (1999)
		97–02	ND	ND	31/340	9.1	1,3,5,6	+(11,12)	V	Mora et al. (2005); Sanchez et al. (2005); Corrales et al. (2009)
	Formosa	09	–	29.1	8/47	17.0	5	ND	ND	Sosa Estani et al. (2009b)
	Santa Fe	76–91	895/6123	14.6	9/341	2.6	1,2,4,7	+(10)	ND	Streiger et al. (1995)
	Tierra del Fuego	01–02	61	ND	3/68	4.4	1,5,9	ND	ND	Mallimaci et al. (2010)
	Tucuman	92–94	927/16842	5.5	26/364	7.1	1,5	ND	ND	Blanco et al. (2000)
		06–07	34/518	6.6	ND	ND	–	–	–	Sosa Estani et al. (2008)
Bolivia	Cochabamba	92–94	444/1606	27.6	22/444	4.9	1,3,6	+(10)	II,V,VI	

(Continued)

Table 22.1 (Continued)

Country	Area	Study Year	Seropositive Pregnant Women		Congenital Cases					References
			n/N	%	*n/N*	%	Diagnostic Methods	Familial Clustering	*T. cruzi* Genotype	
		99–01	809/3879	20.8	47/809	5.8	1,3,6	+(10)		Torrico et al. (2004); Virreira et al. (2006a)
	Santa Cruz	79–80	161/317	51.0	25	–	1,8	ND	ND	Azogue et al. (1985)
		88–89	ND	ND	78 (13)	–	1,7	ND	ND	Azogue and Darras (1991)
		06–07	141/488	28.8	ND	ND	–	–	–	Sosa Estani et al. (2008)
		<09	154/530	29.0	10/154	6.5	1,5,6	ND	ND	Bern et al. (2009b)
	Tarija	01	73/152	48.0	8/149	5.4	5	ND	ND	Brutus et al. (2007)
		02–04	172/508	33.9	8/153	5.2	1	ND	ND	Brutus et al. (2008)
		03–04	1144/2711	42.2	61/1176	5.1	1	+(10)	ND	Salas et al. (2007)
Brazil	Bahia	75–76	47/285	16.5	1/17	5.8	2,7,8	+(10)	ND	Hoff et al. (1978)
		81–82	226/2651	8.5	3/186	1.6	1,4	ND	II (Z2)	Bittencourt et al. (1985a,b)
	Goias	75–04 (14)	145	–	2/278	0.7	5	ND	ND	Rassi et al. (2004)
	Sao Paulo	<99	57	–	3/58	5.17	1,2,4	ND	ND	Nisida et al. (1999)
	National	<04	36/15873	0.2	1/36	2.77	7	ND	ND	Neto et al. (2004)
Chile	Region III	82–83	31/869	3.6	0/3	0.0	4	ND	ND	Mendoza et al. (1983)
	Region IV	<84	68/453	15.0	2/61	3.3	1,4	ND	ND	Thiermann et al. (1985)
		85–87	279/1974	15.6	2/51	3.9	4,5	ND	II, V (Z2a, b)(15)	Aguilera et al. (1988)
		05–08	123/3324	3.7	2/80	2.5	5,6	+(11)	I,V	Apt et al. (2010)
	Santiago	57–68	ND	ND	30	–	2,4	ND	ND	Howard and Rubio (1968)
		<64	13/57	24.0	ND	ND	–	–	–	Apt and Niedmann (1964)
		79	11/402	2.7	2/11	18.2	1,4	ND	ND	Munoz et al. (1982)
		81–82	27/1000	2.7	3/27	11.1	2,4	ND	ND	Tello et al. (1982)

	National	82–90	ND	ND	24/336	7.1	4	+(12)	ND	Schenone et al. (1991)
Honduras	Intibuca	06–07	22/500	4.4	ND	ND	–	–	–	Sosa Estani et al. (2008)
Mexico	Chiapas, Veracruz	05–06	6/145	4.1	0/6	0.0	3,6	–	–	Olivera et al. (2006)
	Guanajuato, Yucatan	06–07	8/988	0.8	ND	ND	–	–	–	Sosa Estani et al. (2008)
	Michoacan	98	ND	ND	1 (16)	–	2	–	ND	Guzman Bracho et al. (1998)
Paraguay	Asuncion, San Pedro	91–92	172/1862	9.2	9/123	7.3	2,3,6,7	ND	ND	Russomando et al. (1998)
	Cordillera, Paraguari	95–04	7802/61091	12.7	104/1865	5.5	1,6,7,9	ND	ND	Russomando et al. (2005)
Peru	Arequipa	01–02	22/3000	0.7	0/22	0.0	1,4,7	–	–	Mendoza Ticona et al. (2005)

ND: not determined.
Methods used for the diagnosis of congenital cases: 1: microhematocrit/strout (blood); 2: fresh examination (blood); 3: hemoculture; 4: xenodiagnostic; 5: postnatal standard serology; 6: PCR (blood); 7: detection of IgM antibodies; 8: placental histopathology; 9: detection of SAPA-specific IgM/IgG antibodies.
Familial clustering: infection found in: 10: both twins; 11: siblings; 12: second generation.
13:<2500 g; 14: retrospective study; 15: in mothers; 16: historical case report.

Table 22.2 WHO Estimations on Pregnant Women and Congenital Cases Infected with *T. cruzi* in Latin American Countries for the Year 2005

Country	Population	Mean Prevalence of *T. cruzi* Infection (%)	Infected Pregnant Women[b] (*n*)	Congenital Cases	
				n	Incidence (%)
South America					
Argentina	38,747,000	4.129	275,900	1,800	0.263
Bolivia	9,182,000	6.752	229,000	1,500	0.573
Brazil	186,405,000	1.019	460,000	5,000	0.135
Chile	16,267,300	0.985	34,600	445	0.181
Colombia	45,600,000	0.956	107,800	1,000	0.104
Ecuador	13,228,000	1.739	58,000	800	0.274
Guyanas	1,397,000	1.288	5,800	20	0.070
Paraguay	5,898,650	2.543	61,000	600	0.342
Peru	27,968,000	0.686	43,700	200	0.032
Uruguay	3,305,700	0.656	5,300	20	0.039
Venezuela	26,749,000	1.159	68,000	600	0.102
Central America					
All Countries[a]	39,656,200	2.034	217,440	1,300	0.123
North America					
Mexico	107,029,000	1.028	243,000	1,100	0.051
Total	522,432,850	1.448	1,808,840	14,385	0.133

Such estimations are calculated from population, prevalence of infection integrating local differences (e.g., rural areas), birth rate and known number of infected neonates or children of *T. cruzi*−seropositive mothers specific to each country.
[a]Belize, Costa Rica, Guatemala, Honduras, Nicaragua, Panama, Salvador.
[b]From 15 to 44 years old.
Source: WHO (2006).

surveys indicate prevalences higher than expected previously (Cruz-Reyes and Pickering-Lopez, 2006; Galaviz-Silva et al., 2009).

22.2.2 Epidemiologic Evolution and Globalization of Congenital Infection with T. cruzi

Two findings are appearing from examination over time of epidemiologic data on congenital *T. cruzi* infection: (i) a trend to reduced incidence in endemic Latin American countries, and (ii) its emergence in nonendemic areas outside Latin America.

Though comparisons of reported epidemiologic data on congenital infection with *T. cruzi* are not easy since the diagnostic methodologies used are largely different (see Section 22.9), studies performed by the same teams at different time periods in Argentina (1987−1993, Freilij et al., 1995) 1995−2004, Altcheh et al., 2005; Table 22.1) and Bolivia (1992−1994 and 1999−2001: Torrico et al., 2004;

Table 22.3 Evolution Over Time of Epidemiological Data on Congenital *T. cruzi* Infection in Cochabamba, Bolivia

	1992–1994	1999–2001	2004–2006
Studied mothers (*n*)	1606	3879	26346
Maternal prevalence (%)	27.6	20.8	16.2
Maternal–fetal transmision rate (%)	4.9	5.8	2.5
Incidence of congenital infection (%)	1.4	1.0	0.3

Source: Torrico et al. (2004, 2007).

2004–2006 Torrico et al., 2007; Tables 22.1 and 22.3) indicate a decrease in incidences of diagnosed cases.

Migration of Latin American people particularly in the last decade has promoted Chagas disease as a global disease, now observed in countries deprived from vectorial transmission (Europe, Canada, Japan, Australia) or with exceptional vectorial transmission to humans as in the USA (Schmunis, 2007; Schmunis and Yadon, 2009). The current trend toward the feminization of such migration (Pellegrino, 2004) is relevant for an increased risk of congenital transmission into nonendemic areas (Buekens et al., 2008; Bern and Montgomery, 2009a; Gascon et al., 2009; Yadon and Schmunis, 2009). Cases of congenital Chagas disease have been reported in the USA (Leiby et al., 1999), Sweden (Pehrson et al., 1981, 1982), and more recently, in Spain (Riera et al., 2006; Munoz et al., 2007, 2009; Flores-Chavez et al., 2008; Carrilero et al., 2009) and Switzerland (Jackson et al., 2009).

22.2.3 *Epidemiological Features Specific to Congenital Infection with* T. cruzi

Congenital transmission of *T. cruzi*, in contrast with toxoplasmosis, can occur in both acute and chronic phases of maternal infection and be repeated at each pregnancy (Bittencourt, 1992; Carlier and Torrico, 2003) and during all of the fertile period of a woman's life (most cases of congenital infection derive from chronically infected mothers, having been infected by insect vectors since childhood by residing in endemic areas of Latin America) (Carlier and Torrico, 2003). Moreover, **transgenerational** (vertical) **transmission** of parasites can occur from an infected mother to her daughter, who in turn transmits parasites to her own infants, etc. All these elements can contribute to the familial clustering of congenital cases observed in siblings of congenital indexed cases (Freilij and Altcheh, 1995; Schenone et al., 2001; Sanchez et al., 2005). Such epidemiological features specific to *T. cruzi* infection suggest a particularly long-term risk of mother-to-offspring transmission through the pool of currently infected pregnant women in endemic as well as in nonendemic areas. This pinpoints congenital infection with *T. cruzi* as an important public health problem that can easily extend in space (through migrations) and time (for reasons mentioned earlier) (Carlier and Torrico, 2003; Raimundo et al., 2010) (see Section 22.2.2).

The observation that a small group of infected mothers transmit parasites to their fetuses, whereas most of them do not, raises the question of the capacity of placenta to contain the parasite transmission, as well as the route and factors controlling such transmission.

22.3 Placental Responses to *T. cruzi* Infection

22.3.1 *Placental Innate Immune Response to* T. cruzi *Infection*

Recent studies have shown that the members of the Toll-like receptors (TLR) family, including TLR2 and TLR4 recognizing pathogen-associated molecular patterns of *T. cruzi* (Tarleton, 2007), are expressed by trophoblast and syncytiotrophoblast, in addition to underlying fibroblasts, Hofbauer phagocytic cells (macrophages), and endothelial cells (reviewed in Patni et al., 2009; Koga and Mor, 2010). Such TLR expression is strongly enhanced during placental infection. Placental sensing through specific intracellular pathways is known to promote an innate immune response with the release into maternal blood of proinflammatory cytokines, chemokines, reactive oxygen, and nitrogen intermediates (Redline, 2006; Abrahams, 2008). In addition, the well-known FcRn, expressed on trophoblastic cells (Simister, 2003), might help to phagocytize parasites **opsonized** by antibodies. Altogether, these innate effector mechanisms activated in placenta might reduce parasitemia into the intervillous space, and limit or prevent maternal−fetal transmission of parasites. *In vitro* studies argue for such a possibility showing the low multiplication rate of parasites in villous explants incubated with *T. cruzi* (Lujan et al., 2004), the killing of parasites by NO produced by placenta (Triquell et al., 2009), and the inhibiting role of placental subfractions on trypomastigote infectivity (Frank et al., 2000). The report of cases with placental inflammation without subsequent congenital infection also argue for the potential efficacy of such infection control by the placental innate immune response (Bittencourt, 1975b, 1992; Moya et al., 1979).

22.3.2 T. cruzi *Invasion and Pathology of Placenta*

Maternal infection can also be associated with much more pronounced placental inflammation (**placentitis**), having pathological instead of protective effects. Intense production of cytokines such as TNFα can have abortive effect (Haider et al., 2009), while at lower levels and associated to other factors, it can induce apoptosis in placental cells, and finally a rupture of the trophoblastic barrier facilitating fetal infection (Redline, 2004, 2006). The histopathological analyses of placentas from abortions, stillbirths, or premature births (dying in the neonatal period), delivered by Brazilian women infected with *T. cruzi* showed important **villitis** with such large areas of trophoblast destruction and necrosis (Bittencourt, 1963, 1975b, 1976, 1988). Further immunohistochemical studies showed important inflammatory responses in such placentas, with infiltrates mainly composed of $CD68^+$ macrophages, $CD8^+$ T lymphocytes, and few NK cells (Altemani et al., 2000).

Other features of such severe villitis were the presence of $MAC387^+$ macrophages and $CD15^+$ granulocytes attached to the sites of trophoblastic necrosis (Altemani et al., 2000), such villous trophoblast overexpressing ICAM-1 (Juliano et al., 2006). A high local production of inflammatory mediators, such as reactive oxygen species, nitric oxide and peroxynitrite, can have deleterious effects on placental vascularization (Myatt and Cui, 2004). This, added to a release of inflammatory cytokines into the umbilical/fetal circulation, may induce long-term damages affecting the developing fetus, as well as a fetal inflammatory response syndrome (Redline, 2004, 2006; Romero et al., 2007) worsening the clinical outcome of congenital Chagas disease, and perhaps contributing to abortion and neonatal mortality.

However, severe villous inflammation is much less marked or not observed in placentas of live congenitally infected neonates, in which necrosis and lysis associated with infiltration of neutrophils and lymphocytes are more frequently detected in chorionic plate (**chorionitis/chorioamnionitis**) and umbilical cord (**funisitis**) (Hoff et al., 1978; Moya et al., 1979; Azogue et al., 1985; Altemani et al., 2000; Carlier, 2005; Fernandez-Aguilar et al., 2005). Such lesions of membranes surrounding the fetus can induce their fragilization and their premature rupture, which is frequently observed in case of congenital infection with *T. cruzi* (Torrico et al., 2004). The *T. cruzi*–associated slight and focused placentitis does not seem to reduce the transfer of protective antibodies from mother to fetus (Dauby et al., 2009).

22.4 Routes of Maternal–Fetal Transmission of *T. cruzi*

22.4.1 The Hematogenous Transplacental Route

The transplacental route is the mandatory mode for transmission of *T. cruzi* present in maternal blood, which bathes the placental intervillous space. Such a route requires parasites to cross the trophoblastic barrier (first placental line of defence) or other placental tissues deprived of trophoblastic defenses, before crossing mesenchymal tissues (second placental line of defense) and finally gaining access to fetal vessels embedded in such mesenchymal tissue (Figure 22.1).

22.4.1.1 Parasite Invasion of Trophoblast

Villi of the human placenta are covered by two layers of trophoblast: an outer layer called syncytiotrophoblast (preventing intercellular penetration; structural barrier) and an inner layer termed cytotrophoblast (Langhans' cells). Extravillous cytotrophoblast also covers nonvillous structures (e.g., the chorionic plate). To a great degree, only the syncytiotrophoblast is interposed between maternal blood and fetal tissues, since the cytotrophoblast cell number decreases during the gestation period (Benirschke et al., 2006).

Some *in vitro* studies mention that *T. cruzi* can easily infect and multiply within human villous trophoblastic cells (Sartori et al., 2002; Shippey III et al., 2005). A parasite-induced disassembly of cortical actin of cell cytosqueletton and the

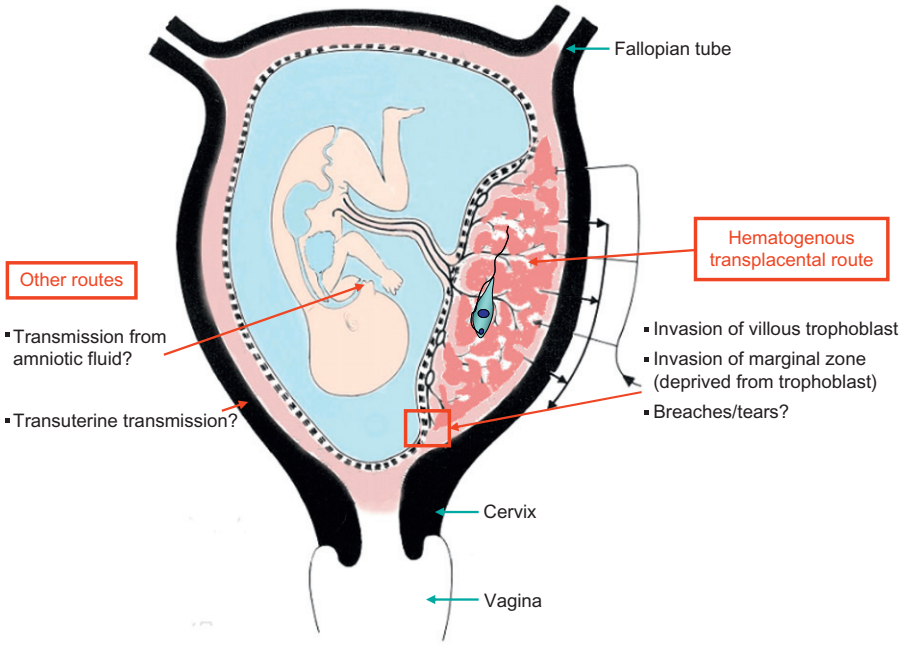

Figure 22.1 Possible routes of maternal−fetal transmission of *T. cruzi.*
Source: Modified from Benirschke, Kaufmann, Baergen, with permission of author and editor.

placental alkaline phosphatase (PALP, also supporting a Fc receptor function) would help (opsonized?) *T. cruzi* to invade and multiply within trophoblastic cells (Sartori et al., 2003; Lin et al., 2005). In a placental perfusion model using blood spiked with live *T. cruzi*, parasite DNA has been identified in the fetal side of placental tissue (Shippey III et al., 2005). However, the presence of DNA does not prove infection on the fetal side, since, as mentioned in Section 22.1, this could correspond to a contaminating transplacental transfer of DNA debris from the maternal side. Moreover, extrapolation of such studies to the *in vivo* situation can be questioned. They used huge amounts of blood trypomastigotes (10^5 to 10^7 for some mg of chorionic villi), largely superior to what can be expected in chronically infected pregnant women, displaying parasitemia below 15 p/mL (see Section 22.6.2) (Virreira et al., 2007). This is up to 3000 parasites in the 200 mL of blood contained into the intervillous space (the total villous weight and surface of placenta at term are estimated at 273 g and 12.5 m^2, respectively; Benirschke et al., 2006).

The histopathological analyses of placentas (from congenital cases) displaying intense *T. cruzi*−associated villitis (see Section 22.3.2) showed parasites in villous trophoblast, as well as amastigotes in villous stromal cells (Bittencourt, 1963, 1975b, 1976, 1988; Bittencourt et al., 1975; Tafuri et al., 1984; Drut and Araujo, 2000). However, in other studies performed in Brazil, Argentina, and Bolivia, also

on placentas of congenital cases, important villitis was uncommon and parasites were not or hardly identified in trophoblast (Hoff et al., 1978; Moya et al., 1979; Azogue et al., 1985; Carlier, 2005; Fernandez-Aguilar et al., 2005). This suggests that trophoblast is likely and more frequently a potential barrier for *T. cruzi* (see Section 22.3.1) and that transmission to fetuses, when it occurs, has to take alternative transplacental routes.

22.4.1.2 Parasite Invasion of Placental Areas Deprived from Trophoblast

In the histopathological studies mentioned earlier, parasites were mainly found in chorionic plate, membranes, and umbilical cords, in association with chorionitis and funisitis (see Section 22.3.2) (Hoff et al., 1978; Moya et al., 1979; Azogue et al., 1985; Carlier, 2005; Fernandez-Aguilar et al., 2005). These observations highly suggest that the parasites present in the intervillous space have encountered a pathway through placental tissues deprived of trophoblastic defences (see Section 22.3.1). Analyses by our team of serial biopsies performed in 19 placentas from infected live Bolivian newborns, showed a particularly high density of parasites at the level of marginal zone, with gradually decreasing densities in the chorionic plate and distant membranes (Carlier, 2005; Fernandez-Aguilar et al., 2005). Indeed, the marginal zone of the placenta joining the membranes to the chorionic and basal plates is known to be constituted with smooth muscle cells embedded in an extracellular fibrinoid matrix (constituted with fibronectin, collagen, laminin) and only covered by a nontrophoblastic maternal epithelium (Nanaev et al., 2000). *T. cruzi* can easily infect and replicate in such muscle and epithelium cells (see Chapter 14), likewise facilitating invasion of the chorionic plate and membranes. *T. cruzi* also expresses membrane receptors for fibronectin (Ouaissi et al., 1984). This might facilitate its adhesion to the matrix present in the marginal zone, but also elsewhere in placenta when such matrix progressively replaces the degenerating syncitiotrophoblast over the course of gestation (Benirschke et al., 2006).

22.4.1.3 Parasite Transmission Through Placental Breaches/Tears

Placental breaches/tears might also facilitate congenital transmission of *T. cruzi* from maternal blood escaping the trophoblastic defences. Such fissures can result from damage induced by strong placental inflammatory responses (see Section 22.3.2), and also appear naturally close to the pregnancy term, particularly during labor (contraction-mediated damages) (Benirschke et al., 2006) (see Section 22.5). Such a transmission mechanism might be relevant for transmission of free trypomastigotes as well as amastigote-infected cells, since this route is currently used for transmission of HIV-infected leukocytes (Biggar et al., 2008), and natural **microchimerism** of maternal–fetal cell frequently occurrs in cord blood (Jonsson et al., 2008). Confirmation of this route of transmission for *T. cruzi* parasites would be of practical importance since elective cesarian section might limit or prevent such transmission, as shown in maternal HIV infection (The European Mode of Delivery Trial Study Group, 1999).

22.4.1.4 Parasite Migration Within Chorionic/Stromal Tissues

Except for the microtransfusions through placental breaches mentioned earlier, *T. cruzi* parasites having crossed the trophoblastic barrier or other placental tissues, are encountered into the chorionic/stromal tissues of villi and/or chorionic plate, constituted by a mixture of fixed connective tissue fibers containing cells, such as fibroblasts, myofibroblasts, and macrophages (Hofbauer cells) (Benirschke et al., 2006). *T. cruzi* trypomastigotes that have not been destroyed by the mesenchymal phagocytic cells (see Section 22.3.1) can undergo further multiplication cycles within such cells, releasing new motile parasites. The latter are susceptible to sequential infection of other cells, finally infecting myocytes and endothelial cells lining fetal vessels embedded in villous chorion, chorionic plate, or umbilical cord, and gaining access to the fetal circulation (Tafuri et al., 1984; Carlier, 2005; Fernandez-Aguilar et al., 2005).

22.4.2 Other Possible Routes of Maternal–Fetal Transmission of T. cruzi *Parasites*

Another possible mode of maternal–fetal transmission might be through parasites released into amniotic fluid (AF) from amniotic cells (Figure 22.1). Indeed, a result of the presence of parasites into the chorionic plate and membranes is a secondary infection of the contiguous layer of amniotic cells (chorioamnionitis) (Carlier, 2005; Fernandez-Aguilar et al., 2005) (see Sections 22.3.2 and 22.4.1). Parasites released into AF might contaminate fetuses by oral or pulmonary routes, or eventually by skin penetration since fetuses bathe in AF and continuously absorb it. Lung and skin infections with *T. cruzi* have been reported in infected macerated fetuses and stillborns (Bittencourt et al., 1975, 1981; Bittencourt, 1975a). However, *T. cruzi* parasites are not found by microscopic observation and parasitic DNA is hardly detected in AF or aspirated gastric fluid content of asymptomatic-infected newborns (Nilo et al., 2000; Virreira et al., 2006b). This suggests that *T. cruzi* parasites are destroyed by the antimicrobial peptides normally contained in AF (Akinbi et al., 2004) and that the AF route of transmission, if it occurs, is likely to be uncommon in humans as in experimentally infected animals (see Section 22.7.2).

The possibility of placental/fetal invasion directly from the uterine wall (transuterine route) remains to be determined, since *T. cruzi* amastigote nests are observed in placental deciduas of mothers having delivered infected neonates (Moya et al., 1979) (Figure 22.1).

Though out of the scope of this chapter, it is interesting to mention that the postnatal transmission of parasites through maternal milk by breast-feeding (see Section 22.5) is likely of limited epidemiologic relevance. Only one report mentions a possible infant contamination during lactation (Medina-Lopes, 1988). Another one indicates the presence of parasites in milk during maternal infection (Mazza et al., 1936), but their detection might have been related to a contamination of collected milk with maternal blood (Jorg, 1992). Other studies do not observe parasites in milk of chronically infected women (Bittencourt et al., 1988; Amato

et al., 1992). Moreover, blood trypomastigotes, by contrast with metacyclic trypomastigotes, cannot survive into the gastric milieu (Hoft, 1996; Hoft et al., 1996), which should prevent their transmission to neonates in the rare cases where parasites could be present in breast milk.

22.5 Timing of Maternal–Fetal Transmission of *T. cruzi*

It is likely that there is little or no transmission of blood trypomastigotes during the first trimester of pregnancy, since the placental intervillous space is not open. Maternal blood supply becomes continuous and diffuse in the entire placenta only after the twelfth week of gestation (Jauniaux et al., 2003). The absence of developmental malformations in live newborns congenitally infected with *T. cruzi* (see Section 22.8.1) also suggests there is no transmission and detrimental interaction of parasites at the early stages of organogenesis in the embryo.

Abortions, stillbirths, and premature births in *T. cruzi*–infected women are more frequent for gestational ages between 19 and 37 weeks of pregnancy, but the proof of congenital infection as responsible of such pejorative outcomes has not been systematically investigated (Bittencourt and Barbosa, 1972; Azogue et al., 1985). The rare reported cases of acute *T. cruzi* infection during pregnancy indicate possible transmission around the twentieth week of pregnancy (Moretti et al., 2005). However, in most pregnant women who are in the chronic phase of an infection having been acquired a long time before pregnancy (see Section 22.2.3), it is impossible to pinpoint the timing of maternal–fetal transmission of *T. cruzi*. Transmission of blood parasites can be expected to occur most frequently during the second and third trimesters of pregnancy (prenatal transmission), and perhaps also closer to delivery and during labor (perinatal transmission) through placental breaches/tears. Comparisons of transmission rates in vaginal and cesarian deliveries remain to be done to appreciate such a possibility of late transmission (see Section 22.4.1).

22.6 Factors Involved in Transplacental Transmission and Development of *T. cruzi* Infection in Fetuses/Newborns

The main factors that permit occurrence and development of a congenital infection are the parasite itself, the mother, and the fetal capacity to respond to parasite invasion.

22.6.1 T. cruzi *Genotypes and Congenital Infection*

T. cruzi parasites are heterogeneous complexes of genetic lineages (see Chapter 19). Such phylogenetic differences might have relevant consequences on congenital transmission and fetal/neonatal pathology. The *T. cruzi* intraspecific

nomenclature has been recently revised to include six main genotypes (some of them being hybrids): TcI (formerly TcI), TcII (formerly TcIIb), TcIII (formerly TcIIc), TcIV (formerly TcIIa), TcV (formerly TcIId), TcVI (formerly TcIIe) (Zingales et al., 2009). Genotypes TcI, TcII, TcIII, TcV, and TcVI have been identified in congenital human infection. The TcV genotype predominates in Bolivia, Argentina, Chile, Paraguay, and the south Brazilian state of Rio Grande do Sul, corresponding to 80−100% of reported congenital cases (Munoz et al., 1994; Virreira et al., 2006a, 2007; Burgos et al., 2007, 2009; Corrales et al., 2009; Puerto et al., 2010; Luquetti, personal communication) The TcII and TcVI genotypes have been reported in neonates from Argentina, Bolivia, and the Brazilian Bahia state (Bittencourt et al., 1985a; Virreira et al., 2006a, 2007; Burgos et al., 2007, 2009), whereas the TcIII has been identified in Paraguay (Puerto et al., 2010). The TcI genotype has been identified in some neonates from Colombia (Falla et al., 2009) and Chile (Apt et al., 2010). Coinfection with different *T. cruzi* genotypes and HIV have been observed in one adult patient congenitally infected with *T. cruzi* (Burgos et al., 2005). Genotypes detected in mothers are found in infected newborns (Munoz et al., 1994; Burgos et al., 2007; Virreira et al., 2007), and also in congenitally infected siblings born in consecutive gestation, confirming that pregnancy does not induce changes in the predominant parasite population (Schijman, 2007; Burgos et al., 2007). The distribution of frequencies in the detected congenital cases is similar to that observed in the local infected population (Virreira et al., 2006a; Burgos et al., 2007; Corrales et al., 2009). So, at this time, by contrast with information collected in experimental murine model (see Section 22.7), there is no clear evidence of a relationship between *T. cruzi* genotypes and congenital infection in humans.

22.6.2 Maternal Parasitic Load and Congenital Infection

Parasitemia in pregnant women seems to be an important factor contributing to congenital transmission of *T. cruzi*. Maternal−fetal transmission occurred in 8 out of the 15 reported cases of pregnant women acutely infected with *T. cruzi* (Bittencourt, 1988; Brabin, 1992; Moretti et al., 2005). This indicates a higher risk of transmission in pregnant women displaying high parasitemia than in chronically infected women in which blood parasites are hardly detectable (53% versus 1−12%, respectively; see Section 22.2.1). Studies by our team indicated a twofold higher frequency of positive hemoculture for *T. cruzi* in chronically infected mothers transmitting parasites than in mothers having delivered uninfected newborns (Hermann et al., 2004), with parasitemia levels estimated by qPCR at 1−12 p/mL (Virreira et al., 2007). This significant association of parasitemia with congenital transmission was confirmed by using a parasite concentration technique in heparinized microhematocrit tubes (see Section 22.9.3 and Chapter 28) (Salas et al., 2007; Brutus et al., 2008). Interestingly, studies using xenodiagnosis in infected women indicated a higher frequency of positive results in the second and third trimesters of pregnancy than in nonpregnant women, but the relation with congenital transmission was not investigated (Storni and Bolsi, 1979; Menezes

et al., 1992). These data allow one to postulate that significant maternal parasitemia in the intervillous space is necessary to cope with the endogenous placental defences (eliminating one part of parasites present in maternal blood; see Section 22.3.1) and to successfully encounter an optimal route of transmission (see Section 22.4.1).

22.6.3 Maternal Immunity and Other Maternal Factors in Congenital Infection

Maternal immunity might be a limiting factor for both transmission and development of infections in the fetus/neonate (see Section 22.6.5). Maternal *T. cruzi*–specific IgG antibodies play a protective role in mothers and in fetuses when antibodies are transferred through the placenta (Miles et al., 1975; Breniere et al., 1983), by contributing to a reduction in parasitemia (see Chapter 24). In the placenta, they probably enhance the uptake of opsonized parasites by the placental phagocytes (see Section 22.3.1).

Activation of innate immune defences in pregnant women might contribute to limit the occurrence and severity of congenital infection. Our team showed that blood mononuclear cells of infected mothers produced higher levels of IL1β, IL6, and TNFα under stimulation with *T. cruzi* or LPS/PHA than uninfected control mothers. This maternal cellular activation upregulated the capacity of their uninfected neonates to produce such cytokines (Vekemans et al., 2000). Further studies reported higher blood TNF levels in mothers displaying detectable parasitemia (Cuna et al., 2009), and lower spontaneous leukocyte release and circulating levels of TNFα in parasite-transmitting mothers than in nontransmitting subjects (Cardoni et al., 2004; Garcia et al., 2008). Altogether these data highly suggest a protective role of such maternal innate defences both in mothers and offspring (Vekemans et al., 2000; Carlier, 2005; Truyens et al., 2005). Results of our team support a probable elimination of opsonized parasites by activated monocytes.

Mothers transmitting *T. cruzi* to their fetuses display lower T-cell–mediated immune responses to parasites and produce less IFN-γ, which probably contributes to an increase in parasitemia (Hermann et al., 2004) (see Section 22.6.2). Persistence of this reduced capacity of T-cell–mediated response after pregnancy (Hermann et al., 2004), as well as the familial clustering of cases of congenital infection with *T. cruzi* (see Section 22.2.3), suggest that some mothers might be predisposed to repeated transmission of parasites, which raises the question of a possible role for genetic factors that favor parasite transmission.

Other maternal factors such as young age and/or primiparity, and/or malnutrition and poverty also favor the congenital transmission of *T. cruzi* (Bittencourt, 1992; Azogue, 1993; Torrico et al., 2004, 2006). Dwelling in areas of high vectorial density exposes pregnant women to multiple reinfections with *T. cruzi* and contributes to increase maternal parasitemia (Torrico et al., 2006) (see Section 22.6.2). This factor appears to lead to increasing morbidity and mortality of congenital Chagas disease in Bolivian studies (Torrico et al., 2006; Brutus et al., 2008), whereas this seems uncommon in Argentina (Sanchez et al., 2005).

22.6.4 Maternal Coinfection and Congenital Infection

Maternal coinfection with *T. cruzi* and HIV results in increasing frequency and severity of congenital Chagas disease (Freilij et al., 1995; Nisida et al., 1999; Sartori et al., 2007; Scapellato et al., 2009), highlighting the important role of maternal immunity and high parasitemia in favoring parasite transmission to the fetus (see Sections 22.6.2 and 22.6.3). Interestingly, recent data have indicated a reduced HIV replication in *in vitro* cultures of human placenta infected with *T. cruzi* (Dolcini et al., 2008).

A recent survey performed in a Bolivian area endemic for both *T. cruzi* and *Plasmodium vivax* infections indicates that chagasic pregnant women displaying positive thick blood smears for *P. vivax* present higher *T. cruzi* parasitemia and rate of *T. cruzi* congenital transmission than mothers infected only with *T. cruzi* (L. Brutus, personal communication).

22.6.5 Fetal/Neonatal Capacity of Immune Responses and Other Fetal/Neonatal Factors in Congenital Infection

A crucial factor to stop, limit, or release the development of fetal/neonatal parasitic infection relates to the capacity of fetus/neonate to mount innate and/or specific immune response(s) against parasites transmitted from their mothers.

The neonatal innate immune system is biased against harmful inflammatory responses that could lead to preterm delivery (Levy, 2007). However, as mentioned earlier, such responses can be induced in neonates by mothers when infected with *T. cruzi* (see Section 22.6.3). If the production of inflammatory cytokines can be observed in uninfected neonates born to infected mothers (Vekemans et al., 2000; Garcia et al., 2008; Cuna et al., 2009), the levels of inflammation markers and activation of NK cells are rather low in congenitally infected newborns (Hermann et al., 2006, 2009; Garcia et al., 2008; Cuna et al., 2009). Altogether these data highly suggest a protective role of such innate defences in uninfected offspring of infected mothers, probably related to the capacity of their activated monocytes to eliminate parasites (opsonized by transferred maternal antibodies; see Section 22.6.3) (Carlier, 2005; Truyens et al., 2005). However, such defences seem lacking or insufficient in congenitally infected neonates.

T-cell immune responses in early life are considered of limited effectiveness owing to the relative immaturity of the immune system. The immune system is initially polarized toward a Th2 immune environment, which appears essential for survival of the fetus (Wilczynski, 2005). Both dendritic cells and T cells present quantitative and qualitative defects during the neonatal period, limiting the development of $CD4^+$ Th1 cell responses essential for the control of intracellular pathogens (Marchant and Goldman, 2005; Willems et al., 2009), as well as for the production of antibodies (Siegrist, 2007). We have demonstrated that neonates congenitally infected with *T. cruzi* can overcome the immaturity of their immune systems. Indeed, such infected newborns are able to mount an adult-like CD8 T-cell−specific immune response producing IFN-γ (Hermann et al., 2002), a

cytokine critical for controlling *T. cruzi* infection (see Chapter 24). Uninfected newborns of infected mothers display much lower specific responses (Neves et al., 1999; Truyens et al., 2005). Parasitic loads in umbilical cord or neonatal blood of congenital cases range from 1 to 125,000 p/mL (Schijman et al., 2003; Virreira et al., 2007), and a relationship has been established between such parasitemia and severity of congenital Chagas disease. Newborns with a compromised capacity to produce IFN-γ display the highest parasitemias and the more severe forms of congenital Chagas disease (Carlier, 2005). This suggests some protective role of T-cell–mediated responses in congenitally infected newborns (Carlier, 2005).

From these observations, it can be postulated that some newborns from *T. cruzi*–infected mothers might naturally auto-cure their congenital infection. This could have important consequences both in interpreting neonatal results of laboratory diagnosis and in management of public health control programs related to congenital *T. cruzi* infection (see Sections 22.9 and 22.11). As for mothers, the familial clustering of congenital *T. cruzi* infections (see Sections 22.2.3 and 22.6.3) suggests that some neonates might be predisposed to a lower capacity of immune responses, raising the question of a possible role of host genetic factors in the susceptibility to congenital infection. Fetal gender is probably not a risk factor for congenital *T. cruzi* infection, since most studies report similar frequency in both genders (Torrico et al., 2004; Altcheh et al., 2005; Sanchez et al., 2005; Salas et al., 2007), whereas only two others consider a predominance in females (Azogue and Darras, 1991; Bittencourt, 1992).

The complexity of mechanisms and factors underlying congenital transmission of *T. cruzi* in humans highlights the interest for information brought by studies in other mammals.

22.7 *T. cruzi* Vertical Transmission in Other Mammals and Experimental Models

22.7.1 *Vertical Transmission in the Natural Mammal Reservoir of* T. cruzi

Various mammals are involved in the sylvatic cycle of *T. cruzi* transmission (see Chapter 11). The possibility of vertical beside vectorial transmission has to be considered as a possible complementary mode of transmission in maintaining the mammal reservoir of *T. cruzi*. Neonatal transmission does not seem involved in the high rate of natural infection of the opossum *Didelphis marsupialis* in endemic areas (this marsupial mammal delivers young after a short gestation time but a long incubation in the marsupial pouch, with breast-feeding for about 100 days) (Jansen et al., 1994). Vertical transmission has been observed in some eutherian placental mammals naturally infected, such as rabbits, dogs (Villela, 1923; Barr et al., 1995), and primates (squirrels, marmosets, tamarins) living in wild conditions or primate centers (Lushbaugh et al., 1969; Eberhard and D'Alessandro, 1982; Sullivan et al., 1993; Maia et al., 2008), whereas there was no evidence that chronic maternal

T. cruzi infection causes fetal loss in baboons (Grieves et al., 2008). However, the importance of this vertical mode in the transmission of *T. cruzi* has not been evaluated on a large scale.

22.7.2 Experimental Models of T. cruzi Congenital Transmission

Mainly mice, rats, and guinea pigs have been studied as potential experimental models for vertical transmission of *T. cruzi*.

22.7.2.1 Mice

The timing of parasite inoculation versus gestation seems an important factor for maternal−fetal transmissibility in mice, since acute infection limits or impairs reproduction of animals (Werner and Egger, 1971; Mjihdi et al., 2002; Solana et al., 2002, 2009). Studies by our team showed that inoculation of mice (BALB/c with TcVI) 1 week before mating, thereby inducing a parasitemia peak (acute phase) on day 19 or 20 of gestation, totally impaired reproduction. Eighty percent of animals did not become gravid due to a preimplantation inhibition of embryo development, and abortion of implanted fetuses was observed in the remaining 20% of female mice (Mjihdi et al., 2002; Id Boufker et al., 2006). Such abortions were not due to fetal infection, but to massive infection of placenta-inducing ischemic necrosis in fetuses and high production of TNF (Werner and Egger, 1971; Mjihdi et al., 2002, 2004). Other experiments with mice (Swiss, C3H, BALB) inoculated with parasites at different times during gestation showed either no or rare maternal−fetal transmission (Apt et al., 1968a; Werner and Egger, 1971; Andrade, 1982; Gonzalez Cappa et al., 1999; Solana et al., 2002), or high transmissibility rate when a blockade of placental phagocytic activity or placental lesions were induced (Werner and Kunert, 1958; Delgado and Santos-Buch, 1978). Congenital infection was confirmed by detection of parasites in direct blood examination, hemoculture, xenodiagnosis, or histological studies in offspring. Transmission would be associated with some *T. cruzi* strains (Delgado and Santos-Buch, 1978; Gonzalez Cappa et al., 1999; Solana et al., 2002), though other strains displaying a placental tropism are not transmitted (Andrade, 1982). Parasites can be occasionally detected in milk of acutely infected mice (Disko and Krampitz, 1971; Miles, 1972), but transmission of infection by this route was not (Disko and Krampitz, 1971) or rarely observed (Miles, 1972).

Chronic infection in pregnant mice (Swiss, C3H, BALB/c) does not (Apt et al., 1968a; Carlier et al., 1987) or rarely induce congenital transmission confirmed by the detection of live parasites in offspring (de Cunio et al., 1980), though IgM antibody response (that can be synthetized in response to free parasitic antigens transferred from mothers; see Section 22.9.3) could be observed in offspring (Cabeza et al., 1980; Solana et al., 2002). Surprisingly, a recent study reported positive PCR in tissues of 33% of offspring (2 weeks old) from BALB/c mice chronically infected with TcI, and in 66% of them when mice were infected with TcIV (formerly IIa) (Hall et al., 2009). However, a positive PCR for *T. cruzi* in offspring

close to birth has to be interpreted cautiously, since parasite DNA fragments might be transferred from mothers without effective transmission of live parasites (see Sections 22.1 and 22.9.3).

22.7.2.2 Rats

Parasite inoculation during gestation in rats (Wistar, AXC, white) showed either no transmission (Apt et al., 1968b) or congenital transmission in 7–9% of offspring as detected by parasitological methods (Davila et al., 1994; Moreno et al., 2003). Parasites were not found in rat AF (Moreno et al., 2003) and postnatal transmission by rat milk was not observed (Davila et al., 1997; Moreno et al., 2003). Interestingly, a study showed the trypanocidal drug benznidazole administrated to pregnant rats, able to cross the placenta and reach the fetus (de Toranzo et al., 1984).

22.7.2.3 Other Animals

Guinea pigs were historically the first mammals to be studied for such transmission, with either negative results (Mayer and Rocha-Lima, 1914), or parasites rarely detected in AF but not in fetuses (Nattan-Larrier, 1921).

Though mice, rats, and guinea pigs have hemochorial placentas as humans do, their layer architecture and/or their maternal–fetal blood flow interrelations are different, in addition to their shorter duration of gestation (Benirschke et al., 2006). This renders difficult extrapolation of data obtained from these animals to the maternal–fetal transmission occurring in humans and should encourage further studies in nonhuman higher primates.

22.8 Clinical Manifestations and Long-Term Consequences of Congenital Chagas Disease

22.8.1 Clinical Manifestations and Mortality Rates of Congenital Chagas Disease

Congenital *T. cruzi* infection, though an acute infection, is frequently asymptomatic at birth, as observed in 40–100% of cases (Streiger et al., 1995; Freilij and Altcheh, 1995; Contreras et al., 1999; Zaidenberg, 1999; Blanco et al., 2000; Carlier and Torrico, 2003; Torrico et al., 2004). Nevertheless, clinical manifestations of congenital Chagas disease can appear within days or weeks after birth (Howard, 1975; Bittencourt, 1976). This is in agreement with the probable high frequency of late parasite transmission during pregnancy (see Section 22.5), reducing the time period for parasite multiplication in fetuses and the induction of clinically evident damage at birth.

Signs and symptoms that can be observed in newborns with congenital Chagas disease are nonspecific. They are generally similar to those reported in other common congenital infections, due to cytomegalovirus and herpes simplex virus

(commonly identified in the acronym TORCH) (Klein et al., 2006). This and the high frequency of asymptomatic cases highlight the mandatory need for laboratory diagnostic tools to detect such infection close to birth (see Section 22.9). *T. cruzi*–infected newborns can exhibit fever, low birth weight (<2500 g), prematurity (gestational age <37 weeks), hepato-splenomegaly, pneumonitis and, more rarely, jaundice (Howard and Rubio, 1968; Saleme et al., 1971; Bittencourt, 1976; Bittencourt et al., 1981; Azogue et al., 1985; Munoz et al., 1992; Freilij and Altcheh, 1995; Zaidenberg, 1999; Blanco et al., 2000; Carlier and Torrico, 2003; Torrico et al., 2004). The premature rupture of membranes frequently observed in congenital Chagas disease (see Section 22.3.2) (Carlier and Torrico, 2003; Torrico et al., 2004) can result in the birth of premature newborns with immature pulmonary function. Pneumonitis in such cases can be more severe and evolve into respiratory distress syndromes (Carlier and Torrico, 2003; Torrico et al., 2004). Growth retardation can be associated with a multisystemic diffusion of parasites in fetus, in addition to being a consequence of placentitis (see Section 22.3.2).

More severe clinical manifestations can also be observed in congenital Chagas disease, such as meningoencephalitis (inducing a large range of signs from slight tremors of face or limbs to generalized convulsions) and/or acute myocarditis (resulting in alterations of cardiac rhythm and cardiomegaly) (Rubio et al., 1967; Saleme et al., 1971; Vieira et al., 1983; Munoz et al., 1992; Freilij and Altcheh, 1995; Torrico et al., 2004). Purpura and edema (anasarca/fetal hydrops in severe forms) can be observed (Howard and Rubio, 1968; Bittencourt, 1976; Torrico et al., 2004). Anemia and thrombocytopenia have been reported as main hematological alteration of congenital Chagas disease (Howard and Rubio, 1968; Saleme et al., 1971; Bittencourt, 1976; Munoz et al., 1992; Zaidenberg, 1999; Contreras et al., 1999; Blanco et al., 2000; Sanchez et al., 2005; Salas et al., 2007). Megaesophagus or megacolon have been rarely reported in congenital cases (Rubio, 1968; Bittencourt et al., 1984; de Almeida and Barbosa, 1986; Atias, 1994; Costa-Pinto et al., 2001). Ocular involvement has been also exceptionally mentioned though the possibility of coinfection with *Toxoplasma* has not been eliminated (Atias et al., 1985; Munoz et al., 1992). No malformations are detected in such infected newborns.

Mortality can occur in the days after birth in untreated severe congenital Chagas disease, with rates below 15% of cases in most studies, but close to 100% when coinfection with HIV occurs (Howard, 1975; Freilij et al., 1995; Nisida et al., 1999; Torrico et al., 2004; Salas et al., 2007) (see Section 22.6.4). The proportions of abortions and stillbirths in women chronically infected with *T. cruzi* were reported either similar (Oliveira et al., 1966; Teruel and Nogueira, 1970) or slightly higher than in uninfected women (Bittencourt and Barbosa, 1972; Bittencourt et al., 1974; Castilho and Silva, 1976; Hernandez-Matheson et al., 1983; Azogue et al., 1985; Schenone et al., 1985). However, the roles of congenital infection versus those of strong placentitis without fetal infection (see Section 22.3.2) in such fatal outcomes have not been systematically investigated.

It is interestingly to note that the oldest clinical reports on congenital Chagas disease from Argentina, Brazil, and Chile, where studies began earlier, indicate the highest morbidity and mortality rates. Such cases presented frequently severe

meningoencephalitis, cardiomyopathy, respiratory distress syndrome, and anasarca (Howard and Rubio, 1968; Saleme et al., 1971; Bittencourt et al., 1975; Szarfman et al., 1975). By contrast, in the more recent studies, the reported cases are more frequently asymptomatic, and meningoencephalitis or cardiomyopathy is preferentially observed in the case of coinfection with HIV (see Section 22.6.4). Though comparisons of reported clinical data are not easy since the casuistics are largely different, this suggests, besides the lower observed incidence of congenital infection (see Section 22.2.2), a trend toward a decrease in severity of congenital Chagas disease in Latin American countries (Torrico et al., 2004, 2006).

Except in women presenting severe cardiac or digestive forms of Chagas disease before pregnancy (Sologuren Acha et al., 2002; Avila et al., 2003), gestation generally does not enhance the development of disease in chronically infected women, even if it might contribute to enhance parasitemia (see Section 22.6.2). This allows considering congenital infection and placentitis as being the main consequences of *T. cruzi* infection during pregnancy.

22.8.2 Long-Term Consequences of Congenital Infection with T. cruzi

Untreated congenital *T. cruzi* infection, whatever the neonatal morbidity, can develop into chronic chagasic myocardiopathy or digestive megaviscera 25–35 years after birth (Prata, 2001; Carlier et al., 2002) (see Chapter 27). However, evolution toward severe cardiopathy seems less frequent than in subjects residing in endemic areas and infected by vectorial route (Storino et al., 2002). Untreated cases with meningoencephalitis can suffer severe neurologic sequelae (Howard, 1975). The transgenerational transmission of parasites (see Section 22.2.3) is also a potential long-term consequence of congenital infection.

Another unexpected effect of such congenital infection is the **imprinting** of the fetal/neonatal immune system, which can lead to long-term consequences on later immune responses (see Section 22.6.5). Fetuses can be exposed to parasites and/or parasitic antigens and/or parasite-specific antibodies (antibody idiotypes are working as antigen surrogates). These elements transferred from mothers can prime specific immune responses or induce specific immune tolerance susceptible to increased resistance or susceptibility to subsequent homologous reinfection (Carlier and Truyens, 1995; Petersen, 2007). Indeed, some degree of parasite- or idiotype-specific responses have been observed in uninfected children of *T. cruzi*–infected mothers (Eloi-Santos et al., 1989; Neves et al., 1999) whereas, as mentioned earlier (see Section 22.6.5), congenitally infected neonates mounted strong type 1 T-cell immune response to *T. cruzi* (Hermann et al., 2002). However, the long-term consequences of such priming in relation to further reinfection(s) and development of human Chagas disease remain to be investigated (de Andrade et al., 1995). Moreover, such imprinting can also affect heterologous immune responses, particularly those to vaccines (Labeaud et al., 2009). Recent studies by our team showed that in comparison to infants born from *T. cruzi*–uninfected mothers, those suffering congenital *T. cruzi* infection developed stronger type 1 immune responses to hepatitis B, diphtheria, and tetanus vaccines, as well as an enhanced antibody

production to hepatitis B vaccine. Those born to infected mothers but remaining uninfected displayed stronger type 1 response to BCG (Dauby et al., 2009).

22.9 Laboratory Diagnosis of Congenital Infection with *T. cruzi*

The laboratory diagnosis of congenital infection with *T. cruzi* involves, first, the detection of infection in pregnant women and, second, the confirmation of infection in newborns of positive mothers. Detailed aspects of the laboratory diagnosis of Chagas disease are covered in Chapter 28. This section will focus on the specific aspects of the biological diagnosis of congenital *T. cruzi* infection in newborns.

22.9.1 Detection of Infection in Pregnant Women

Positive results to two standard serological tests detecting the presence of *T. cruzi*–specific antibodies (IHA, IF, or ELISA) are necessary to confirm a latent chronic infection in pregnant women, as in other patients (see Chapter 28) (Andrade and Gontijo, 2008). Such tests are generally available at a low cost at the primary health care level. They should be performed as soon as pregnancy has been diagnosed, if possible, though they can be performed at any time during pregnancy, including at the time of delivery, or in umbilical cord blood (detecting transferred maternal antibodies) (Munoz et al., 1982; Carlier and Torrico, 2003; Sosa-Estani et al., 2008). Sometimes more rapid and simple tests (as immunochromatographic-, immunodot-, immunofiltration-tests; see Chapter 28) are necessary (e.g., when a pregnant woman enters care just before delivery without previous serodiagnosis, or at primary health care level in rural endemic areas). Positive results obtained with these screening tests need further confirmation with standard serological tests (Sosa-Estani et al., 2008).

22.9.2 In Utero Detection of Fetal Infection

As mentioned in Section 22.4.2, PCR rarely detects *T. cruzi* DNA in amniotic fluid collected close to or at delivery (Virreira et al., 2006b) and amniocentesis is not recommended for the prenatal diagnosis of *T. cruzi* congenital infection (Carlier and Torrico, 2003; Virreira et al., 2006b).

Fetal blood sampling (cordocentesis to be performed by experienced clinical practitioners) for standard parasitological or molecular testing has been rarely used for the prenatal diagnosis of congenital *T. cruzi* infections (Okumura et al., 2004).

22.9.3 Detection of Neonatal Infection

Blood samples can be collected either at birth, from the umbilical cord (the most easy to collect, without trauma for newborns and mothers) or later in neonates by peripheral venipuncture (from heel, arm, or finger). In case of symptoms suggesting meningoencephalitis, cerebrospinal fluid can also be collected in neonates.

22.9.3.1 Parasitological Tests

Living *T. cruzi* trypomastigotes can be detected by direct microscopic examination of fresh blood samples or fixed blood smears using standard procedures (see Chapter 28). If results are negative, concentration techniques can be employed, such as thick smear or microscopic examination of buffy coat from centrifuged heparinized microhematocrit tubes. The latter technique is a recommended method, since it is more sensitive than a fresh smear, rapid, cheap, affordable, and can detect around 40 p/mL using 4–6 tubes) (Freilij et al., 1983; Carlier and Torrico, 2003; Torrico et al., 2005). The sample has to be examined within 24 h to avoid a decrease of sensitivity due to parasite lysis. Examination of parasites can be done by direct microscopic examination of buffy coat after cutting the tube (Freilij et al., 1983; Mora et al., 2005). However, to avoid possible contamination of the examiner, buffy coat can also be examined directly in the tube without previous rupture using immersion oil (Woo, 1969; La Fuente et al., 1984) or by rotating it (Torrico et al., 2004, 2005). The alternative Strout concentration method (so-called microstrout using Eppendorf tubes) can also be used (Moya et al., 1989; Azogue and Darras, 1995).

Indirect parasitological methods such as hemoculture (detection limit around 20 p/mL) and artificial xenodiagnosis can also be used for detecting low parasitemias in congenital *T. cruzi* infection (Moya et al., 1989, 2005; Carlier and Torrico, 2003; Torrico et al., 2004). However, they are generally more expensive and weeks are needed before obtaining results.

Though all these methods need well-skilled personnel and regular quality controls, the detection of parasites in blood definitively confirms congenital infection. In the case of negative results at birth, examination of another biological sample of the same neonate week(s) or month(s) after birth is required (this increases the sensitivity of detection when congenital transmission occurs later in pregnancy) (Carlier and Torrico, 2003).

22.9.3.2 PCR Assays

Molecular tests are able to detect low amounts of *T. cruzi* DNA in blood samples for the diagnosis of congenital infection (Russomando et al., 1992, 1998; Virreira et al., 2003; Schijman et al., 2003; Mora et al., 2005; Diez et al., 2008; Burgos et al., 2009). However, if protocols and primers have been recently compared and standardized (TDR, 2009), validation of their use for clinical diagnosis is still under evaluation. Critical information is still lacking on the stability of parasite DNA in maternal and umbilical cord blood. This complicates the interpretation of PCR results showing low intensity amplicons in umbilical cord, since trace amounts of parasite DNA transmitted from the mother might be detected instead of live parasites (see Section 22.1). Moreover, a self-cure in some infected fetuses having mounted an efficient immune response cannot be excluded (see Section 22.6.5). This probably explains the high maternal–fetal transmission rates reported from studies considering only results of PCR tests close to birth (Russomando et al., 1998; Diez et al., 1998; Garcia et al., 2001; Mora et al., 2005). Indeed, if a DNA negative test might be considered as excluding a possible congenital infection, a positive test at birth has to be confirmed

on subsequent samples of neonate (Diez et al., 2008). Quantitative (real time) PCR that estimates parasite DNA levels might be useful in validating equivocal PCR results (Schijman et al., 2003; Virreira et al., 2007).

22.9.3.3 Detection of T. cruzi—Specific IgM and IgA Antibodies

Detection of antibody isotypes not transferred by mothers has been proposed for the diagnosis of congenital *T. cruzi* infection (Stagno and Hurtado, 1970, 1971; Szarfman et al., 1973, 1975; Lorca and Thiermann, 1991; Lorca et al., 1995; Aznar et al., 1995; Di Pentima and Edwards, 1999). However, such antibody isotypes are not present in all parasitologically positive newborns, and are also detected in uninfected newborns of infected mothers (Reyes et al., 1990; Lorca and Thiermann, 1991; Aznar et al., 1995; Lorca et al., 1995; Russomando et al., 1998, 2005; Di Pentima and Edwards, 1999; Carlier and Torrico, 2003; Rodriguez et al., 2005). Such detection is presently not recommended for the diagnosis of congenital *T. cruzi* infection.

22.9.3.4 Detection of Particular Antigenic Specificities Recognized by IgG Antibodies

Immunoblot assays using trypomastigote excretory secretory antigens (TESA blot) allow to detect antibodies (IgG and IgM) recognizing some *T. cruzi*−specific antigenic bands in congenital infection, not detected in chronic infection cases (Umezawa et al., 1996). Dotblot assays would allow to identify congenital cases by detecting IgG antibodies recognizing shed acute-phase antigen (SAPA) synthesized by infected fetus/neonates and not transferred from mother's (undetectable in mother's blood) (Reyes et al., 1990). However, further studies indicated the SAPA-specific antibodies also being detected in chronic patients (Breniere et al., 1997), including most mothers.

22.9.3.5 Detection of Parasite Soluble Antigens

Detection of *T. cruzi* soluble antigens in urines and serum by capture ELISA test has been proposed for diagnosis of congenital cases. However, these tests did not detect all infected cases (Freilij et al., 1987; Corral et al., 1996).

22.9.3.6 Placental Histopathology

Standard histopathological or immunoenzymatic studies, PCR analyses or *in vitro* cultures of placental biopsies have also been considered for the diagnosis of congenital *T. cruzi* infection. However, placental parasitism can be either not detected in congenitally infected neonates, or, conversely, observed in uninfected newborns (Bittencourt, 1976; Azogue et al., 1985; Azogue and Darras, 1995; Carlier and Torrico, 2003; Fernandez-Aguilar et al., 2005). Indeed, the presence of parasites in the placenta does not confirm compulsorily a congenital infection, since the placental defences are able to contend parasitic infection before it occurs in neonates (see Section 22.3.1). So, placental analysis is presently no longer recommended for the diagnosis of congenital *T. cruzi* infection.

22.9.4 *Infant Detection of* T. cruzi *Congenital Infection*

If newborns of mothers infected with *T. cruzi* display negative results with the above-mentioned parasitological (or PCR) tests, or if it was not possible to perform such tests, detection of specific antibodies using standard serological assays (as for diagnosis in mothers; see Section 22.9.1) can be carried out in infants when IgG antibodies transferred from the mother have been eliminated. Different studies concord to indicate there are no more maternal antibodies 8–9 months after birth (Moya et al., 1989; Carlier and Torrico, 2003; Altcheh et al., 2005; Chippaux et al., 2009). A negative serological result at 8 months of age indicates the absence of congenital infection. Conversely, a positive serological result at this time indicates that the infant is currently infected. The congenital origin of the *T. cruzi* contamination 8 or more months after birth can be established in areas where possibilities of vector or other transmission routes have been ruled out (i.e., in nonendemic areas or in areas previously endemic but having developed vector and blood bank control programs) (Carlier and Torrico, 2003).

Detection of SAPA-specific IgG antibodies (see Section 22.9.3) in infants after 3 months of age might be an alternative for obtaining more quickly a diagnosis of congenital infection, since such antibodies disappear earlier than the other IgG antibodies transferred from the mother (detected by standard serology) (Russomando et al., 2005; Mallimaci et al., 2010). However, corresponding reagents are not commercially available and larger studies have to be carried out to validate this procedure.

22.9.5 *Recommendations*

There is now a consensus to consider the detection of blood parasites at any time after birth, and/or a positive serology after 8 months of age, as the gold standard for the diagnosis of congenital *T. cruzi* infection (conclusions of the WHO technical group IV on Congenital CD, in press). An early diagnosis allows an easier follow-up with the newborn, as well as a rapid initiation of the treatment. Nevertheless, generally speaking, each health system has to evaluate the best strategy allowing the earliest possible diagnosis of *T. cruzi* congenital infection (Carlier and Torrico, 2003; PAHO [Pan American Health Organization] and CLAP [Latin American Center for Perinatology and Human Development], 2004; Carlier et al., 2009).

22.10 Treatment of Congenital Infection with *T. cruzi*

The treatment of *T. cruzi* infection/Chagas disease is reviewed in detail in Chapter 30. This paragraph will focus on the pediatric application of such treatment. In order to limit the morbidity and mortality of acute infection and prevent the development of further chronic disease at adult age (see Section 22.8), there is a consensus to treat with standard trypanocidal drugs all congenital *T. cruzi* infection detected in newborns or infants as soon as the diagnosis has been confirmed. Both drugs, benznidazole (Russomando et al., 1998; Blanco et al., 2000; Schijman et al., 2003; Torrico et al., 2004; Luquetti et al., 2005a) and nifurtimox (Moya

et al., 1985; Freilij and Altcheh, 1995; Blanco et al., 2000; Schijman et al., 2003; Altcheh et al., 2005), can be used to treat congenital cases. Though no comparative and randomized trials have been achieved for congenital infection, it seems that that they have similar curative effects (Schijman et al., 2003). The recommended doses for benznidazole and infurtimox are 5−10 mg/Kg/day and 10−15 mg/Kg/day, respectiovely (in 2 divided oral subdoses) in neonates and infants till one year of age, with a recommended 60 days duration of treatment (such duration cannot be inferior to 30 days). The use of lower doses is recommended for infants above one year (Carlier et al., 2009). Such a prolonged treatment regimen with doses adapted to increasing body weight is not easy to carry out since pediatric formulations of these drugs are still unavailable despite the fact that they have been urgently requested (Carlier and Torrico, 2003; Sosa-Estani et al., 2005). Precautions have to be taken to obtain appropriate dosages, since available tablets have to be crushed and used as suspension. Full adherence of mothers to scheduled treatment requires a strong relationship between the pediatrician and affected families (Suarez et al., 2005).

Side effects currently seen in adults (see Chapter 30) are not observed in neonatal treatment with benznidazole (Russomando et al., 1998; Blanco et al., 2000; Torrico et al., 2004) and are rare using nifurtimox (Moya et al., 1985; Blanco et al., 2000). The chromosomal clastogenic effect of nifurtimox and benznidazole initially reported in children (Moya and Trombotto, 1988) has never been further confirmed. Negative standard (conventional) serology is required to confirm cure, and this result generally needs 3−16 months after the initiation of treatment to ascertain (Moya et al., 1989; Freilij and Altcheh, 1995; Russomando et al., 1998; Blanco et al., 2000; Chippaux et al., 2009). The use of other markers (e.g., the anti-F2/3 antibodies) or conventional PCR or or qPCR (see Section 22.9.3) can shorten the timing needed to obtain negative results and to assess cure (Russomando et al., 1998; Schijman et al., 2003; Altcheh et al., 2003; Burgos et al., 2009), but such tests remain to be validated at a larger scale. Therapeutic efficacy is around 90−100% in most studies if treatment is applied before 1 year of age (Freilij and Altcheh, 1995; Russomando et al., 1998; Blanco et al., 2000; Carlier and Torrico, 2003; Schijman et al., 2003; Torrico et al., 2004).

22.11 Prevention and Control of Congenital *T. cruzi* Infection

Primary prevention (**prophylaxis**) of fetal infection with *T. cruzi* aims to prevent infection of pregnant women. This can be obtained by limiting the risk of contamination through vectorial contacts (see Chapters 10 and 21) or blood transfusion (see Chapter 23), and by treating infected girls before they enter into their child bearing years (see Chapter 30) (Sosa-Estani et al., 2009a).

Secondary prophylaxis would aim to avoid maternal−fetal parasite transmission from a previously infected pregnant woman using trypanocidal safe drugs. However, the potential teratogenic effects of both currently used trypanocidal drugs,

benznidazole and nifurtimox, are not known. Their side effects in adults are unacceptable during pregnancy and their curative efficacy is limited in the chronic phase of infection presented by most infected pregnant women (see Chapter 30). For all these reasons, the treatment of *T. cruzi* infection during pregnancy is not recommended (Carlier and Torrico, 2003; PAHO [Pan American Health Organization] and CLAP [Latin American Center for Perinatology and Human Development], 2004). So, there is presently an international consensus to consider that the best control strategy is to associate the detection of congenital infection based on laboratory diagnosis (neonatal parasitological screening in newborns of infected mothers and/or infant serological screening after 8 months of age; see Sections 22.9.3–22.9.5), with the systematic treatment of positive neonates (see Section 22.10) (Carlier and Torrico, 2003; PAHO [Pan American Health Organization] and CLAP [Latin American Center for Perinatology and Human Development], 2004). The cost/benefit of this control strategy has been evaluated and shown to be much cheaper than the cumulative costs of managing chagasic patients over many years (Billot et al., 2005). Obviously, its benefit remains fully effective if infants are reintegrated in areas where vectorial transmission has been controlled. Mathematical models have been proposed to estimate the time period it will take to eliminate congenital transmission in regions where vectorial transmission was reduced to close to zero (Raimundo et al., 2010). Such a control strategy has been applied successfully in different endemic countries (Blanco et al., 1999, 2000; Russomando et al., 2005; Luquetti et al., 2005a; Torrico et al., 2007) and is presently also recommended in some nonendemic countries (Gascon and Pinazo, 2008; Brutus et al., 2009). Restriction of breast-feeding in infected mothers has no rational base (see Section 22.4.2).

22.12 Conclusions

Maternal–fetal transmission of *T. cruzi* can have severe outcomes by compromising survival and fetal/neonatal growth and/or lead to severe clinical forms of chronic infection later in adult life if an infant remains untreated. Congenital *T. cruzi* infection can be found worldwide since such transmission can occur in endemic areas, as well as nonendemic areas receiving immigrants from endemic regions. Though often asymptomatic at birth and neglected, this congenital infection must be considered an important public health problem requiring the development of reasonable prevention or control strategies, based on a deep understanding of mechanisms and multiple involved factors.

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Glossary

Chorionitis, chorioamnionitis inflammation of the placental chorion and/or the amniotic cell layer.
Funisitis inflammation of umbilical cord.
Hematogenous coming from blood.
Imprinting (in the context of infection in a pregnant woman) modulating effects of mater nal infection on the fetal/neonatal immune system.
Maternal−fetal transmission (a synonym of "congenital" transmission) prenatal (*in utero*) and/or perinatal (at the time of delivery) transmission.
Microchimerism (in the maternal−fetal context) mixture of maternal and fetal cells with an extremely low percentage of one of both cell types.
Opsonization covered by antibodies.
Placentitis placental inflammation.
Prophylaxis prevention.
Transgenerational transmission a synonym of "vertical transmission" more frequently used when occurring in more than two successive generations.
Vertical transmission (a synonym of "mother to child" transmission) transmission from one generation to the next, including prenatal, perinatal (see "maternal−fetal transmis sion" for the definitions of these terms), and postnatal (by breast feeding) transmission; see also "transgenerational transmission."
Villitis inflammation of villous placenta.

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23 Other Forms of Transmission

Mireille Hontebeyrie[1,], Simone Frédérique Brenière[2] and Christine Aznar[3]*

[1]Institut Pasteur, Paris, France, [2]Institut de Recherche pour le Développement (IRD), Montpellier, France, [3]Université des Antilles et de la Guyane, Cayenne, French Guyana

23.1 Introduction

In addition to the natural transmission of *Trypanosoma cruzi* by vectors (Chapter 21) or from a mother to a newborn (Chapter 22), the parasite may be transmitted by transfusion of infected blood, at the time of an organ transplant from an infected donor or to an infected recipient, by a laboratory accident with contaminated samples, or by an oral route. Presently, safe policies have been implemented to control the blood supply in blood banks in endemic countries and in most of the nonendemic countries where there are many Latin American migrants and to control the status of a donor or receiver of an organ transplant. To prevent laboratory accidents, severe measures exist to avoid the risks of contamination, and working conditions are highly managed. Oral transmission seems to be developing particularly in the Amazonian area because of the custom to drink juice from fruits of palm trees in which generalist triatomines are living. Education of the population is absolutely essential to control this kind of transmission. These four modes of *T. cruzi* contamination are discussed in this chapter.

23.2 Blood Bank Transmission

In the past, transmission of *Trypanosoma cruzi* by blood transfusion has been very extended in endemic areas because of the lack of controls in blood banks. Due to the persistence of the parasite in the patient, infected people may be responsible for parasite transmission through blood donation throughout their life even when they are asymptomatic and unaware of their infected status. In rural endemic areas, transfusion from arm to arm has been practiced in the doctor's office. In urban areas, blood bank control was not completely effective until the 1980s (Pinto-Dias and Brener, 1984). To sterilize contaminated blood, gentian violet was added to the blood supply. This resulted in a black color in the samples, which created fear in the patients sometimes leading to their refusal of the transfusion (Nussenzweig et al., 1953).

*E mail: mirhor@free.fr

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Furthermore, in hospitals in cities where serological diagnosis was available, contaminated bloods were tested and eliminated when serologically positive. Following the spread of HIV, blood bank controls for HIV, Hepatitis C, and *T. cruzi* became obligatory in almost all South American endemic countries (Schmunis, 1991, 1999; Schmunis et al., 2001; Schmunis and Cruz, 2005; Ponce et al., 2005). At that time, there was uncertainty regarding which *T. cruzi* serological tests were more effective (i.e., more sensitive). Different antigens were tested in different multicentric assays as well as in research laboratories (Levin et al., 1991; Carvalho et al., 1993; Vergara et al., 1992; Gomes et al., 1999; Umezawa et al., 2004; Pirard et al., 2005). However, some cases of false negative serology were described in Bolivia (Brenière et al., 1984). Recently, molecular diagnoses were developed that are better at detecting the parasite in the blood (Degrave et al., 1988; Avila et al., 1993; Wincker et al., 1994; Wincker et al., 1997; Brenière et al., 2002).

In many of the endemic countries, blood transmission of Chagas disease strongly decreased in the 1990s after the development of the detection of *T. cruzi*-specific antibodies in blood banks (Moncayo, 2003; Moncayo and Silveira, 2009; Schmunis, 2007). The blood transmission of *T. cruzi* was interrupted in several countries, in particular, Uruguay (1997), Chile (1997), and Brazil (2006). The interruption of blood transmission in Andean countries and Central America is still expected to be occurring in 2010. However, no policies or procedures existed for testing for *T. cruzi* antibodies in blood bank samples in countries considered as nonendemic, i.e., without vectors (Schmunis, 1991).

Due to the continuous immigration of Latin American people from South and Central America to the USA, Europe, Asia, and Australia, unexpected cases of *T. cruzi* infection were detected first in the USA (Navin et al., 1985; Grant et al., 1989; Nickerson et al., 1989; Kerndt et al., 1991; Milei et al., 1992; Galel and Kirchhoff, 1996; Di Pentima et al., 1999) and then in Europe (Frank et al., 1997; Flores-Chavez et al., 2008), Canada (Steele et al., 2007), and Asia (Ueno et al., 1995). Thus, the transmission of Chagas disease has become a real public health concern in blood banks in nonendemic countries. Almost 2% of the Latin American immigrants in the USA or Europe were estimated to be infected with *T. cruzi*. In Spain, where the number of immigrants is very high, the prevalence may reach 5% in the Latin American immigrants (Gascon et al., 2009; Schmunis and Yadon, 2009). Most of these people were asymptomatic for Chagas disease and did not know that they were carrying the parasite. Recently, Hontebeyrie and Aznar found that a 17-year-old French male in Paris was seropositive for *T. cruzi*. The physician requested a test for *T. cruzi* antibodies after observing flu-like clinical symptoms, perhaps knowing that the patient was adopted from northwest Argentina. Then, the twin brother was found to be *T. cruzi* positive without any clinical signs of Chagas disease. Both were adopted as newborns. They were probably infected congenitally from an infected mother (see Chapter 22).

In fact, 70% of *T. cruzi*-infected people were believed to be in a chronic phase and asymptomatic, and the acute phase of the infection was most often not detected due to a lack of specific clinical symptoms. However, many of the immigrants from Latin America were regular blood donors in the USA, where donors are paid for their blood. After cases of blood transmission of acute Chagas disease occurred in the USA

(Leiguarda et al., 1990; Cimo et al., 1993; Leiby et al., 1999; Leiby et al., 2002; Young et al., 2007), regulations on blood banks were organized by the CDC (Centers for Disease Control) (Galel et al., 1995; Kirchhoff et al., 2006). The USA was the first country to make tests for *T. cruzi* obligatory, thereby acting against the spread of Chagas disease by blood transfusion. Recently, European countries have created safety policies to avoid *T. cruzi* transmission by blood supply, first in Spain (2005) and next in other countries (Reesink, 2005; Kerleguer et al., 2007; Gascon et al., 2009; Guerri-Guttenberg et al., 2009; El Ghouzzi et al., 2009). For example, circulation of *T. cruzi* was suspected in a blood bank in Cayenne, French Guiana, resulting in its closure in 2005 (Aznar et al., 2004) while a testing policy was created. In addition to the blood transfusion problem with immigrants infected by *T. cruzi*, the diagnosis of chagasic cardiomyopathy is a real problem because it is unrecognized in nonendemic regions, and it may be confused with idiopathic cardiopathy (for review, see Bern et al., 2007).

Contamination was particularly problematic when patients were immunocompromised, for example, with AIDS or leukemia (Ferreira et al., 1997; Sartori et al., 1998; Rivera et al., 2004). Additionally, organ-transplanted patients were more susceptible to the parasite due to their weakness and the effect of immunosuppressive drugs. Several cases are reported in the literature (Jost et al., 1977; Kohl et al., 1982; Leiguarda et al., 1990; Añez et al., 1999; Sartori et al., 2007; Flores-Chavez et al., 2008). Reactivation may lead to meningoencephalitis (Leiguarda et al., 1990) or acute myocarditis. Many of these patients developed severe organ dysfunction and died. Control of *T. cruzi* infection in blood banks was recently extended to nonendemic countries (Wendel, 2010). People who have resided for some months in endemic areas cannot give their blood without serological *T. cruzi* control. It is assumed that the efficiency of blood donor screening programs could be improved by screening only blood donors who were born in Latin America or who have traveled in Latin America for extended periods, using a single enzyme immunoassay. For this purpose, a good questionnaire has to be implemented (Appleman et al., 1993; O'Brien et al., 2007). It is important to note that classical serological kits used to diagnose *T. cruzi* infection in Latin America cannot systematically be used in nonendemic countries because of a possible different cut-off between the pool of normal uninfected sera from endemic and nonendemic countries (Aznar et al., 1997; Verani et al., 2009). Increasingly, molecular diagnosis in endemic and nonendemic countries is performed to confirm the risk of *T. cruzi* infection. A polymerase chain reaction (PCR) method for circulating blood has been developed but one needs to pay attention to false negatives due to inhibitory effects of some blood components. Moreover, it should be noted that some strains of *T. cruzi* give a very low parasitemia that may be under the threshold of detection of this method (Brenière et al., 2002; Añez et al., 1999). Indeed, in the chronic phase, the parasites persist in organs or tissues and are safe from detection.

There were early attempts to sterilize blood before transfusion by adding gentian violet (Nussenzweig et al., 1953). However, this practice was discontinued because patients would not accept the dark color of blood treated with gentian violet. Studies are currently researching antiparasitic drugs to sterilize blood infected by *T. cruzi* (Moraes-Souza et al., 2002; Silva et al., 2007).

23.3 Organ Transplants

Persons receiving an organ transplant from an infected donor are also at risk for Chagas disease (Riarte et al., 1999; Altclas et al., 2005; D'Albuquerque et al., 2007; Forés et al., 2007; Martin-Davila et al., 2008; Sousa et al., 2008; Kun et al., 2009). Due to immunosuppressive treatment in the organ receiver, a small number of parasites present in the graft were able to develop very quickly. In this case, a curative treatment must begin immediately. Additionally, an infected patient who is receiving an organ transplant may develop a high parasitemia and clinical signs of Chagas disease when immunosuppressive treatment is implemented before the graft. In the past, many cases of heart graft on patients infected by *T. cruzi* led to mortality. This occurred primarily in endemic countries before systematic control of blood for *T. cruzi* infection. However, heart transplantation is recommended for patients with chronic Chagas heart disease doing a survey of the parasitemia and PCR control in the myocardium (Freitas et al., 2005; Diez et al., 2007; see Bestetti and Theodoropoulos, 2009 for review). If parasites are reactivated, treatment with a trypanocidal drug has to begin immediately. In general, patients recover, and mortality from *T. cruzi* reactivation in heart transplantation is estimated to be as low as 0.7% (Bocchi and Fiorelli, 2001). Recently, a donor and receiver of a liver transplantation were serologically tested for *T. cruzi*, and the results were negative. The receiver developed signs of congestive heart failure 10 months later, when *T. cruzi* seropositive diagnosis and the presence of amastigotes in myocardial biopsy specimens confirmed an acute Chagas disease. The patient was immediately treated with benznidazole, and the patient's response was excellent (Sousa et al., 2008). The problem also occurred in nonendemic countries and the impacts differed according to the transplanted organ. Indeed, the consequences of heart transplantation seem much more serious than kidney or liver transplantation from the same donor.

It should be a top priority of governments to prevent the donation of blood and organs from *T. cruzi*-infected donors. Obviously, detection and treatment of acute and chronic cases of Chagas disease, as well as congenital infection, have to be implemented in nonendemic areas.

23.4 Laboratory-Acquired Contamination

Persons working in research or clinical laboratories are at risk of being infected with *Trypanosoma cruzi* through the handling of materials containing viable parasites (e.g., infective trypomastigotes, infective amastigotes, or metacyclic trypomastigotes). Among them, the most frequent accidental laboratory-acquired contaminations resulted from needle-stick injuries during experimental infection of mice or from transmission by aerosol or droplets of infected materials (*T. cruzi* tissue culture supernatants, triatoma feces, and infected blood) by skin or mucosal contact (Coudert et al., 1964; Brener, 1984; Hofflin et al., 1987; Herwaldt, 2001). Other sources of laboratory contamination were the spraying of parasites by droplets or the breaking of a tube containing living parasites. In particular, special tubes for cryogenic preservation frequently rupture when they are thawed, and this is very dangerous. Contamination may

be due to a very low quantity of parasites and the parasite may be present also in dry droplets of infective culture or contaminated blood present on a bench. There are several recommendations to decrease the risk of these accidents: the wearing of a facial mask, disposable gloves, and lab coat. Additionally, the epimastigote cultures are often considered to be noninfective; however, old cultures that are enriched in metacyclic trypomastigotes are infectious.

Laboratory-acquired contamination can be prevented by wearing gloves, a mask, by making cultures in a biosafe laboratory (L3 type), and by using appropriate facilities for animals. In general, laboratories should be confined and entry should be controlled by security following the risk classification of this parasite. National and international ethical committees have developed requirements. For example, specific authorization to work with this parasite may be required in several countries in Europe.

T. cruzi multiply in the human host from a very few number of parasites. If one suspects that there has been contact with a mucosa or that one's skin has been scratched by a needle containing infected blood, it is necessary to immediately initiate preventive treatment to stop multiplication of the parasite in the body. For example, the person should be treated with benznidazole as soon as possible in accordance with the directions for use. If the treatment begins within 24 h of the accident and continues for 2 weeks, the development of the parasite will generally be stopped. However, some strains of *T. cruzi* are less sensitive to this drug and need to be treated for 2 months. In addition, if the diagnosis occurs after the development of the parasite and there are clinical symptoms such as chagoma, fever or myalgy, the treatment must last 2 months. Sometimes, the treatment must be discontinued due to secondary effects. In case of an allergic reaction, it is possible to complete the treatment with a low dose of corticoid. Circulating parasites need to be searched for during the fever period by direct examination of the blood or leuko-concentration as well as the detection of IgM-specific antibodies (during the first month after the presumed infection) followed by IgG-specific antibodies. The contaminated person may have to use investigational molecular methods like PCR, which allows a very early and sensitive parasitic diagnosis. A person has been cured when tests for detection of the parasite and for the specific antibodies are both negative. It is best to confirm the negative results of the serology or the molecular diagnosis with a second test.

It is important for people working with *T. cruzi* to have a control serum taken at the moment of recruitment, and several frozen aliquots of this sample should be kept to avoid repeated freezing and thawing. Trypanocidal drugs have to be available in each laboratory that works with infectious *T. cruzi*. However, it is important to note that some countries are not authorized to trade these drugs, and in this case, the laboratory needs a governmental agreement to import them. This is true in most countries in Europe that need to import benznidazole from Brazil or Argentina.

23.5 Oral Transmission

Oral transmission was unknown for a long time even though it was described in 1913 by Brumpt (1927), and in 1927, young rats were infected by *T. cruzi* after

infected triatomines were deposited on their oral mucosa. In 1921, Nattan-Larrier (1921) gave blood-containing trypomastigotes to rats, which led to an infective rate of 66%. This mechanism of natural transmission by an oral route was proposed for wild animals eating infected triatomines (Dias, 1933) as well as domesticated animals (Dias, 1935). Then, Talice (1944), Torrico (1950), and Diaz-Ungria (1965) confirmed the transmission of *T. cruzi* by an oral route through eating triatomine feces. Since 1961, several experimental infections were performed. Mayer et al. (1961), fed rats milk contaminated by the feces of infected *Triatoma infestans*, and thus described experimental infection with *T. cruzi* by a digestive route. Baretto et al. (1978) infected mammals by an oral route; Schenone et al. (1982) infected *Rattus norvegicus* with trypomastigotes by an oral route and showed the effectiveness of the digestive route in the transmission of *T. cruzi*; Jansen and Deane (1985) described the infection of rodents after eating food contaminated by parasites present in the anal glands of *Didelphis marsupialis*. Calvo et al. (1992) in Mexico infected mice and young rats by an oral route with *T. cruzi* in blood, in triatomines' feces, in infected triatomines, and from *T. cruzi* obtained from an acellular culture medium. In this study, the authors confirmed that an oral route is a probable way of transmission of *T. cruzi* because they reached an infective rate of 80% for mice and 70% for rats with an inoculum containing live triatomines. When they infected mice and rates with *T. cruzi* from *in vitro* cultures, only 30−40% were parasite positive. Ribeiro et al. (1987) found high infective rates when *Didelphis albiventris* took food from *T. cruzi*-infected rodents. Camandaroba et al. (2002) studied the infectivity of *T. cruzi* strains of different biodemes (Peruvian strain, biodeme type I, Z2b, *T. cruzi* IIa and IIb, and the Colombian strain, Type III, Z1, *T. cruzi* Ia and Ib) in mice subjected to infection by a digestive route (force-fed). These findings showed (i) the pathogenicity of the biodeme type III by intragastric infection, represented by strains adapted to the sylvatic cycle, and (ii) the importance of the oral route in geographical regions in which there are conditions for the contamination of food or beverages (Shikanai-Yasuda et al., 1991; Valente et al., 1999). Taken together, all these data on the natural or experimental infections of wild or domesticated animals show the possible existence of an oral transmission by ingestion of *T. cruzi* either (i) from other infected animals, or (ii) from urines or secretions of animals living near the houses like opossums, or (iii) from food contaminated by feces of hematophagus reduviidae or secretions of wild reservoirs, or (iv) from entire triatomines.

Transmission by food contaminated by *T. cruzi* is only possible if the parasite can survive for some time in this food. Ferreira et al., (2001) in a technical report showed that pasteurization inactivates *T. cruzi* trypomastigotes in human milk, and that the nutritional value of the milk is preserved. Another study evaluated the survival of *T. cruzi* in sugar cane used to prepare fresh cane juice and found that the parasite survived up to 24h after initial contamination, suggesting that inadequate storage of the cut cane in areas endemic for Chagas disease can represent a risk to health (Cardoso et al., 2006).

In humans, the possibility of the oral transmission of *Trypanosoma cruzi* was recently considered when microepidemic or epidemic episodes of acute Chagas disease were observed in areas without domiciliated triatomines or with a low level of

domestic infestation. However, Silva et al. (1968) reported the first outbreak of acute Chagas disease in 1968. These authors described 17 simultaneous cases with acute myocarditis in March 1965 in a rural school from Teutônia, situated in Estrêla, state of Rio Grande do Sul, Brazil. An inquiry found that the time of infestation (5 days) was from February 25 to March 3 depending on the patient. The first clinical signs appeared between March 13 and 22, from 13 to 19 days after the ingestion. During this epidemic period, 5 people died before day 40. Evidence suggesting an oral contamination included the following: (i) the appearance of 17 serious cases at the same moment, (ii) the lack of cutaneous or mucosal injury, (iii) the lack of triatomines in the school, and (iv) the presence of a *Didelphys* spp. contaminated by *T. cruzi* in the school. The authors favored the hypothesis that foods contaminated by the fluids of the anal glands of the opossum were ingested.

In the Amazonian region, the first acute human cases of Chagas disease were reported in 1939 by Floch, 1941 by Floch and Tasque (1941), and in 1948 by Floch and Camaim (1948) in French Guiana and next in 1969 by Shaw et al. (1969) in Belem, the capital city of the state of Pará, Brazil. In this region, reports of acute cases are more common, but it is important to differentiate isolated cases from grouped cases. When serious acute cases appeared at the same time in a family, a community, or during a common meal (meetings, celebrations), a systematic inquiry was needed to identify the cause of the contamination. Several cases were documented in this way. In October 1986, Shikanai-Yasuda et al. (1991) described that 7−22 days after a meeting at a farm in Catolé do Rocha, in Paraíba state, Brazil, there were 26 cases of acute Chagas disease with a febrile illness associated with bilateral eyelid and lower limb edema, mild hepatosplenomegaly, lymphadenopathy, and occasionally a skin rash. Only one 74-year-old patient died. In this outbreak, it seemed that an infected opossum could have deposited infective anal gland secretions over the sugar cane crusher. Between 1988 and 2005, Neves Pinto et al. (2008) observed in Pará, Amapá, and Maranhão, Brazil, 233 cases including 183 (78.5%) that were parts of outbreaks (mean of 4 individuals), probably due to oral transmission. In those cases, the most frequent clinical signs were fever, headache, myalgia, pallor, dyspnea, swelling of the legs, facial edema, abdominal pain, myocarditis, and exanthema. These grouped cases appeared mostly in July (19 cases) and August (25 cases), with the highest numbers in October (40 cases) and November (43 cases). This seasonality suggests an oral transmission because at that time, there was a very high production and consumption of fruit juice from palm trees, sugar cane, and other sources. In 2005 in Santa Catarina, Brazil, sugar cane juice was implicated as the source of infection in an outbreak that caused several deaths (Streindel et al., 2008). From 1968 to 2005, a total of 437 cases of acute Chagas disease were reported in the Brazilian Amazon region. Among these cases, 311 were related to 62 outbreaks in which the suspected contamination of patients was the consumption of açaí, the fruit of a palm of the family Aracaceae (Valente et al., 2006).

Between January and November 2006, 178 cases of Chagas disease were reported in Pará state, Brazil (Nóbrega et al., 2009), and 11 cases occurred in Barcarena. All patients had symptoms only in September and October. Among these 11 cases, five

were staff members at a health post who shared a meal at a staff meeting on September 15. The only suspected cause was the consumption of açaí juice on September 15. In French Guiana, an outbreak with nine cases of acute Chagas disease was observed in a littoral village in November 2005. In this case, the "Comou" juice from the fruit of *Oenocarpus bacaba* was suspected (Blanchet et al., in preparation). The consumption of water contaminated by triatomine feces was believed to be the cause of seven cases of acute Chagas disease in the Southwest of Bahia, where the lethality was 28.6% (Dias et al., 2008). In December 2007, a considerable outbreak of Chagas disease (128 confirmed cases) occurred in a municipality school in Caracas, a nonendemic zone in Venezuela (ProMED-mail, 2007).

All these acute cases were attributable to microepidemics of orally transmitted infection by contaminated food. Coura et al. (2002) reviewed the potential sources of food contamination. The sources include triatomine bugs or triatomine feces, raw meat from infected wild mammals carrying pseudocysts or blood-form trypomastigotes, and anal gland secretions of infected opossums. Additionally, palm juice presses lit at night may be open to contamination by light-attracted triatomine bugs, and thus represent a risk of oral transmission. In 2006, Pinto-Dias (2006) described practical rules for the prevention of oral transmission of Chagas disease. He proposed that all parasite forms, including trypomastigotes, epimastigotes, and probably amastigotes, coming from contaminated mammals or vectors might be responsible for oral transmission. All the types, zymodemes ZI, Z3 (Amazonas-Paraiba), and Z2 (Santa Catarina) have been associated with human contamination by an oral route. The consumption of crude or undercooked meat even if very unusual might be a source of oral transmission.

23.5.1 Prevention of Oral Transmission

Over the last few years, centers of investigation have been developed in the Amazonian region and elsewhere, which has led to an increase in knowledge on vectors, reservoirs, and symptomatology of acute cases found in these areas where few sporadic cases or chronic cases have been reported. Working with experts, the PAHO (OPS) and WHO (OMS) (OPS and OMS, 2006) investigated the theme of the transmission of *T. cruzi* from food. In 2006, the experts concluded that the oral transmission of *T. cruzi* was an important consideration that warranted inclusion in the National Programs of Prevention and Control of Chagas disease. The risk factors involved in this type of oral transmission are directly bound to the consumption of foods contaminated by the parasite. These foods are either naturally contaminated or contaminated during their preparation.

In 2007, the Ministry of Health of Brazil with the departments of survey and prevention, in conjunction with the Evandro Chagas Institute, FUNASA, ANVISA, the Ministry of Agriculture and Fishing, and other institutions, proposed measures to prevent and survey the preparation and production of foods likely to be contaminated by *T. cruzi,* including açai juice, which is very important to the economy of northern Brazil. At the industrial level, it was recommended that açai juice should be pasteurized; for the small producers at the family scale, recommendations were

proposed to improve hygiene and minimize the contamination risk for the juice at the moment of collection, during transportation, and while handling the palm fruits. All methods of information, communication, and education were taken into account to inform the population of the risks related to contamination by an oral route.

This type of *T. cruzi* oral transmission is very important, and it is more difficult to eradicate than vector transmission because some cases of oral contamination have been detected in areas with a very low rate of parasite infestation or in areas that are considered to be nonendemic.

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24 Protective Host Response to Parasite and Its Limitations

Carine Truyens and Yves Carlier*

Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles, Bruxelles, Belgium

24.1 Introduction

This chapter will highlight the complexity of the immune response toward *Trypanosoma cruzi*, which displays two main features. First, this protozoan parasite is present under two different forms, the extracellular trypomastigote (unable to multiply but disseminates in biological fluids throughout the body) and the intracellular amastigote (the multiplicative form able to invade a wide variety of cell types), requiring different effector mechanisms to be controlled. Second, the vertebrate host is usually unable to eliminate the parasite and the acute infection evolves in chronic infection characterized by parasite persistence at low levels in tissues.

The experimental mouse model of infection is widely used for immunological studies as its progression is very similar to that of human infection, resulting after the acute parasitemic phase, in chronic parasite persistence (Petry et al., 1989). A lot of studies have been performed in mice to decipher the mechanisms mounted by the host in order to establish an immunological control of infection, as well as those used by the parasite to escape from such immune response. This later point is of particular interest as parasite persistence in the vertebrate host largely accounts for the immunopathological development of severe, life threatening, cardiac or digestive pathology in 30−40% of patients (see Chapter 25). However, experimental data cannot be systematically extrapolated to natural human infection since mouse and human immune systems display subtle differences (Mestas et al., 2004). More and more data become available in human infection, and special attention will be paid to separate human data from experimental data obtained in mice. This chapter deals with current knowledge on innate and adaptive immune responses involved in the control of *T. cruzi* infection, while the immunopathological aspects are discussed in the next one. Refer also to previous reviews on immune responses to *T. cruzi* infection, mentioned in Brener (1980); Brener et al. (1997); Reed (1998); Tarleton (2007); Sathler-Avelar et al. (2009); Padilla et al. (2009a).

* E mail: ctruyens@ulb.ac.be

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24.2 Innate Immune Response in *T. cruzi* Infection

24.2.1 Role of Soluble Components of the Innate System

To eliminate microbial invaders as rapidly as possible before they cause damages, mammals are endowed with an arsenal of molecules ready to act quickly, namely the complement system, natural antibodies, and antimicrobial peptides, the latter being rapidly produced mainly by epithelial cells and leukocytes.

The *complement* system is one of the microbicidal weapons physiologically present at high level in serum and ready to rapidly eliminate every encountered microbe entering a host. The *complement* cascade is activated through three major pathways (Lambris et al., 2008; Pangburn et al., 2008; Zipfel and Skerka, 2009): (i) the alternative pathway (AP) activated by microbial molecules, (ii) the lectin pathway (LP) activated by the mannose-binding lectin (MBL) and by ficolins bound to microbes, and (iii) the classical pathway (CP) activated by immune complexes, depending on the presence of microbe-specific Ab (of IgM or IgG isotypes). All these pathways lead to activation of central enzymes of the complement cascade, the C3 convertases (C3bBb for AP and C4bC2b for LP and CP), and the covalent attachment of the ensuing C3b fragment to the surface of the target. This results in the immediate production of molecules that directly lyse the microbe, and indirectly favor its clearance by phagocytes and potentiate innate and adaptive immune responses.

T. cruzi trypomastigotes are able to activate the complement system through the AP (Kipnis et al., 1985; Krettli and Pontes de Carvalho, 1985) and, as recently shown, the LP, through recognition of parasite *N*-glycans by MBL and ficolins (Cestari et al., 2009). Amastigotes, when released after host cell disruption (Ley et al., 1988), are also potent activators of nonclassical pathway(s) (Iida et al., 1989).

However, trypomastigotes and amastigotes are endowed with various molecules rendering them resistant to complement lysis (Section 24.5.1), while epimastigotes are not infective since they are highly susceptibility to complement lysis (Norris, 1998). The natural resistance of birds to *T. cruzi* infection is based on the capacity of their complement systems to lyse trypomastigotes (Kierszenbaum et al., 1981). In line with this, the administration of normal chicken serum induces a marked decrease of parasite blood levels in experimentally infected mice (Budzko et al., 1975).

Natural IgM antibodies, present in the serum of individuals in absence of known antigenic stimulation, have been shown to be involved in early recognition of microbes (Baumgarth et al., 2005). They may provide the host with rapid and broad protection against pathogens. No information is available on the role of such Ab in resistance to *T. cruzi* infection, except that *Xid* mice, which display a different natural Ab repertoire due to a mutation affecting the development of B cells, are more resistant to *T. cruzi* infection than wild animals (Santos-Lima et al., 2001). However, humans might benefit the presence of others with Ab, known as *natural anti-α-Gal Ab*, directed against a particular carbohydrate structure [Galα(1→3)Gal-Gal1β(1→4)-GlcNAc]. They are produced in response to the continuous exposure to normal intestinal flora (Galili et al., 1993; Macher and Galili, 2008). Though of relatively low avidity, these Ab bind to α-galactosyl residues of mucins abundantly

present on the trypomastigote surface (Pereira-Chioccola et al., 2000). As similar Ab produced during *T. cruzi* infection have been shown to lyse trypomastigotes (provided they are not covered by acid sialic residues) (Pereira-Chioccola et al., 2000), the question arises if natural anti-α-Gal Ab might also lyse parasites. Though speculative, this might contribute to restrain the number of trypomastigotes susceptible to enter host cells, before they cover themselves with acid sialic residues. Natural anti-α-Gal antibodies are known to be restricted to humans and Old World monkeys. Whether other mammalian species also harbor potentially lytic antibodies of different specificities is unknown.

Antimicrobial peptides are conserved molecules protecting against various pathogens by reducing their viability or killing them in addition to having immunomodulatory properties (Guani-Guerra et al., 2010). The sole study dealing with this in *T. cruzi* infection shows that human *defensin α-1*, rapidly produced by infected epithelial cells, induced irreversible damages to trypomastigotes and amastigotes through pore formation, leading to reduced infectivity (Madison et al., 2007).

24.2.2 Recognition of T. cruzi by Pattern Recognition Receptors

24.2.2.1 Receptors Involved and T. cruzi Ligands

Pattern recognition receptors (PRRs), which are germline encoded, recognize conserved molecular motifs (termed pathogen-associated molecular patterns (PAMPs)) shared by different type of microbes. Upon engagement, they activate the transcription of various genes involved in inflammation and antimicrobial responses in the target cell. They are mainly expressed by cells involved in immune responses and play a pivotal role in the rapid induction of innate immune responses, as well as in activation, maturation, and shaping of adaptive immune response, coordinating various aspects of responses to pathogens (Medzhitov, 2007).

The most studied PRRs are the Toll-like receptors (TLRs), which are localized either at the cell surface or within endosomes. To date, 13 TLR proteins have been described, forming 10 dimeric receptors, with slight differences in human and mouse (Beutler, 2009). On ligand recognition, TLRs dimerize and undergo conformational changes required for the subsequent recruitment of cytosolic adaptor molecules, either MyD88 or TRIF, depending on the TLR. This leads to activation of various signalling pathways and activation of transcription factors. The Myd88 pathway is mainly responsible for inflammatory cytokine expression induced by activation of NF-κB and MAPKs, while the TRIF pathway is essential for mounting a type I IFN response as well as for contributing to activation of NF-κB (Trinchieri and Sher, 2007). An important property of the PRR system is that no single class of pathogen is sensed by only one type of PRR. Amongst TLRs, TLR2, TLR4, and TLR9 have to date been implicated in the recognition of *T. cruzi*-derived components (Gazzinelli and Denkers, 2006; Tarleton, 2007).

TLR2 recognizes the glycosylphosphatidylinositol (*GPI*) *anchors* of surface mucins of blood trypomastigotes (tGPI). Both the glycan core and the lipid moiety (and particularly the unsaturated fatty acid at the *sn*-2 position of glycerol) of tGPI,

but not the phosphatidylinositol moiety, seem to be required to induce an inflammatory response (Almeida and Gazzinelli, 2001; Campos et al., 2001; Ropert et al., 2002). The heavily glycosylated mucins of *T. cruzi* are among the most abundant surface molecules of trypomastigotes (Buscaglia et al., 2006; Nakayasu et al., 2009). They make up a dense surface coat, which likely conceals their *GPI* anchors. This raises the question as to how these anchors, and moreover their lipid moiety buried in the plasma membrane, are accessible for TLR recognition. Soluble tGPI purified *in vitro* also possess the ability to trigger *TLR2* signalling (Ropert et al., 2002). However, if such active tGPI are naturally released by parasites remains to be demonstrated, though they possess a *GPI*-PLC (Andrews et al., 1988; Redpath et al., 1998; Mortara et al., 2001). In addition, the structural requirements for bioactivity of tGPI (see earlier) are incompatible with the site of cleavage of PLC. Other possibilities might be the accessibility of *GPI* anchors on the vesicles profusely shed by trypomastigotes and enriched in glycoproteins and in desaturated fatty acids (Agusti et al., 2000; Buscaglia et al., 2006; Trocoli Torrecilhas et al., 2009), or the "presentation" to TLRs by heat shock proteins (HSP) as described elsewhere (Tsan and Gao, 2009). This latter mechanism has not yet been addressed in *T. cruzi* infection and would merit consideration, knowing that the gene of the *GPI*-PLC is adjacent to that of a parasite HSP (Redpath et al., 1998). *TLR2* functions as a heterodimer with either TLR1 or TLR6. The observation that macrophages lacking TLR6 expression fail to respond to tGPI indicates that the heterodimer *TLR2*−TLR6 is engaged by *T. cruzi* (Gazzinelli and Denkers, 2006). In addition, a growing number of "accessory molecules" like CD14, CD36, MBL are identified for modulating TLR signalling by interacting with TLR-ligands and the receptor (Akashi-Takamura and Miyake, 2008). Likewise, CD14 has been shown to amplify many *TLR2* responses and has been suggested to be involved in *TLR2* recognition of tGPI (Camargo et al., 1997a). Metacyclic trypomastigotes and epimastigotes also express *GPI*-anchored mucins. Their interaction with TLRs has not been proven but is assumed as they also trigger inflammatory responses in macrophages. They are, however, 100- to 1000-fold less potent than tGPI. This may be related to structural differences (Almeida and Gazzinelli, 2001; Ropert et al., 2002). Tc52, an intracellular trypanothione-glutathione thioltransferase shed by trypomastigotes and amastigotes (Moutiez et al., 1995), has also been reported to bind to *TLR2* (Ouaissi et al., 2002). However, the molecular basis for this recognition is not known.

TLR4 is responsible for the recognition of ceramide-containing GPI anchors expressed at the parasite surface, known as glycoinositolphospholipids (*GIPLs*) (Oliveira et al., 2004). It is worth noting that *GIPLs* are abundant on epimastigotes but rare on trypomastigotes (Pereira-Chioccola et al., 2000). This calls into question the *in vivo* relevance of *GIPLs* as *T. cruzi* PAMPs recognized by mammalian TLRs.

TLR9 has more recently been shown to recognize *T. cruzi DNA*, in relation with the presence of unmethylated CpG motifs known to interact with this TLR (Bafica et al., 2006; Bartholomeu et al., 2008). Amazingly, the immunostimulatory *DNA* sequences that mostly stimulate human and mouse cells are positioned in different *T. cruzi*-specific genes (Bartholomeu et al., 2008). In resting cells *TLR9* is located in the endoplasmic reticulum (ER). So that target *DNA* would encounters the receptor,

it has to move into an endo-lysosomal compartment in which *TLR9* is translocated from the ER (Latz et al., 2004). In *T. cruzi*-infected cells, the authors show that parasite *DNA* becomes available to *TLR9* when parasites are destroyed in the lysosome-fused vacuoles during parasite uptake by phagocytes. Access to such sequences later on, after parasites have escaped in the cytoplasm of the host cell, through the mechanism of autophagy (Virgin et al., 2009), would merit consideration.

As explained earlier, dimerization of TLR triggered by ligand binding is a necessary step to initiate signal transduction. Dimerization of TLR2, -3, and -4 takes place after a host neuraminidase (activated by TLR engagement) removes α2-3 sialic acid residues from the glycosylated TLR molecules. Removal of these residues allows two TLR molecules to move closer and dimerize. Interestingly, a recent study shows that the *T. cruzi* enzyme trans-sialidase *(TS)* is able to directly induce *dimerization* of TLR4, thereby activating downstream signalling (Amith et al., 2010). This suggests that the parasite might activate TLRs independently of PAMP recognition.

The family of PRRs is steadily growing and also includes surface *C-type lectins receptors* (CLRs) such as dectins, the *mannose receptor* (MR) and DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin), as well as cytosolic protein sensors like the nucleotide-binding oligomerization domain (NOD)-like receptors (*NLRs*, also termed nucleotide-binding domain, leucine-rich repeat-containing) and the retinoic acid inducible gene I (RIGI)-like receptor (*RLRs*) in Medzhitov (2007); Willment and Brown (2008); Mogensen (2009); and Fukata et al. (2009). Little information is to date available about interaction of *T. cruzi* with such receptors. It has been shown that amastigotes, but not trypomastigotes, bind to a CLR, most likely the MR, through glycoproteins of the trans-sialidase family (Kahn et al., 1995). It is interesting to mention that the MR, though expressed at cell surfaces, is mainly located within the endocytic pathway. It is present in macrophages, dendritic cells (DCs), and some other cells like cardiomyocytes. It is thought to induce NF-κB activation and the production of inflammatory or immunosuppressive cytokines, depending on the context (Taylor et al., 2005; Willment and Brown, 2008). In addition, recognition of *T. cruzi* through the *NLRs NOD1* and *NOD2* has recently been disclosed (Silva et al., 2010). These receptors are mainly expressed by Ag-presenting cells and epithelial cells and are to date known to bind particular peptide−lipidic moieties derived from bacterial peptidoglycans (Strober et al., 2006; Fukata et al., 2009). On the other hand, sensing of *T. cruzi* through the *NLRs* RIGI and MDA5, recognizing viral dsRNA seems unlikely (Chessler et al., 2008).

24.2.2.2 Innate Recognition and Control of T. cruzi *Infection*

Studies of the course of infection (parasitemia and mortality) in mice selectively deficient for different PRRs show that TLRs as well as NOD1 (Silva et al., 2010), significantly contribute to the development of effector mechanisms that eliminate parasites during acute infection. Amongst TLRs, TLR9 plays a dominant role, though TLR2 likely synergizes with TLR9 (Campos et al., 2004; Bafica et al., 2006). The adaptor molecule Myd88 is used by various TLRs as well as IL1 and IL18 receptors to transduce signals. In *T. cruzi* infection, it has been observed that

mice deficient for Myd88 or for both TLR9 and TLR2 similarly fail to control infection. This suggests that combined TLR2 and TLR9 deficiencies totally account for Myd88 deficiency and that no other Myd88-dependent pathways are crucial to control acute infection (Bafica et al., 2006). TLR4 has also been shown to play a minor role in such control (Oliveira et al., 2004, 2010), suggesting that the spare TLR4 ligands of trypomastigotes are recognized *in vivo* or that "bystander" activation of TLRs by parasite sialidase is working *in vivo*.

In addition to this, mice in which all TLR-dependent pathways are abolished (by simultaneous deleting MyD88 and TRIF) present still higher susceptibility to *T. cruzi* infection than mice deficient for Myd88 alone, while knocking out the TRIF pathway alone does not have a significant effect (Koga et al., 2006). This shows that the Myd88 pathway predominates on the TRIF one, although this latter synergizes with the former. The TLR(s) involved in TRIF pathway activation by *T. cruzi* has not been identified but might be TLR4. It is also possible that currently unknown parasite components are recognized by TLR4 or TLR3, both of which use the TRIF-dependent pathway.

Despite such an evident role in the control of acute infection, TLR engagement by *T. cruzi* is not exclusive for induction of protection. Indeed, IL12 production (a protective cytokine) can be produced independently of any TLR engagement in infected mice, but its release is upregulated by TLR-ligation (Koga et al., 2006; Kayama et al., 2009). Thus, the role of TLR recognition in *T. cruzi* infection is likely the amplification of responses initiated by other receptors, rather than actually initiating them.

In addition, and supporting this hypothesis, Tarleton suggests that *T. cruzi* does not activate host PRRs as rapidly as do other pathogens (Padilla et al., 2009b). This may relate to the initial round(s) of intracellular parasite multiplication at the site of infection, which take several days before trypomastigotes are released to infect other cells. During this period, *T. cruzi* PAMPs would not be exposed to PRRs. Indeed, free *T. cruzi* ADN (TLR9 ligand) would not be available since parasites are not killed (microbicide potential of host cells are still not at all or insufficiently induced), and trypomastigotes bearing or shedding tGPI (TLR2 ligand) are still not produced. Moreover, GPI anchors of metacyclic trypomastigotes, the initial infective form in case of vectorial transmission, are far less powerful activators of TLR than tGPI (see earlier). After some days, the strong increase in parasite load, along with the release of parasites and of damage-associated molecular patterns from death host cells, would facilitate potent innate immune recognition necessary for the initiation of adaptive immune responses. So, *T. cruzi* would be a rather stealth invader avoiding recognition by components of the innate immune system until the infection is well established.

24.2.3 Role of Cells of the Innate Immune System

24.2.3.1 Natural Killer (NK) Cells

NK cells (non-T non-B lymphocytes) are vital effector cells of innate immunity. They are known to be rapidly activated by cytokines (among which IL12 and IL18 are the

most potent) produced by DCs and monocyte/macrophages having encountered pathogens. Constitutive expression of a complex repertoire of surface receptors also enables them to directly sense infected cells without prior sensitization. Activated NK cells then interact with DCs, monocyte/macrophages, and T cells through direct contact and/or cytokine release (primarily IFN-γ), thereby shaping innate and adaptive immune responses. They also rapidly become effector cytotoxic cells able to eliminate target cells (Korbel et al., 2004; Vivier et al., 2008; Empson et al., 2010).

Production of IFN by *T. cruzi*-infected mice was previously suspected in 1970 by Rytel et al., who showed IFN-like antiviral activity to be detectable in the circulation of acutely infected mice (Rytel and Marsden, 1970). Antiviral activity rapidly peaks during the first days of infection (Rytel and Marsden, 1970; Sonnenfeld and Kierszenbaum, 1981). Parallely, the presence of activated NK cells very early in infected mice was assumed by Hatcher et al. (1981). Both cytotoxic and IFN-γ producing NK cells are quickly induced upon infection (first days) (Hatcher et al., 1981; Cardillo et al., 1996; Hamano et al., 2003). Interestingly, IFN-γ release by NK cells is of short duration (a few days) while the development of cytotoxic activity is more gradual and persists longer (Une et al., 2000). However, the kinetics and magnitude of NK cell responses may vary between mouse strains, in relation to the resistant or susceptible phenotype to the infection (Antunez and Cardoni, 2000).

Experiments in mouse *T. cruzi* infection shows depletion of NK cells lead to higher parasitemia and an increased mortality rate (Rottenberg et al., 1988; Cardillo et al., 1996; Sakai et al., 1999; Lieke et al., 2004). The beneficial role of NK cells relies on both IFN-γ release (Cardillo et al., 1996; Lieke et al., 2006) and cytotoxic activity (Lieke et al., 2004), though cytotoxicity does not seem to be of major importance (Une et al., 2003). Mouse cytotoxic NK cells can directly kill trypomastigotes *in vitro*. Trypanolysis requires contact between NK cell and parasite followed by degranulation (Lieke et al., 2004). The effector molecule(s) are not clearly identified. It is not perforin (Lieke et al., 2004), while granulysin might be involved in humans (this molecule is present in granules of cytolytic NK cells in humans but absent in mice) (Jacobs et al., 2003). Indeed, porcine granulysin (homologue of human granulysin) lyses trypomastigotes *in vitro*. Whether cytotoxic NK cells also kill *T. cruzi*−infected cells has not been demonstrated. Numerous studies show the cytotoxic potential of NK cells from *T. cruzi*−infected mice against NK-sensitive tumor target cells like mouse YAC1 (Une et al., 2000; Lieke et al., 2004), but they did not test in parallel the action of NK cells on *T. cruzi*−infected cells. Only one study indicates that IL2-activated mouse NK cells (LAK cells) are unable to kill *T. cruzi*−infected macrophages (Zychlinsky et al., 1990). On the other hand, granulysin, or a fragment of it released extracellularly, is able to kill *in vitro* some intracellular parasites (Jacobs et al., 2003). Though, NK cells are currently recognized as the major innate source of innate IFN-γ in mouse that activates nitric oxide (NO) production by macrophages as well as by other cells like fibroblasts, to limit *T. cruzi* replication during the early acute phase of the infection (Rottenberg et al., 1988; Cardillo et al., 1996; Lieke et al., 2006).

Induction of the capacity of NK cells to lyse extracellular trypomastigotes is dependent on IL12 and type 1 IFNs release by other cells, without a role for IFN-γ

(Une et al., 2003; Lieke et al., 2004). At odds, a study shows that induction of NO in infected fibroblasts by NK cells is mediated by IFN-γ but not IL12 (Lieke et al., 2006). Curiously, the involvement of IL12 in IFN-γ production by NK cells in *T. cruzi* infection has to date not been undoubtedly demonstrated. However, various studies strongly support it, like the kinetics of IL12 and IFN-γ levels in infected mice, and the observation that IFN-γ production by NK cells requires the indirect action of parasites on other cells, presumably the source of IL12 (Cardillo et al., 1996; Antunez and Cardoni, 2000; Une et al., 2003). We have nevertheless observed that IFN-γ production by human NK cells cultured with *T. cruzi* was strongly dependent on IL12 release by monocytes (unpublished data). If parasite molecules also contribute to directly activate NK cells is not reported, except the oligosaccharide moiety of *T. cruzi* GIPLs (more abundant on epimastigotes) that *in vitro* induce proliferation of a NK cell line (Arruda Hinds et al., 2001).

Beneficial effects of NK cells may also rely on their known ability to direct the development of protective Th1 cells (Vivier et al., 2008). However, the importance of the NK-derived IFN-γ for development of a Th1 response in *T. cruzi* infection remains uncertain. Indeed, early NK depletion in mouse infection does not affect IFN-γ mRNA levels produced later on, indicating no severe impairment in Th1 development (Une et al., 2000). Nevertheless, NK cells are at least necessary to properly activate a particular Th1 $CD4^+$ T cell-mediated protective mechanism, related to expression of HSP65 in macrophages (see Section 24.2.3.4) (Sakai et al., 1999).

NK cells may in some cases provide help to B cells for Ab production against T-independent Ag (Li et al., 2007). One study indicates that B cells from chronically infected mice may produce *in vitro* increased amounts of Ig upon help from an NK cell line (Arruda Hinds et al., 2001).

Thus, the main protective function of NK cells in *T. cruzi* mouse is to stimulate effector functions, such as generation of NO, in the early stage of infection when parasites are multiplying exponentially inside cells and spreading in the blood.

Few data are available on NK cells in human Chagas disease. We have observed phenotypic and functional modifications of circulating NK cells in *T. cruzi* congenitally infected newborns, suggestive of a previous in utero activation of these cells by the parasite (Hermann et al., 2006). Sathler-Avelar et al. (2003) report a transient increase of circulating immature NK cells (CD56 $CD16^+$ lymphocytes) during the late acute phase of infection in children. In the chronic phase of the infection, circulating levels of mature $CD56^+CD16^+$ NK cells are similar to that found in uninfected individuals (Sanchez et al., 2002). Potentially cytotoxic NK cells have also been identified in chronically infected patients. Indeed, cytotoxicity against NK-sensitive tumor cells is generated when PBMC from patients are cultured *in vitro* with *T. cruzi* epimastigote lysate, which was not the case for cells from control subjects (Brodskyn et al., 1996).

24.2.3.2 Innate Lymphocytes

Innate lymphocytes encompass particular subsets of B and T lymphocytes, namely the B1 B cells, invariant natural killer T (iNKT) cells, and a subset of γδ-T cells.

Their hallmark is the expression of semi-invariant, germline-encoded Ag receptors, allowing recognition of conserved antigenic structures of self or microbial nature. Germline receptors and high copy numbers ensure rapid and massive innate responses, making them important regulators of immune responses, through release of soluble mediators and/or contact with other cells of the immune system (Bendelac, 2006). B1 lymphocytes are discussed in the context of polyclonal activation (Section 24.4.3).

iNKT cells constitute a conserved sublineage of αβ-T cell expressing TCRs recognizing glycolipids. Most of them express an invariant TCR alpha chain (Vα14 in mice and Vα24 in humans), and a restricted set of TCR beta chains (Vβ2, 7 and 8 in mice, Vβ11 in humans). They are restricted by the monomorphic MHC class-I-like molecule CD1d that presents self and exogenous glycolipids. When activated, they regulate other immune cells through cell−cell interactions and rapid prolific cytokine production. iNKT cells are also endowed with cytotoxic properties (Kronenberg, 2005; Diana and Lehuen, 2009; Wu et al., 2009; Godfrey et al., 2010).

T. cruzi infection activates iNKT cells early during mouse *T. cruzi* infection to produce both IFN-γ and IL4 (Duthie and Kahn, 2002a; Duthie et al., 2005a). However, no parasite CD1d-restricted Ag could be identified. This suggests that iNKT cell activation may be indirectly induced by host glycolipids made accessible during the infection in conjunction with activating cytokines, as explained elsewhere (Wu et al., 2009). Yet, IL12, but not IL18 or MyD88-dependent TLR signals, is required for activation of iNKT cells in *T. cruzi* infection (Duthie et al., 2005a).

A protective role for IFN-γ-producing iNKT cells is suggested by the observation that the absence of iNKT cells in Ja18 $^{-/-}$ mice (deficient in iNKT cells) and in CD1d $^{-/-}$ mice (deficient in both iNKT and variant NKT cells) is associated with increased parasitemia and mortality rate in the acute phase, while activation of iNKT cells by administration of the glycoplipid α-galactosylceramide (α-GalCer), a potent activator of iNKT cells, prior to *T. cruzi* infection, reduces the parasitemia (Duthie and Kahn, 2002a,b; Duthie et al., 2005c, 2006).

The mechanism of iNKT-dependent protection is not precisely deciphered. iNKT cells are known to be able to activate NK cell functions through IFN-γ production (Carnaud et al., 1999). In murine *T. cruzi* infection, iNKT cells augment the number of NK cells in the liver but not in the spleen. The importance of such an effect for the control of infection is not known. In any case, iNKT cells do not increase NK cell cytotoxic capacity (Duthie et al., 2005c). The observation that the absence of T lymphocytes (and thus of NKT cells) does not impede NK-IFN-γ production triggered by *T. cruzi* also supports that iNKT cells are not directly involved in NK cell activation. Another study, performed in mice receiving α-GalCer to hyperactivate iNKT, suggests that iNKT cell control of parasite multiplication relates to their ability to produce IFN-γ and to improve T and B lymphocyte responses (Duthie and Kahn, 2002a). In line with this, iNKT cells of *T. cruzi*-infected mice seem to favor specific IgG response against GPI-anchored but not other parasite molecules (Duthie and Kahn, 2002b). Thus, as iNKT cells are rapidly activated in an MHC-unrestricted way, they might promote an earlier and stronger IgG response against the GPI-anchored molecules coating the parasite surface, as compared to IgG directed against other Ag that

depend on help given by conventional $CD4^+$ T cells (Schofield et al., 1999). This might favor parasite opsonization and clearance, while limiting the production of Ab directed against functionally important molecules for the parasite that favors its survival (see Sections 24.3.1 and 24.5.3).

In addition, iNKT cells likely dampen the inflammatory response (production of IFN-γ, TNF-α, and NO) of variant NKT cells and this seems to protect mice from mortality. Thus, iNKT cells likely exert opposing regulatory effects on T and B lymphocytes in one way, and on variant NKT cells the other way to limit parasite multiplication and improve survival, respectively (Duthie et al., 2005b).

On the other hand, in absence of IFN-γ (IFN-γ knockout mice), α-GalCer treatment exacerbates parasitemia through IL4 production (Duthie and Kahn, 2002a,b; Duthie et al., 2006). This suggests that NKT cells may have a beneficial or a harmful role depending on the amount of IFN-γ originating from other cells that may fluctuate during infection.

Gamma-delta (γδ) T lymphocytes express germline-encoded TCR displaying a limited Ag recognition repertoire. Their mechanism of activation is at present not precisely known but does not require classical Ag presentation in the context of MHC. They recognize endogenous molecules expressed by stressed cells and pathogen-derived molecules and play an important role in immunosurveillance against tumors and infections. Subsets expressing defined TCR chains present particular tissue distribution. In this way, γδ-T cells expressing the Vγ9δ2 TCR recognize small phosphorylated molecules like biphosphonates produced endogenously by infected cells or by some pathogens (such cells are present in humans but absent in mice). Other subsets expressing a Vδ1 TCR in association with various γ chains are found preferentially in mouse or human epithelia. In addition, it seems to be an association between chain usage and cytokine production. For instance, mouse Vγ1 and Vγ4 γδ-T cells are prone to produce IFN-γ and IL17, respectively. Innate γδ-T cells generally present an "activated-yet-resting" phenotype allowing them to mount rapid and robust responses. They regulate both innate responses by cross-talk with DCs, NK cells, and neutrophils, and adaptive ones by interacting with conventional lymphocytes (Carding and Egan, 2002; Thedrez et al., 2007; Hamada et al., 2008).

γδ-T cells have been shown important in regulating responses to various infections (Beetz et al., 2008). The few data available to date in mouse *T. cruzi* infection indicate they exhibit either detrimental or beneficial effect, probably in relation to subsets presenting different properties in different tissues. Two studies suggest a detrimental role for γδ-T cells in acute infection, though the importance of the harmful effect on parasitemia and mortality varies according to the model (Santos-Lima and Minoprio, 1996; Cardillo et al., 1998). Such effects might rely on their ability to restrain IFN-γ production by αβ-T cells and the humoral immune response (Cardillo et al., 1993, 1998). Another study identifies Vγ1 γδ-T cells as involved in resistance to the infection (Nomizo et al., 2006) apparently through upregulation of type 1 immune responses. Indeed, depletion of this γδ-T cell population leads to increased activation of conventional $CD4^+$ αβ-T cells that produce more IFN-γ and less IL4 (while IL10 release remain unchanged), as well as upregulation of CD40L expression on $CD4^+$ and $CD8^+$ T cells. In addition, liver

γδ-T cells (of unidentified γδ chain usage) might also confer a local protective effect. The liver of *T. cruzi* acutely infected mice is infiltrated with high numbers of CD3$^+$ CD4 CD8 γδ-T cells (Sardinha et al., 2006). This population is heterogeneous, with an important proportion of them coexpressing markers of NK cells, suggesting the presence of a particular subset of NKγδ-T cells previously described in the liver (Emoto and Kaufmann, 2003). Importantly, these γδ-T cells produce IFN-γ, which could contribute to the particularly efficient control of parasite multiplication in liver as compared to other organs. CD4 CD8 γδ-T cells have also been reported to be increased in the spleen (Minoprio et al., 1989). A shared observation in all these studies is that γδ-T cells seem important in regulating the response of conventional αβ-T cells to *T. cruzi* infection.

Studies on γδ-T cells have to date not been reported in human infection. However, we possess some preliminary results indicating that *T. cruzi* can prime human Vδ2-T cells for IFN-γ production (unpublished data).

24.2.3.3 Polymorphonuclear Cells and Platelets

Neutrophils are the first leukocytes to gain access to the skin in case of intradermal trypomastigote inoculation in BALB/c mice (Monteon et al., 1996; Chessler et al., 2009). They also infiltrate infected organs as heart and skeletal muscle (Molina and Kierszenbaum, 1988), liver (Sardinha et al., 2006), and brain (Da Mata et al., 2000) during murine acute and chronic infection, as do *eosinophils* in skeletal muscle (but not in myocardium) (Monteon et al., 1996). Neutrophils and eosinophils are however far less numerous than infiltrating macrophages.

Neutrophils seem to play a rather important role in the control of infection by their ability to rapidly eliminate parasites as well as shaping the adaptive response. They are not involved in early intradermal IFN response (Chessler et al., 2009), but produce various cytokines/chemokines during the acute phase. The profile of soluble mediators they release differs between mouse strains. Thus, neutrophils from infected BALB/c express predominantly type 1 cytokine (IFN-γ, IL12p40, TNF-α) and Th1 chemoattractive chemokines (CXCL9/MIG and MIP1α), while neutrophils in C57BL/6 express only TNF-α and MIP1α (Chen et al., 2001). Importantly, neutrophils have been shown to play a major role in the development of resistance to *T. cruzi* in BALB/c mice and susceptibility in C57BL/6 mice by modulating the expression IL2, IFN-γ, IL12p40, TNF-α, and IL10, but not IL4, in the spleen, thereby balancing Th1 and Th2 responses (Chen et al., 2001).

Neutrophils are also known to rapidly release, upon degranulation in response to infection or tissue injury, endogenous peptides called alarmins. These peptides can recruit and activate other leukocytes like DCs and consequently enhance innate and adaptive immune responses (Yang et al., 2009). Though this has not been studied, we may assume alarmins play a role in adaptive immune response in *T. cruzi* infection. Indeed, lactoferrin, belonging to the group of alarmins, is increased in the circulation of acutely infected mice (Lima et al., 1988) and favors the uptake and killing of trypomastigotes by human monocyte and mouse macrophages (Lima and Kierszenbaum, 1987a,b).

Human neutrophils contribute to parasite clearance by taking up and killing trypomastigotes by producing reactive oxygen species (ROS). This occurs at low levels but is strongly amplified when parasites are opsonized with Ab (Docampo et al., 1983). Unactivated human neutrophils, as well as eosinophils, are able to quickly uptake extracellular amastigotes and kill them when they are still in the phagolysosomal vacuole (Villalta et al., 1984). This phenomenon is significantly enhanced by granulocyte-macrophage colony-stimulating factor GM-CSF (Villalta et al., 1986), a cytokine produced rather early during the acute infection in mouse (Olivares et al., 1996). Killing is mediated by myeloperoxidase activity and H_2O_2 in neutrophils and at least through major basic protein (MBP) in eosinophils. In this way, neutrophils may clear amastigotes liberated in infected tissues (Villalta and Kierszenbaum, 1983; Villalta et al., 1984). Interestingly, uptake of amastigotes by neutrophils is linked to their ability (not shared by trypomastigotes) to capture at their surface host lactoferrin, which then interacts with LF receptors on neutrophils (Lima et al., 1988). Similarly, parasites bind host fibronectin that favor its entry into cells (Ouaissi, 1988; Ouaissi and Capron, 1989).

Eosinophils probably do not play a central role in the control of *T. cruzi* infection. One report mentions that they increase in the liver in IFN-γ-deficient mice, but their role remains to be determined (Sardinha et al., 2006).

Mast cells are known to respond to IFN-γ (Lykens et al., 2010) and to produce soluble mediators involved in inflammation and fibrosis. Some mast cells have been identified in cardiac tissue of *T. cruzi*-infected mice (Postan et al., 1994; de Oliveira et al., 2007) in association with histamine liberation (Pires et al., 1992), and in organs from chronically infected patients (Cabral et al., 2002; Lima Pereira et al., 2007). Their role in the immune response to infection has not been really addressed but they might be involved in the pathogenesis. Mouse mast cells have nevertheless been reported to be able to kill, *in vitro*, extracellular trypomastigotes in the presence of specific Ab (ADCC) (Tambourgi et al., 1989).

It is now recognized that *platelets* play an active role in immune responses. They can express TLRs and CD40L, interact with leukocytes and endothelial cells, produce numerous inflammatory mediators that serve to initiate and modulate innate immune functions, and are armed for pathogen elimination (Yeaman, 2010; Semple and Freedman, 2010). Platelets are able to directly lyse trypomastigotes *in vitro* (Umekita and Mota, 1989) and help in eliminating blood parasites in cooperation with specific Ab, complement, and macrophages *in vivo* (Takehara and Mota, 1991). However, the real contribution of platelets in parasite clearance *in vivo* is difficult to evaluate (Umekita and Mota, 1990; Fernandes et al., 1992), since thrombocytopenia is associated with *T. cruzi* acute infection in mouse, resulting from platelet destruction after removal of acid sialic residues by parasite TS (Tribulatti et al., 2005).

24.2.3.4 Monocytes/Macrophages

Activated macrophages and their inflammatory products play key roles in host defenses against pathogens. Macrophage activation is a complex process involving

signals from cytokines, chemokines, and PAMPs. IFN-γ mainly, but also type 1 IFNs (and more recently disclosed IL27), activate macrophages through STAT1 signalling. At low doses, IFNs do not actually activate macrophages but prime them, or prepare them to make an increased response to other cytokines or IFNs and to microbial products. Full activation of macrophages requires synergistic action of cytokines (especially IFN-γ) and microbial products. To avoid toxicity associated with excessive activation, STAT1 concurrently induces the expression of the inhibitory proteins SOCS (suppressor of cytokine signalling), whereas TLR engagement induces the production of the well-known macrophage deactivator IL10 (Hu et al., 2008).

Macrophages are probably the most extensively studied cells in *T. cruzi* infection as they simultaneously act as host cells (harboring parasite multiplication and participating to its control when activated) and as Ag-presenting cells.

Activation of Macrophages and Production of Cytokines and NO

The source of IFN for macrophage priming in *T. cruzi* infection has not precisely been identified. We may hypothesize that type 1 IFNs produced by dermal fibroblasts at the site of parasite entry (Vaena et al., 2002) and IFN-γ rapidly produced by iNKT cells (see earlier) play this role. NK cells do not drive the early IFN response (Chessler et al., 2009) and play a role in a second step, like IFN-γ produced by macrophages themselves (Bastos et al., 2007). Granule proteins released by neutrophils infiltrating the site of parasite entry before macrophages (see earlier) might also participate in early macrophage activation (Soehnlein et al., 2009).

Little data allow knowing if *T. cruzi* actually induces cytokine release by unprimed macrophages residing in tissues, what would make these cells true initiators of the immune response. One *in vitro* study shows that infected mouse peritoneal macrophages produces small amounts of IL1β and TNF-α, but does not specify if the cells are resident (i.e., unprimed) or not (Perez et al., 2005). Another reports that *T. cruzi* elicits the production by resident macrophages of an IFN-γ-inducing activity, likely IL12 (Aliberti et al., 1996). A more recent study indicates that macrophages may themselves produce IFN-γ in the presence of IL12 (Bastos et al., 2007). Concerning human cells, circulating monocytes from healthy individuals (presumed unprimed) accumulate mRNA for IL1β, IL6, and TNF-α when infected with *T. cruzi in vitro* (Van Voorhis, 1992) while no protein production could be detected for TNF-α, IL12, and IL10 (Souza et al., 2004).

However, *T. cruzi* clearly activates previously primed mouse macrophages to produce IL1, IL12, TNF-α, IL10, and the microbicidal product NO (Camargo et al., 1997a,b; Ramos-Ligonio et al., 2004). Whether infected macrophages also produce IL18 and IL27 (other pro-Th1 cytokines than IL12) has not been addressed but may be supposed as these cytokines are found in infected mice (Antunez and Cardoni, 2001; Hamano et al., 2003) and macrophages are known to be an important cell source of these cytokines (Tsutsui et al., 2004; Goriely et al., 2009). In *T. cruzi*−infected humans, circulating monocytes do not show an activation phenotype in early acute infection (Sathler-Avelar et al., 2003) while monocytes from chronically infected patients produce IL12, TNF-α, and IL10 (Gomes et al., 2003; Souza et al., 2004).

GPI anchors of trypomastigotes (tGPI), Tc52 shed by trypomastigotes and amastigotes, the major GPI-anchored surface glycoprotein of amastigotes (Ssp4) and a surface mucin of metacyclic trypomastigotes (AgC10) are all *T. cruzi* molecules able to directly activate primed macrophages and thus susceptible to initiate early innate responses (de Diego et al., 1997; Fernandez-Gomez et al., 1998; Almeida and Gazzinelli, 2001; Ramos-Ligonio et al., 2004). It is worth noting that, the pattern of cytokines induced, and if NO production is triggered or not, vary according to the parasite molecule in relation to engagement of different receptors GPI mucins (Almeida and Gazzinelli, 2001) and Tc52 act through TLR2 while AgC10 binds to surface L-selectin (CD62L) and induces Ca^{2+} mobilization (Alcaide et al., 2010) as well as to differential regulation of gene expression. Indeed, upon IL12 or IL18 priming, induction of NOS2 for NO production depends on IFN-γ, whereas expression of TNF-α is IFN-γ-independent (Bastos et al., 2007). Macrophage activation is evidently strengthened later on during the infection, when higher amounts of IFN-γ are produced by T lymphocytes (Rodrigues et al., 2000; Guinazu et al., 2007).

IFN-γ has been shown by our team and others to be the most important cytokine for the control of *T. cruzi* infection (Torrico et al., 1991; Cummings and Tarleton, 2004). This cytokine acts by tuning on numerous genes in various cells (Schroder et al., 2004), including macrophages. IFN-γ-activated macrophages help to control *T. cruzi* infection through their trypanocidal action and indirectly by activating or regulating other cells, mainly NK cells and T lymphocytes, which we will discuss next.

Control of Parasite Multiplication and Trypanocidal Action of Macrophages

One of the best-known actions of IFN-γ on macrophages is the induction of the *NO* synthase NOS2 (or iNOS for inducible NOS), catalyzing the conversion of L-arginine into NO, an essential cytotoxic and cytostatic factor to many intracellular pathogens (MacMicking et al., 1997; Mayer and Hemmens, 1997). *T. cruzi* itself and TNF-α, produced at high levels during acute infection (Truyens et al., 1999), upregulate NO production by IFN-γ-activated macrophages (Metz et al., 1993). Type 1 IFNs also contribute to NOS2 induction (Costa et al., 2006). High amounts of NO (micromolar levels), together with ROS generated by NADPH oxidase (activated upon parasite phagocytosis), mediate intraphagosomal peroxynitrite-dependent killing of parasites before they escape in the cytoplasm of the macrophage (Peluffo et al., 2004; Piacenza et al., 2009), contributing to drastically reduce the growth of parasites (Gazzinelli et al., 1992; Munoz-Fernandez et al., 1992b). NO released extracellularly probably also limits the entry of parasites into cells by killing extracellular trypomastigotes (Vespa et al., 1994) and by inhibiting cruzipain (the major cysteine protease of *T. cruzi,* which plays a key role in the penetration of the parasite into host cells) (Venturini et al., 2000). *In vivo*, the administration of various NOS2 inhibitors to infected mice supports a critical role for NO in the control of *T. cruzi* acute infection (Vespa et al., 1994; Holscher et al., 1998). Recently, Lykens et al. have definitively confirmed the pivotal role of IFN-γ-induced NO production by macrophages by showing that all transgenic mice, of which only macrophages were impaired to respond to IFN-γ, did not control the parasitemia and died (Lykens et al., 2010).

However, in some circonstances NO may not be optimally produced. Indeed, when macrophages are "alternatively" activated and secrete IL10 and TGF-β, NO production is downregulated. IL10 inhibits the activating effect of IFN-γ on NOS2, and TGF-β preferentially induces the activity of ornithine decarboxylase. This enzyme utilizes L-arginine (the substrate of NOS2) and subsequently limits NO synthesis, in addition to supply polyamines that are essential for intracellular parasite replication. This results in persistent intracellular parasite growth (Nunes et al., 1998; Freire-de-Lima et al., 2000; DosReis and Lopes, 2009) in Peluffo et al. (2004), Noel et al. (2004).

Another point is that requirement for NO in the control appears to vary with the virulence of the parasite strain (NO is dispensable to control the multiplication of low-virulence parasites; Marinho et al., 2007) and the stage of infection (chronically infected mice can survive when NOS2 activity is inhibited) (Saeftel et al., 2001). Other data strongly suggest that, at odds with the results obtained with NOS2 inhibitors (the specificity of which has later come into question), NOS2 deficient mice control the infection like wild animals, clearly indicating the existence of other mechanisms of control that may compensate the absence of NO (Romanha et al., 2002; Cummings and Tarleton, 2004).

In line with this, Knubel et al. showed *T. cruzi* infection to upregulate the expression of indoleamine 2,3-dioxygenase (*IDO*) in mouse macrophages (Knubel et al., 2010). This enzyme catalyzes tryptophan catabolism, leading to the production of metabolites known to have immunoregulatory properties as well as limiting proliferation of some intracellular pathogens. The authors show IDO also to limit *T. cruzi* amastigotes multiplication in macrophages and to display an important role *in vivo* in the control of mouse infection.

Another recently revealed IFN-dependent mechanism of control of *T. cruzi* multiplication by mouse macrophages concerns the induction of IRG47 and LRG47 proteins, belonging to the family of "immunity-related GTPases" (*IRGs*) (Santiago et al., 2005; Koga et al., 2006). IRG proteins constitute a particularly powerful system to fight intracellular pathogens. They might regulate some factors involved in phagosome maturation or trigger macrophage autophagy, a newly described mechanism for intracellular control of pathogens (Howard, 2008). Santiago et al. (2005) and Koga et al. (2006) showed these IRGs (induced by IFN-γ (LRG47 or Irgm) as well as IFN-β (IRG47 or Irgd)) to be able to limit *T. cruzi* multiplication *in vitro* and *in vivo*.

Macrophages also mediate IFN-independent control of parasite multiplication through *GM-CSF* production. High levels of GM-CSF are produced during acute infection in mouse by resident macrophages and participate *in vivo* in the control of the infection (Olivares et al., 1996). GM-CSF itself, and TNF-α induced by GM-CSF, also activate autocrine production of NO and ROS that limit infection (Reed et al., 1987; Olivares and Vray, 1995), thereby improving IFN-γ induced control of parasite multiplication. The role of GM-CSF in regard to IFN-γ is difficult to assess, but it might at least contribute to early innate control of parasite multiplication, together with IRG47 induced by IFN-β (see earlier), before adaptive immune responses take place.

IFN-γ and TNF-α also activate *in vitro* NO-dependent trypanocidal action in human monocytes (Munoz-Fernandez et al., 1992a). However, as induction of NOS2 in human monocytes is much slower than what occurs in the murine counterpart, it is expected that more parasites escape in the cytoplasm before being killed in the phagosome (Piacenza et al., 2009). The NO-dependent control of infection might therefore be less efficient in humans than in mouse. No information is currently available about IRGs proteins in human Chagas disease. Their role is probably reduced since the IRG gene family contains only two members in humans (IRGC and a truncated gene IRGM) versus multiple copies in most mammalian species (Bekpen et al., 2009).

Modulation of Innate and Adaptive Immune Responses by Macrophages

We will consider here the role of key cytokines known to be produced mainly by macrophages, considered to be important in the regulation of ongoing innate and adaptive immune responses.

"*Classically*" IFN-γ-activated macrophages mediate the control of intracellular pathogens by expressing genes like NOS2 and producing IL12, which drives type 1 responses. In the presence of IL10, glucocorticoïds, or apoptotic cells, macrophages are "*alternatively*" activated (with considerably variations according to the genetic background of the host; Lopes et al., 2007). Such macrophages produce mainly IL10 and TGF-β, which antagonize the protective action of IFN-γ, exert anti-inflammatory functions by lowering the production of proinflammatory cytokines, and are involved in tissue remodelling (Noel et al., 2004).

Higher susceptibility of mice receiving anti-*IL12*-neutralizing Ab or deficient in IL12p35 highlights the role of this cytokine in resistance to *T. cruzi* infection (Aliberti et al., 1996; Hunter et al., 1996; Muller et al., 2001). This mainly relates to the ability of IL12 to promote IFN-γ and TNF-α production by other cells that synergize for NO production (Munoz-Fernandez et al., 1992b; Hunter et al., 1996), and to drive trypanolytic action of NK cells (see earlier). *IL18* also contributes to trigger IFN-γ (Muller et al., 2001). Its early production, combined with IL12, improves resistance to infection (Antunez and Cardoni, 2001). *IL27* is also involved in protection (Hamano et al., 2003), by limiting Th2 responses (which favors Th1-dependent control of parasite multiplication) as well as the production of the the proinflammatory cytokines IL6 and TNF-α (released at high levels during acute infection; Truyens et al., 1994, 1999) involved in mortality.

IL10 is normally produced (even by classically activated macrophages) to limit the potential harmful effects of excessive inflammatory cytokines (Hu et al., 2008). If produced in higher quantities by alternatively activated macrophages, it prevents the action of IFN-γ. IL10 has been shown to regulate *in vitro* the interplay between *T. cruzi*−infected macrophages and NK cells. Indeed, it limits the action of IL12 on NK cells, but reversely IFN-γ released by NK cell restrains macrophage IL10 production (Cardillo et al., 1996). *In vivo*, the balance between IFN-γ and IL10 expression is crucial in modulating the resistance or susceptibility to *T. cruzi* infection. Some data point to a pivotal role of host genetic background for driving preferentially classical or alternative activation of macrophages. Indeed, cruzipain

induces in BALB/c mice alternative macrophage activation, which is associated with low IL12 and IFN-γ response and increased IL10 production (Stempin et al., 2002; Giordanengo et al., 2002), while in C57BL/6 mice it generates a predominant Th1 response (Guinazu et al., 2007).

Other immunosuppressive or anti-inflammatory mediators like active *TGF-β* and *PGE2* are also produced during the acute phase of experimental infection. Their production is triggered by uptake of apoptotic lymphocytes (Freire-de-Lima et al., 2000; Lopes et al., 2000). In addition, PGE2 is induced by TNF-α and NO (Pinge-Filho et al., 1999). TGF-β has a complex but beneficial for the parasite role in *T. cruzi* infection. Indeed, *T. cruzi* activates and utilizes this cytokine to invade host cells bearing TGF-β receptors and to differentiate from amastigotes into trypomastigotes (Ming et al., 1995; Hall and Pereira, 2000a; Waghabi et al., 2005a,b). Furthermore, TGF-β can both prevent IFN-γ-activation of macrophages for NO production and limit the protective effect of already activated macrophages, meaning that this cytokine should exert its action whenever it is produced and active (Silva et al., 1991). On the other hand, PGE2 counter-regulates IFN-γ and TNF-α release but inversely accentuates NO production, while it seems not to affect levels of IL12 and IL10 (Pinge-Filho et al., 1999). Thus, PGE2 limits the production of two cytokines known to have adverse effects when present at very high levels (Jacobs et al., 1996; Truyens et al., 1999) while preserving the NO-dependent trypanocial action, which should be beneficial for the host. Yet it exerts a detrimental effect by inhibiting lymphocyte proliferation and IL2 production (see Section 24.4.1) (Michelin et al., 2005).

Role of Macrophage Apoptosis in *T. cruzi* Infection

Mouse resident macrophages rapidly undergo apoptosis when infected *in vitro* with *T. cruzi*, favoring parasite persistence (see Section 24.5.2) (Freire-de-Lima et al., 1998; de Souza et al., 2003). This mechanism might have been counter-regulated *in vivo* through increased production of antiapoptotic molecules like intracellular HSP65 (Sakai et al., 1999) and the serum protein alpha-2 macroglobulin (A2M) (de Souza et al., 2008). In addition, A2M not only limits apoptosis, but also enhance uptake and killing of trypomastigotes and amastigotes by macrophages (Araujo-Jorge et al., 1990). Protective effects of these molecules are underlined by the correlation between their levels *in vivo* and resistance to *T. cruzi* infection (Araujo-Jorge et al., 1992; Sakai et al., 1999). HSP65 is known to be induced by IFN-γ and TNF-α. In *T. cruzi* infection, it has been shown that HSP65 induction is mediated by $CD4^+$ T cells that have to be properly activated by NK cells, while $CD8^+$ and γδ-T cells are not involved. Thus, the beneficial effect of HSP65 can only take place when $CD4^+$ T cells are activated (Sakai et al., 1999).

Role of Macrophage as Ag-Presenting Cells

Two studies with mouse macrophages report *T. cruzi* infection to cause impaired Ag presentation to $CD4^+$ T cells, leading to a reduction of their capacity to proliferate (Plasman et al., 1995; La Flamme et al., 1997). This is related to impeded ability to take up and catabolize exogenous Ag, decreased expression of MHC

class II molecules, and/or defective capacity of macrophages to adhere to T cells (Section 24.4.1), but likely not to a defect in delivery of costimulatory signals (Frosch et al., 1997; La Flamme et al., 1997). Reduced expression of MHC class II molecules has also been observed in infected human monocytes (Louie et al., 1994).

As for the effect of *T. cruzi* on the expression of MHC class I molecules, *in vitro* infection of mouse macrophages with *T. cruzi* does not inhibit MHC class I Ag presentation to cytotoxic $CD8^+$ T cells (Stryker and Nickell, 1995; Buckner et al., 1997). However, another study interestingly suggests that MHC I-dependent Ag presentation capacity may take time to become efficient, through a delay in immunoproteasome synthesis, as well as in MHC class I mRNA synthesis and cell surface expression (Bergeron et al., 2008). Such delay results from transient inhibition by the parasite in macrophage of protein tyrosine phosphatase. Thus, in the first hours after invasion, inhibition of MHC class I-dependent Ag presentation by the parasite could potentially hinder early CTL-mediated immunity long enough in order to facilitate parasite establishment inside the host (Bergeron et al., 2008).

24.2.4 DCs and the Initiation of the Adaptive Immune Response

DCs are sparsely but widely distributed in tissues where they act as sentinels, specialized for the capture, processing, and presentation of Ag to T cells. They are proposed to be more efficient for activating naïve T cells in secondary lymphoid organs than macrophages and thereby play a central role in initiating adaptive immune responses. Depending on the profile of PRRs and other receptors engaged at the encounter with a pathogen, DC subsets will express costimulatory molecules for T cell activation and produce defined cytokines, leading to different types of immune responses or to T-cell tolerance (Shortman and Naik, 2007; Hume, 2008; Banchereau et al., 2009).

The number of Langerhans cells (dermal DCs) transiently decrease in the epidermis at the site of *T. cruzi* inoculation, and increases in the dermis as well as in the draining lymph node where clustering of parasite-laden DCs with lymphocytes are seen (Nargis et al., 2001). The number of DCs also increases drastically in spleen of acutely infected mice, but data suggest that they might not be located in the correct site for activating T cells is not sure (Chaussabel et al., 2003).

T. cruzi can readily infect mouse DCs and induce their maturation *in vitro* and *in vivo*, as indicated by increased surface expression of CD40, CD80, CD86, and MHC molecules of class I and II. Parallely, they produce IL12 and are appropriate to induce a strong type 1 response (i.e., IFN-γ release by $CD4^+$ and $CD8^+$ T cells) (Aliberti et al., 2003; Monteiro et al., 2006, 2007; Cobb et al., 2009; Kayama et al., 2009). The costimulatory action of CD80 and CD86 on CD28 expressed by lymphocytes has been shown to be critical for inducing protective immune response against *T. cruzi* in mouse (Miyahira et al., 2003; Martins et al., 2004).

TLRs engagement on DCs is not absolutely required to develop a Th1 response to *T. cruzi* infection *in vivo*. Indeed, IFN-γ is produced by $CD4^+$ T cells in the absence of any functional TLR in Myd88 $^{-/-}$ and TRIF $^{-/-}$ mice (Kayama et al., 2009).

Intracellular Ca^{2+} mobilization is rather the activating signal of DCs (Monteiro et al., 2006; Scharfstein et al., 2007; Kayama et al., 2009). Ca^{2+} mobilization may result from parasite entry into the cell. Indeed, only live parasites are able to activate DCs, a lysate being not active (Kayama et al., 2009). Interestingly, Ca^{2+} mobilization is also triggered by bradykinin produced upon the action of the *T. cruzi* protease cruzipain on kininogen (Scharfstein et al., 2000, 2007; Monteiro et al., 2006). This constitutes a new mechanism by which a pathogen may turn on immunity. Ca^{2+} mobilization then triggers IFN-γ production in DCs through successive translocation of the transcription factors NFATc1 and T-bet (Kayama et al., 2009), known to be crucial for activating competent DC that will induce type 1 responses (Lugo-Villarino et al., 2003). Nevertheless, TLR2 is an important "add-on" as it indirectly potentiates the bradykinin-dependent activation of DC. Indeed, kininogens, the substrate of cruzipain, are released in the vicinity of locally infected cells only after plasma leakage has occurred from blood capillaries. This event takes place only after neutrophils or macrophages have been activated by the parasite through TLR2 for producing CxC chemokines (Scharfstein et al., 2007; Schmitz et al., 2009).

Similarly to what is observed in experimental models, *T. cruzi* activate human DCs by inducing the expression of CD83 and CD86 and the production of IL12, TNF-α, and IL6 (Cuellar et al., 2008). In line with this, our own unpublished data (manuscript in preparation) indicate that *T. cruzi* infection of blood cells from healthy individuals induces or increases the expression of the costimulatory molecules CD40, CD80, CD86, and CD83 on myeloid DCs. Human DCs generated *in vitro* from monocytes of chronically infected patients have also been shown to induce IFN-γ in autologous $CD8^+$ T cells (Albareda et al., 2006).

Some parasite molecules that activate DCs have been identified besides the cruzipain; purified HSP70 (Planelles et al., 2002; Cuellar et al., 2008) and Tc52 (Ouaissi et al., 2002) have been shown to activate human monocyte-derived DCs. Although Tc52 is known to bind to TLR2, whether upregulation of activation markers results from TLR2 engagement remains unclear. Yet, this receptor is involved in cytokines/chemokines production triggered by Tc52 (IL6, IL8, MCP1, MIP1α).

An interesting point is that activation of pro-type 1 DCs varies with the host genetic background (Planelles et al., 2003) as well as with parasite virulence (Alba Soto et al., 2003). Highly virulent strains of *T. cruzi* preferentially trigger the production of the immunosuppressive cytokines TGF-β (high amounts) and IL10 (low amounts) which limit the ability of DCs to induce T cell proliferation (Poncini et al., 2008). IL10 (but not TGF-β) restrains deactivation procedure, while both impede the action of differentiated DC on T cells. Thus, the balance between IL12, IL10, and TGF-β modulate the magnitude or the quality of the ensuing T cell response. IL10-producing DCs have also been detected in chagasic patients (Cuellar et al., 2008).

If some studies indicate that *T. cruzi* limit LPS-induced maturation of mouse and human DCs (Van Overtvelt et al., 1999, 2002; Brodskyn et al., 2002; Poncini et al., 2008), another one interestingly shows that on the contrary, LPS (a TLR4 ligand) upregulates IL10 (but not IL12 or TGF-β) production by *T. cruzi*-infected

DCs (Poncini et al., 2008). This suggests that coinfections with pathogens bearing TLR4 ligands, or leakage of such ligands from the intestinal flora, which occurs in some pathologies (Nagata et al., 2007), might modulate the course of the infection.

24.3 Adaptive Immune Response: Induction, Characterization, and Role of the T-Cell Response

24.3.1 T-Cell Epitopes

The low frequency of specific T cells and overall to the large size of the parasite genome encoding a huge quantity of proteins have made difficult the identification of T cell epitopes of *T. cruzi*. Nevertheless, epitopes presented by *MHC class II* molecules to mouse or human $CD4^+$ T cells have been identified in the catalytic domain of *T. cruzi* TS (Fujimura et al., 2001), in the variant SA85-1.1 of TS (Millar and Kahn, 2000a), in cruzipain (Giordanengo et al., 2002) and in KMP11 (Cuellar et al., 2009). A particular epitope of SA85-1.1 seems to be a major Th1 inducer (Millar and Kahn, 2000a), while cruzipain preferentially induces Th2 cells (Giordanengo et al., 2002).

$CD8^+$ T cell responses appear to be broadly induced to an array of parasite Ag (Laucella et al., 2004; Alvarez et al., 2008). Hence, TSA1 (trypomastigote surface antigen-1), ASP1 and -2 (amastigote surface proteins), FL-160 (flagellar-associated antigen family specific of trypomastigotes) belonging to the TS family of *T. cruzi* have been detected as target Ag of mouse and human $CD8^+$ T cells (Wizel et al., 1997, 1998; Low et al., 1998; Fonseca et al., 2005). Other human *MHC class I*–binding peptides have been found in the parasite cruzipain, the calcium-binding protein (CaBP), LYT1 protein (involved in the invasion process of host cells (Manning-Cela et al., 2002)) (Laucella et al., 2004; Fonseca et al., 2005) and ribosomal protein (Garcia et al., 2003). Interestingly, despite the fact that parasites concomitantly express a large number of polymorphic proteins of the TS family, the group of Tarleton has observed that infected individuals (human or mouse) highly focus their $CD8^+$ T cell response on a restricted repertoire of TS epitopes. This is especially evidenced in acutely infected mice, likely due to their inbred nature, with more than 30% of the $CD8^+$ T-cell response being specific for two major groups of trans-sialidase epitopes, TSKb20 and TSKb21 found in many variants of the proteins constituting the TS family (Martin et al., 2006; Padilla et al., 2007, 2009a).

24.3.2 Induction of $CD4^+$ and $CD8^+$ T Cells in Mouse Infection

The T cell response to *T. cruzi* exhibits a mixed type 1/type 2 cytokine production profile. Some data in mouse infection support that IFN-γ-producing $CD8^+$ T lymphocytes would develop simultaneously or even earlier than $CD4^+$ Th1 cells, while Th2 $CD4^+$ T cell response might appear somewhat later on (Une et al., 2000;

Kumar and Tarleton, 2001; Lopes et al., 2007). However, initial $CD8^+$ T cell activation is rather slow as compared to other acute viral or bacterial infections, as it is not evident until more than 1 week after parasite inoculation (Martin et al., 2006; Tzelepis et al., 2006). Lymphocyte apoptosis induced by various pathways (Section 24.4.1) appears to be an important mechanism for regulating the response of the different T cell subsets (Guillermo et al., 2007). Recently, $CD4^+$ and $CD8^+$ T cells producing IL17A early in *T. cruzi* mouse infection have been detected. As expected, this IL17, restrains the development of the type 1 immune response (Matta Guedes et al., 2010).

The decision for T cells to produce type 1 or type 2 cytokines is known to be predominantly governed by the cytokines in the microenvironment and, to some extent, by the strength of the interaction between TCR and Ag (Zhou et al., 2009). As explained earlier (Section 24.2.3.1), the contribution of NK-derived IFN-γ for development of a type 1 response is debated. Other factors such as IL12 and IL18 also contribute in establishing a type 1 environment during *T. cruzi* acute infection (IL12 exerting it effects earlier than IL18) (Muller et al., 2001; Une et al., 2003).

$CD8^+$ T cell response may develop in *T. cruzi*-infected mice in the absence of help from $CD4^+$ T cells (Padilla et al., 2007). Nevertheless, unhelped $CD8^+$ T cells fail to reach the frequencies achieved in the presence of CD4 T-cell help necessary to the control of the infection. The increase in parasite load in acute infection is likely a critical parameter governing the $CD4^+$ T cell-dependent expansion of specific $CD8^+$ T cells (Tzelepis et al., 2007). Expansion of $CD8^+$ T cells in *T. cruzi*-infected mice also requires other signals that induce the expression of the transcription factor T-bet in $CD8^+$ T cells themselves. These signals have to date not been indentified but seem different from IFNs and $CD4^+$ T cell-independent (Cobb et al., 2009).

24.3.3 Role of T Lymphocytes in the Control of the Infection

The critical dependence on both $CD8^+$ and $CD4^+$ T cell-mediated responses for control of acute *T. cruzi* infection in mouse is unequivocal. Indeed, genetically modified knockout mice that do not express either MHC class I or II antigens, CD4 or CD8, or mice depleted of T cells using anti-CD4 or anti-CD8 Ab, are highly susceptible to infection compared to wild-type mice (Tarleton et al., 1992, 1994, 1996; Rottenberg et al., 1993; Padilla et al., 2009a).

The protective effect of $CD4^+$ Th1 cells relates to IFN-γ activation of trypanocidal action of macrophages (though they are not the unique cell type to release IFN-γ), and to their help for inducing $CD8^+$ T cells (see earlier) as well as the B cell switch to produce protective Ab isotypes (Hoft et al., 2000) (Section 24.3.2). It has also to be kept in mind that when in apoptosis, T cells trigger alternative activation of macrophages (cf. Section 24.4.2). Therefore, infiltrating $CD4^+$ T cells in infected tissues, even if displaying a protective Th1 phenotype, may actually have a harmful effect by exacerbating the parasite load in tissues (DosReis, 2000).

Even if IFN-γ released by $CD8^+$ T cells also contribute to the control of the infection (Tarleton et al., 1992), its early production seems to be dampened by

apoptosis (Guillermo et al., 2007). Thus, the main protective function of $CD8^+$ T cells is likely related to their cytotoxic properties. However, the contribution of perforin or granzyme B in killing infected cells likely depends on parasite virulence and dose (Kumar and Tarleton, 1998; Nickell and Sharma, 2000; Henriques-Pons et al., 2002; Muller et al., 2003).

Studies of the group of Tarleton have provided a lot of information on $CD8^+$ T cells in the chronic phase of infection (Padilla et al., 2009a). Briefly, parasite-specific $CD8^+$ T cells present an effector phenotype ($CD44^{hi}$ $CD62L^{lo}$ $CCR7^{lo}$ $CD127^{lo}$), remain highly functional (Martin and Tarleton, 2005) and hold down the number of infected cells in tissues, as indicated by the exacerbation of the infection in case of T cell depletion or administration of immunosuppressive drugs (Tarleton et al., 1994; Bustamante et al., 2008). However, the infection appears to be rarely, if ever, completely resolved in either experimentally infected animals or in humans. The group of Tarleton has shown that protective $CD8^+$ T cells presenting a phenotype of central memory cells persited after cure in treated mice (Bustamante et al., 2008; Padilla et al., 2009a). The mechanisms underlying the inability to totally eliminate *T. cruzi*-infected cells in the absence of treatment are to date not precisely known. It is not related to T cell exhaustion at least in mouse (Martin and Tarleton, 2005; Bustamante et al., 2008), while TGF-β or regulatory $CD4^+$ T cells are likely not pivotal in limiting the control (Kotner and Tarleton, 2007; Martin et al., 2007). In humans however, as the infection lasts so many years, a weakening of $CD8^+$ T cell response can be observed (Padilla et al., 2009a).

24.3.4 Regulatory T Cells

Although multiple T cell subsets can exert immunoregulatory effects, the major regulatory T cell population is phenotypically characterized as $CD4^+CD25^{hi}$ $Foxp3^+$ T cells, named "Treg." They do not produce effector cytokines like IL2, IL4, and IFN-γ, but mediate immunoregulation through bystander effects on various cell types (T and B lymphocytes, DCs, macrophages, NK and NKT cells, etc.) by producing cytokines such as IL10, TGF-β, IL35, consuming IL2, expressing CTLA4 that blocks costimulation, inducing apoptosis or cytolysis, and so on (Shevach, 2009). Few data are available about Treg in *T. cruzi* infection. Graefe et al. show increased expression of CTLA4 on $CD4^+$ T cells in acutely infected mice. Interestingly, level of expression differ between mouse strain and is related to susceptibility, likely by limiting IFN-γ production (Graefe et al., 2004). Data in humans are given hereunder.

24.3.5 Characterization of T Cells in Asymptomatic Chronically Infected Humans

The *global level of circulating $CD4^+$ and $CD8^+$ T cells* is not affected in the indeterminate, asymptomatic chronic phase of the infection as compared to uninfected individuals (Dutra et al., 1996a; Albareda et al., 2006). They present a similar to

slightly increased ability to proliferate in response to the mitogen PHA, indicating that this function is not altered in this phase of infection (Cetron et al., 1993; Michailowsky et al., 2003). In addition, they express activation markers as CD45RO and HLA-DR (Dutra et al., 1994).

Various successive differentiation states (early, intermediate, and late) of activated T lymphocytes may be identified by expression of surface markers as CD45RA/RO (receptor displaying protein tyrosine phosphatase activity that regulates TCR signalling), CD27 and CD28 (costimulatory receptors), CD62L and CD197 (L-selectin and CCR7, respectively, homing receptors for entry in secondary lymphoid tissues). Phenotypically distinct subpopulations are generally associated with different functional characteristics in term of cell cycle, survival, migration, cytokines/chemokines production, and cytotoxic activity (Appay et al., 2002; Appay and Rowland-Jones, 2004; Ahmed et al., 2009; Kim et al., 2009; Parish et al., 2009). Recent studies aiming to characterize circulating T lymphocytes in chronically infected patients show that they harbor a lower proportion of early ($CD45RA^{-}$ $CD27^{+}$ $CD28^{+}$) differentiated $CD4^{+}$ and $CD8^{+}$ T cells than controls (Dutra et al., 1996b; Albareda et al., 2006, 2009). This is surprising as the permanent presence of parasites in the host might be expected to continuously generate newly activated lymphocytes. In line with this, live parasites effectively induce *in vitro* the expansion of specific $CD8^{+}$ T cells expressing CD27 and CD28 (Albareda et al., 2006). Various hypotheses might underlie such discrepancy between *in vivo* and *in vitro* observations. Most likely specific early activated T cells are too little numerous *in vivo* to modify the global proportion of circulating cells. Alternatively, it might reflect a blockage or a defect of T cell activation in chronic infection not observed *in vitro*, or the impossibility of early activated T cells to recirculate (preventing their detection *ex vivo*). Supporting this, the proportions of activated $CD8^{+}$ T cells expressing CCR7 (marker for homing in lymph nodes) may be increased in chronic chagasic as compared with uninfected individuals (Albareda et al., 2006).

Meanwhile, higher levels of late ($CD45RA^{+\text{ or }-}$ $CD27^{-}$ $CD28^{-}$) differentiated $CD4^{+}$ and $CD8^{+}$ T cells are present in chronically infected patients (Dutra et al., 1996b; Albareda et al., 2006, 2009). They present some features also seen in persistent viral infections (Appay et al., 2002; Appay and Rowland-Jones, 2004). Indeed, late differentiated $CD4^{+}$ T cells express higher levels of CD57, a marker that increases with the number of cell divisions and reflects what is called "replicative senescence" (Brenchley et al., 2003; Wood et al., 2009), as well as of caspase 3, a key mediator of apoptosis, compared with uninfected controls, while $CD8^{+}$ T cells are not particularly prone to apoptosis (Albareda et al., 2006). In addition, $CD4^{+}$ T cells have not upregulated the expression of CD122 (beta-chain of IL2 and IL15 receptors of intermediate affinity) and express slightly higher levels of IL7R compared to controls. This suggests that they might not have acquired properties of true memory cells, since IL7 and IL15 are known to play a role in Ag-independent maintenance of memory T cells.

Specific T. cruzi T lymphocytes are evidenced in a majority of chagasic patients by their proliferative response to various parasite extracts (Morato et al., 1986;

Cetron et al., 1993; Dutra et al., 1996a, 2000; Michailowsky et al., 2003). Likewise, IFN-γ-positive specific T cells can be detected in 78% of chronically infected patients after short culture (16−20 h) of their PBMC with amastigote lysate. The frequency of specific T cells ranged between 0.014−1.57% and 0.002−2.56% for $CD4^+$ and $CD8^+$ IFN-γ-positive T cells, respectively (Laucella et al., 2004; Albareda et al., 2009). Specific $CD4^+$ T cells have been further shown to display a phenotype of early ($CD27^+$ $CD28^+$) and intermediate (CD27 $CD28^+$) differentiated cells (Albareda et al., 2009). The phenotype of circulating specific $CD8^+$ T cells is not further described. However, an *in vitro* study indicates live parasites to induce the expansion of specific $CD8^+$ T cells expressing CD27 and CD28 (Albareda et al., 2006).

The use of *T. cruzi* MHC I peptide linked to HLA molecules (tetramers) allows the detection of specific $CD8^+$ T cells only in few patients (Low et al., 1998), likely as a consequence of the low frequency of specific cells. Clonal deletion due to persistent Ag stimulation or sequestering of responsive T cells at sites of active parasite replication (so they are not available in blood), might contribute to explain the low frequency. Moreover, the absence of continuous exposure to *T. cruzi* reinfections (cf. individuals living from many years in a region free of vectorial transmission) still decreases the frequency of specific $CD8^+$ T cells, suggesting that reinfections might be important to boost the level of parasite-specific $CD8^+$ T cells (Laucella et al., 2004). This may have implications for pathology.

Data on Treg in human *T. cruzi* infection are in (Sathler-Avelar et al., 2009). While a low frequency of $CD4^+CD25^{hi}$ Treg lymphocytes is found in *T. cruzi*-infected children with indeterminate clinical disease (inferior to that in noninfected children), increased levels of circulating Treg are found chronically infected asymptomatic adults. Interestingly, Treg levels inversely correlate with those of activated $CD8^+$ T cells, suggesting that their expansion might protect from tissue damages (Vitelli-Avelar et al., 2005, 2006; Araujo et al., 2007).

24.4 Adaptive Immune Response: The B Cell Response and Production of Antibodies

24.4.1 Targets of T. cruzi*-Specific Ab*

T. cruzi infection is characterized by the production of both parasite-specific and unspecific Ab arising from polyclonal B-cell stimulation (Brener, 1980; el Bouhdidi et al., 1994; Minoprio, 2001). Polyclonal activation will be discussed further in this chapter as well as in Chapter 25 about pathology. *T. cruzi*-specific antibodies recognize surface, internal, and shed molecules. In human infection, it has been observed that the proportion of Ab directed against surface/shed Ag is higher in the acute phase of infection, while those recognizing internal Ags are more prevalent in the chronic phase (Umezawa et al., 1996).

Molecules of the TS family (expressed by trypomastigotes but not or poorly by amastigotes) are major targets of Ab. The major B epitope is a repetitive sequence

located at the C-terminal part of TS molecules, called shed acute phase antigen (SAPA). SAPA-specific Ab are already detected during acute infection in humans and mice (Affranchino et al., 1989; Frasch, 1994, 2000). Other Ab directed against the enzymatic catalytic site of TS molecules (in both humans and mouse) neutralize the enzymatic activity of TS (Leguizamon et al., 1994a,b; Pitcovsky et al., 2002). Ab produced against enzymatically inactive TS molecules (TS family encompasses both active and inactive molecules) also inhibits the catalytic action of active TS (Cremona et al., 1999).

Cruzipain, the major cysteine protease of *T. cruzi* involved in cell invasion, is also strongly immunogenic and elicit Ab in mouse and human infection (Zuniga et al., 1999; Duschak et al., 2001). Some others *T. cruzi*-specific Ab neutralize parasite molecules like CRP (complement-regulatory proteins) (Norris et al., 1989; Beucher and Norris, 2008), T-DAF (Tambourgi et al., 1995), trypanosomal immunosuppressive factor (TIF) (Kierszenbaum et al., 1994), HSP70 (Engman et al., 1990; Krautz et al., 1998), the flagellar calcium-binding protein (FCaBP 24 kDa) (Godsel et al., 1995), and the adhesion sequence RGD (Truyens et al., 1995). They regulate the interaction between *T. cruzi* and the host, favoring either the parasite or the host (see later and Section 24.5).

Additionally, and in humans only (cf. Section 24.2.1), Ab directed against α-galactosyl residues abundantly expressed on surface mucins of trypomastigotes strongly increases during infection, due to the shedding of these molecules (Towbin et al., 1987; Almeida et al., 1994; Pereira-Chioccola et al., 2000). They are similar to the natural "anti-α-Gal" Ab but their level and avidity to α-Gal epitopes is strongly enhanced.

24.4.2 Isotypes of Specific Ab

Specific Ab of IgG, IgM, and IgA classes are produced during *T. cruzi* infection. IgG production depends on the help from both $CD4^+$ T cells and MHC class I restricted cells (Tarleton et al., 1996). The levels of Ab classes vary during the course of infection (Grauert et al., 1993; Medrano-Mercado et al., 1996). In *human* infection, IgA appears first followed by IgM and IgG. Ab levels peak during the acute phase (IgA earlier than IgM). IgA and IgM are generally no more detectable in the chronic phase, while IgG persist all life in untreated patients. *IgG1* subclass predominates amongst IgG, followed by IgG3. Such isotypes are known to be associated with type 1 responses to intracellular pathogens (Snapper and Paul, 1987; Garraud et al., 2003). Slight differences of isotypic profiles are however observed according to *T. cruzi* Ag used for detection as well as to the clinical form of chronic disease (Scott and Goss-Sampson, 1984; Morgan et al., 1996; Cordeiro et al., 2001; Flechas et al., 2009). A similar kinetics is observed in *mouse T. cruzi* infection, though IgM Ab persist in the chronic phase of the infection (probably related to the short time span is as compared to years-long chronic phase in humans) (el Bouhdidi et al., 1994). In mouse infection, the IgG-specific response is also dominated by an isotype known to be driven by Th1 immune responses, i.e. *IgG2a*, which accounts for 70% of all Ab. We have however shown that the

production of this isotype might also be IFN-γ-independent in infected mice (Markine-Goriaynoff et al., 2000). IgG1-specific Ab, known in mice as being associated with Th2 responses, are also produced, though at lower levels than IgG2a (el Bouhdidi et al., 1994). Both isotypes recognize roughly a similar set of parasite Ag (Brodskyn et al., 1989), though we showed that IgG1 were globally of higher avidity than IgG2a (el Bouhdidi et al., 1994). Diversity of *T. cruzi* genotypes influences the balance between IgG2a and IgG1 (dos Santos et al., 2009). IgG3 and IgE Ab isotypes have also been observed (at lower levels) in mouse and human *T. cruzi* infection (el Bouhdidi et al., 1994; Laderach et al., 1996; Mineo et al., 1996; Motran et al., 1998).

24.4.3 Effector Mechanisms of Ab

The production of specific Ab in *T. cruzi* infection starts rather late. High levels of Ab are reached when parasitemia starts to decrease and they have an important protective role in the transition from acute to chronic phase of infection, assessed by transferring immune serum from chronically infected to naïve challenged mice (Krettli and Brener, 1976). Different mechanisms account for their role in clearance of extracellular trypomastiogtes (Umekita and Mota, 2000).

24.4.3.1 Lysis of Extracellular Trypomastigotes

T. cruzi-specific Ab found in chronic infection have been shown to lyse trypomastigotes *in vitro*. Two main types of Ab present this capacity, both directed against surface molecules of the parasite. The first type of Ab are directed against parasite molecules such as CRP and T-DAF that prevent the lytic action of complement (see Section 24.5.1). By binding, they shut down the protection, allowing the activated complement system to lyse parasites (Krettli et al., 1982; Krautz et al., 2000). The action of such Ab thus results from the capacity of their $F(ab')_2$ fragment to neutralize CRP and T-DAF, and not to their ability to activate the complement cascade via their Fc fragment. It is thus not mandatory they are of an isotype able to activate the CP of complement, as *T. cruzi* itself activates nonclassical pathways. However, this type of lytic Ab likely contribute poorly to eliminate circulating parasites *in vivo*. Indeed, trypomastigote clearance from the circulation of infected mice is not reduced in C5-deficient animals (Umekita et al., 1988). The second type of lytic Ab bind to parasite surface α-Gal epitopes, inducing membrane deformations that lead to lysis of trypomastigotes (Pereira-Chioccola et al., 2000). However, *in vivo* the parasite covers itself very quickly with sialic acid residues that mask α-Gal epitopes (see Section 24.5.3), preventing the binding of anti-α-Gal Ab. We may therefore suppose that anti-α-Gal Ab have only a very short time to act *in vivo* and might again only weakly contribute to parasite elimination.

Other Ab directed against membrane molecules have been described or suggested to mediate lysis of trypomastigotes, but their precise mechanism of action is not established (Krautz et al., 2000). Ab against the glycoprotein GP90 specific of metacyclic trypomastigotes (regulating parasite invasion in host cells (Yoshida,

2006)) lyse parasites although it is not known to regulate complement activation or to bear α-Gal epitopes (Mortara et al., 1988; Gonzalez et al., 1991). As they are directed against metacyclic trypomastigotes, they might play a role in vectorial reinfections.

It is worth noting that the susceptibility of trypomastigotes to lytic Ab that neutralize complement-regulatory molecules seems to vary between parasite strains, likely in relation to their ability to "eliminate" surface bound Ab by shedding and/or interiorization: parasites that remove more efficiently the immune complexes from their membrane are less susceptible to complement-mediated Ab lysis (Krettli et al., 1979). In addition, different CRP molecules are synthesized by a single parasite strain and differentially recognized by Chagasic patient sera (Beucher and Norris, 2008). Strain variations to lysis by GP90-specific Ab has also been observed, probably due to different levels of GP90 expression or expression of isoforms, as GP90 also belong to a multigenic family (Gonzalez et al., 1991; do Carmo et al., 2002).

Finally, one report suggests that polynuclear cells expressing Fc receptors might lyse extracellular trypomastigotes by antibody-dependent cellular toxicity (ADCC) (Okabe et al., 1980).

24.4.3.2 Clearance of Ab-Opsonized Parasites

Trypomastigotes are easily removed from the mouse circulation in the presence of Ab collected during the chronic phase of the infection. The effect of such Ab is dependent on their Fc fragment (Umekita et al., 1988) and expression of FcγR on phagocytic cells, upregulated during *T. cruzi* infection as shown by our team (Araujo-Jorge et al., 1993a,b). These receptors are also known to be induced by IFN-γ and downregulated by IL10 or TGF-β (Nimmerjahn et al., 2006). As expected, the Ab isotypes able to bind to these FcγR, i.e., IgG2a, IgG2b, and IgG1, have a preferential role in parasite clearance (Brodskyn et al., 1989). In addition, coating of parasites with Ab also leads to deposition of complement components on their membrane (see earlier), allowing their uptake by phagocytic cells expressing complement receptors (Krettli et al., 1979).

Different cell populations may be responsible for the elimination of Ab and complement-opsonized parasites. Tissue infiltrating macrophages, neutrophils, and even eosinophils have been shown to contain parasites in various stages of degeneration after immune clearance (Umekita and Mota, 2000). The liver and the spleen appears to be primary organs of immune clearance of *T. cruzi* (Scott and Moyes, 1982; Sardinha et al., 2010). Vascular endothelial cells activated to produce NO, in conjunction with platelets adhering to trypomastigotes, have also been proposed to mediate parasite elimination in the presence of Ab (Shaw et al., 1991; Umekita and Mota, 2000).

We may speculate that the fate of opsonized parasite cleared from the circulation by entry into phagocytic cells may differ along with the immune environment. Mouse IgG1 and IgG2 have been shown to possess a similar clearance ability of blood trypomastigotes (Brodskyn et al., 1989). IgG2, known to be associated with a type 1 T cell

response, should favor the entry into IFN-γ-activated phagocytes able to kill parasites, while conversely, IgG1, generally reflecting a Th2-skewed response, would allow entry into alternatively activated cells, favoring the persistence of parasites (cf. Section 24.2.3.4). On the other hand, a study of the *in vitro* uptake of bloodstream forms of *T. cruzi* by peritoneal macrophages has revealed appreciable strain differences in ensuing uptake and handling of parasites (Krettli et al., 1979).

24.4.3.3 Other Mechanisms

Various Ab directed against functionally important molecules for *T. cruzi* survival in its host are expected to modulate the host−parasite interactions and have a protective effect. Apart from vaccination assays, that are biased through adjuvant use and vaccine scheme as compared to the natural infection, data supporting the protective effect of such Ab in the natural infection are scarce. Hence, cured patients possess TS-neutralizing Ab, suggesting their beneficial role (Leguizamon et al., 1997).

Recently, a protective effect of autoantibodies reactive with apoptotic cells (induced by systemic exposure to apoptotic cells) produced during *T. cruzi* infection in mouse has been described (Montalvao et al., 2010). Fc-dependent phagocytosis by macrophages of apoptotic lymphocytes opsonized by these Ab increases the production of TNF-α while decreasing production of TGF-β by infected macrophages, which leads to a better control of intracellular parasite multiplication (cf. Section 24.1.3). Opsonization with these Ab will thus counteract the deleterious effect of engulfment of apoptotic lymphocytes (mediated by another pathway than FcR) on the control of the infection. Their beneficial effect is also shown *in vivo*.

24.5 Deregulations of T and B Lymphocyte Responses

24.5.1 Defective Lymphocyte Responses and Immunosuppression in T. cruzi*-Infected Mice*

Reports from 25 to 30 years ago already suggested that acute *T. cruzi* infection in mice was characterized by suboptimal cellular and humoral immune responses. Deficient lymphocyte responses are particularly pronounced during the acute parasitemic phase of infection (though subsiding during the chronic phase) and affects both *T. cruzi*-specific and unspecific responses (Cunningham et al., 1978; Rowland and Kuhn, 1978; Maleckar and Kierszenbaum, 1984; Tarleton and Kuhn, 1985). Multiple mechanisms have been described, resulting from both *direct and indirect action of parasites on lymphocytes*.

24.5.1.1 Deficient IL2 Production

An important *defect of B and T lymphoproliferative responses* to mitogens and Ag, including *T. cruzi* Ag, is seen during the acute phase of experimental infection in mice, with a partial recovery in the ensuing chronic phase (Cunningham et al.,

1978; Maleckar and Kierszenbaum, 1984; Tarleton and Kuhn, 1985; Vandekerckhove et al., 1994). Poor proliferation is largely associated with a scarce production of the central cytokine *IL2* (Harel-Bellan et al., 1983, 1985; Reed et al., 1984; Tarleton and Kuhn, 1984). This deficit is not due to a restricted number of precursor cells potentially able to produce IL2 or to deficient IL1 production (Tarleton, 1988a,b). It is mainly associated with low IL2 mRNA levels resulting from weak gene transcription and not from accelerated decay (Soong and Tarleton, 1992; Majumder and Kierszenbaum, 1995), in relation to decreased levels of some transcription factors involved in IL2 gene transcription (AP1>NF-κB, NFAT>Oct-1) (Soong and Tarleton, 1994).

If the low levels of transcription factor results from active suppression and/or from insufficient/inadequate activation is to date not elucidated. However, as IL2 optimal production requires both TCR and costimulatory signals (Gaffen and Liu, 2004), we may hypothesize that T lymphocytes receive inadequate activating signals through TCR and/or costimulatory receptors, due to insufficient activation of Ag-presenting cells (cf. Section 24.2.3.4), as well as altered peptide presentation (see later). In line with this, defective signalling through CD3 and the costimulatory molecule Thy-1 (CD90) associated with reduced T cell proliferation has been reported (de Oliveira and Gattass, 1991; Lopes and Dos Reis, 1994). Besides, parasite molecules have been shown to reduce proliferation of spleen cells through inhibition of IL2 production (TIF [Kierszenbaum et al., 1994] and AgC10 (Alcaide and Fresno, 2004). The molecular basis of immunosuppression (IS) mediated by TIF has been studied on human cells (Kierszenbaum et al., 1998). AgC10, a GPI-anchored parasite mucin, has been shown to bind to L-selectin (CD62L) on T cell. This transduces a signal that inhibits phosphorylation of the protein tyrosine kinase ZAP70 and its substrate SLP-76 involved in TCR signalling. In turn, this leads to an inadequate signal to mobilize the transcription factors for IL2 expression (NFAT>AP1 » NF-κB) (Alcaide and Fresno, 2004).

Administration of exogenous IL2 does only partially restore lymphocyte proliferation (depending also of parasite concentration: more difficult to restore at high concentration) (Kierszenbaum et al., 1993) and the capacity to mount a normal Ab response. This probably relies on the slightly reduced membrane expression of IL2Rα (CD25) on lymphocytes from acutely infected mice, and the small transient increase of soluble IL2R levels that may partially neutralize the action of IL2 (Pakianathan and Kuhn, 1992; Lopez et al., 1993). The ceramide moiety of *T. cruzi* GIPLs have been shown to prevent expression of CD25 on both $CD4^+$ and $CD8^+$ T cells activated with anti-CD3 Ab (Gomes et al., 1996).

However, impairment of the IL2 network does not exclusively account for lymphocyte defective responsiveness, and adherent cells have been shown to participate in IS (Kierszenbaum, 1982; Harel-Bellan et al., 1983, 1985; Tarleton, 1988a,b).

24.5.1.2 Suppression by NO-Producing Myeloid Adherent Cells

Myeloid adherent cells, thought to be macrophages, have been suspected early to be involved in *suppression of lymphocyte proliferation* in lymphoid organs of

infected mice (Kierszenbaum, 1982; Harel-Bellan et al., 1985; Cerrone and Kuhn, 1991; Cerrone et al., 1992; Vandekerckhove et al., 1994). The group of Kuhn et al. suggested two subpopulations of macrophages being implicated: one abundant subset able to suppress both mitogen- and primary antigen-specific responses but unable to inhibit cells once they are activated or primed, and another minor subset, able to suppress the response of activated or primed T cells by the inhibiting IL2 production (Cerrone et al., 1992). However, these macrophages subsets have not been further characterized.

Suppression of proliferative responses in *T. cruzi*-infected mice has next been shown to largely depend on increased NO production by adherent spleen cells activated by IFN-γ and TNF-α during the acute phase (Abrahamsohn and Coffman, 1995; Goni et al., 2002). Immature myeloid cells ($CD11b^+$ $GR1^+$ $Lyc6^+$ CD8a) have been identified as responsible for IS. The NO-dependent mode of action is elsewhere (Bronte et al., 2003; Talmadge, 2007). Moreover as described below, NO is also involved in apoptosis of lymphocytes.

24.5.1.3 Apoptosis of Lymphocytes

Apoptosis of B and T lymphocytes is observed in *T. cruzi* infection and particularly pronounced during the acute phase (Guillermo et al., 2007; Lopes et al., 2007). Apoptosis of B cells is observed in bone marrow, while T apoptosis occurs in both primary and secondary lymphoid organs.

T. cruzi infection in mice induces the *apoptosis of immature B cells* ($CD19^+$ HSA^{high} IgD^-) (HSA = heat-stable Ag = CD24a in mouse), leading to their drastic reduction in bone marrow. Although it occurs only during the parasitemic phase of the infection, it does not result from a direct action of parasites on B cells and is Fas/FasL-independent. PGE2 production by $CD11b^+$ myeloid cells is thought to be involved in this process (Zuniga et al., 2005; Acosta Rodriguez et al., 2007). In addition, splenic B cells, though activated in some extent, express high levels of Fas and are prone to apoptosis during the acute phase of infection (Zuniga et al., 2000). It is likely that such immunologic disorders, occurring early in infection, contribute to delay the production of Ab, which are indeed detectable only from the descending phase of the parasitemia onward, when mice start to recover from B hypoplasia.

T. cruzi acute infection is also associated with a severe *atrophy of the thymus*, largely reflecting intense lymphocyte depletion, particularly of cortical thymocytes, bearing the phenotype $CD4^+$ $CD8^+$ (Savino, 2006). This depletion actually corresponds to massive cortical thymocyte *apoptosis*, which is independent from Fas-FasL pathway (Henriques-Pons et al., 2004). It is worth mentioning that parallely to be depleted in the thymus, double positive $CD4^+$ $CD8^+$ cells are abnormally released outside the thymus. As mentioned by the authors, this implies that such cells have bypassed intrathymic negative selection and bear a potential autoreactive phenotype that apparently differentiates in the periphery into $CD4^+$ or $CD8^+$ single-positive cells, opening the way to autoimmune reactions (Mendes-da-Cruz et al., 2003).

Various pathways account for T and B apoptosis in secondary lymphoid organs (Guillermo et al., 2007), involving *Fas-FasL* pathway and *TNF*-α in mesenteric

lymph nodes (de Meis et al., 2006), while apoptosis of *spleen* cells in the acute phase is preferentially induced by high amounts of *NO* (Martins et al., 1998). In addition, apoptosis of $CD4^+$ T cells, but not of $CD8^+$ T cells, in chronically infected mice, is likely related to the physiological process of *activation-induced programmed cell death* (AICD) mediated by the Fas-FasL (Lopes et al., 1995; Nunes et al., 1998). Though IFN-γ is crucial for the production of high amounts of NO, it also triggers in *T. cruzi*-infected mice the synthesis of a protein likely favoring hematopoiesis, namely Irgm1 (or LRG47) (Santiago et al., 2005). As this protein is induced later than NO, it might help to compensate the loss of lymphocytes occurring in acute phase, facilitating the development of the immune response.

Interestingly, besides these diverse mechanisms varying with cell type and location, the parasite *trans-sialidase* contributes to lymphocyte apoptosis (Leguizamon et al., 1999; Mucci et al., 2002). Mucci et al. have shown the intact enzyme activity of TS to be required for inducing apoptosis. The sialylation of the host mucin CD43 by the parasite TS is thought to initiate a process leading to TNF-α/p55-mediated apoptosis (Mucci et al., 2002, 2005, 2006). *T. cruzi* TS is shed in the host early during the infection, precisely when the induced damage to the immune system becomes evident. The action of circulating enzyme is then controlled by neutralizing Ab (cf. Section 24.3.1), which prevent further damage and allows recovery since the effect of the TS on the immune system is reversible (Leguizamon et al., 1999; Mucci et al., 2002). Amazingly, the authors also show that TS-induced thymocyte apoptosis occurs only in male mice as the presence of androgens is required. This indicates that deletion of thymocytes (altered in their sialylation pattern) might be related to the known differences in the immune response among sexes (Mucci et al., 2005).

24.5.1.4 Altered Peptide Presentation

The group of Kahn et al. identified another important mechanism responsible for suboptimal-specific response in *T. cruzi* infection, related to the abundant expression and shedding by parasites of molecules of the *TS* superfamily. Thus, each trypomastigote simultaneously expresses many variants of these polymorphic proteins sharing related epitopes (Kahn et al., 1999). For instance, the *SA85* subfamily of TS is composed of more than 100 proteins presenting 70−80% homology to each other at the amino acid level. The authors have observed that the variant SA85-1.1 is unable to induce IL2 production and $CD4^+$ T cell proliferation in acutely or chronically infected mice, although it is immunogenic, i.e., inducing T cell response when administered to mouse in the presence of adjuvant (Millar et al., 1999). The defect does not result from a deficient Ag presentation or from inhibition of $CD4^+$ T cells by NO (Millar and Kahn, 2000b). One possible explanation is that some variant epitopes act as altered epitopes. Such kind of epitopes are presented to and recognized by T cells but, due to a lower affinity of the peptide−TCR interaction, they induce incomplete or anergic T-cell rather than optimal responses (Uhlin et al., 2006). Supporting this hypothesis, they have isolated from infected mice, $CD4^+$ T cells directed against the "defective" variant

SA85-1.1. These T cells are effectively unable to proliferate or produced IL2. However and interestingly, they produce IFN-γ but not IL4. This indicates that SA85.1.1 acts as a partial agonist, inducing a polarized Th1 response without proliferation (Millar et al., 1999). Importantly, this epitope was further characterized as a major $CD4^+$ T cell Ag in mouse (Millar and Kahn, 2000a). This suggests that in *T. cruzi* infection, altered peptide presentation may have a substantial impact in biasing the global response to suboptimal (though Th1) levels. The simultaneous expression of multiple related TS molecules is also thought to delay the production of some protective Abs (Pitcovsky et al., 2002). More generally, these results underline the benefit for *T. cruzi* to express simultaneously a high number of polymorphic molecules, some of them being able to limit the immune response, thereby favoring its survival.

24.5.1.5 Contribution of Immunoregulatory Cytokines

The contribution of immunosuppressive cytokines produced during *T. cruzi* infection was also studied. Kierszenbaum suggested nearly 30 years ago the involvement of prostaglandins by treating infected mice with indomethacin (Kierszenbaum, 1982). During acute *T. cruzi* infection, TNF-α triggers PGE2 production (Pinge-Filho et al., 1999), known to be a potent downregulator of lymphocyte proliferation by inhibiting IL2 production (Minakuchi et al., 1990) and the expression of its receptor. On the other hand, PGE2 may induce the expression of NO synthase and the synthesis of NO in macrophages (Gaillard et al., 1992). PGE2 seems to be involved in early inhibition of lymphocyte proliferation (during the ascending phase of parasitemia), likely in relation to both properties of PGE2 to inhibit IL2 production and induce NO release (Pinge-Filho et al., 1999). However, during the descending phase of parasitemia, PGE2 is no more implicated in inhibition of proliferation, although it reached its maximal levels, indicating that other mechanisms follow. Later on, PGE2 regains its immunosuppressive role.

On the contrary, IL10 and TGF-β do not seem to significantly regulate *T. cruzi*-associated IS (Abrahamsohn and Coffman, 1995). This is surprising, taking into account their well-known inhibitory effect on NO production, also showed *in vitro* in *T. cruzi*-infected macrophages (Gazzinelli et al., 1992) and their potential to inhibit Ag- or mitogen-driven lymphocyte proliferation (Wahl et al., 1988; Ding and Shevach, 1992). However, IL10 might in certain conditions favor rather than inhibit NO production (Jacobs et al., 1998).

24.5.1.6 Other Host or Parasite Factors or Cells Involved in IS

Reed et al. have pointed to a deficient production of GM-CSF and IL1 by macrophages to be responsible for depressed ability to mount an Ab response to heterologous Ag during the chronic phase of infection in mouse (Reed et al., 1989, 1990).

Ouaissi et al. (Schoneck et al., 1994) described an intracellular protein shed by parasites (trypomastigotes and amastigotes), *Tc52*, that totally inhibits proliferation of mouse spleen cells. This protein might account for early IS since it is detected in

the serum of infected mice before specific Ab are produced (Ouaissi et al., 1995). The IS activity has been mapped in the C-terminal portion of the protein (residues 432−445), which in parallel also downregulates the ability of spleen cells to produce various cytokines (IFN-γ, IL2, IL10, IL4) (Borges et al., 2003). This protein possesses binding properties for cysteine and glutathione (GSH). It is hypothesized that it inhibits lymphocyte response by limiting the availability of these molecules, known to be important for optimal immune responses (Ouaissi et al., 1995).

Splenic *γδ-T cells* have been reported to inhibit nonspecific lymphocyte proliferation and IFN-γ production by αβ-T cells. Interestingly, depletion of γδ-T cells improves the capacity of infected mice to mount a (nonspecific) humoral immune response (the specific Ab response has not been still studied). This was associated with an increased control of the parasitemia. The mechanism of suppression is not known. It has only been shown to be independent of IL2 restriction and not mediated by IL10 or TGF-β, but these aspects have never been studied further, and might look at odds with another study of the same group, showing protective effect of a subpopulation of γδ-T cells (Cardillo et al., 1993, 1998; Nomizo et al., 2006).

24.5.2 IS in Infected Humans

Argibay et al. showed that some proteins from the two major families of trypomastigote surface molecules, i.e., the trans-sialidases and the mucins, inhibited *in vitro* T cell proliferation in response to xenogenic or PHA stimulation (Argibay et al., 2002; Vercelli et al., 2005). The effect of the studied proteins (recombinant TS (Leguizamon et al., 1999) and TcMUC-e2) was not related to apoptosis but likely to defective CD25 (IL2-Rα chain) expression. They also showed *O*-glycosylation and sialylation of TcMUC-e2 to be required for the immunosuppressive effect of this molecule (Argibay et al., 2002). However, the suppression induced by TcMUC-e2 was only partial, since lymphocytes retained their ability to express activation markers (though it was somewhat delayed), as to secrete IFN-γ. Additionally, the inhibition of lymphoproliferation was reversed by addition of exogenous IL2, suggesting that higher amounts of this cytokine might overcome the effect of CD25. Suppression of Ab responses in chronically infected humans has also been reported (Cunningham et al., 1980).

24.5.3 Polyclonal Activation

The host has to face a second difficulty in the development of the B-cell response. Indeed, it is known from a long time that *T. cruzi* infection induces in mouse an important *polyclonal activation of B lymphocytes* (Ortiz-Ortiz et al., 1980; D'Imperio Lima et al., 1985), responsible for the production of large amounts of immunoglobulins that lack parasite specificity. This response, initiated during the acute phase of the infection, persists in late stages of the chronic infection and is dominated by *IgG*, though other isotypes are also induced (D'Imperio Lima et al., 1985; el Bouhdidi et al., 1994). Polyclonal activation is thought to constitute an immune evasion mechanism because it masks/deviates the specific responses,

thereby limiting the control of infection. Moreover, it might participate to enlarge the B repertoire to self-antigens, favoring the production of autoreactive Ab, of which a dramatic consequence is the induction of tissue damages, responsible for the pathology of the chronic infection (see Chapter 25).

T. cruzi-induced B-cell polyclonal activation appears to be due to both T helper cell-dependent regulation and to a T-independent, mitogenic effect of parasites on B cells (D'Imperio Lima et al., 1985; Minoprio et al., 1987). Several molecules presenting B mitogenic properties *in vitro* on mouse cells have been identified: Gao et al. showed that *T. cruzi TS*, and particularly its C-terminal long tandem repeat (LTR or SAPA), is a T-independent B-cell mitogen, mainly for conventional B2-cells (the effect is mediated through Bruton's tyrosine kinase Btk activation); it stimulates secretion of nonspecific Ig both *in vitro* and *in vivo*, through an IL6 independent way (Gao et al., 2002). Minoprio et al. have identified and characterized another B-cell mitogen from *T. cruzi*: the *proline racemase* (TcPRAC). This enzyme catalyzes the interconversion of L- and D-proline enantiomers and was the first identified eukaryotic proline racemase. The enzyme isoform released by the infective forms of the parasite is responsible for the B mitogenic property, which is independent from the racemase activity itself but might depend on conformational epitopes displayed by the enzyme when in its open form (Reina-San-Martin et al., 2000; Minoprio, 2001; Buschiazzo et al., 2006). Minoprio also identified another parasitic B mitogen: *Tc24*. This protein, of which the function is unknown, is shed by parasites and is a potent B-cell activator in the presence or absence of T cells. Moreover, a single injection of rTc24 to mouse induces a polyclonal, nonspecific, B-cell response. It induces mainly IgM (Da Silva et al., 1998). Montes et al. described parasite mitochondrial *malate* (MDH) and *glutamate dehydrogenase* (GDH) to activate naïve B cells in a T-independent way to proliferate and produce IgM. Interestingly, GDH acted by inducing the production by myeloid $CD11b^+$ cells of BAFF (B lymphocyte activating factor of the tumour necrosis factor family), IL6 and IL10 (Montes et al., 2002, 2006). Finally, parasite *GIPLs* induce IgM and IgG production by purified mouse B cells (Bento et al., 1996). The effect is not so pronounced and such glycolipids are not abundant on trypomastigotes. However, they more markedly potentiate Ig secretion induced by cytokines or cross-linking of their surface IgD.

In mouse experimental infection, the B1 B cells ($CD5^+$, T independent) have been shown to play an important role in the production of unspecific Ig. In their absence (*Xid* mice), the polyclonal B activation is strongly reduced. Interestingly, this was associated with a reduced parasitemia in acutely infected animals, sustaining the harmful role for polyclonal B stimulation in the control of the acute infection (Minoprio et al., 1991), though other mechanisms likely contribute to the improved control in *Xid* mice. Indeed, they are also relatively deficient in IL10 ($CD5^+$ B cells are important producers of IL10), which likely allows a better IFN-γ-dependent control of the infection (Minoprio et al., 1993). Moreover, the Bruton's tyrosine kinase (Btk), deficient in Xid mice, has been shown later on to also be expressed by other cells of the innate and adaptative immune system, which complicates the interpretation of the results (Brunner et al., 2005). Finally, B1 cells have been shown to play an essential

role in establishing the fetal and adult natural Ab repertoires. It has been suggested that the skewing of such repertoire in *Xid* mice might be beneficial for the host in the case of *T. cruzi* infection if for instance it contains Ab able to neutralize mitogenic parasite molecules (Santos-Lima et al., 2001).

24.6 Escape Mechanisms of *T. cruzi* from the Immune Responses

24.6.1 Resistance to Complement Lysis

Since they activate the complement system (Section 24.1.2), trypomastigotes must evade this powerful mechanism of killing before entry into host cells and again after they emerge. Hence they possess membrane-bound proteins that confer them a certain degree of resistance to complement lysis, enabling them to survive in the bloodstream. Some of them are known from many years (Tomlinson and Raper, 1998) while others have recently been discovered.

Three types of trypomastigote surface glycoproteins inhibit the formation or the enzymatic activity of C3 convertase and/or accelerate its decay: the family of GPI anchored 160 kDa complement regulatory proteins (*160 CRP*), a subfamily of the trans sialidase like proteins (Norris et al., 1991; Beucher and Norris, 2008); the trypomastigote decay accelerating factor (*T DAF*) (Tambourgi et al., 1993); and a glycoprotein named *gp58/68* (Fischer et al., 1988). 160 CRP and T DAF display functional analogy to human DAF CD55). These molecules impede mainly the AP of complement activation, though CRPs have been shown to also inhibit the classical C3 convertase.

The parasitic *calreticulin*, found on the surface of trypomastigotes (shed in the extracellular medium and recaptured at the surface by an unknown mechanism), which interacts with soluble human C1q. This results in strong impairment of C1q mediated CP, probably by subtracting the C1q molecule from proteolytic cleavage that initiates the cascade of complement activation (Ferreira et al., 2004; Valck et al., 2010).

A protein of 27−32 kDa recently described in metacyclic trypomastigotes (Cestari et al., 2008), highly homologous to the mammal complement C2 receptor inhibitor trispanning gene (*CRIT*) that inhibits the cleavage of C2 involved in AP and LP (Inal et al., 2005). Interestingly, CRIT may have been acquired by the parasite through horizontal gene transfer (Inal, 2005).

Two additional mechanisms account for complement resistance of *T. cruzi* (Tomlinson and Raper, 1998). First, the trypomastigote covers its surface with sialic acid residues it takes from host molecules, thanks to TS (Frasch, 2000). Sialylated molecules are known to downregulate the activation of the AP of complement (Pangburn et al., 2008; Zipfel and Skerka, 2009). Trypomastigotes that have been desialylated and deprived of the means of resialylation show an increased susceptibility to lysis by human serum, which correlates with enhanced deposition of C3 on the parasite surface (Tomlinson et al., 1994). Second, trypomastigotes continuously shed membrane molecules, including the acceptor(s) of C3b, thereby also limiting the risk of activation of the complement cascade normally leading to target lysis (Norris and Schrimpf, 1994; Tomlinson and Raper, 1998).

Resistance to complement-mediated lysis is developmentally regulated. Like trypomastigotes, *amastigotes* (that might be found in the circulation when host cells disrupt before transformation into trypomastigotes (Ley et al., 1988)), are also resistant, although they are potent activators of the complement system in the absence of Ab (Iida et al., 1989). Interestingly, the mechanism of escape is totally different from trypomastigotes, as they do not express the above-mentioned trypomastigote complement-regulatory molecules (Norris et al., 1989; Tomlinson and Raper, 1998). Instead, they resist lysis by preventing the insertion of the lytic membrane attack complex (MAC or C5b−9) into their surface membrane. The mechanism by which this is achieved is unknown, but the amastigote membrane might possess an inhibitor functionally analogous to the mammalian inhibitor of MAC formation, CD59.

24.6.2 Intracellular Survival

Resident macrophages at the site of parasite invasion are among the first cells to be invaded. Internalization of trypomastigotes activates the NADPH oxidase generating ROS, and also triggers a modest production of low doses of NO, independently of IFN-γ. Thereafter, cytokines induces NOS2 synthesis that boosts trypanocidal action of macrophages. While it is not known if *T. cruzi* possesses enzymes able to decompose NO, the parasite is endowed with an "armada" of antioxidant enzymes, distributed among different cellular compartments, including several peroxidases, peroxiredoxins, the parasite particular trypanothione and tryparedoxin, etc. (Irigoin et al., 2008; Piacenza et al., 2009). They allow the parasite to partially resist to the trypanocidal action of phagocytes. Interestingly, transformation of epimastigotes to metacyclic trypomastigotes in the vector is accompanied by an increase in the expression of several antioxidant enzymes, preparing the parasite to deal with the first waves of oxidative burst when infecting its vertebrate host. Apart from this well-known strategy, *T. cruzi* takes advantage when low amounts of NO are produced in resident macrophages, which actually favor parasite proliferation instead of impeding it (Rottenberg et al., 1996; Peluffo et al., 2004). Next, ceramide-containing components of *T. cruzi* (like GIPLs of trypomastigotes and GPI anchors of amastigotes) rapidly induce apoptosis of resident macrophages, an effect reinforced by IFN-γ. This leads to release amastigotes in the extracellular medium before they are killed, that may infect other cells (Freire-de-Lima et al., 1998; de Souza et al., 2003). In association with lymphocyte apoptosis which triggers alternatively activated macrophages less able to control parasite replication, it strongly serves parasite persistence.

As if not enough, some amastigotes preferentially invade nonactivated macrophages, i.e., not armed to kill the parasite and more permissive for parasite replication than activated ones. This may be related to their use of the MR for entry, the expression of which is decreased on IFN-γ-activated macrophages (Kahn et al., 1995). Trypomastigotes do not bind to the MR, conferring to amastigotes an advantage to achieve the persistence of the infection.

The question still remains as to why *T. cruzi* is controlled at most tissues sites but is able to persist, albeit at very low levels, specifically in muscle cells, some neurons

and adipose tissue. Muscle cells produce less NO and more polyamines than phagocytes, which favors parasite multiplication (Peluffo et al., 2004). Additionally, myoglobin and neuroglobin is known to scavenge toxic free radicals and reactive oxygen and nitrogen species (Herold and Fago, 2005). Besides, parasite molecules like cruzipain and some TS induce, in infected cardiomyocytes for instance, the production of antiapoptotic molecules. This will protect the host cell from apoptosis induced by oxidative stress, TNF-α and Fas-FasL pathway is expected to help the completion of its multiplicative cycle by increasing the life span of the host cell (Aoki et al., 2006; Chuenkova and PereiraPerrin, 2009). Survival of *T. cruzi* in muscle cells is also associated with the fact that the parasite does not induce NF-κB-dependent gene expression in these cells, contrary to what happens in epithelial cells, endothelial cells, and fibroblasts (Hall and Pereira, 2000b). At last, it is proposed that other particular metabolic features of muscle and adipose tissue, which preferentially store and utilize fatty acids as energy sources, provides a survival advantage for *T. cruzi* in these cells (Padilla et al., 2009a).

24.6.3 Escape from the Action of Ab

T. cruzi can limit the Fc-dependent effector functions of specific Ab bound to its surface by two mechanisms. First, cruzipain is able to digest human IgG; the Fab fragment is only slightly degraded, but Fc is extensively hydrolyzed to small peptides, impairing its binding to FcR (Bontempi and Cazzulo, 1990; Berasain et al., 2003). Second, it expresses on its surface an Ig-binding factor for mammalian IgG and IgM through their Fc fragment (Campos-Neto et al., 2003). *In vitro* experiments point to an active role of this molecule in inhibiting Ig-mediated attachment and penetration of *T. cruzi* into macrophages.

TS enzymatic activity assures survival of *T. cruzi* in various ways. During the acute phase, TS molecules bearing SAPA repeats are abundantly released by the parasite. The immunodominance of SAPA prevents the early formation of neutralizing Ab directed against the catalytic site, situated away on the molecule. This might profit to the parasite during the acute period of infection, allowing TS to transfer sialic acid to the surface molecules of the parasite and to infect the mammalian host cells. Later on, when SAPA-specific Ab are generated, SAPA sequences on TS molecules are masked and neutralizing Ab against the TS catalytic region are produced. Neutralization of TS activity by Ab after the acute period of infection might prevent massive cell invasion by the parasite and thus allow the survival of the infected host (Frasch, 2000). Hence, *T. cruzi* might take advantage of either the absence or the presence of TS-neutralizing Ab. By transferring acid sialic residues on the mucin coat of the parasite, TS also confers resistance to lytic anti-α-Gal Ab (Pereira-Chioccola et al., 2000).

24.6.4 Being a Stealth Parasite, Dampening and Disturbing Immune Responses

As already explained in this chapter, *T. cruzi* seems to slow down the initial development of an optimal immune response, for instance by delaying its recognition by

TLRs (Section 24.2.2.2). Moreover, the parasite uses its TS enzyme to resialylate $CD8^+$ T cell surface, thereby dampening Ag-specific $CD8^+$ T cell response, what might favor its own persistence in the mammalian host (Padilla et al., 2009a). The simultaneous expression of many variant TS encoded epitopes is also thought to mediate immune evasion (Padilla et al., 2009a). It could rely on competition for MHC-dependent presentation on APCs, or to altered peptide presentation. Interestingly, the patterns of dominant $CD8^+$ epitopes vary with parasite strain (Martin et al., 2006), suggesting that this may contribute to variable virulence, depending on the potential to escape the immune response.

24.7 Integrated Dynamic of the Immune Response

From the data in this chapter, we can propose the following scheme for the development of the protective immune response.

Main steps of the innate immune response in *T. cruzi* infection (step numbers are indicated in Figure 24.1):

1. At the site of parasite entry, fibroblasts are infected, which triggers the production (by around 24 h) of IFN β, through a yet unidentified TLR independent pathway.
2. Neutrophils rapidly infiltrate the site and start to phagocyte and kill parasites.

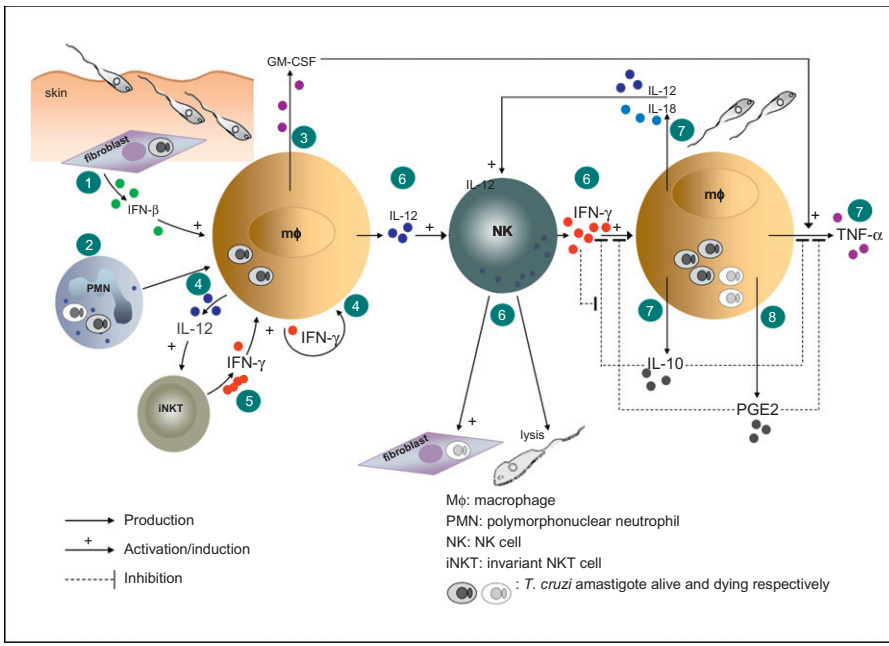

Figure 24.1 Development of the innate immune response in *T. cruzi* infection.

3. They are followed by macrophages which, upon infection, initiates the intracellular pro duction of ROS (not shown) and produce GM CSF (able to lyse trypomastigotes not shown).
4. Macrophages are primed by IFN β and produce low amounts of IL12 and IFN γ, trig gered by intracellular amastigotes (via TLR9 and NODs); granule proteins released by neutrophils also contribute to macrophage activation.
5. We may speculate that IL12, in conjunction with an unidentified glycolipid likely pre sented by infected cells expressing the nonclassical MHC molecule CD1d (not shown), activates iNKT cells to produce IFN γ.
6. This supply of IFN γ increases IL12 release (and perhaps low amounts of IL18) by infected macrophages, which will activate NK cells to produce IFN γ and to induce kill ing of trypomastigotes and NO dependent killing in macrophages and other cells like fibroblasts.
7. Infection is spreading; the still higher supply of IFN γ, in conjunction with the release of extracellular trypomastigotes which activate TLR2 through their GPI anchors, synergize with intracellular parasites acting on TLR9 and NODs for the induction of inflammatory (TNF α, IL6) and anti inflammatory (IL10) cytokines, as well as trypanocidal molecules (NO, ROS, and IRGs not shown); IFN γ also upregulates IL18 production by infected macrophages. TNF α synergizes with IFN γ for inducing NO synthesis (not shown), while GM CSF boosts the production of TNF α, ROS, and NO; thereby, the control of intracellular parasite multiplication increases more and more, as well as the inflammatory reaction, both events being balanced by IL10.
8. TNF α and NO induce the production of PGE2, which downregulates IFN γ and TNF α, weakening the control of parasite multiplication.

In the meantime, infected DCs mature and express costimulatory molecules (1) (step numbers are indicated in Figure 24.2). They migrate into lymph nodes to activate lymphocytes. However, for reasons not completely identified, linked to the surreptitious establishment of infection, the adaptive response develops rather late as compared to what is observed in viral infections. $CD8^+$ T cells would be first activated (2) independently of $CD4^+$ T cell help. Then Th1 lymphocytes differentiate (3) and help activating additional $CD8^+$ T cells. Subsequently, Th2 cells are generated and activate B lymphocytes to produce Ab (4). Fas-induced apoptosis is involved in the regulation of successive activation of various lymphocyte subsets. Th17 T cells also differentiate and reduces the magnitude of the Th1 response (5). Meanwhile, the parasite load increases in the host and give rise to an intense production of pro- (IFN-γ, TNF-α), and anti-inflammatory (mainly IL10 and PGE2) cytokines that compete for improving or dampening the control of the acute phase.

Progressively, the *effector mechanisms* for eliminating parasites take place. Mainly, amastigote killing (6) results from the activation, in infected cells, of the enzymes NOS2 and IDO, leading to NO synthesis (as well as peroxynitrite in macrophages [mϕ]) and tryptophan catabolism, as well as the induction of IRGs proteins. IFN-γ, produced by activated $CD4^+$ Th1 and $CD8^+$ T cells, in synergy with TNF-α, is pivotal in governing this event (5). Cytotoxic $CD8^+$ T cells also contribute to eliminate infected cells (7). Ab-opsonized trypomastigotes are taken up by activated phagocytes and cleared from the circulation (8).

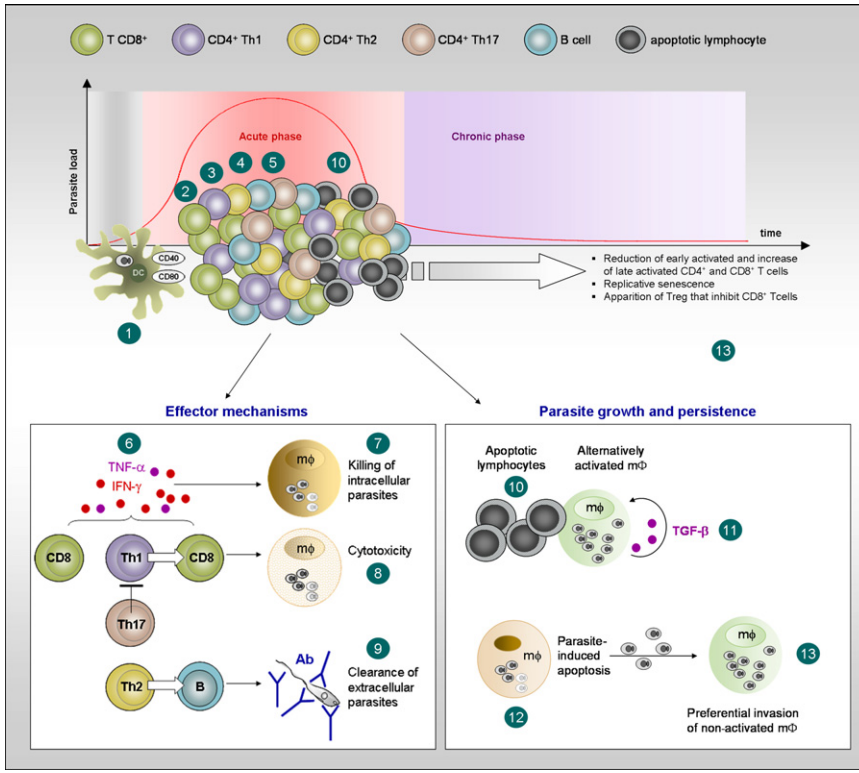

Figure 24.2 Adaptive immune response in *T. cruzi* infection.

Yet, the *response is suboptimal* since activated lymphocytes probably do not reach a sufficient number to eliminate all parasites. Indeed, their proliferation is impeded by various mechanisms. *T. cruzi* coexpresses a huge number of polymorphic molecules bearing similar but not identical T cell epitopes. Some of these epitopes are less potent in triggering lymphoproliferation (altered peptide presentation). Later on, immunosuppressive cytokines (TGF-β and PGE2) are produced as a result from high TNF-α and NO production in the acute phase. The situation is worsened by high levels of *lymphocyte apoptosis* (9) which evidently restrains the number of effector lymphocytes. Apoptosis results from activation-induced cell death, high NO production and the direct action of parasite molecules. The pronounced polyclonal lymphocyte activation occurring in the acute phase, also due to parasite molecules, enlarges the pool of activated cells potentially undergoing apoptosis.

Lymphocyte apoptosis has another dramatic consequence for the host. Indeed, upon uptake by macrophages, it triggers the production of TGF-β (10) which, in association with IL10, induces *alternatively activated macrophages*. These macrophages not only turn off NO synthesis, thereby limiting the control of the infection, but favor the production of polyamines that facilitates parasite growth.

Altogether, these events allow the host to eliminate most but not all parasites, which persist in tissues, overall in cells having reduced killing capacity like cardiomyocytes. The host enters the chronic phase of infection.

High levels of Ab persist during the chronic phase, allowing the host to maintain a low parasite burden. Yet, the parasite is able to induce the apoptosis of infected macrophages (11). This releases amastigotes in the extracellular medium before their transformation into trypomastigotes. As amastigotes present the advantage (for the parasite) to preferentially invade unactivated macrophages (12), this contributes to parasite persistence. Quite later on in the chronic phase, the T cell response progressively declines, as shown in humans by the reduction of early activated T cells, replicative senescence and the generation of regulatory T cells that dampens the action of $CD8^+$ T cells (13).

24.8 Conclusion

Control of *T. cruzi* infection requires the activation of multiple immune effector mechanisms in relation to the presence of both extracellular trypomastigotes and intracellular amastigotes. Protection is mainly governed by IFNs (initially type 1 IFNs, then IFN-γ). However, it is worth noting that neutrophils and iNKT cells seem to have an important role in the very initial steps of the infection, without forgetting the essential contribution of NK cells and macrophages later on. The parasite has evolved an impressive number of mechanisms allowing it to establish slowly as well as to escape such complex immune response. Its ability is to temporarily limit Ag presentation through MHC class I molecules (delaying the recognition of infected host cells by cytotoxic lymphocytes) and to favor the release of amastigotes (through host cell apoptosis) that invade preferentially unactivated macrophages in particular. Actually, the parasite is most generally on the winning side as the host does not reach eliminating it.

The superfamily of trans-sialidases and cruzipain are the main parasite molecules regulating host invasion. They also regulate the host immune response in conjunction with parasite PRR-ligands, not all identified to date. Variations in the expression of these polymorphic molecules between parasite genotypes are more and more recognized as determining the virulence of *T. cruzi*. The host genetic background also accounts for susceptibility or resistance to infection. Indeed, the host aptitude to rapidly produce enough IFN-γ and IFN-inducing cytokines like IL12 and IL18 governs its ability to restrain the amplitude of the parasitemic phase of the infection (resistant phenotype), while more susceptible hosts release earlier higher quantities of counter-regulating cytokines like IL10.

Amongst microbicidal molecules produced by host cells, NO has for long been identified as pivotal in infected mice, since the parasite is well equipped to resist to ROS. However, it has to be kept in mind that NO also exerts dramatically adverse effects for the host by inducing IS and lymphocyte apoptosis. This dampens the immune response and induces alternatively activated macrophages unable to control the infection. Metabolites produced by the enzyme IDO have recently been shown to

also participate in parasite control. In addition, IRGs have been identified as other IFN-γ-induced effector proteins. Though they are less numerous in humans than in mice, their mechanisms of action merits further consideration.

The importance for the host of limiting the parasite load during the acute phase of the infection is mainly to avoid a too strong inflammatory reaction susceptible to be very deleterious and lethal if not contained. Indeed, the production of harmful inflammatory cytokines is generally related to the parasite burden.

Thus, *T. cruzi* seems to be a real magician that adapts to many adverse situations. To date it is not known why the host does not reach eliminating the parasite and remains life-long infected. Late chronic infection in humans is associated with the progressive disappearance of early activated T cells and waning of T cell responses, at least in the absence of reinfections. The underlying mechanisms should be addressed, keeping in mind that it remains to know if the host would actually benefit from a better efficient immune response in the long term, since it might have pathological consequences in some chronically infected individuals, as it is discussed in the next chapter.

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25 Pathological Consequences of Host Response to Parasite

Mireille Hontebeyrie[*,1], *Carine Truyens*[2] *and Simone Frédérique Brenière*[3]

[1]Institut Pasteur, Paris, France, [2]Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium, [3]Institut de Recherche pour le Développement (IRD), Montpellier, France

25.1 Introduction

The most severe pathology induced by *Trypanosoma cruzi* concerns the heart disease that affects 20−30% of the infected patients and leads to sudden death. Only 5−10% suffer from megaesophagus, megacolon, or peripheral neuropathies. The pathophysiology of Chagas heart disease is still not totally understood and two main mechanisms have been proposed: (i) parasite-dependent myocardial damage and (ii) impaired immunological responses associated to molecular mimicry (epitope common between host and parasite molecules) (for review see Marin-Neto et al., 2007). If the clinical features were well known since the beginning of the twentieth century, it remains to explore the mechanisms underlying the development of this chronic disease. Moreover, the recent researches have mostly focused on mechanisms of protection mediated by $CD8^+$ T cells and definition of candidates for vaccination. Due to the restrictive rules of ethical approaches in humans, we will discuss the validity of the mouse model to learn about the pathological consequences of host-immune response to *T. cruzi*.

25.2 Pathological Disorders in Humans

25.2.1 Acute Phase

Acute *T. cruzi* infection in man is characterized by general clinical signs (fever, edema, myalgia, tachycardia, asthenia, etc.) and most of the time it is not diagnosed in endemic areas due to lack of specific symptoms and poor medical care. Many people are unaware of the disease and of its parasite's transmission by the bugs located in the houses. Facial edema representing a local inflammation at the site of entry of the

*E mail: mirhon@free.fr

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parasite is often observed and was described by Romaña (Romaña, 1935) as chagoma. The localization of this edema at the palpebral site is called "sign of Romaña." Only few cases of specific symptoms are represented by acute myocarditis or meningoencephalitis occurring in this stage of the disease (Chagas, 1911, 1916; Vianna, 1911; Torres, 1917; Köberlé, 1957, 1968; Rassi et al., 1958; Brener, 1980; Carlier et al., 2002). In a recent case of oral transmission, meningoencephalitis has been observed (Medeiros et al., 2008).

The main characteristic of the acute phase that lasts from 2 to 4 months is the large multiplication of the parasites able to invade a very large kind of cells (Brener, 1973; Melo and Brener, 1978; Burleigh and Andrews, 1995; Tan and Andrews, 2002; Andrade and Andrews, 2005) and parasitemia. The immune response allows the control of the parasitemia at least partially because some parasites persist in tissues and can multiply far from the action of specific antibodies (Krettli and Brener, 1976). This specific response is concomitant to a polyclonal B cell activation eliciting polyreactive antibody response (Van Voorhis, 1992; Grauert et al., 1993; Dutra et al., 2000). T cells are also activated (Dutra et al., 2000).

At the level of global organism, local inflammation spreads in organs where the parasites multiply mainly in heart and central or peripheral nervous system. Recently, it has been observed that genetically distinct populations of *T. cruzi* display different tissue tropisms and are responsible for different evolutions of the disease (Melo and Brener, 1978; Macedo and Pena, 1998; Dos Santos et al., 2009). Generally the acute phase is followed by an indeterminate phase that may last long or evolves in a symptomatic chronic phase with severe dysfunctions of the heart or the digestive tract. The peripheral nervous system may also be attacked (Palmieri et al., 1984). Spontaneous cure from *T. cruzi* infection is extremely rare (Dias et al., 2008).

Parasite molecules are potentially able to polyclonally activate the B cell compartment soon after the infection have been described (Hernandez-Munain et al., 1992; Cordeiro da Silva et al., 1998; Montes et al., 1999; Reina-San-Martin et al., 2000; Gao et al., 2002). Until now, few data are available on the role of these molecules in human acute phase of infection. But the mouse model suggests strongly that these generally secreted molecules are able to induce production of nonspecific antibodies. This will be discussed below.

25.2.2 Indeterminate and Chronic Phases

After parasitemia is partially controlled, the patients enter in a second phase of the disease, the chronic phase. It may be asymptomatic indeterminate phase or symptomatic chronic phase with lesions of the heart, the digestive tract, or the central or peripheral nervous system (Köberlé, 1957, 1968) (for review see Teixeira et al., 2006; Pittella, 2009). Most of the interest was applied to the chronic Chagas heart disease that concerns 20−30% of the infected people and is the most frequent cause of cardiomyopathy in Latin America.

Chronic Chagas heart disease is related to a late progressive inflammatory reaction, with focal accumulation of mononuclear cells leading to tissue destruction, neuronal damages, microvascular dysfunctions, and fibrosis (for review, see Marin-Neto et al.,

2007; Rassi et al., 2009). Two main nonexclusive hypotheses could explain this pathology: (i) a unique role for the *parasite* present in the heart as demonstrated by polymerase chain reaction (PCR) (Jones et al., 1993; Brandariz et al., 1995; Vago et al., 1996) or (ii) an *autoreactive mechanism* correlated to molecular mimicry between parasite and host molecules (Van Voorhis and Eisen, 1989; Levitus et al., 1991; Aznar et al., 1995; Ferrari et al., 1995; Belloti et al., 1996; Kalil and Cunha-Neto, 1996; Kaplan et al., 1997) (for review see Gironès et al., 2005). The first hypothesis is partially supported by the decrease of heart disease following treatments that lead to disappearance of parasites located in heart tissues (Viotti et al., 1994; Bahia-Oliveira et al., 2000) (for review see Urbina and Docampo, 2003) and indirectly by the relationship between high levels of acute parasitemia and evolution of the chronic phase toward chronic Chagas heart disease. The second one is supported by the findings of epitopes common to *T. cruzi* and host molecules involved in the heart function or nerve tissues (Levin et al., 1989; Van Voorhis and Eisen, 1989; Levin et al., 1990; Mesri et al., 1990; Kerner et al., 1991; Levitus et al., 1991; Cunha-Neto et al., 1995, 1996). Indeed, antibodies or T cells specific for parasite molecules bearing common epitopes to the host cells ("molecular mimicry") have been described and sometimes associated to the chronic pathology of Chagas disease (Sterin-Borda et al., 1988; Levin et al., 1990; Mesri et al., 1990; Levitus et al., 1991; Cunha-Neto et al., 1996; Gironès et al., 2001).

Some data support that the pathology would rely on a boosted specific immune response triggered by the parasites present in tissue lesions. Vitelli-Avelar et al. (2005) have observed that symptomatic chagasic patients harbored less NK cells than asymptomatic infected individuals. As NK cells help limiting the parasite load (see Chapter 24), their decrease might contribute to slightly enhance tissue parasite numbers. This could indirectly lead to the development of a stronger specific lymphocyte immune response, boosted by parasites. Additionally, they reported a decrease of regulatory $CD4^+CD25^+$ ("Treg") and NKT cells in the circulation of chronically infected patients with cardiac or digestive pathology as compared with asymptomatic individuals (Vitelli-Avelar et al., 2005). Regulatory lymphocytes limit the action of other T lymphocytes during the chronic infection. Their decrease was associated with a slight increase of activated $CD8^+$ T cells. Focus has been put on $CD8^+$ T cells specific for T cell epitopes from different parasite proteins (Alvarez et al., 2008). These T cells are important for specific control of parasitized cells (Padilla et al., 2009). Enhanced $CD8^+$ T cells' activity could account for stronger cytotoxic activity and tissue damage. In accordance, tissue lesions are infiltrated with $CD8^+$ T cells (Reis et al., 1993). Besides, heart cross-reactive $CD8^+$ T cells generated during the infection likely contribute to worsen the tissue destruction (Cunha-Neto and Kalil, 2001). Indeed, T cell clones specific of a cross-reactive epitope between cardiac myosin and *T. cruzi* antigen B13 were identified (Cunha-Neto et al., 1996; Abel et al., 1997; Iwai et al., 2005) and may be associated with chronic Chagas heart disease. However, it is not clear if these $CD8^+$ T cells are directly responsible for pathology or for eliminating the parasitized cells, decreasing the parasite load.

Aside from this, Gomes et al. (2003) reported that $CD4^+$ T cells producing high levels of IFN-γ and low levels of IL-10 are present in patients with cardiac

disease and may be correlated with the development of severe form of chronic Chagas heart disease, though this result was not confirmed in another study of the same group (Fiuza et al., 2009). Similarly, Cunha-Neto and Kalil (2001) have described a higher production of IFN-γ by $CD8^+$ T cells from symptomatic patients. Abel et al. (2001) also suggested that the ability to mount a vigorous IFN-γ response may be associated with the development of cardiomyopathy. Thus, IFN-γ might display a dual role in the course of *T. cruzi* infection, protective during the acute phase (Bahia-Oliveira et al., 2000), at the contrary of what occurs in the chronic pathology. It would be interesting to study if the higher IFN-γ production associated with the pathology relates to the preferential expansion of some T cells expressing particular T cell receptor (TCR) beta chain (BV) families shown to occur in *T. cruzi* infection (Sunnemak et al., 1988; Leite de Moraes et al., 1994a; Anez et al., 1999; Costa et al., 2000; Fernandez-Mestre et al., 2002; Tekiel et al., 2005), and of which the production of cytokine may be regulated in a tissue-specific way and participate in tissue parasite persistence (Vogt et al., 2008). In addition, a higher proportion of IFN-γ-producing circulating T lymphocytes from patients with cardiac disorders expressed the chemokine receptor CCR5, known to bind chemokines like MIP1-α (CCL3), MIP1-β (CCL4), and RANTES (CCL5) (Gomes et al., 2005). This should allow higher infiltration of these lymphocytes into parasitized tissues known to express the ligand chemokines. In these cases also, the studies do not allow to conclude to a pathological effect of these circulating lymphocytes.

The stronger activity of parasite specific and autoreactive lymphocytes in patients with heart failure is coupled with increased antigen-induced apoptosis resulting from increased expression of TNF-α and Fas-FasL on lymphocytes, and lower proliferative capacity of lymphocytes (Rodrigues et al., 2008). Lymphocyte apoptosis is known to trigger alternative activation of macrophages, rendering them less able to control parasite multiplication and even appropriate to favor parasite growth (see Chapter 24 and Peluffo et al., 2004; Noel et al., 2004). Moreover, TGF-β produced by these macrophages is suspected to participate in heart fibrosis and in the hypertrophic response of cardiomyocytes (Araujo-Jorge et al., 2008). Altogether, this will still worsen the situation. Finally, in the most severely affected patients, the capacity to produce IFN-γ is nearly abolished (Laucella et al., 2004). This might be linked to exhaustion of memory ($CD45RO^+$ $CCR7^+$) $CD4^+$ T cells assumed to occur in severe chagasic cardiopathy, resulting in the loss for these T cells of their ability to respond to specific *T. cruzi* stimulation (Fiuza et al., 2009).

While the immune response in patients suffering from Chagas disease becomes more and more characterized, studies on the global profile of cytokines produced by lymphocytes from *T. cruzi*-infected humans either asymptomatic or with cardiac dysfunctions (Vitelli-Avelar et al., 2008), reviewed in Dutra et al. (2005), are sometimes contradictory and difficult to integrate in a comprehensive model of immune deregulations leading to pathology. Knowledge of the underlining mechanisms associated with the establishment and maintenance of these distinct clinical outcomes is still missing.

25.3 The Mouse Model of *T. cruzi* Infection

First of all, mouse model reproduces a great number of features of Chagas disease: acute and chronic stages, polyclonal activation, specific antibodies, CD8 protective T cells, autoreactivity and autoantibodies, and inflammatory pathology in different tissues and organs. Although, the mouse model is not totally correlated to the human Chagas disease, it may be very useful to help formulate a hypothesis relevant to the disease. We will describe the different domains in which the mouse model is very close to the human disease and useful to approach mechanisms of chronic pathology.

25.3.1 Polyclonal B and T Cell Activation

One of the most important immune disorder occurring rapidly after the entry of the parasite in the host is the large *polyclonal activation* (Ortiz-Ortiz et al., 1980; D'Imperio Lima et al., 1985, 1986; Minoprio et al., 1986a,b). This massive nonspecific response might be responsible for the first step of autoreactive B cells induction (Minoprio et al., 1988). $CD5^+$ B cells are increased and play a role in the induction of this polyclonal activity as deficiency in these cells increases resistance of mice to the infection (Minoprio et al., 1991). Activation of $CD5^+$ B cells was shown in humans (Dutra et al., 2000). Interestingly, this nonspecific B cell response is accompanied by a specific anti-*T. cruzi* IgM and IgG response able to exert the first step of parasite control by decrease of parasitemia (El Bouhdidi et al., 1994). T cells are also concerned by polyclonal activation (Minoprio et al., 1986a,b, 1989a,b). Cytotoxic T cells, nonspecific for the parasite might be the first step in lyzing cells after autoreactive recognition of self-peptides on host cells (Gironès et al., 2007). It is also worth noting that such important T cell activation *in vivo* may be responsible for the unresponsiveness observed *in vitro* as measured, for example, by production of or response to IL-2 (Harel-Bellan et al., 1983, 1985; Bandeira et al., 1987).

The expression of MHC-class II antigens is increased in spleen, lymph nodes, and peritoneum of the infected mice, during acute and chronic phase of experimental infection (Behbehani et al., 1981; Spinella et al., 1989). These class II molecules are mainly expressed on large activated B cells that secrete Ig2a and IgG2b while conventional autoantibodies are generally of IgM isotype (D'Imperio Lima et al., 1985, 1986; Spinella et al., 1992). This is in accordance with our previous work on polyclonal B cell activation (Minoprio et al., 1988). Treatment of mice with an anti-MHC class II (Ia molecules) antibody drastically decreased the production of IgG2a and IgG2b but not of IgM autoantibodies (Spinella et al., 1989). Moreover, the anti-*T. cruzi* specific antibody response was totally abolished and associated with a higher parasitemia, confirming the role of specific antibodies to control the parasites (Krettli and Brener, 1976). The intense expression of MHC-class II molecules on activated B cells as well as on skeletal muscle fibers could play a role in their capacity to present antigenic epitopes to CD4 T cells, specific of *T. cruzi* or of cross-reactive host molecules (Krieger et al., 1985; Janeway et al., 1987; Cabeza Meckert et al., 1991). Altogether, these data are in accordance with a role for $CD4^+$ T cells in the tissue pathology of chronic *T. cruzi* infection.

Recently, parasite molecules with a potential to activate B cells have been cloned and characterized (Hernandez-Munain et al., 1992; Cordeiro da Silva et al., 1998; Montes et al., 1999; Reina-San-Martin et al., 2000; Gao et al., 2002). Most of them are surface-secreted parasite molecules. Particularly, one is a proline racemase (Reina-San-Martin et al., 2000) and the other is a *trans*-sialidase (Gao et al., 2002). Actually, the B cell activators are related to high level of secretion of Ig, mainly autoreactive IgG and also IgM. Hypergammaglobulinemia composed of a majority of IgG directed against natural antigens (Coutinho et al., 1995) may have further pathological consequences. Indeed, abolition of polyclonal lymphocyte activation correlates with resistance to infection and pathology development of the acute phase (Minoprio et al., 1987, 1991, 1993; Santos Lima and Minoprio, 1996). Although treatment by anti-CD4 T cells was shown to abolish the B cell polyclonal activation, data are not available on the interactions between T cells and B cell polyclonal activation during acute or chronic stages of the infection. However, in the context of an increase of MHC-class II antigens on B cells and possible presentation of host's epitopes (cross-reactive epitopes between host and parasite molecules), aberrant antibody responses can emerge during indeterminate or chronic phase as recently described in humans. Indeed, molecular mimicry may be a mechanism that induces autoreactive antibodies with pathological effects during the chronic phase of the *T. cruzi* infection. The following cross-reactive antigens have been described in the sera of chronic patients as well as of chronically infected mice. A parasite antigen FL-160 carries common epitope with mammalian nervous tissue (Van Voorhis and Eisen, 1989). Antibodies against a ribosomal P protein of *T. cruzi* recognize an epitope of the second loop of β1-adrenergic receptor on cardiocytes (Levin, 1991; Levitus et al., 1991; Ferrari et al., 1995). These antibodies have a physiological role on cardiocytes by favoring their beating (Ferrari et al., 1995; Kaplan et al., 1997; Sepulveda et al., 2000; Malher et al., 2001). Antibodies against cardiac myosin are able to recognize the parasite antigen B13 (Cunha-Neto et al., 1995). Another common epitope was described by Gironès et al. (2001), named Cha, shared by two *T. cruzi* proteins, SAPA and TENU2845. It has the property to induce IgG antibodies in a T-cell dependent manner, arguing for a molecular mimicry dependent response.

25.3.2 Acute Pathology

Acute infection of different strains of mice by *T. cruzi* is characterized by parasitemia and inflammatory reactions in tissues where the parasites are multiplying intracellularly (Russo et al. 1989). Infiltrating cells are mainly composed of neutrophils and differ from the granulomatous infiltrates of the chronic phase. On the contrary of what is occurring in humans, acute phase may be lethal for several strains of mice, named susceptible. Overproduction of TNF-α has been involved in this susceptibility (Starobinas et al., 1991; Truyens et al., 1995) while IFN-γ was associated to resistance to infection (Torrico et al., 1991; Minoprio et al., 1993; Abrahamson and Coffman, 1996).

The entry of the parasite into the mouse triggered off an important remodeling of the lymphoid organs. B and T cells activation results in splenomegaly and

swelling of peripheral and subcutaneous lymph nodes (Minoprio et al., 1986b; Russo et al., 1996; De Meis et al., 2009). However, the thymus, a primary lymphoid organ parasitized by *T. cruzi*, rapidly enters in atrophy with loss of thymus weight, decrease in cell number, and depletion of double positive $CD4^+CD8^+$ thymocytes (Savino et al., 1989; Leite de Moraes et al., 1991, 1992). These changes are accompanied with high levels of IL-10 and IFN-γ (Leite de Moraes et al., 1994b). Altered expression of galectin-3, fibronectin or chemokines CXCL12 and CCL4, extracellular ATP as well as increased levels of endogenous glucocorticoids are reported to explain the thymus atrophy in *T. cruzi* experimental infection (Mantuano-Barradas et al., 2003; Mendes-da-Cruz et al., 2006; Silva-Monteiro et al., 2007; Pérez et al., 2007) (for review see De Meis et al., 2009). It has also been reported that the *T. cruzi trans*-sialidase has the capacity to promote apoptosis of double positive thymocytes (Leguizamon et al., 1999). Equally, atrophy of mesenteric lymph nodes occurred during acute *T. cruzi* infection with decreased B and T lymphocyte numbers (Antunez et al., 1997; De Meis et al., 2006). As parasites multiply in all these lymphoid organs, it is interesting to remind that trypanocidal treatment of infected mice during acute infection prevented thymic atrophy and decrease of double positive thymocytes (Olivieri et al., 2005). That is a strong argument for the role of *T. cruzi* in inducing thymus' remodeling. Until now, no data are available on the mechanisms of this atrophy or on the responsible parasite molecules. An important observation during acute phase of *T. cruzi* infection is the inhibition of IL-2 production, a crucial growth factor for T lymphocytes, in spleen but not in peripheral lymph nodes (Harel-Bellan et al., 1983). However, it is not clear if suppression of IL-2 production measured *in vitro* depends on the regulation by $CD25^{hi}CD4^+$ Treg cells *in vivo* or on anergy presented by *in vivo* activated T cells when they are stimulated *in vitro*.

25.3.3 $CD4^+$ T Cell-Dependent Chronic Pathology

In mice, the acute phase lasts 2 months as we measured the apparition of chronic phase by diminution of parasitemia and persistence of intracellular parasites (Ben Younes Chennoufi et al., 1988a), restoration of the IL-2 response (Harel-Bellan et al., 1985), regeneration of thymus (Leite de Moraes et al., 1992), resistance to reinfection, apparition of granulomatous infiltrates in organs and tissues (Barreira et al., 1982; Saïd et al., 1985; Ben Younes Chennoufi et al., 1988b). While $CD4^+$ T cells have a protective role in the acute phase of the infection (Minoprio et al., 1987, Russo et al., 1988a,b, 1996; Rottenberg et al., 1990; Tarleton et al., 1994), they play a pathological role in mouse chronic infection (Hontebeyrie-Joskowicz et al., 1987; Gironès et al., 2007). We need to be very careful with the definition of the chronic stage in mouse. Every model using one strain of parasite and one strain of mouse should be precisely defined as a pathological model. There are some reports where it is clear that some confusion exists between pathology of acute phase and of chronic phase that are so different. In our model, 60 days postinfection represented the breaking point between the two phases as measured by restoration of IL-2 production, recovery of thymus, resistance to reinfection, etc. So, we designed

peripheral neuropathy in mouse infected by *T. cruzi* (Saïd et al., 1985) and were able to passively transfer it by inoculation of $CD4^+$ T cells (Hontebeyrie-Joskowicz et al., 1987) isolated from lymph nodes or circulating blood. Isolation from peripheral blood was consistent with the need for these T cells to reach the tissue or organ where they may exert their pathological effect. These T cells acted through a specific *T. cruzi* delayed-type hypersensitivity (DTH) reaction. Further, $CD4^+$ T cell lines against *T. cruzi* or peripheral nerve extract were established and shown to mediate a DTH reaction against both *T. cruzi* and peripheral nerve extracts, suggesting common epitopes between the parasite and the peripheral nerve tissue. Moreover, when these CD4 T cell lines were inoculated into the sciatic nerve of a normal mouse, they provoked inflammatory granulomas and typical demyelination as observed in chronically infected mice (Saïd et al., 1985; Hontebeyrie-Joskowicz et al., 1987). One of these T cells line, named G-05, was further characterized by the secreted cytokines and shown to be of Th2 type (Spinella et al., 1990). Interestingly, by passive transfer to naïve mice, G-05 induced a B cell polyclonal activation with an isotypic pattern similar to that described in infected mice (Minoprio et al., 1986b; Spinella et al., 1992). Altogether, these results are in favor of the development of aberrant T cells during the chronic phase of *T. cruzi* infection able to reproduce their pathological features in presence of IL-4 and IL-10. However, as a rule, DTH reactions are generally mediated by Th1 cells secreting IFN-γ, but not by Th2 cells (Hontebeyrie-Joskowicz, 1991). We may wonder if this discrepancy (DTH mediated by Th2 T cells) would be a peculiar consequence of chronic *T. cruzi* infection allowing aberrant T cells to emerge to maintain the chronic inflammatory granulomatous infiltrates. This remains to be confirmed. Also, the molecular characterization of the epitope bound to the TCR of this T cell line has not been achieved. However, we assume that this is not the main point: probably the junction epitope−ligand (MHC-class II-TCR) is weak, allowing a degenerated recognition of host and parasite molecules and the activation of autoreactive cells by cross-reaction between parasite and self-epitopes. The essential point lies in the characteristics of the TCR and of the secreted cytokines defining the G-05 line. It is essential to keep in mind that atrophy of thymus during the acute phase and its recovery during chronic phase may have important consequences in the establishment and/or maintenance of the potentially new T cell repertoire of the chronic stage, both at the thymic and peripheral levels (Correia-Neves et al., 2001).

Another approach of the role of $CD4^+$ T cells in the pathological response of chronically infected mice has been developed by Ribeiro dos Santos et al. (1992). Grafts of syngeneic newborn hearts are totally accepted by normal recipients while they are rejected in mice chronically infected with *T. cruzi*. This rejection is dependent on $CD4^+$ T cells. More recently, they developed a $CD4^+$ T cell line from infected mice, specific to heart and *T. cruzi* antigens and able to induce heart inflammation and syngeneic heart transplant rejection (Ribeiro-Dos-Santos et al., 2001). Unlike our results, these T cell lines were of Th1 type, secreting a large quantity of IFN-γ and IL-2, cytokines generally related to protection. The most relevant difference may be the inability of these Th1 T cell lines to migrate into the recipient mouse, requiring inoculation into an infected or immunized nu/nu mouse.

We remind that in our experiments, the T cell lines were isolated from blood or lymph nodes and were able to circulate after intravenous inoculation into normal naïve recipients. Nevertheless, these results together indicate a role for autoreactive $CD4^+$ T cells in the chronic pathology of Chagas disease as shown also in humans through molecular mimicry between cardiac myosin and B13-antigen (Cunha-Neto et al., 1996; Abel et al., 1997).

25.4 Strains of Parasite and Pathology

T. cruzi remains the first actor of Chagas disease and in this sense it is obvious it has an important role to play in the commitment of the pathological symptoms. The story begins in the 1980s with the discovery through analysis of isozymic polymorphism of remarkable genetic variability among *T. cruzi* strains isolated from vectors, mammals, and humans. Then, these studies showed that *T. cruzi* evolved mainly clonally, and the strains were divided into two main lineages *T. cruzi* I and *T. cruzi* II (see Part 4 The causative agent: *T. cruzi)*. The genetic distances were so great between the two lineages that several authors suggested the hypothesis of an impact of the genetic variability of *T. cruzi* on the development of the disease and clinical forms: acute, asymptomatic, cardiac, and digestive. Firstly, in Venezuela (Miles et al., 1981), where Chagas disease is mainly asymptomatic, it was found that *T. cruzi* "Z1" predominated (lineage *T. cruzi* I) and was radically differed from "Z2" (lineage *T. cruzi* II) found in patients from Central and Eastern Brazil, which usually suffered of "mega" syndromes, cardiac and/or digestive. According to the new nomenclature, six discrete typing units (DTU) were designed, DTUs TcI−TcVI, Z1 corresponding to TcI and Z2 found in Brazilian patients to TcII (Zingales et al., 2009). In Bolivia, a different situation was described. Strains belonging to the two lineages were found circulating sympatrically, but none of the DTUs (TcI and TcV) had a special relationship with the clinical forms: asymptomatic, cardiac, or digestive pathologies (Brenière et al., 1985, 1989). Similarly, the same DTUs (TcI and TcV) were described in domestic cycle in Argentina, but there, the results suggested a differential clinical prognostic, being TcV derived infections more benign than those with TcI. Particularly, the risk of cardiac lesion was greater for patients infected by TcI than by TcV (Montamat et al., 1996; Blanco and Montamat, 1998). In Chile, molecular typing was conducted among 23 acute and chronic patients where TcV was mainly detected after isolation of the strains, whatever their clinical status, showing a low correlation between the *T. cruzi* strain and the clinical prognosis of Chagas disease (Muñoz et al., 1994). Later, the observation in Bolivia of patients with mixture of *T. cruzi* clones and the demonstration of strain selection (TcI over TcV) during the isolation process (Bosseno et al., 2000) has lead the authors to develop another typing strategy and identifying the DTUs directly in blood without the isolation and culture steps. The detection of *T. cruzi* strains by PCR followed by molecular typing was introduced and applied in a Bolivian hyperendemic region where TcI and TcV circulate in equal proportions in the vector *T. infestans*. Unexpected results were observed: TcV was selected over TcI in the

blood whatever might be the age of the patient, except in acute cases (parasites detected in blood by microscopy), where TcI and TcV had equal proportions than in vectors (Brenière et al., 2002). Interestingly, previous works carried out in Brazil showed similar frequencies of TcI and TcII in acute patients whatever the clinical forms, while TcII was the only strain detected in chronic patients (Luquetti et al., 1986). The two works agreed showing that, in a sympatric context, the strains of both lineages infected humans, but that strains belonging to *T. cruzi* II lineage only, persisted in the bloodstream during asymptomatic and chronic infections. Moreover, in Bolivian pregnant women, DTU TcV only was identified and this DTU was detected in congenital cases (Virreira et al., 2007).

From the point of view of strict pathology, the debate remains open with little literary support. However, a recent study of 37 strains isolated from chronic chagasic cardiopathic and noncardiopathic patients in Chile shows no association of cardiac disease with TcI while TcV and/or TcVI (DTUs belonging to *T. cruzi* II lineage) were significantly associated with noncardiopathic patients (Venegas et al., 2009). Based on the current researches it is impossible to conclude. However, strains belonging to TcI could be regarded as more likely to cause heart disease than strains belonging to *T. cruzi* II lineage. Moreover, strains of *T cruzi* II lineage were detected in endemic areas where digestive pathology is important while this pathology is scarcely reported in the north of the Amazon where mainly DTU TcI is circulating in domestic and sylvatic cycles. The story is not over; several factors make these studies difficult: (i) the methods to detect and identify DTUs do not detect all the strains that infect a patient (intracellular and circulating forms), (ii) genetic diversity is such, especially in the TcI DTU, as differences in virulence and pathogenesis are likely within the same DTU (see e.g., Brenière et al., 1989), (iii) the consequences of multiple infections of several *T. cruzi* clones of same DTU or different ones are unknown, and (iv) similarly, the outcome of reinfection (highly likely in hyperendemic area) is ignored. In the absence of appropriate identification of *T. cruzi* virulence factors (many factors have been described), of their differential expression in DTUs and of mechanisms involved in pathogenesis, the study of the consequences of genetic variability strains of *T. cruzi* on the clinical prognosis of the disease remains difficult.

25.5 Perspectives

Since more than 30 years, the hypothesis of autoimmunity is considered for chronic Chagas disease (Cossio et al., 1974; Acosta and Santos-Buch, 1985; Schmuñis, 1987; Kierszenbaum, 1989; Eisen and Kahn, 1991; Engman and Leon, 2002; Tarleton, 2003), especially for the most severe manifestation: the chronic Chagas heart disease. Actually, many studies were performed to sustain this hypothesis and successful outcomes reached to demonstrate molecular mimicry and presence of autoreactive T and B cells with potential pathological impact.

Really, nobody may be unaware of the parasite, *T. cruzi*, as the first and main component responsible for Chagas disease. Treatment of acute infection, if the

parasite strain is sensitive to the drug, leads to complete cure of the infected patient without any aftereffect. It is the best evidence for the role of *T. cruzi* in Chagas disease. Unfortunately, diagnosis of acute infection is rare because people often are unaware of the disease, due to general nonspecific clinical signs. Moreover, the available drugs are sometimes ineffective because of the resistance of certain strains. So, the first goals are (i) to develop easy and cheap methods of diagnosis accompanied with appropriate education, mainly at school for the children, and (ii) to develop research for new drugs. A peculiar interest has to be carried out to negative serology in patients with evidence of the disease (Tarleton et al., 2007).

Another possible approach to control acute phase of *T. cruzi* infection is the search for vaccine candidate(s). Today several parasite molecules have been improved in research of vaccine candidates, although it will be very difficult to test the validity of a vaccine faced with a chronic disease that is lasting for the lifetime. Correlation between decrease of parasitemia during acute phase and decrease of severity of the clinical signs in chronic phase may be an encouraging result for vaccination. The assay performed in mouse by Garg and Tarleton with TS genes supports this hypothesis (Garg and Tarleton, 2002).

However, most of infected people to date are chronically infected or in indeterminate phase or, for 30−40% of them with severe cardiomyopathy. It is important to improve our knowledge of the mechanisms of this severe pathology in order to find the better treatment or prevention of the chronic Chagas heart disease. We are convinced that the polyclonal activation, and disturbances at the level of lymphoid organs induced by parasite molecules in the early acute phase are crucial points for the development of the chronic pathology. Based on this, to eliminate the parasites (or at least reduce the acute parasite burden) before they have the capacity to persistently multiply in the host's tissues or organs, it is possible to consider to vaccine against parasite molecules responsible for the polyclonal activation. Two molecules correspond to this definition and both are surface proteins shed into the bloodstream: the proline racemase (TcPRAC or TcPA45) (Reina-San-Martin et al., 2000; Minoprio, 2001) and the *trans*-sialidase (TS) (Gao et al., 2002). Recently, Bryan and Norris (2010) showed the possibility to induce a specific immune response against TcPRAC that prevented mitogenic activation of B cells without limiting the parasite-specific immune response. Coimmunization with TcPRAC and with another parasite molecule necessary for invasion (CRP, complement regulatory protein) is possible. This opens a novel strategy of immune-protection against *T. cruzi* with several molecules. It will be interesting to study parasites KO for the polyclonal activators to better understand the real impact of their action on the development of chronic stage of the disease, and particularly of the possible activation of autoreactive T cells.

Knowing the key molecules inducing the polyclonal activation in the acute phase, it is of first importance to understand what are the key events that allow chronic parasite persistence. To survive and establish chronic infection, the parasite has to evade from the host's immune system and to delay the specific responses to maintain itself as long as possible. Little attention has been paid for the crucial changes in lymphoid organs at the first step of the disease (acute phase). We know by the works of Savino's group that thymus and mesenteric lymph nodes are subjected to a

severe atrophy during the acute phase of infection in mouse. These two organs are the place for maturation of T cells and CD5 B cells, respectively. Recovery of these organs will take place in an organism that is infected by *T. cruzi*, with an immune system polyclonally activated, and in presence of tissue's destruction. An important question is to know how autoreactive T cells may emerge, that is, what are the mechanisms allowing the maturation and selection of an aberrant T cell repertoire leading to an autoimmune-like manifestation. Are these aberrant T cells selected at the beginning of the chronic stage? What is the step that will determinate the commitment to chronic disease versus the persistence of an indeterminate stage?

In conclusion, the complexity of interactions between the mammalian host and *T. cruzi* needs further studies to elucidate the multifactorial process to chronic Chagas disease depending (i) on the parasite and its genomic heterogeneity and (ii) on host genetic factors as well as environmental factors. Studies in experimental infection should be better designed and focused on very few standards considering the strain of mouse and the strain of *T. cruzi*. Too many reports differ on these two parameters rendering difficult the comparison of the results and giving a vague image of the mechanisms underlying the *T. cruzi*-induced pathology.

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26 Human Genetic Susceptibility to Chagas Disease

*Michel Tibayrenc**

Institut de Recherche pour le Développement (IRD)/Centre National de la Recherche Scientifique (CNRS), Montpellier, France

26.1 Introduction of Key Concepts

- This chapter discusses some specific key concepts in the field that are still under debate. The most important of these is that it is misleading to focus exclusively on those **genes** that are assumed *a priori* to be more directly linked with susceptibility to Chagas disease (candi date genes) without considering the general genetic background of the individuals or the populations under survey. It is more desirable to draw a general population genetic frame work of the populations under study, and then to try and identify the genes of interest and to follow their functioning and dynamics within that framework. This approach has been successfully applied to the study of pathogenic agents (see, e.g., Tibayrenc, 1995).
- Susceptibility to infectious diseases is a **phenotype**. Generally speaking, we know very lit tle about how **genotypes** generate phenotypes. As a matter of fact, the overall functioning of the human genome still is poorly understood in many species. Indeed, in the present state of knowledge, even knowing the whole sequence of a genome is poorly informative about the way this genome generates given phenotypes. As an example, knowing the whole sequence of the human genome (Craig Venter et al., 2001) and of the chimpanzee genome (The Chimpanzee Sequencing and Analysis Consortium, 2005) gives little expla nation about why the phenotypes of humans and chimpanzees are so different. Only 1.23% of base pairs are different between the two genomes. This means that very limited sequence differences are compatible with drastic phenotypic differences. Possibly major genomic rearrangements (MGRs) play an important role in these phenotypic differences (see below).
- MGRs (macro deletions and macro duplications) are indeed now suspected to play a major role in phenotypic expression (Check, 2005) in general (either normal or pathologi cal) and in susceptibility to transmissible diseases in particular.
- It is probable that genetic susceptibility to Chagas disease and to other infectious dis eases, like many phenotypic characteristics, generally involves multigene mechanisms rather than isolated genes.
- Even if we knew the whole sequence of all the genes involved, this does not mean that we could understand the precise way they command susceptibility to Chagas diseases.

* E mail: Michel.Tibayrenc@ird.fr

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Indeed, interaction between the many genes involved and between this genetic background and environmental parameters could be very complex.

- Geographic and climatic zones have a huge impact on the spread of transmissible diseases, since they drive the distribution of pathogen species. This effect should be amplified for vector borne diseases, including Chagas disease. As a matter of fact, geography and climate also have a considerable impact on vector distribution.
- When human genetic susceptibility to infectious diseases in general, and Chagas disease in particular, are considered, the population level is as or more important to survey than the individual level. As a matter of fact, the target of **natural selection** is populations as much as or more than individuals. The medical relevance of race/ethnic diversity is a subject of hot debate (Cho and Sankar, 2004). However, many scientists (Jorde and Wooding, 2004) consider that this is quite relevant. This could especially be the case for transmissible diseases, including Chagas disease.
- Population genomics is the new challenging discipline aiming at combining the necessity of the population scale and the high resolution power of **genomics**. This is made more and more accessible thanks to the improvement of genomic technology at lower costs. A further desirable step is to include genetic expression at this approach (population proteomics). This is the very goal of the Human Variome Project (http://www.humanvariomeproject.org/).
- Lastly, while exploring the genetic characteristics of the host (and the pathogen, and the vector in vector borne diseases) involved in the transmission and severity of infectious diseases, it is vitally important to analyze the role played by the environment. It is generally considerable, and socioeconomic factors are a leading cause of epidemics. This is especially true for Chagas disease, which has been identified as a disease of underdevelopment (Gentillini, 1999). It is therefore indispensable to explore the respective role of the innate and the environment. This is not easy, for environmental causes could mimic genetic causes, and they considerably interact.

The above-summarized concepts, many of which have already been previously exposed (Tibayrenc, 2007), lead to drastically different approaches than the classical approach relying on analysis of a few "candidate genes," chiefly at the individual level.

26.2 General Recalls about Research on Human Genetic Susceptibility to Infectious Diseases

26.2.1 "Classical" Approach

It is hypothesized that there are genetic differences among individuals in terms of susceptibility to transmissible diseases. This hypothesis is explored by analyzing the "sibling risk" (ls): the statistical risk for the sibling to exhibit a given pathology when the other sibling does. In the case of transmissible diseases, ls is lower than in the case of autoimmune diseases such as type I diabetes (Cooke and Hills, 2001), but it is not negligible. This genetic component is by definition the **heritability** of susceptibility to infectious diseases.

The two main approaches to identify the responsible genes are (i) candidate genes and (ii) linkage studies. In the candidate gene approach, a given gene or

family of genes is suspected because it is associated with biological mechanisms that are frequently involved in infectious processes. Genes involved in immunological processes are frequently candidate genes. This is most specifically the case for the genes that code for **cytokine** molecules. Genes of the **major histocompatibility complex** (MHC; **human leukocyte antigens** (HLAs) in humans) are very often taken as candidate genes when searching for the heritability of transmissible diseases. This is definitely the case for Chagas disease.

Other putative candidate genes are those that exhibit a similar function in animal models. However, frequently, comparable **gene sequences** sometimes commend drastically different functions in different animal species (Blackwell, 1996).

In the linkage approach, a specific region of the genome is identified throughout genome mapping with a broad set of **microsatellite** or other high-resolution markers such as **single-nucleotide polymorphisms (SNPs)** and linkage analyses based on twin sib pair/family/pedigree studies. This kind of high throughput screening is becoming easier and cheaper, and the number of microsatellite and SNP markers available is now high (see below: the Hap Map project). The progress of **bioinformatics** is of considerable help in analyzing the results of linkage studies. The null hypothesis is a total lack of **linkage disequilibrium**, which shows random **recombination** between the marker used and the phenotype explored (here genetic susceptibility to infectious diseases, more specifically to Chagas disease). If the recombination rate is significantly lower than 0.5, the linkage hypothesis is retained. This shows that the region identified by this molecular screening contains genes that are involved in the pathological trait considered. Unfortunately, such candidate regions could harbor hundreds of genes.

To explore linkage, **nonparametric tests** have the advantage of not being dependent upon a working hypothesis on genetic inheritance. However, their resolution power is lesser than that of parametric tests. The more commonly used parametric linkage test is the lod (logarithm of the odds) score (Morton, 1955).

Lod = $\log_{10}$ (probability of data if disease and marker are linked)/(probability of data if disease and marker recombine freely).

26.2.2 The HLA System, a Gold Mine for Candidate Genes

The HLA system is the name of the MHC in humans. The HLA genes are a "**superlocus**" of chromosome 6. HLA genes code for **antigens** (Ag) that specifically interact with **T cells** to expose foreign antigens to the immune system. One distinguishes HLA Ags of classes I, II, and III. Class I Ags are labeled A, B, and C. They specifically interact with $CD8^+$ T cells ("killer" T cells). Class II Ags comprise DP, DM, DOA, DOB, DQ, and DR Ags. They specifically interact with $CD4^+$ "helper" T cells. Class III Ags interact with the complement system.

The HLA genes are by far the most variable genes of the human genome (at least when coding sequences are concerned). Each gene could have as many as hundreds of different **alleles**. Intense selective pressure due to infectious diseases and, more specifically, **balanced selection** are generally invoked to explain this impressive variability, which survived the important **bottleneck effect** undergone

by the human species 150,000 years ago. An alternative explanation is **sexual selection**: females could be able to identify males who harbor HLA genes similar to their own HLA genes (Wedekind et al., 1995). The strong selective pressure undergone by HLA genes makes some human HLA alleles look genetically more similar to some *Macacus* monkey alleles than to other human alleles (Ayala and Escalante, 1996). The **coalescence** time of such alleles is ancient, prior to the divergence among Catarrhines (Old World monkeys).

HLA typing traditionally relied on serotyping. However gene sequencing has greatly increased the resolution power of this typing. The nomenclature of HLA alleles is constantly updated by an international consortium (see IMGT/HLA Database; http://www.ebi.ac.uk/imgt/hla/). The coding is as follows: HLA-name of the **locus** numbers that specify the allele (Marsh et al., 2005).

A novel approach called sequence feature variant type (SFVT) has been proposed by Karp et al. (2010). It classifies HLA Ags into biologically relevant smaller sequence features (SFs) and their variant types (VTs). SFs correspond to combinations of amino acid sites defined according to structural and functional information and polymorphism. VTs for each SF are defined according to all recorded polymorphisms in the HLA locus considered.

26.2.3 Problems Encountered in Analyzing Human Genetic Susceptibility to Infectious Disease

The ideal conditions to identify the genes responsible for a given disease are as follows: (i) when the pathological trait (phenotype) is clearly defined. This is precisely the case for Chagas disease and for its different clinical manifestations; (ii) when the heritability is strong; (iii) when only one gene, or a few genes, are responsible of the trait; and (iv) when the Mendelian inheritance of the involved genes is known. Such favorable conditions are the exception rather than the rule (Cooke and Hill, 2001). It is probable that the genetic background of transmissible diseases (and of many other pathological processes) generally involves many genes, with complex interactions among them and between them and the environment. Also, in phenotypic expression, MGRs could play an important role, possibly more that the gene sequences themselves (see earlier; Check, 2005). Lastly, as underlined in the introduction, the individual approach could not be enough, and should be completed by studies at the population scale. Natural selection acts as much, or more at the population level than at the individual level. With infectious diseases, interaction phenomena among members of a given group play a major role. The term “herd immunity” (Anderson and May, 1990) has been coined to designate a group’s overall level of immune protection. Let us consider the case where almost all members of a human population are vaccinated against a given infectious disease. The few individuals who are not vaccinated are protected by the other individuals because epidemics cannot spread within the group. Such group phenomena most probably play an important role in the evolution and dynamics of genetic susceptibility to infectious diseases. Unfortunately, they are not sufficiently considered by the classical research on susceptibility genes. It is therefore crucial to consider

the population/ethnic level of analysis, because obviously different populations and ethnic groups, which constitute chiefly mating groups, have undergone different selective pressures (Tibayrenc, 2007).

26.3 State-of-the Art with Chagas Disease

26.3.1 Clinical/Phenotypic Diversity of Chagas Disease

Chagas disease should be a favorable scenario for the analysis of human genetic susceptibility. It strikes considerably diversified human populations of European, African, Amerindian, and mixed ancestry, which should make it possible to explore in depth the role played by population/ethnic diversity. Moreover, the well-defined clinical forms of the disease provides many different clearly defined phenotypic categories (Gentillini, 1999), which could be explored by linkage studies. The disease starts with an acute phase, which can be either asymptomatic, or symptomatic with a severe septicemic syndrome. The mortality rate at this stage is about 10%. Patients who have survived enter the undetermined phase, characterized by the facts that: (i) the parasite hides in the host's cells; (ii) the serological reactions (explored by indirect immunofluorescence and ELISA test) are generally positive; and (iii) symptoms are absent. Most of the patients remain asymptomatic. About 30% develop a symptomatic Chagas disease, of which the main clinical forms are: (i) cardiac (the majority of the cases; it leads to a severe cardiac insufficiency of bad prognosis); (ii) digestive (megacolon, megaesophagus, about 3% of the cases); and (iii) cardiac plus digestive. A limited percentage of symptomatic chagasic patients exhibit negative serological tests (Brenière et al., 1984). Some patients have electrocardiogram abnormalities and have no clinical symptoms. Lastly, there are considerable differences in susceptibility to antichagasic drugs from one patient to another (Toledo et al., 2003). There is, therefore, an abundant set of clearly defined clinical forms/phenotypic classes in Chagas disease. This ideal situation could match that of leprosy (Abel et al., 1998) for the possibility of defining clearly identified phenotypes. These different pathological phenotypes are not evenly distributed over the geographical era of distribution of Chagas disease. Different countries exhibit huge differences.

26.3.2 Results

In spite of these favorable conditions, our knowledge on the possible impact of human genetic diversity in the transmission of the disease and of its clinical forms is still limited. By comparison, the results for leishmaniasis are much more informative (Blackwell et al., 2009). Even the very cause of chronic Chagas disease is still debatable. Kierzenbaum (1985) has hypothesized that it was a pure autoimmune disease. However, it is now generally accepted that the parasite is still present in the host's cells even during the chronic phase of the disease and is the cause

of a chronic inflammatory response. This has been demonstrated by both the classical **xenodiagnosis** and PCR experiments. Obviously, in the search for genes of susceptibility, this debate is of considerable relevance.

To our knowledge, few people have taken advantage of the existence of clearly defined chagasic phenotypes to undergo linkage studies. The bulk of the results gathered about human genetic susceptibility to Chagas disease concern the analysis of candidate genes, with many studies dealing with genes of the HLA system.

26.3.3 General Heritability of Some Chagasic Traits

A Brazilian study has suggested a possible familial component in chagasic cardiopathy (Zicker et al., 1990). However, an Argentinean study has given a different result (Morini et al., 1994). Large extended pedigree analyses have shown an apparent heritability of some serological traits. Chagasic seropositivity heritability in Brazil is 0.556, which is high (Williams-Blangero et al., 1997). The levels of IgA and IgG in Brazilian patients also have a heritability of 0.33 (Barbossa et al., 1981). These results could confirm personal observations from some Bolivian regions, where 100% of the triatomine bugs collected (all *Triatoma infestans*) harbored the parasite, while 50% of 10-year-old children were still seronegative. All houses harbored hundreds of *T. infestans* at that time, and surely all children have been bitten hundreds or thousands of times since birth. However, only some of them had become chagasic, which suggests a genetic variability concerning the ability to become chagasic.

26.3.4 Role of the HLA System

As noted above, an important part of the results dealing with human genetic susceptibility to Chagas disease, especially the most recent ones, concerns the possible role of the HLA system. It is now known that this supergene is a major factor in the transmission, severity, and clinical diversity of Chagas disease.

The pioneering work by Apt (1988) concerned 124 Chilean seropositive patients, distributed into patients with chronic Chagas cardiomyopathy (CCC) and asymptomatic patients (ASY). Fernandes-Mestre et al. (1998) found a decreased frequency of DRB114 and DQB10303 in chagasic patients by comparison with controls, suggesting independent protective effects to the chronic infection in this population. There was also a higher frequency of DRB101, DRB108, and DQB10501 and a decreased frequency of DRB11501 in the CCC patients. Faé et al. (2000) found no significant association between HLA and Chagas infection. However, they found that males had a higher probability to exhibit CCC. This negative result has been falsified by many further studies. Layrisse et al. (2000), by comparing 113 seropositive patients (CCC versus ASY), found that the HLA-C03 allele was a risk factor for CCC.

Nieto et al. (2000) surveyed 172 Peruvian patients (85 sero (+) with CCC = 33, ASY = 52; 87 sero (+) controls) for the variability of the HLA-DRB1 and DQB1 genes. No **allelic frequency** differences were observed between CCC and ASY patients. On the other hand, the DRB114-DQB10301 haplotype was significantly

linked to seronegativity, which suggests that this haplotype has a protective role against Chagas disease.

Visentainer et al. (2002) compared 35 CCC patients with 72 control patients in Brazil. They limited their study to patients of Caucasian origin. Homogenizing the ethnic origin of the patients under survey lowers the risk of genetic stratification due to allelic frequency differences among different populations. They found a positive association between CCC and HLA-DR2.

Cruz-Robles et al. (2004) surveyed a total of 193 Mexican patients. Among them 66 were sero (+) (either CCC or ASY) and 127 were sero ( ) controls. Their results suggested that HLA alleles are linked to the development of chronic Chagas disease and CCC. HLA-DR4 and HLA-B39 alleles could be associated directly with the infection by the parasite, whereas HLA-DR16 could be a marker of susceptibility to CCC and HLA-A68 might be protective against CCC.

Moreno et al. (2004) surveyed 104 sero (+) patients and 60 sero ( ) controls. They looked for the presence of genotype and linkage disequilibrium on microsatellite loci linked to the HLA, interleukin (IL)-2, IL-2Rβ chain, IL-4, IL-10, and natural resistance-associated macrophage protein 1 (NRAMP1). Significant allelic frequency differences were observed between sero (+) patients and controls at the microsatellite loci HLA D6S291 and IL-10. The results suggested epistasis between the HLA and IL loci that could be linked to susceptibility to Chagas disease.

Ramasawmy et al. (2006a) surveyed the variants of BAT1, a putative anti-inflammatory gene (localized in the HLA class III region) in 154 CCC and 76 ASY patients, all sero (+). They found that some BAT1 were predictive of the occurrence of CCC.

The same authors (Ramasawmy et al., 2008) analyzed the variants in the promoter region of the human inhibitory κB-like (IKBL/NFKBIL1) gene. The gene pertains to the MHC class I region. A total of 245 patients (169 CCC and 76 ASY) were surveyed. Subjects who were **homozygous** for the 62A allele had a threefold risk of having CCC compared with those having the TT genotype. Moreover, the haplotype 262A 62A was prevalent in CCC patients.

Borrs et al. (2009) surveyed 152 Argentinean subjects (71 sero (+) individuals and 81 control individuals) for the polymorphism of the second **exon** of HLA-DRB1. The DRB11103 allele was prevalent in the control group, which suggests a protective role of this allele. On the other hand, DRB10409 and DRB11503 had a significantly higher frequency in sero (+) patients, and CCC patients showed a specific higher frequency of DRB11503.

26.3.5 Other Genetic Factors Involved in Genetic Susceptibility to Chagas Disease

Calzada et al. (2001a) compared 85 sero (+) patients (32 CCC and 53 ASY) with 87 sero ( ) controls. They found an association between the polymorphism of the CCR5 59029 promoter polymorphism and differential susceptibility to CCC. The same authors (Calzada et al., 2001b) studied 168 Peruvian patients (83 sero (+)

with 32 CCC and 51 ASY; 85 sero ( ) controls) for the polymorphism of the NRAMP1 gene. They found no differences between: (i) sero (+) and sero ( ) subjects; (ii) CCC versus ASY patients.

Messias-Reason et al. (2003) also took care of matching the ethnic and geographical origin of their subjects (see Visentainer et al., 2002). They surveyed 100 sero (+) individuals (CCC: 57; ASY: 43) and 100 sero ( ) controls. They found a positive association between CCC and complement C3 and BF allotypes, and a negative association between CCC and sero (+) on one hand, and the BFS haplotype. No significant links were found for the C3, BF, CAA, CAB, and C2 haplotypes.

Ramasawmy et al. (2006b) surveyed the monocyte chemoattractant protein-1 (CCL2/MCP-1) gene polymorphism in 245 sero (+) patients (169 CCC and 76 ASY). They found that subjects harboring the CCL2 2518AA genotype had a fourfold greater risk of developing CCC.

Flórez et al. (2006) explored the association between the IL1A, IL1B, and IL1RN gene polymorphisms and Chagas disease in 260 sero (+) Colombian patients (130 CCC and 130 ASY). They evidenced that the presence of the IL1B + 5810G allele was associated with an increased risk for CCC.

Drigo et al. (2007) surveyed 246 patients (166 CCC and 80 ASY), matched for age and geography. They found no association between pathology and tumor necrosis factor-α polymorphisms. This result was in disagreement with another study from the same authors (Drigo et al., 2006), which showed that CCC patients having the TNF2 or TNFa2 alleles have a significant shorter survival time compared to patients who harbor other alleles.

Similarly, Campelo et al. (2007) found that the TNFa2, TNFa7, TNFa8, TNFb2, TNFb4, TNFd5, TNFd7, and TNFe2 alleles were overrepresented, whereas the TNFb7 and TNFd3 alleles were underrepresented when 162 chagasic patients were compared with 221 control patients.

Robledo et al. (2007) found no association between Chagas disease and the variants of the protein tyrosine phosphatase nonreceptor 22 (PTPN22) gene in 316 chagasic patients versus 520 healthy controls in Peru and Colombia.

Zafra et al. (2007) analyzed the possible influence of a 3′ untranslated region (3′ UTR) polymorphism of the IL12B gene on Chagas disease in 460 Colombian subject (sero ( ): 200; sero (+): 260, with 130 CCC and 130 ASY). They found a significant higher frequency of the IL12B 3′ UTR CC genotype and of the IL12B 3′ UTR C allele in CCC patients.

The same authors (Zafra et al., 2008) analyzed the variability of toll-like receptor 2 and 4 genes in 475 Colombian patients (143 CCC, 132 ASY, 200 sero ( ) controls). No frequency differences were found between chagasic patients and controls.

Ramasawmy et al. (2007) explored the variability of the gene coding for lymphotoxin-α in 169 CCC patients and 76 ASY patients. Homozygosity for the LTA +80C and LTA +252G alleles was significantly more frequent in CCC patients than in ASY patients. Haplotype LTA +80A 252A appeared to have a protective effect on CCC, whereas haplotype LTA +80C 252G was linked to susceptibility to CCC.

Costa et al. (2009) found that the IL-10 gene polymorphism and IL-10 expression play an important role in the susceptibility to CCC. Calzada et al. (2009) explored the transforming growth factor beta 1 (TGFβ1) gene polymorphisms in 626 Peruvian and Colombian patients (CCC = 172; ASY = 175; sero ( ) controls = 279). The frequency of the high TGFβ1 producer genotype 10 C/C was significantly higher in chagasic patients than in control sero ( ) patients.

Ramasawmy et al. (2009) screened 169 CCC and 76 ASY patients for their variability in the MAL/TIRAP gene, which expresses an adaptor protein in the toll-like receptor pathway. Contrary to Zafra et al. (2008), who found no association between Chagas disease and genes of the toll-like receptor pathway, these authors evidenced a protective role against CCC of heterozygosity in the S180L variant of the gene under survey.

Cruz-Robles et al. (2009) surveyed 86 individuals sero (+) (58 CCC and 28 ASY) 50 individuals sero ( ) with idiopathic dilated cardiomyopathy (IDC) and 109 control individuals for the distribution of IL-1B and IL-1 receptor antagonist (IL-1RN) polymorphisms. Sero (+) individuals had a higher frequency of the CC genotype of the IL-1RN.4 polymorphism. CCC patients had an increased frequency of the C allele and of the CC genotype of this polymorphism.

26.4 Conclusion: Perspectives

At one time, research on human genetic susceptibility to Chagas disease was behind that of other transmissible diseases. This is less true today. Many valuable studies have been published in recent years, made easier by the impressive progress in genomic technology and bioinformatics. Many candidate genes, pertaining either to the HLA system or to other systems, have proved to play a role in genetic susceptibility to Chagas disease and to its clinical forms. Conversely, some negative results have been produced too. It will be worthwhile to go on with this search for putative candidate genes, which will be made easier thanks to the constant progress of genomics and bioinformatics.

Here are some proposals to launch research toward new avenues.

26.4.1 Getting Out of the Candidate Gene Approach

Most recent studies deal with candidate genes, either from the HLA system or from other genetic systems (interleukins, toll-like receptor pathway, lymphotoxin-∝). To my knowledge, no recent studies have relied on general association studies using general screening of the genome by a large set of microsatellites/SNP markers and looking for linkage between these markers and chagasic phenotypes. However, the fact that chagasic phenotypes are well defined along with progress in genomic mapping (Genome-Wide Association Studies) and bioinformatic analysis could make this approach powerful.

26.4.2 Possible Role of MGRs

As exposed in the introduction, in generating normal and pathological phenotypes, the sequences of the genes themselves could not be the only parameter to take into account. Major insertions/deletions/duplications/inversions could play a major role too (Check, 2005). This has been poorly explored in the case of infectious diseases. To my knowledge, no studies of this kind have been conducted in the case of Chagas disease.

26.4.3 Drawing First a General Population Genetics Framework

As exposed in the introduction, the direct analysis of candidate genes independently from the general genetic background of the population could lead to missing much relevant information. It could be informative to try to link candidate gene analyses and linkage studies with general population genetic approaches, and to associate the research on human genetic susceptibility to Chagas disease with the current international consortium researches such as the Hap Map (stands for International Human Haplotype Map) project (International Hap Map Consortium, 2003; http://www.hapmap.org/), the Human Genome Diversity Project (Cann et al., 2002; http://www.stanford.edu/group/morrinst/hgdp.html), or the Genographic Project sponsored by National Geographic (https://genographic.nationalgeographic.com/genographic/index.html).

26.4.4 Introducing the Parameter of Ethnic Diversity

Latin America is rich in ethnic diversity, with a large range of populations of Amerindian, African, Caucasian, and mixed ancestry. These different populations, who have been put together only at historical times, have undergone drastically different selective pressures in the past, chiefly from infectious diseases (Haldane, 1949). Where Chagas disease is concerned, it is obvious that persons of European or African ancestry can be considered as "naive" populations. Even among Amerindian people, not all have been evenly exposed to the risk of chagasic infection. The introduction of the ethnic parameter is all the more desirable, since due to differential selective pressures exerted by transmissible diseases, the association between HLA diversity and infectious diseases differs among ethnic groups (Alves et al., 2006).

It is noticeable that not one of the recent publications on the topic has explicitly aimed at comparing different ethnic groups for their differential genetic susceptibility to Chagas disease. Even with the mere goal of avoiding unknown genetic stratification within the populations under survey, ethnic diversity should be taken into account in this research. Modern typing tools (see **ancestry informative markers**; Pfaff et al., 2001) make it possible to finely discriminate among different ethnic groups. Again, the international population genetic programs cited here could help considerably in this effort.

26.4.5 Integrated Genetic Epidemiology of Chagas Disease

Long-called for (Tibayrenc, 1998a,b), this approach aims to consider at the same time the impact on the transmission and severity of infectious diseases, of the host, pathogen, and vector's genetic diversity, and the coevolution phenomena between them. It is very distressing that, in spite of our fine knowledge on *Trypanosoma cruzi* genetic diversity and evolution, people working on human genetic susceptibility to Chagas disease never consider the most probable role played by the parasite on Chagas pathogenicity. The reciprocal is true: parasitologists are largely ignorant of the results gathered by human genetics on Chagas disease. Entomologists are a third separate group. However, the impressive diversity of vector species could also play an important role in Chagas pathogenicity.

A multidisciplinary approach joining these three lines of research is therefore sorely needed to explore in depth the role played by genetic characters in the transmission and severity of Chagas disease. It is obvious that this research should consider environmental parameters too, with the help of specialists of human sciences.

Glossary

Allele different molecular forms of a given gene.

Allelic frequency the ratio of the number of a given allele to the total number of alleles in the population under survey.

Ancestry informative marker (AIM) the subset of genetic markers that are very different in allelic frequencies among populations of the world. Most polymorphisms are shared among all populations, and for most loci, the most common allele is the same in each population. An ancestry informative marker is a unique combination of genetic markers that occurs mostly in particular founder population sets but may also be found at varying levels across all or some of the populations found in different parts of the world (Pfaff et al., 2001) (definition from DNAPrint Genomics' website; http://www.dnaprint.com/welcome/home/index.php, with permission).

Antigen a molecule that can be targeted by the immune system. It binds specifically to an antibody, and can be bound by a major histocompatibility complex (MHC) and presented to a T cell receptor.

Balanced polymorphism/balanced selection genetic polymorphism that persists in a population because the **heterozygous** individuals for the alleles concerned have a higher fitness than either homozygous individual.

Bioinformatics the technology of informatics as applied to biological research, more specifically to genomic and evolutionary analysis. Bioinformatics is the management and analysis of data using advanced computing technologies. Bioinformatics is particularly important as a companion topic to genomics research, because of the large amount of complex data genomics research generates. It is also widely used in the analysis of genomic expression (postgenomics, **proteomics**).

Bottleneck effect a particular case of genetic drift that occurs when the size of a population is drastically reduced. Through bottleneck events, many alleles can be randomly lost from the gene pool.

Coalescence time time elapsed between the common ancestral copy (one gene in one individual) and two or more copies of a given gene at the present time.

Cytokine an intercellular messenger protein (lymphokines, monokines, interleukins, interferons, tumor necrosis factor), which is released by lymphocytes and macrophages. Cytokines permit communication among cells of the immune system and between immune system cells and cells belonging to other tissue categories. They play an important role in the defense against pathogens.

Exon the DNA sequence of a gene that codes for proteins.

Gene a DNA sequence coding for a given polypeptide. More broadly: any given DNA sequence.

Gene sequence (or genetic sequence or DNA sequence) can be compared to a series of letters corresponding to the primary structure of a DNA molecule or strand. The possible letters are A, C, G, and T, which correspond to the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine, and thymine). This coded sequence corresponds to the basic genetic information. A DNA sequence may code for proteins. In this case, it directly monitors the succession of amino acids that constitute the primary structure of the protein. Many DNA sequences have no known coding function.

Genomics molecular analysis of the whole genome of species instead of isolated genes.

Genotype genetic constitution of a given organism; see also **Phenotype**.

Heritability proportion of variation in a given character among individuals in a population that can be attributed to genetic effects. The heritability of most common diseases is considered to be 40%.

Heterozygous in a diploid organism, the two copies of a given gene in one individual have a different molecular structure. A heterozygous individual harbors two different alleles of the same gene.

Homozygous in a diploid organism, the two copies of a given gene in one individual have an identical molecular structure.

Human leukocyte antigens (HLA) cell surface proteins detected by blood testing that exhibit considerable diversity among individuals and determine an individual's leukocyte type. HLA is the name for the human major histocompatibility complex.

Linkage disequilibrium nonrandom association of genotypes occurring at different loci; see also **Recombination**.

Locus The physical location of a given gene on the chromosome. By extension, in the genetic jargon, the gene itself (plural: loci).

Major histocompatibility complex (MHC) a complex of genes found in mammals that determine the histocompatibility antigens found on cell surfaces; see also **Human leukocyte antigens (HLA)**.

Microsatellite a short DNA sequence, usually 1−4 bp long, that is repeated together in a row along the DNA molecule. In humans, as in many other species, there is considerable variation among individuals (widely used in forensic applications for individual identification) and among different populations in the number of repeats. Numbers of repeats for a given locus define microsatellite alleles. There are hundreds of places in human DNA and in most other species that harbor microsatellites. Microsatellites are fast evolving markers with a high resolution level, and are found in many different organisms.

Natural selection process first described by Charles Darwin, which favors certain genotypes to the detriment of others over generations because they are better adapted to survive and therefore have more abundant offspring. It is entirely driven by the interaction of an organism with the environment.

Nonparametric test statistical test for which a distribution curve cannot be designed, either because the parameters of the equation are unknown or because the statistical model cannot be represented by any equation.

Phenotype all observable characteristics of a given individual or a given population distinct from the genome.

Proteomics all the proteins expressed by the genome of a given organism. Also, the study of the expression, function, and interaction of proteins, it is widely used to explore patho genic processes (by comparing gene expression in healthy and sick individuals). Proteomics explores gene expression at the scale of the entire genome and is the logical complement of genomics.

Recombination reassortment of genotypes occurring at different loci through the processes of meiosis and crossing over. Random recombination leads to a total lack of linkage dis equilibrium. Free recombination results in the expected probability of a given multilocus genotype being the product of the observed probabilities of the single genotype of which it is composed.

Sexual selection selection that promotes traits that increase success in mating, sometimes to the detriment of the overall fitness of the organism.

Single-nucleotide polymorphism (SNP) polymorphisms of one letter variations in the DNA sequence. SNPs contribute to differences among individuals and populations. Most of them have no effect, others cause subtle differences in countless features, such as appearance, while some affect the risk for certain diseases. SNPs are widely used as high resolution population markers and are the basic tool used in the so called Hap Map project. SNPs constitute approximately 90% of all human genetic variations, and SNPs with a minor allele frequency of $\geq 1\%$ occur every 100−300 bases throughout the human genome.

Superlocus a set of genes sharing common functional properties, localized in a given region of the genome.

T cells or T lymphocytes correspond with a group of white blood cells that play a key role in cell mediated immunity (see **HLA**).

Xenodiagnosis a classical and efficient method of diagnosis for Chagas disease. Triatomine bugs reared in the laboratory and fed on chicken (so that one is certain that they are *Trypanosoma cruzi* free) are allowed to bite a patient suspected of having Chagas disease. Three weeks later, the dejections of the triatomine bugs are checked for presence of try panosomes. Xenodiagnosis can work in a chronic case where circulating forms of the par asite are very rare. Triatomine bugs act as biological incubators for the parasite.

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27 Clinical Phases and Forms of Chagas Disease

Anis Rassi[1], *Joffre Marcondes de Rezende*[1], *Alejandro O. Luquetti*[1] *and Anis Rassi Jr*[2,*]

[1]Faculty of Medicine, Federal University of Goias, Goias, Brazil, [2]Anis Rassi Hospital, Goias, Brazil

27.1 Introduction

Chagas disease is characterized by an acute and a chronic phase of infection. In the acute phase most patients have the unapparent (asymptomatic) form, while the remaining infected individuals usually show a nonspecific febrile disease. In the chronic phase two well-defined forms of disease are distinguished: indeterminate (latent, preclinical) and determinate (clinical), which is subdivided into cardiac, digestive (usually expressed as megaesophagus and/or megacolon), and cardiodigestive forms. Cardiac disease is further classified into stages, and esophageal Chagas disease into groups (Figure 27.1).

Any febrile illness in an endemic area should lead to suspicion of acute Chagas disease, which is confirmed by the demonstration of parasites in the peripheral blood. The chronic phase is suspected from clinical findings, mainly in the cardiac and digestive systems, supported by epidemiological evidence and confirmed serologically.

Chagas disease is clinically silent in most patients (mainly in the acute phase, but also during the chronic phase), and the diagnosis should be confirmed by the results of laboratory tests. Very often the diagnosis is made fortuitously; for example, when individuals donate blood, during health screening examination, during self-referral testing, and in patients with a strong positive family history or epidemiological antecedents.

It has been estimated that more than 95% of all acute cases of Chagas disease are not diagnosed (Teixeira, 1977). This observation is based on the patient's past medical history: when chronic chagasic individuals are asked about their signs and symptoms of acute infection, almost all cannot reply. In the chronic phase, it is well known that at least half of the infected individuals do not have either manifestations

*E mail: arassijr@terra.com.br

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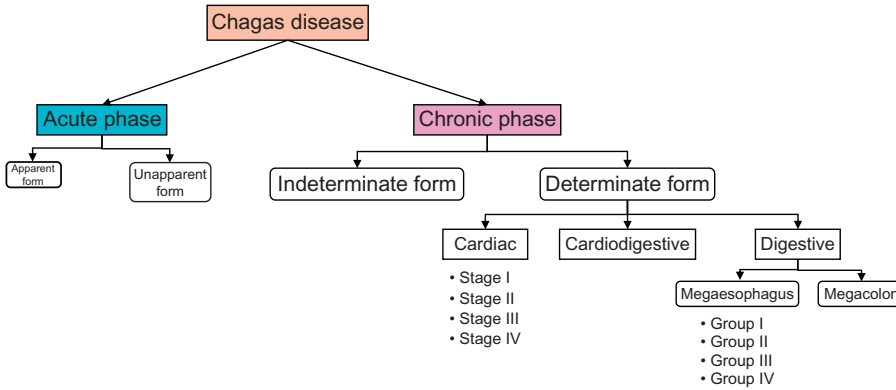

Figure 27.1 Chagas disease: clinical phases, forms, stages, and groups. Evolutionary and temporal relationships are not represented.

of disease nor visceral lesions that can be identified by routine tests, such as the electrocardiogram (ECG), chest radiography, and barium studies of esophagus and colon.

The acute and chronic phases of Chagas disease are associated with distinct clinical findings that do not overlap. The initial phase of infection with *T. cruzi* lasts for 4–8 weeks, and the chronic phase persists for the host's life span. Symptomatic manifestations of the acute phase usually disappear within 60 days, even if the infection is not treated with a trypanocidal agent. However, manifestations of the chronic phase, if present, persist, and in some patients become more severe with time. There is no well-defined laboratorial marker, patient characteristic, or clinical measure that reliably predicts Chagas disease progression.

There are few or no geographic differences in the clinical findings of the acute phase, but in the chronic phase the differences are remarkable. The benefit of specific treatment is more accentuated and easily demonstrable when given during the acute phase. Once at the chronic phase, the response will depend on the time elapsed since the acute infection (Cancado, 2002; Coura and Castro, 2002). Evolution to death is rare in the acute phase but common during the chronic phase in patients with severe cardiac lesions.

As a large proportion of individuals are asymptomatic during the chronic phase, they are not referred to as patients, but as infected persons. Most individuals with the chronic form of the disease are actively working, and do not know that they are infected. For those who find out about their infection, most may continue working at the same job. Some individuals (e.g., 15% in the 21- to 30-year-old age group) may have cardiac symptoms that need further investigation, and may incapacitate them for work. Very few (approximately 2%) in the same age group need to retire because of severe cardiomyopathy (Table 27.1).

In relation to the subclassification of individuals in the chronic phase, Carlos Chagas proposed dividing them into two large groups: those without clinical manifestations and without abnormalities in routine tests, and those with symptoms and/or other abnormalities in one or more tests. This classification was convenient for its simplicity,

Table 27.1 Chronic Phase: Theoretical Model of the Clinical Form of 100 People Infected with *T. cruzi* According to Age (*T. cruzi* II, Central Brazil)

Age Group (years)	Indeterminate Form	Cardiac Form		Digestive Form	Cardiodigestive Form	Total
		Mild/ Moderate	Severe[a]			
≤10	96	2	1	1	0	100
11–20	90	2	1	5	2	100
21–30	75	7	2	10	6	100
31–40	60	15	3	12	10	100
41–50	55	18	4	13	10	100
≥51	50	21	5	13	11	100

[a]Severe cardiac form includes sustained ventricular tachycardia, complete atrioventricular block, atrial fibrillation, echocardiogram with an ejection fraction <35%, cardiomegaly, and congestive heart failure.

feasibility, and applicability to most endemic regions since no sophisticated or expensive tests were needed. It was also noted early that a certain number of patients with Chagas disease would progress from one group to the other each year. The importance of this classification was supported by the results of autopsy studies (Lopes et al. 1982), which showed that in patients with sudden cardiac death a previous abnormal ECG was seen in all. Moreover, in patients with a normal ECG, the probability of severe cardiopathy in the short and medium term is almost zero. Since the disease may progress, annual follow-up should be carried out to search for new ECG abnormalities that, if present, indicate the need for further investigation with other tests for proper risk stratification and work capacity evaluation (Rassi et al., 2009a,b).

This multistep evaluation is affordable, cost-effective, and has been applied in several specialized outpatient clinics in Latin America. Obviously, each case needs to be assessed with regard to the patient's occupation, daily work performed, and other potential risks. For example, a worker who lifts heavy weights every day, if classified with the indeterminate form, needs to be further investigated by an exercise testing or perhaps a **24-h Holter monitoring**. These tests are usually not indicated for individuals who work solely at home or as executives.

27.2 Acute Phase

The acute phase of vector-borne Chagas disease is observed mainly in the first or second decades of life. Clinical manifestations appear around 8–10 days after the penetration of the parasite (Rassi et al., 2000). In transfusion-transmitted Chagas disease this period may be longer (20–40 days). The acute phase is not clinically recognized in most cases. The experience of those who work in endemic areas is that there is one diagnosed acute case for every 100 chronic patients. When the concentration of the inoculum is small, an atypical clinical picture, not recognized by the patient or the physician, may ensue. Alternatively, the disease may appear as a chronic infection since the beginning.

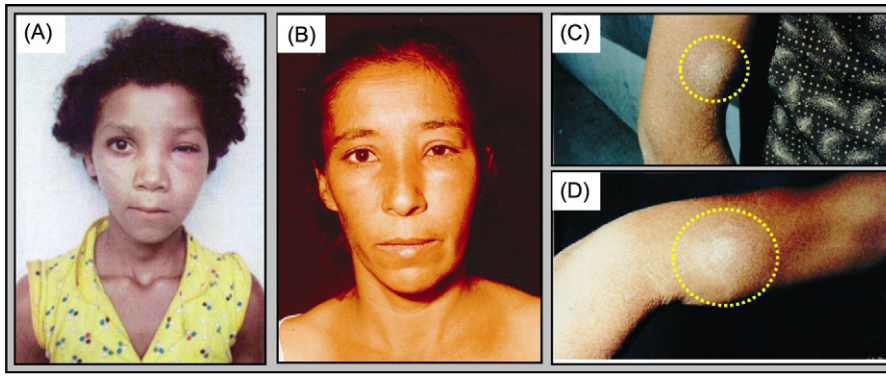

Figure 27.2 Signals of portal of entry. (A) and (B) Romaña's sign; (C) and (D) chagoma in the arm (circle).
Figures C and D have been reproduced from Rassi et al., 2010 (copyright Elsevier Inc).

Romaña's sign is the most typical sign of portal of entry of the parasite. It is characterized by a painless swelling of one or both eyelids of one eye (Figure 27.2A and B).

The eyelids turn a bluish color, and conjunctival congestion and hypertrophy of satellite lymph nodes (usually preauricular) frequently occur. The edema may spread to half of the face; sometimes dacryoadenitis and diminished conjunctival secretion are observed. Inoculation chagoma is another sign of portal of entry (through the skin), characterized by a maculonodular erythematous lesion, consistent, painless, surrounded by swelling and increased volume of satellite lymph nodes, more often found on open areas and sometimes ulcerated (Figure 27.2C and D).

Fever is a constant sign, frequently accompanied by malaise, asthenia, anorexia, and headache. Fever is usually higher in children, may be continuous or intermittent, and the temperature may be more elevated during the afternoon.

Lymph node enlargement, hepatomegaly, splenomegaly, and subcutaneous edema are the principal systemic signs, together with cardiac and neurologic alterations. Lymph node enlargement is frequent, of slight or moderate intensity, isolated or contiguous, with a smooth surface, painless, hard and nonadherent, and not fistulous. Hepatomegaly and splenomegaly are also frequent, with characteristics similar to that of lymph nodes.

The edema, whose exact mechanism is unknown, may be generalized or restricted to the face and lower limbs. It is seen most frequently in children. Meningoencephalitis and myocarditis (sometimes with associated pericarditis) are the most severe neurological and cardiological manifestations (Rassi et al., 2000).

ECG and radiological alterations are not frequently observed during the acute phase if compared with the histopathological findings, but they may become more evident when these tests are repeated (Figure 27.3).

A disproportional increase in heart rate may be seen in the recovery phase, when fever is no longer present, a finding well described by Carlos Chagas, who also

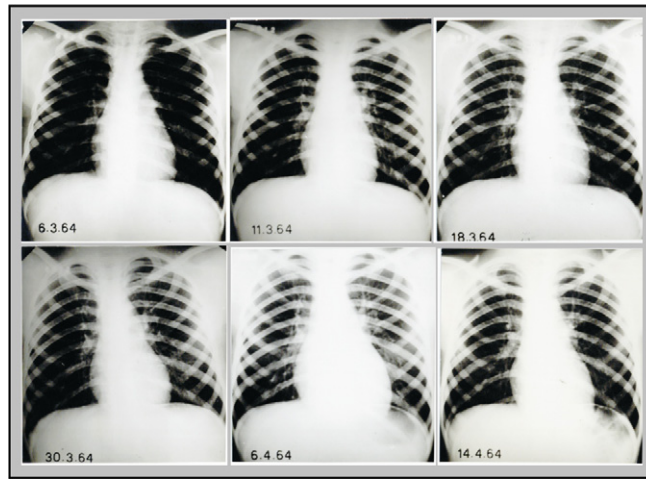

Figure 27.3 Acute phase of Chagas disease. Importance of serial radiography in the detection of cardiac involvement, which is observed only on chest radiography of 6.4.64 (April 06, 1964).

noticed the rarity of cardiac rhythm disturbances in the acute phase, in contrast with their high prevalence during the chronic phase. Other investigators subsequently confirmed these observations. The most common ECG alterations during the acute phase are: sinus tachycardia, **low QRS voltage**, **primary ST-T changes** (Figure 27.4), prolonged electrical systole, and **first-degree atrioventricular block**.

The chest radiograph may reveal variable degrees of global cardiomegaly (Rassi et al., 2000). Echocardiography was recently introduced, which explains the lack of information about its performance during the acute phase (since most cases were reported before this method was available). Nevertheless, a recent publication (Pinto et al., 2008) described the echocardiographic findings in 108 of 158 patients during the acute phase of the disease. The main abnormalities were variable degrees of pericardial effusion, mitral or tricuspid valve regurgitation, and concentric hypertrophy of the left ventricle, often with more than one abnormality seen in the same patient.

Other less frequent manifestations observed mainly in Argentina are squizotripanides (a morbilliform, urticariform, and macular exanthema) (Mazza et al., 1941), orchiepididymitis (*T. cruzi* in the fluid produced by the vaginal tunics), and hematogenic chagomas, described by Mazza and Freire (1940). Hematogenic chagomas are flat formations or nodules on the skin and subcutaneous tissues, without color alteration, nonadherent, painless, and of variable size (the size of a coin or larger) that according to Lugones (2001) are more palpable than visible.

The mortality in the acute phase used to be around 5% of all symptomatic cases, often as a consequence of meningoencephalitis or myocarditis. However, nowadays this percentage has decreased as the result of use of specific drugs. Spontaneous cure, although exceptional, may occur as has been described by Zeledón et al. (1988) and Francolino et al. (2003).

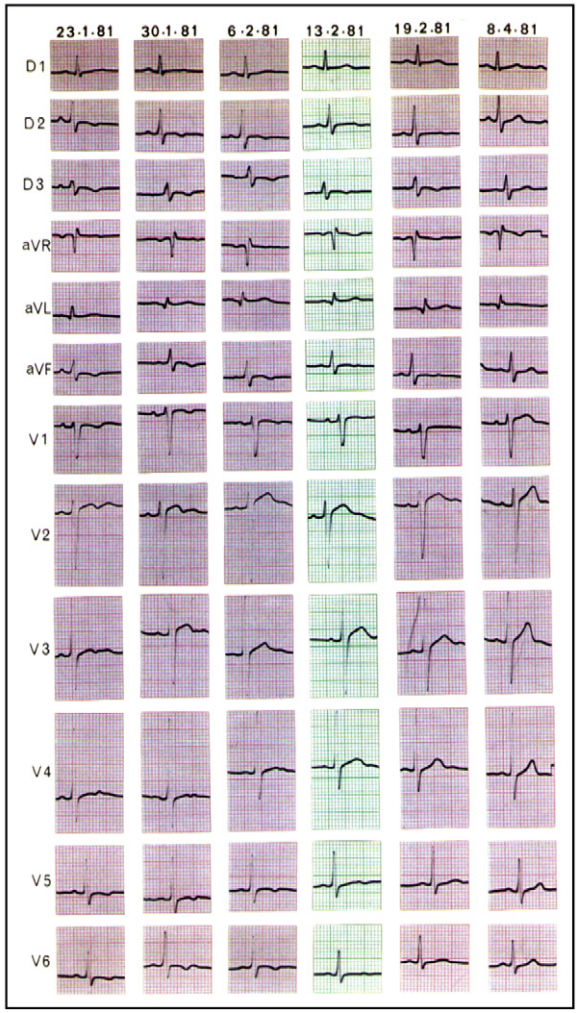

Figure 27.4 Acute phase of Chagas disease. Primary ST T changes on the 12 lead ECG that start to normalize on the ECG registered on 13.2.81 (February 13, 1981).

Mild to moderate leucocytosis may occur during the acute phase of the disease, with lymphocytosis (atypical lymphocytes), plasmocytosis, and relative neutropenia. Eosinophilia may be observed during the evolution of the disease. The hemosedimentation rate is slightly increased and C-reactive protein is also elevated. Plasma protein electrophoresis usually shows hypoalbuminemia and increased levels of alpha2- and gamma-globulins. When meningoencephalitis is present, the spinal fluid shows hypercellularity with lymphocytosis (<100 cells/mL), low glucose level, and a slight increase in protein level. It is possible to find trypomastigote forms of *T. cruzi* after centrifugation and specific staining (Rassi et al., 2000).

The natural evolution of the acute phase in about 90−95% of infected individuals is to pseudocure, when all clinical symptoms and signs disappear spontaneously in

approximately 2 months. A direct progression from the acute phase to a clinical form of Chagas disease has been recorded in a few patients (5−10%).

Diagnosis in the acute phase is based on the demonstration of the parasite in peripheral blood, via a wet smear, or after staining (thick smear) or via concentration methods (Strout, microhematocrit).

Identification by these methods is generally possible only during the initial weeks of the disease. Other methods used for diagnosis are skin biopsy of a suspected chagoma, lymph node, and skeletal muscle. **Xenodiagnosis** with an early examination of the parasites (5−10 days after the blood meal) and the search for specific IgM class antibodies by indirect immunofluorescence are alternative methods.

27.3 Chronic Phase

The chronic phase begins 2−3 months after the initial infection when the clinical manifestations (if any) of the acute phase disappear, and parasitemia falls to undetectable levels. In most cases, the chronic phase presents as an indeterminate form, which may evolve to the cardiac, digestive, or cardiodigestive forms after years or decades. The diagnosis is made by serological tests, such as indirect hemagglutination, indirect immunofluorescence, and ELISA, all of which have high sensitivity and acceptable specificity. The historical complement fixation reaction (Guerreiro-Machado) is no longer used because of its complexity and because it is no more sensitive or specific than the other tests. According to WHO (2002) recommendation, diagnosis should be based on the positivity of at least two of the tests mentioned above.

Demonstration of the parasite in blood may be performed by xenodiagnosis, with the classic (4 boxes with 10 triatomines in each) or the artificial method; the latter has several advantages (is more comfortable for the patients, avoids skin reactions to the triatomine bites, and has equal or superior sensitivity than the classical xenodiagnosis). Another method for parasitological diagnosis is hemoculture, which shows positivity in about 50% of cases. Positivity of these techniques increases when the examination is performed two or more times.

The polymerase chain reaction (PCR) method is useful for diagnosis of the chronic phase, mainly in those cases with dubious results on serology.

27.3.1 Indeterminate Form

The concept of the indeterminate form was not based on histological findings, but on the fact that visceral lesions could not be detected through clinical examination and complementary routine exams in a significant proportion of patients in the chronic phase of Chagas disease. According to a consensus of experts published in 1985, during the first meeting of applied research in Chagas disease (Araxá, Brazil), in order to be classified in the indeterminate form, the patients should meet all the following criteria: positive serological and/or parasitological tests; absence of signs and symptoms of disease; normal 12-lead ECG; and normal

radiological examination of chest, esophagus, and colon. This strict definition provides a good opportunity to categorize patients in epidemiological surveys. In cross-sectional studies conducted in endemic areas, about half of the patients with chronic Chagas disease have the indeterminate form. Recognition of the indeterminate form delimits a group of patients with a favorable prognosis, low morbidity, capable of performing any type of activity, and having the same mortality as that of the general population.

A serious conceptual error frequently observed in the medical literature is to assume that Chagas disease has three phases (acute, indeterminate, and chronic), instead of considering "indeterminate" as one of the forms of the chronic phase. By definition, "chronic" is the time period that follows any acute condition, and where patients with the indeterminate form are situated. Otherwise, we should not consider as being at the chronic phase of the disease an asymptomatic individual who has been infected with *T .cruzi* for a long time.

Although a variable proportion of patients with the indeterminate form present some structural and/or functional abnormalities when they are fully evaluated by more sensitive methods (usually for research purpose), such as ergometry, 24-h Holter monitoring, vectocardiography, echocardiography, radioisotopic techniques, cardiac magnetic resonance imaging, hemodynamic study, electrophysiologic study, endomyocardial biopsies, autonomic tests, and esophageal and colonic manometric studies, these abnormalities are often subtle and frequently isolated. Moreover, they can occasionally be found also in healthy individuals, and are not related to a decrease in life expectancy. Of note, most studies that have assessed tests of higher sensitivity in patients with chronic Chagas disease did not include the use of contrast media in the examination of the esophagus and colon because of the difficulty of carrying out such tests in asymptomatic individuals. So, it is quite possible that some of these patients had the digestive and not the indeterminate form of the disease.

Some of us studied 103 patients with the indeterminate form, who performed an exercise testing, an echocardiogram, and a 24-h Holter monitoring and had their results compared with that of 20 healthy controls (Rassi et al., 1991). All chagasic patients fulfilled the rigid definition of the indeterminate form and had a normal barium swallow and enema done. The 2D echocardiogram only left ventricular systolic function was analyzed was normal in all patients of both groups. The ambulatory Holter monitoring showed frequent or complex ventricular arrhythmias in 20% of chagasics versus 25% of controls. In the exercise testing, the prevalence of ventricular arrhythmias or an **abnormal inotropic or chronotropic response** was 16% in the chagasics versus 10% in normal individuals. This controlled study suggests that chagasic patients with the indeterminate form have similar performance when compared with normal population. Discrepancies in the results of studies investigating the role of highly sensitive tests in patients with the indeterminate form may reflect variations in sample size, lack of a normal control group, differences in the definition of abnormal responses, lack of radiological study of esophagus and/or colon, and use of plain abdominal X-ray in substitution of a barium enema.

Although patients with the indeterminate form (inclusive of those with any abnormality on more sensitive tests) have good prognosis, epidemiological studies in endemic areas have shown that 1−3% of them evolve each year from the indeterminate to a clinical (determinate) form of the disease (Dias, 1989; Storino et al., 1994). Whether these abnormalities detected by more sensitive techniques are a reliable early marker of disease progression or an innocent bystander remains to be determined. The subsequent follow-up of patients with the indeterminate form should rely on annual history, physical examination, and 12-lead ECG tracing.

An individual chronically infected with *T. cruzi* remains in the indeterminate form, generally for a period of 10−30 years. Nevertheless, the finding of older people (far from endemic regions for decades) with antibodies against *T. cruzi* and no evidence of visceral involvement indicates that a proportion of the infected people (50−60%) remain in this form for longer periods of time, or even for life.

There have been few pathological studies focusing on individuals with the indeterminate form. Necropsy studies of patients who died from accidental causes revealed mild myocarditis with scattered small foci of interstitial infiltration by lymphocytes, macrophages, and plasma cells, together with a limited reduction in the number of cardiac neurons and myenteric plexuses that are insufficient to produce clinical manifestations (Lopes et al., 1981). Intact parasites are rarely seen, but *T. cruzi* DNA can be demonstrated in the samples of myocardium, by PCR (Jones et al., 1993) or other techniques, even in the absence of local inflammation. Whether these lesions represent sequelae of the acute phase, a parasite−host state of equilibrium, or are cumulative, progressing to diffuse myocardial damage remain elusive.

27.3.2 Cardiac Form

The cardiac form is the most serious and frequent manifestation of chronic Chagas disease. It develops in 20−30% of individuals and manifests as three major syndromes that may coexist in the same patient: arrhythmic, heart failure, and thromboembolism (systemic and pulmonary) (Rassi et al., 1992, 2000). Clinical presentation varies widely according to the extent of myocardial damage.

Arrhythmias are very common and of different types, frequently in association (Figure 27.5), and cause palpitations, presyncope, syncope, and **Stokes-Adams syndrome**; sometimes arrhythmias are asymptomatic.

Frequent, complex, ventricular premature beats, including **couplets** and runs of **nonsustained ventricular tachycardia**, are a common finding on 24-h Holter monitoring or stress testing (Figure 27.6). They correlate with the severity of ventricular dysfunction, but can also occur in patients with relatively well-preserved ventricular function. Episodes of nonsustained ventricular tachycardia are seen in approximately 40% of patients with mild wall motion abnormalities and in virtually all patients with heart failure, an incidence that is higher than that observed in other cardiomyopathies (Rassi et al., 1995).

Sustained ventricular tachycardia is another hallmark of the disease. This life-threatening arrhythmia can be reproduced during **programmed ventricular stimulation** in approximately 85% of patients and seems to result from an

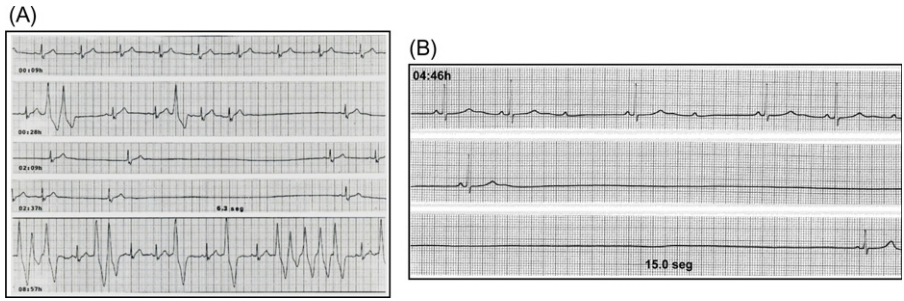

Figure 27.5 Associated arrhythmias in patients with Chagas heart disease (24 h Holter monitoring). (A) Sinus pauses and nonsustained ventricular tachycardia; (B) second degree atrioventricular block (Mobitz 2) and sinus pause of 15.0 s duration (during sleep).

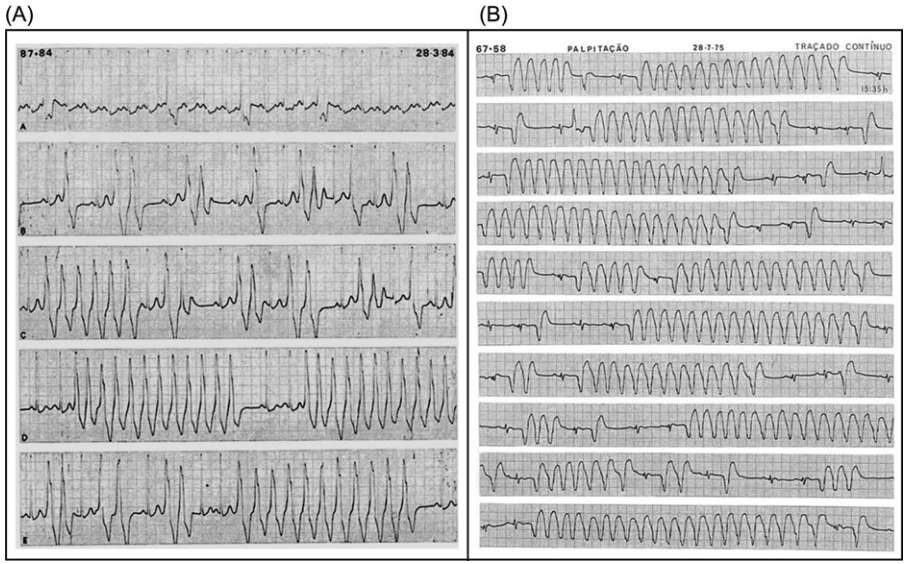

Figure 27.6 Frequent episodes of nonsustained ventricular tachycardia on stress testing (A) and on 24 h Holter monitoring (B) in patients with Chagas heart disease.

intramyocardial or subepicardial re-entry circuit usually located at the inferior–posterior–lateral wall of the left ventricle (Sosa et al. 1998; D'Avila et al., 2002).

Heart failure is often a late manifestation of Chagas heart disease. It is usually biventricular with a predominance of right-sided failure (peripheral edema, hepatomegaly, and ascites more prominent than pulmonary congestion) at advanced stages. Nocturnal paroxysmal dyspnea, cardiac asthma, and acute pulmonary edema are all rare. **Gallop rhythm** is infrequent. Once cardiomegaly appears, a systolic murmur of functional mitral or tricuspid regurgitation may be heard. Isolated left

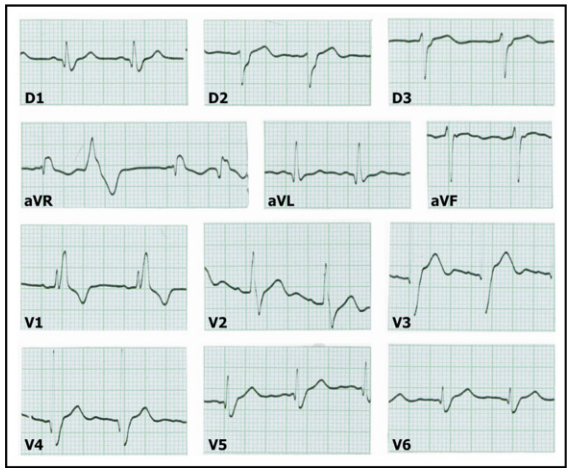

Figure 27.7 ECG of a patient with Chagas heart disease showing the three most typical alterations: complete right bundle branch block, left anterior fascicular block, and ventricular extrasystole.

heart failure can be seen in the early stages of cardiac decompensation (Rassi Jr et al., 2000; Marin-Neto et al., 2010). Heart failure of chagasic etiology is associated with higher mortality than is heart failure from other causes (Freitas et al., 2005). Systemic and pulmonary embolisms arising from mural thrombi in the cardiac chambers are quite frequent (Oliveira et al., 1983). Clinically, the brain is by far the most frequently recognized site of embolisms (followed by limbs and lungs), but at necropsy, embolisms are found more frequently in the lungs, kidneys, and spleen. Chagas disease is an independent risk factor for stroke in endemic areas (Carod-Artal et al., 2005).

Sudden death is the main cause of death in patients with Chagas heart disease, accounting for nearly two-thirds of all deaths, followed by refractory heart failure (25−30%) and thromboembolism (10−15%) (Rassi et al., 2001). Sudden cardiac death can occur even in patients who were previously asymptomatic. It is usually associated with ventricular tachycardia and fibrillation or, more rarely, with **complete atrioventricular block** or **sinus node dysfunction**. Leading causes of death vary depending on the stage of disease, with a clear predominance of sudden death at early stages, and a slight predominance of death from pump failure at advanced stages.

Electrocardiographic alterations are varied, but the most frequent and important are ventricular premature beats (monomorphic or polymorphic, isolated or in pairs), complete right bundle branch block (CRBBB), left anterior fascicular block, primary ST-T changes, Q waves, different degrees of atrioventricular block, manifestations of sinus node dysfunction (sinus bradycardia, sino-atrial block, and sinus arrest), **atrial fibrillation**, and nonsustained or sustained ventricular tachycardia. All these alterations may be isolated or associated. A frequent association is CRBBB and left anterior fascicular block, and when this occurs in an endemic area, it strongly suggests chronic Chagas heart disease (Figure 27.7).

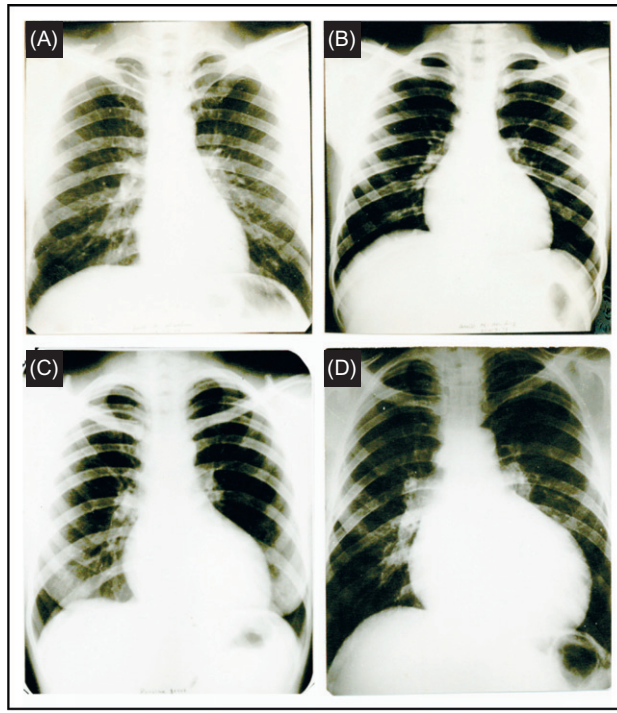

Figure 27.8 Chest radiography of four patients with Chagas heart disease. (A) normal; (B) mild cardiomegaly; (C) moderate cardiomegaly; (D) severe cardiomegaly without pulmonary congestion.

On radiological examinations, the cardiac size is generally normal in the initial phase of the cardiopathy and even when important electrocardiographic changes are present. The cardiac size may be slightly, moderately, or severely increased, in all chambers (Figure 27.8). In nearly half of the cases with heart failure, the manifestations of pulmonary congestion are poor or even absent.

The echodopplercardiogram may be abnormal even in patients with a normal ECG and normal chest radiograph. Echo shows wall motion abnormalities in two main areas of the left ventricle: the apex and the posterior−inferior wall. The most characteristic findings are apical aneurysms (with or without thrombi) (Figure 27.9) and akinesia or hypokinesia of the posterior wall of the left ventricle (with preservation of the atrioventricular septum).

Ambulatory ECG monitoring (Holter system) is an excellent method for investigating patients with Chagas heart disease (Rassi and Perini, 1979; Rassi et al., 1985, 1990). It may be used to identify complex ventricular arrhythmias, evaluate antiarrhythmic therapy, diagnose transitory arrhythmias, identify the association of tachyarrhythmias with bradyarrhythmias, evaluate people for job activities, and

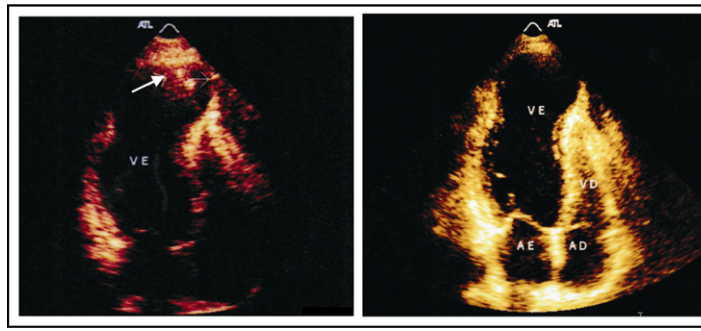

Figure 27.9 Two dimensional echocardiogram showing left ventricular apical aneurysm with (arrow) and without thrombus.

establish whether an artificial pacemaker is working properly. It is performed routinely for a 24-h period. In some cases a special device (event recorder) is used that registers ECG activity for several days.

The exercise testing evaluates the functional capacity of the patient, qualifies and quantifies ventricular premature beats, and may verify the efficacy of antiarrhythmic drugs.

Intracardiac electrophysiological studies evaluate the sinus node function, identify the location of atrioventricular and intraventricular blocks precisely, and investigate the inducibility of ventricular tachyarrhythmias, as well as their place of origin. It is of great value in the evaluation of presyncope and syncope of unknown origin, in indicating the need for an artificial pacemaker, as well as evaluating alternative methods of treatment of sustained ventricular tachycardia (e.g., **transcatheter ablation**). Because of its invasive nature, it should be performed only in selected patients or after all other noninvasive methods have been tried, such as Holter monitoring and exercise testing.

Chagas heart disease can be divided schematically into four stages (Rassi et al., 2010) (Table 27.2).

In stage I, patients are usually symptom free and show mild and nonspecific ECG alterations. Left ventricular systolic function is preserved, but diastolic abnormalities may be found. Complex ventricular arrhythmias on 24-h Holter monitoring are rare. In stage II, manifestations include more specific conduction abnormalities, most frequently right bundle branch block, left anterior fascicular block (or both), complex ventricular arrhythmias, and segmental left ventricular wall motion abnormalities. Manifestations of later stages (III and IV) are: (a) sinus node dysfunction, usually leading to severe bradycardia; (b) high-degree heart block; (c) pathologic Q waves, low QRS voltage, and atrial fibrillation, compatible with extensive areas of myocardial fibrosis; (d) pulmonary and systemic thromboembolic phenomena due to thrombus formation in the dilated cardiac chambers or aneurysm; (e) cardiomegaly; and (f) progressive dilated cardiomyopathy with marked impairment of systolic function and congestive heart failure.

Table 27.2 Stages of Chronic Chagas Heart Disease[a]

	Cardiac Symptoms[b]	NYHA Class	ECG Changes	Chest X-ray (Cardiomegaly)	24-h Holter (Complex VA[c])	2D Echocardiogram		Thrombo-embolism	Sustained VT	Sudden Death
						LV Wall Motion Abnormalities	LV Apical Aneurysm			
Stage I	Absent or minimal	–[d]	Not specific[e]	Absent	Rare	Rare	Very rare	Very rare	Rare	Rare
Stage II	Fairly common	I/II	RBBB±LAFB, mono VPBs, diffuse ST T changes, 1°, 2° AV block	Absent or mild	Common	Absent or segmental	Common	Fairly common	Common	Common
Stage III	Common	I/II/III	+ Q waves, poly VPBs, advanced AV block, severe bradycardia, low QRS voltage	Mild to moderate	Very common	Segmental or diffuse (mild to moderate)	Common	Fairly common	Common	Common
Stage IV	Common	II/III/IV	+ Atrial flutter/fibrillation	Moderate to severe	Very common	Diffuse (severe)	Fairly common	Common	Fairly common	Fairly common

2D = two-dimensional; AV = atrioventricular; ECG = electrocardiogram; LAFB = left anterior fascicular block; LV = left ventricular; mono = monomorphic; NYHA = New York Heart Association; poly = polymorphic; RBBB = right bundle branch block; VA = ventricular arrhythmias; VPBs = ventricular premature beats; VT = ventricular tachycardia.

[a]Schematic classification based on the authors' own experience and review of the literature with the aim of helping physicians better understand the heterogeneous clinical course of chronic Chagas disease; minor interchanges in some characteristics among the different stages of cardiac disease are possible.

[b]Palpitations, presyncope, syncope, atypical chest pain, fatigue, and edema.

[c]Couplets and/or episodes of nonsustained VT.

[d]data not applicable.

[e]Incomplete RBBB, incomplete LAFB, mild bradycardia, minor increase in PR interval, minor ST-T changes.

Risk Factor	Points
NYHA class III or IV	5
Cardiomegaly (chest X-ray)	5
Segmental or global WMA (2D echo)	3
Nonsustained ventricular tachycardia (24-h Holter)	3
Low QRS voltage (ECG)	2
Male sex	2

Total points	Total Mortality		Risk
	5 years	10 years	
0–6	2%	10%	Low
7–11	18%	44%	Intermediate
12–20	63%	84%	High

Figure 27.10 Prognostic factors in Chagas heart disease. Rassi score for prediction of total mortality. Echo = echocardiogram; NYHA = New York Heart Association; WMA = wall motion abnormality.

Improved understanding of prognostic factors in Chagas heart disease has helped clinicians to identify patients' risk, choose appropriate treatment, and direct patient counseling. Some of us used a rigorous multivariate analysis to develop a risk score for mortality prediction in 424 outpatients from a regional Brazilian cohort and the score has been validated (Rassi et al., 2006; Rocha and Ribeiro, 2006) successfully in two external cohorts. Several demographic, clinical, and noninvasive variables were tested, and six were identified as independent predictors of mortality and were assigned points according to the strength of their statistical association with the outcome. From addition of the points to provide the risk score, patients can be classified into groups of low, intermediate, and high risk (Figure 27.10).

Subsequently, two systematic reviews (Rassi, et al., 2007b; Rassi et al., 2009b) integrated the results of all previous studies in which multivariable regression models of prognosis were used and a clearly defined outcome (all-cause mortality, sudden cardiac death, or cardiovascular death) was analyzed. According to these reviews, the strongest and most consistent predictors of mortality are **New York Heart Association (NYHA) functional class** III or IV, cardiomegaly on chest radiography, impaired left ventricular systolic function on echocardiogram or cineventriculography, and nonsustained ventricular tachycardia on 24-h Holter monitoring. On the basis of these findings, a risk stratification model for mortality, which can assist treatment in patients with Chagas heart disease is proposed in Table 27.3.

With the exception of some peculiarities, the general principles that guide the symptomatic treatment of chronic Chagas heart disease are the same as those established for heart disease of other causes. Bradyarrhythmias are treated with pacemaker implantation. As a general rule, a pacemaker is indicated for all patients with symptomatic bradyarrhythmias or for those at high risk of complete atrioventricular block. The electrode should be placed in the subtricuspid zone (Korman and Jatene, 1977) avoiding the apex of the right ventricle, which may be thin, fibrotic, and contain thrombus. However, the management of rhythm disorders does not end with the

Table 27.3 Stratification of Risk of Death Associated with Chagas Heart Disease and Recommended Therapy

Risk of Death	Risk Factor			Recommended Treatment
	NYHA Class III/IV	LV Systolic Dysfunction (Echo) and/or Cardiomegaly (Chest X-ray)	Nonsustained VT (24-h Holter)	
Very high	Present[a]	Present	Present	ACE inhibitor, espironolactone, amiodarone, diuretics, digitalis, betablocker,[b] cardiac transplant,[c] ICD?
High	Absent	Present	Present	ACE inhibitor, amiodarone diuretic,[c] betablocker,[b] ICD?
Intermediate	Absent	Present	Absent	ACE inhibitor, betablocker, diuretic,[c] Antiparasitic drug?
	Absent	Absent	Present	Amiodarone Antiparasitic drug?
Low	Absent	Absent	Absent	Antiparasitic drug[c]

ACE = angiotensin-converting enzyme; echo = echocardiogram; ICD = implantable cardioverter-defibrillator; LV = left ventricular; NYHA = New York Heart Association; VT = ventricular tachycardia.
[a]Nearly 100% of patients with Chagas heart disease in NYHA class III or IV also have LV systolic dysfunction on echo and nonsustained VT on 24-h Holter monitoring.
[b]If clinically tolerated.
[c]For selected patients.
This table has been adapted from Rassi et al., 2010 (copyright Elsevier Inc).

implant of a pacemaker. Ventricular arrhythmias should also be promptly searched in patients with a pacemaker using the 24-h Holter monitoring and/or an exercise testing (Figure 27.11), and treated accordingly (Rassi et al., 1995).

Ventricular arrhythmias are treated mainly with amiodarone; sotalol and beta blockers are second choice drugs. Propafenone and mexiletine have been used in some patients based exclusively on their antiectopic activity. Quinidine, procainamide, and disopiramide do not have adequate antiarrhythmic activity; nevertheless, the use of procainamide when given intravenously for the treatment of paroxysmal ventricular tachycardia is highly effective. Monomorphic sustained ventricular tachycardia may also be amenable to percutaneous endocardial or pericardial ablation using catheter-delivered radiofrequency or cumulative high-energy fulguration in selected patients with mappable arrhythmia. The use of implantable cardioverter-defibrillators in patients with Chagas heart disease is hampered by the lack of controlled data to establish precise indications and efficacy (Rassi, 2007a) as well as by socioeconomic limitations. When cardiac failure is present, it may be necessary to use higher doses of diuretics; angiotensin-converting enzyme inhibitors, espironolactone, and digoxin are also commonly used, and in special situations, beta blockers are used (Rassi et al.,

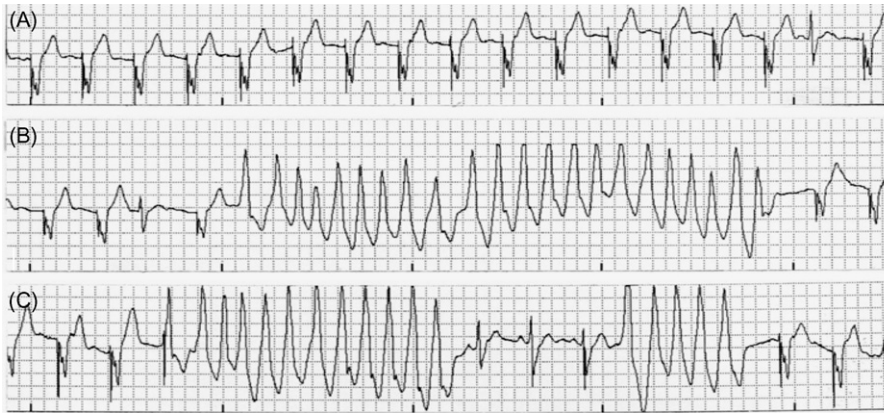

Figure 27.11 (A) A 24 h Holter monitoring in a patient with Chagas heart disease after pacemaker implantation (B and C) showing episodes of nonsustained ventricular tachycardia.

2010). In some individuals, such as those with intractable cardiac failure following optimized treatment, a cardiac transplant is necessary; in this situation, the patient should usually receive specific treatment before or shortly after the surgical procedure. Palliative procedures, such as dynamic cardiomyoplasty and partial left ventriculectomy, are contraindicated because of unsatisfactory results. **Cardiac resynchronization** is another form of treatment for heart failure, and is mostly for patients with left bundle branch block. However, remarkably few data support its use in patients with right bundle branch block, which is much more common in patients with Chagas heart disease. The potential benefit of transplantation of bone marrow cells for treatment of Chagas heart failure is being assessed in a multicenter randomized controlled trial sponsored by the Brazilian Health Ministry (Tura et al., 2007).

Because of the high occurrence of thromboembolic phenomena in Chagas heart disease, oral anticoagulants are recommended for patients with atrial fibrillation, previous embolic episodes, and apical aneurysm with thrombus. If poor social and economic factors limit the implementation of anticoagulant therapy, because of the increased risk of bleeding, the use of acetylsalicylic acid is considered a reasonable alternative.

27.3.3 Digestive Form

The digestive form of Chagas disease is characterized by alterations in the motor, secretory and absorptive functions of the gastrointestinal tract. Lesions of the enteric nervous system are pivotal in the pathogenesis of Chagas digestive megasyndromes. The myoenteric plexus of Auerbach, which is located between the longitudinal and circular muscular layers of the digestive tract, is the main one affected (Köberle, 1957). Although most of the damage to the neurons of this

plexus and the nervous fibers occurs during acute infection, further neuronal loss occurs slowly over an extended period of the chronic phase. There is a marked reduction in the number of nervous cells of the Auerbach's plexus as demonstrated by Köberle (1961) in quantitative studies performed on several segments of the digestive tract, in humans, and in experimental animals infected with *T. cruzi*. Denervation occurs to variable degrees, is irregular and noncontinuous, and probably depends on both parasite and host factors.

Variations in *T. cruzi* strains related to the pathogenicity for the enteric nervous system probably explain the regional differences associated with Chagas disease (i.e., why the digestive form is seen only in some geographical areas), its unpredictable evolution, and its multiplicity of clinical manifestations.

The medical literature indicates a higher prevalence of the digestive form in the central region of Brazil. It has also been described in other countries of South America, but is not seen in countries above the equatorial line, where only a few cases with esophageal motor alterations have been described (Rezende, 1976).

Prevalence studies of the chagasic digestive form in Brazil (the country with the highest rates) have been performed based on radiological findings of the esophagus as an indicator of the involvement of the digestive tract. In seven radiological surveys performed in endemic areas and blood banks by abreugraphy (roentgenphotography, a radiograph of 35 or 70 mm, used as screening for tuberculosis), the prevalence rates of esophagopathy ranged from 7.1% to 18.3% (mean 8.8%) among 3073 infected individuals (Rezende and Luquetti, 1994).

Although intrinsic denervation can be found along the entire digestive tract, with variable intensity and distribution, the esophagus and the distal colon, because of their physiology, are the most frequently involved segments. As a consequence of denervation, motor uncoordination and **achalasia** of the sphincters occur, making it difficult for these segments to empty semisolid material, and leading to dilatation with time; this is the pathophysiological mechanism underlying chagasic megaesophagus and megacolon.

It has been observed that the frequency of megaesophagus is higher than that of megacolon in outpatient clinics or institutions caring for chagasic patients.

A survey conducted at the Hospital das Clinicas of the Federal University of Goias, between 1976 and 1997, showed that 1761 patients had megaesophagus at the first consultation (56.8% males and 43.2% of females; ages ranging from 2 to 102 years, of whom 75% were 20−70 years old). The association with megacolon was investigated by barium enema in 765 patients, and 365 of them (45.5%) also had megacolon (Rezende and Moreira, 2004).

Serological tests for Chagas disease were performed on 1271 sera from patients with megaesophagus, including 362 with associated megacolon. Positive results were found in 91.3% of patients with megaesophagus and in 99.4% of patients with associated megacolon (Rezende and Luquetti, 1994).

Association of the cardiac and digestive form may vary according to peculiarities in each region and these data have limited value. In a region of central Brazil, this association was evaluated in 1313 patients. A normal ECG was found in 48.2% ($n = 633$), nonspecific ECG alterations were found in 21.1% ($n = 277$), and

characteristic alterations (i.e., CRBBB) in 30.7% ($n = 403$). Only 14/403 had severe alterations. The more frequent ECG abnormalities were CRBBB with or without left anterior fascicular block and ventricular premature beats (Rezende and Luquetti, 1994).

The clinically more interesting entities in the digestive form are esophagopathy and colopathy. In those cases with higher denervation, evolution is to ectasic forms of megaesophagus and megacolon.

27.3.3.1 Megaesophagus

The main complaints presented by patients at consultation are dysphagia, regurgitation, and esophageal pain. Other less frequent symptoms are hiccups, pyrosis, and hypersalivation accompanied by parotid hypertrophy. Malnutrition is observed with the progression of the disease.

Dysphagia is often the first and the most frequent symptom in the natural history of idiopathic achalasia or chagasic megaesophagus. It may be mild, moderate, or severe, and the intensity varies with type of food ingested, its temperature, and the emotional status of the patient.

Regurgitation may be active, occurring during or immediately after meals with the conscious participation of the patient, or passive, when the patient is lying in bed sleeping, generally at night. The regurgitated material is expelled through the mouth and narines and may enter the respiratory tract, causing coughing and suffocation. Regurgitation is a common cause of pulmonary complications, mainly aspirative bronchopneumonia.

Esophageal pain may be spontaneous or associated with food ingestion, when it is called odynophagia. Spontaneous pain is localized at the level of the xiphoid appendix or below the sternum and propagates in an ascending direction up to the base of the neck, and radiates to the inter scapulovertebral region and upper limbs. The pain is of the burning type, constrictive, tearing or colicky, and is alleviated or abolished with the ingestion of water or other liquids.

Radiological examination is essential to confirm the diagnosis and to stage the disease (based on the morphofunctional characteristics of the esophagus), which is very important for the selection of the most appropriate therapy. Although there are several radiological classifications, we recommend the one by Rezende et al. (1960), which identifies four groups (Figure 27.12).

Group I: Normal diameter of esophagus; minimal contrast retention; presence of a resid ual air column above the contrast;
Group II: Moderate dilatation, with some contrast retention; increase in uncoordinated motor activity; relative hypertony of the inferior third of the esophagus;
Group III: Large increase in diameter and great contrast retention; hypotonic esophagus with weak or absent motor activity;
Group IV: Large increase in volume, atonic, elongated esophagus, lying on the right dia phragmatic dome.

Radiological examination should be combined with fluoroscopy to assess esophageal motility and emptying. The examination is performed with the patient in the

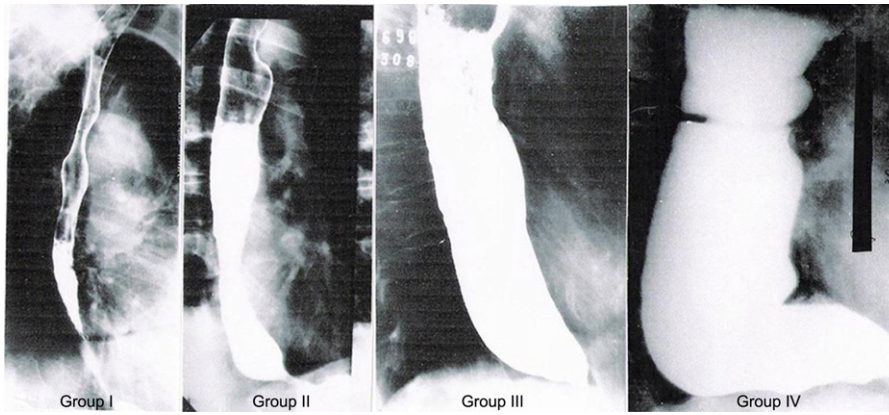

Figure 27.12 Radiological classification of chagasic megaesophagus in four groups according to the stage of disease (Rezende et al., 1960).

upright position, preferentially at right anterior oblique view. In this position, the cardiac shadow projects to the front and the column to the back, allowing for better discrimination of the contrasted esophageal image.

The amount of contrast medium should be sufficient to generate an ideal height and pressure column that promotes the passage of contrast to the stomach, when the shape, diameter, wall contour, and mainly the contractile activity of the esophagus can be clearly seen. It is also important to search for eventual tertiary waves, which are uncoordinated and nonpropulsive contractions. The distal esophagus should be carefully observed. The last X-ray should be done after all the contrast medium has passed through the cardia.

For the detection of cases in group I, in which the diameter of the esophagus is normal, a special technique, known as retention test (analysis of two consecutive radiographs, the first immediately after ingestion of barium, and the second 1 min later), is recommended. The test is positive when incomplete emptying is observed on the second radiograph producing a level of residual contrast, and the esophagus remains open by the presence of an air column. A differential diagnosis should be established with other diseases that may display the same image, such as presbyesophagus, hiatus hernia, esophagitis, systemic sclerosis, neoplasia, and the use of anticholinergic drugs before the barium swallow.

There is significant correlation between symptoms and the evolution of megaesophagus. In group I, dysphagia is generally the only complaint. In group II, besides more severe dysphagia, there is active regurgitation and esophageal pain. In groups III and IV, passive regurgitation and malnutrition are frequent.

The evolution of chagasic esophagopathy to worsening of symptoms and progression of radiological and manometric parameters is not uniform in all cases; and if it occurs, it evolves slowly over time.

In a longitudinal study performed in an endemic area (Mambai, Goias State, Brazil) the prevalence of megaesophagus was assessed in 1006 individuals with

positive serology. A chest abreugraphy (roentgenphotography, 70 mm) was taken after a barium meal of 75 mL contrasted the esophagus; a second abreugraphy was done 1 min later. Megaesophagus was detected in 71 (7%) of these patients: 43 were classified as group I, 18 as group II, 5 as group III, and 5 as group IV. Twenty-one patients with megaesophagus were reexamined 25 years later with the same technique. Progression was observed in 10 cases (Peñaranda-Carrillo et al., 2006).

Esophageal-gastro-duodenal endoscopy should be routinely performed after the radiological examination of the esophagus for evaluation of the mucosa and detection of associated lesions, mainly esophageal cancer, and cancer of the esophageal-cardio-tuberosity region.

For the differential diagnosis of chagasic megaesophagus with other esophagopathies, if there are any doubts, esophageal manometry is indicated using at least three pressure channels. The abnormalities seen in patients with chagasic megaesophagus vary depending on the stage of the disease. There is a loss of peristaltic activity at the body of the esophagus (aperistalsis), with synchronic waves and failure or incomplete aperture of the lower esophageal sphincter with ingestion (achalasia). In groups III and IV contractions are of low amplitude.

Manometry is particularly useful in the differential diagnosis of the hyperkinetic forms of megaesophagus and diffuse esophageal spasm, and for evaluation of the basal pressure of the lower sphincter of the esophagus before and after dilatation or surgery.

Idiopathic achalasia and chagasic megaesophagus are both risk factors for the development of esophageal cancer, because of chronic irritation of the mucosa caused by residual food. The prevalence of this association varies widely from 0.4% to 9.3% (Rezende and Moreira, 2004). The duration of the disease may be more important than the age of the patient. Higher prevalence of cancer in megaesophagus has been described by surgical services, probably because patients who need surgery are those with more advanced disease.

Treatment of chagasic megaesophagus and idiopathic achalasia may be clinical, instrumental, or surgical. Clinical treatment is indicated only for group I, or when other types of treatment are contraindicated for groups II, III, or IV. Clinical treatment is based on hygiene and dietary measures, and, eventually, the use of drugs such as isosorbitol dinitrate or nifedipine given before meals to relaxes the lower esophageal sphincter.

Instrumental treatment may be performed by dilatation of the cardioesophageal junction with a mercury-filled dilator (bougies of Hurst or Maloney) to temporarily alleviate the dysphagia, or by a pneumatic or hydrostatic balloon that reduces the pressure of the lower esophageal sphincter and usually shows satisfactory results. For older patients, the injection of botulinum toxin into the muscular layer of the esophagus−gastric transition may be performed as an alternative procedure and has an effect that lasts, on average, for 1 year.

The preferential treatment for groups II and III is surgery, using the Heller extramucosal cardiomyotomy technique via video-laparoscopy. For group IV, different types of surgery, including resection of the esophagus with cervical esophagogastric anastomosis, are indicated.

27.3.3.2 Megacolon

The prevalence of megacolon is hard to estimate, because of difficulties related to its diagnosis, which involves the realization of a barium enema. Megacolon is seldom the only manifestation of the digestive tract; in most cases it is associated with megaesophagus.

The most common symptoms are constipation, meteorism, **dyskesia**, and less often, abdominal colicky pain. Constipation may even be absent in 25−30% of individuals who have radiological dilatation of the colon (Rezende and Luquetti, 1994; Vaz et al., 1996).

On physical examination, an increase in the abdominal volume is observed. Since the distal colon is the most affected segment, the distended sigmoid occupies a large part of the abdominal cavity and may be localized by palpation and percussion outside its normal topography.

Prolonged retention of feces in the distal colon leads to formation of **fecaloma**, which may be diagnosed by simple abdominal palpation, as an elastic tumor that can be molded by pressure. Rectal examination will detect a fecaloma at the rectal ampulla.

Radiological examination is necessary to confirm the diagnosis, and should begin with a noncontrasted plain abdominal radiograph, which may show increased intestinal air and, if fecaloma is present, a bread-like image.

After the noncontrasted X-ray, a barium enema is performed, which usually involves the use of intestinal cleansing or purgatives, as well as the introduction of air into the colon to achieve double contrast. These maneuvers, however, modify the original morphology of the colon and may induce false results. The colon is an elastic organ with capacity for distension or contraction, depending on the fecal contents as well as the endogenous or exogenous stimuli. Purgatives are irritants and increase the tonus and enterocolic contractility. The introduction of air into the colon causes distension of its wall that is proportional to the injected pressure, increasing the diameter of the distal colon, mainly the sigmoid colon. As a result, a false picture of the anatomical dimensions of the distal colon is obtained.

To avoid these pitfalls, a simplified technique is recommended, which has been shown to be satisfactory for the diagnosis of chagasic megacolon in endemic regions (Ximenes et al., 1984). Barium enema is performed without previous preparation and double contrast, using 300 mL of barium sulfate diluted up to 1200 mL with water. This preparation is delivered at a height of 1 m with the patient in ventral decubitus position, without any pressure effect. Then the patient moves to the right lateral decubitus position for 5 min. The first radiograph is taken in the dorsal decubitus position, and the second in the ventral decubitus position, using a 30×40 mm X-ray film. Another film, 24×30 mm, is taken with the patient in the right lateral decubitus position to image the rectum. The distance between the source of X-rays and the film (focus-film distance) should be 1 m. The presence of fecaloma is not an obstacle to this simplified technique. If there is suspicion that another disease of the colon is present, enema should be repeated with the conventional technique.

When the colon is largely dilated, the diagnosis is easy. When it is not, doubts may arise, because there is no clear-cut division among normal and abnormal patterns. The diameter and the dimension of sigmoid and rectal ampoule as well as the total length of the colon varies widely in normal subjects and in infected people. For this reason, the limits of normality need to be established for a given population. In an endemic region of Central Brazil, the application of the technique mentioned above in 72 nonchagasic individuals allowed to establish the following values as the upper limits of normal for radiological films: 7 cm for the diameter of sigmoides in an anteroposterior view; 11 cm for rectum diameter; and 70 cm for the length of distal colon, including rectum and sigmoides. By employing these parameters, the prevalence of megacolon in 225 infected individuals in this area was 6.2% (Hernandez et al., 2002).

Dilation is usually located at the distal colon, including sigmoid and rectum (Figure 27.13A). Rarely a dilatation is found in other segments or in the entire colon (Figure 27.13B). Very often dilatation is associated with an increase in colon length, the dolicomegacolon.

Obviously, diagnosis of the nondilated colopathy cannot be performed by radiological examination and requires other methods, such as manometry and pharmacological tests of denervation.

Differential diagnosis should be made with other colonic dilatations of obstructive or functional origin, such as neoplasias, stenosis, extrinsic compressions, and rectosigmoid endometriosis. Among dilatations of functional origin, the psicogenic megacolon of the infancy, the andine megacolon (without lesions of the myoenteric plexus), the toxic megacolon that occurs as a complication of inflammatory bowel

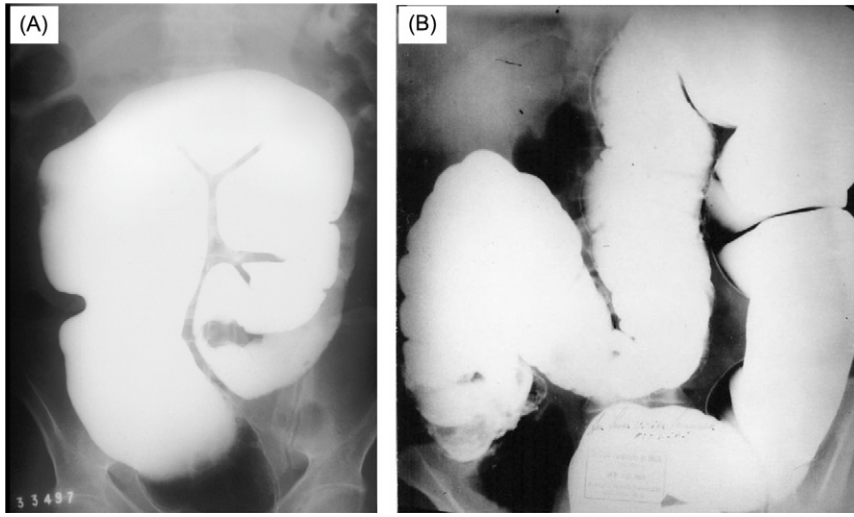

Figure 27.13 Chagasic megacolon. (A) Dilatation is mainly at the rectum and sigmoid. (B) Total megacolon (rare).

diseases, and the atonic colon due to the action of drugs leaving to a secondary dilatation should always be remembered.

Differential diagnosis with Hirschsprung disease, also called congenital megacolon, is usually easy because chagasic megacolon is exceptional in low-age children.

Two other complications, apart from fecaloma formation, may occur: the fecal impactation and sigmoid **volvulus**, both with a clinical syndrome of intestinal occlusion. Fecal impactation may be solved with fecaloma emptying. Volvulus, depending on the degree of torsion and the aspect of the mucosa, may be treated by endoscopy distortion. If signals of suffering of mucosa are present at the local of torsion, surgical treatment is indicated.

Differently from megaesophagus, cancer of colon is rarely seen in patients with megacolon.

Treatment of megacolon may be clinical or surgical. In oligosymptomatic patients, when constipation is mild to moderate, a treatment based on osmotic laxatives (saline, lactulose, macrogol 3350) or emollients (mineral oil) is indicated, together with appropriate hygienic and dietary measures. An additional aid may be the inclusion of glycerol in enemas or in suppositories. The same conservative procedure is indicated for patients waiting for surgery and for those with a high surgical risk.

Surgical treatment is indicated in symptomatic patients with persistent constipation and clear evidence of dilatation of the distal colon in the radiological examination, as well as in those with previous complications. There are several surgical techniques, but the most frequently used (because of the results) is the resection of the dilated segment and retrorectal lowering of the colon, leaving the rectum without function (technique of Duhamel-Haddad).

27.3.3.3 Other Organs of the Digestive Tract

Other segments and organs of the digestive system may be compromised in Chagas disease, causing functional alterations that may be detected by different investigation methods, but with a lower impact than the lesions involving esophagus and colon (review by Rezende and Moreira, 2004).

Chagasic gastropathy was initially suspected based on clinical evidences only (Porto, 1955). Gastric involvement is found in nearly 20% of patients with megaesophagus. On radiological examination the gastric volume is extremely variable, and the absence of air in the stomach of patients with advanced megaesophagus is very typical (Figure 27.14).

In patients with the digestive form, hypersensitivity of the muscle layer of the gastric wall in response to cholinergic pharmacological stimuli, as well as alterations in both motility and secretion, may be detected by several methods. In these cases there is rapid gastric emptying for liquids and delayed for solids. A lower adaptative relaxation of the stomach in response to distension is also usually found. An alteration of the gastric electric rhythm has recently been demonstrated by electrogastrography (Rezende Filho et al., 2005).

Another alteration seen in patients with Chagas disease is a lower basal and stimulated (under different stimuli such as histamin, histalog, pentagasrtrin, and

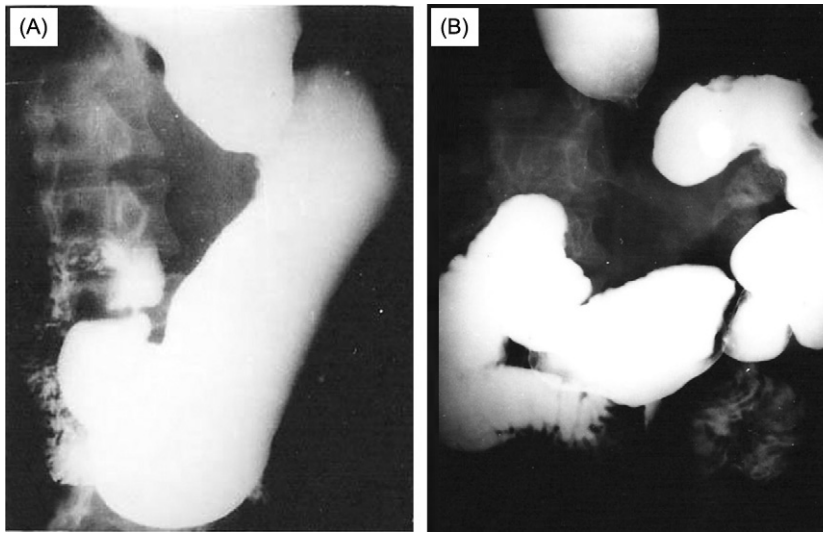

Figure 27.14 (A) Megaesophagus group IV associated with megastomach; (B) megaesophagus of group III associated with dilatation of stomach, bulb, and duodenal archade.

calcium ion infusion) gastric acid secretion. However, when a stimulus with a cholinergic substance is added, an increase of chloridric acid or pepsin secretion is obtained. This demonstrates that hyposecretion is mainly due to intrinsic denervation of the stomach and not due to reduction in the number of secretory cells. Fast and postprandial hypergastrinemia is another finding.

Besides these secretory and motor alterations, chronic gastritis of variable intensity is frequently found. Multiple etiopathogenic factors may be involved, such as biliary duodenogastric reflux and infection by *Helicobacter pylori.*

Hypertrophy of pyloric muscle is usually seen in cases with severe difficulty in gastric emptying, formerly known as pylorus achalasia. In these cases pyloroplastia is indicated as a complement to cardiomyotomy for the surgical treatment of megaesophagus.

Duodenum is, after the esophagus and colon, the segment that most often shows dilatation (Figure 27.15).

Megaduodenum is nearly always associated with other visceromegaly. The dilatation may be localized only at the bulb (megabulb), at the second and third segments, or involve the entire duodenal arcade. Even when no dilatation is present, dyskinesia and hyperreactivity to cholinergic stimuli are common, due to enteric denervation. Symptoms caused by megaduodenum may be confused with dyspepsia of gastric origin, of the dysmotility type.

Histopathological studies have shown less degree of denervation at the small intestine than at esophagus and colon. Dilatation of jejunum or ileum characterizing megajejunum or megaileum is rare, with few published cases (Figure 27.16).

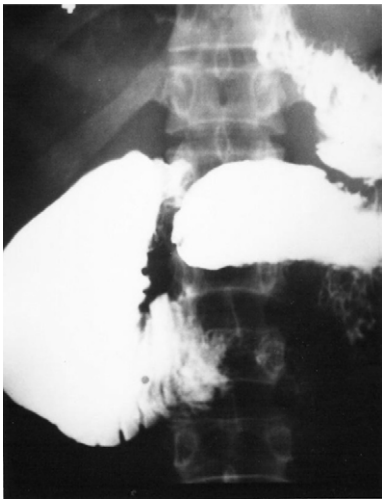

Figure 27.15 Chagasic megaduodenum.

Figure 27.16 Megajejunum in a patient with megaesophagus of group II.

The evidence of chagasic enteropathy is not readily apparent by clinical means, but may be detected using specific investigations. Motor alterations have been described in radiological and manometric studies. Patients with other manifestations of the digestive form usually show abnormalities in the interdigestive motor migratory complex. As a consequence, it is possible to have an increase in bacterium flora growth, which in some cases is similar to that seen in the syndrome of stagnant loop.

Studies in patients with the digestive form have also demonstrated an abnormal increase in the absorption of glucose and other sugars. As a consequence, the tolerance to glucose oral test may show abnormal glycemic curves, with transitory hyperglycemia in the first hour. In association with hyperabsorption of glucose a modest

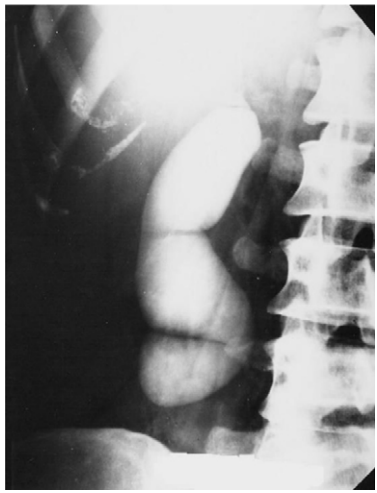

Figure 27.17 Colecistomegaly in a chagasic patient.

hypoabsorption of fats may be seen, although not sufficient to reduce the fecal fat excretion rate. Both alterations are partially due to abnormalities in gastric emptying.

An intrinsic denervation of the gallbladder may also be observed, leading to motor alterations in gallbladder filling and emptying. Manometric alterations were also described at the Oddi sphincter. Nevertheless, colecistomegaly and choledocho dilatation are not frequent (Figure 27.17).

A higher prevalence of colelithiasis in chagasic patients with megaesophagus and/or megacolon has been described.

Salivary glands, mainly parotids, are hypertrophic in patients with megaesophagus, a common finding in any obstructive esophageal disease as a consequence of the esophageal−salivary reflex that produces hypersalivation. Chagasic patients also have a higher sensitivity of salivary glands to mechanical stimuli of mastication and to the pharmacological stimuli by pilocarpin. Interestingly, the hypersalivation and parotid hypertrophy persist in esophagectomized patients, showing that these alterations are not exclusively due to the esophagus−salivary complex, but that there may also be some inervation involvement of salivary glands in Chagas disease (Figure 27.18).

Regarding the exocrine pancreas, its functional capacity is preserved in relation to direct stimuli of the organ. However, in consequence of alterations in the delivery of duodenojejunal hormones, a secretory deficiency by indirect stimuli may be found.

27.3.4 Cardiodigestive Form

The association of heart disease with megaesophagus or megacolon, or both defines the cardiodigestive form of Chagas disease. In most countries the development of megaoesophagus usually precedes heart and colon disease, but the exact prevalence of the cardiodigestive form is not known because of the scarcity of appropriate studies.

Figure 27.18 Hypertrophic parotids in a patient with chagasic megaesophagus.

27.4 Concluding Remarks

Individuals with Chagas disease may be seen in two distinct phases: acute, seldom diagnosed as such, and chronic. The diagnosis of the chronic phase is based mainly on the presence of IgG antibodies against *T. cruzi* in patients with high suspicion of the disease or in those with a compatible clinical syndrome. Chronic Chagas infection is silent for life in more than half of the individuals. In order to define the clinical form of disease, a complete search for cardiovascular and gastrointestinal symptoms and a resting 12-lead ECG are essential. Although barium swallow and enema are needed for final diagnosis of the digestive form, these tests are usually not recommended as standard practice for patients without gastrointestinal symptoms. Asymptomatic patients with a normal ECG and no gastrointestinal tract or cardiovascular symptoms have a favorable prognosis and should be followed up every 12−24 months, since about 2% of these patients progress to a clinical form of the disease each year. There are no known markers of disease progression. Patients with ECG changes consistent with Chagas heart disease should undergo a routine cardiac assessment to establish the stage of disease. Ambulatory 24-h Holter monitoring is used to detect arrhythmias; combined chest radiography and 2D echocardiography refine the assessment of cardiac size and function, and provide additional prognostic. If complaints of dysphagia or constipation are present, the routine contrasted X-rays are indicated. Most patients can be followed by a general practitioner. Labor restrictions are limited according to the degree of heart involvement. Of note, even if a reasonable proportion of patients with Chagas heart disease die early, a similar number may have irrelevant disease until elderly ages.

Glossary

24-h Holter monitoring (ambulatory Holter monitoring) a portable device used for 24 h that continuously records the patient's ECG during usual daily activity.

Abnormal chronotropic response inadequate increase in heart rate during exercise testing.

Abnormal inotropic response inadequate increase in systolic blood pressure during exercise testing.

Achalasia an esophageal motility disorder in which the smooth muscle layer of the esophagus loses normal peristalsis (muscular ability to move food down the esophagus), and the lower esophageal sphincter fails to relax properly in response to swallowing.

Atrial fibrillation an abnormality in the heart rhythm that involves irregular and often rapid beating of the heart and is related to thromboembolic phenomena.

Cardiac resynchronization (biventricular pacing) a treatment for heart failure that uses a three lead biventricular pacemaker implanted in the chest. The pacemaker sends tiny electrical impulses to the heart muscle to coordinate (resynchronize) the pumping of the chambers of the heart, improving the heart's pumping efficiency. Both ventricles are paced to contract at the same time. This can reduce the symptoms of heart failure.

Complete atrioventricular block also known as third degree heart block, it is a rhythm disorder in which the impulse generated in the sinus node in the atrium does not propagate to the ventricles.

Couplets two ectopic beats occurring one after the other.

Dyskesia (dyschezia) difficulty in defecation.

Fecaloma a tumor made of feces.

First-degree atrioventricular block a disease of the electrical conduction system of the heart in which the PR interval is lengthened beyond 0.20 s.

Gallop rhythm a usually abnormal rhythm of the heart on auscultation. It includes three or four sounds, thus resembling the sounds of a gallop.

Intracardiac electrophysiological study placement of multiple catheter electrodes into the heart for the diagnosis and management of selected cardiac conditions. This procedure has been used mainly for identifying the mechanisms, site, and severity of brady or tachyarrhythmias.

Low QRS voltage voltage of entire QRS complex in all limb leads of the ECG <5 mm.

New York Heart Association functional class a functional classification of heart failure into four stages according to the type of activity causing shortness of breath: I (intense physical activity); II (moderate physical activity); III (mild physical activity); IV (rest).

Nonsustained ventricular tachycardia a period of three or more ventricular ectopic beats lasting less than 30 s.

Primary ST-T changes ST T wave changes that are independent of changes in ventricular activation and that may be the result of global or segmental pathologic processes that affect ventricular repolarization.

Programmed ventricular stimulation a minimally invasive procedure which tests the electrical conduction system of the heart to assess its electrical activity and conduction pathways.

Sinus node dysfunction a group of abnormal heart rhythms presumably caused by a malfunction of the sinus node (the heart's primary pacemaker).

Stokes-Adams syndrome sudden collapse into unconsciousness due to a disorder of heart rhythm in which there is a slow or absent pulse resulting in syncope (fainting) with or without convulsions.

Transcatheter ablation an invasive procedure used to remove a faulty electrical pathway responsible for some cardiac arrhythmias. Catheters are advanced toward the heart and high frequency electrical impulses are used to induce the arrhythmia, and then ablate (destroy) the abnormal tissue that is causing it.

Volvulus a bowel obstruction in which a loop of bowel has abnormally twisted on itself.

Xenodiagnosis procedure allowing the feeding of laboratory reared triatomine bugs (known to be infection free) the blood of patients suspected of having Chagas disease; after sev eral weeks, the bug feces are checked for the presence of *Trypanosoma cruzi*.

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28 Diagnosis of *Trypanosoma cruzi* Infection

Alejandro O. Luquetti[*,1] *and Gabriel A. Schmuñis*[2]

[1]Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Brazil, [2]Former Officer of Communicable Disease Program, Regional office of the World Health Organization for the Americas

28.1 Introduction

This review describes the different methodologies and techniques that made possible the laboratorial diagnosis of Chagas disease since the first test was described in 1913. It has been a long journey and still there are questions without answer.

With the globalization of the infection by the protozoan *Trypanosoma cruzi* with thousands of infected individuals all over the world (Schmunis, 2007), the role of the laboratory is even more important than before. We will describe here how the possible few cases of recent *T. cruzi* infection that may appear today in special circumstances would be diagnosed using parasitological techniques. Taking into account that the vast majority of infected individuals are at the chronic asymptomatic phase, their only marker would be the presence of *T. cruzi* antibodies, mainly of the IgG class. Therefore, their accurate serological diagnosis is a must, either for exclusion or for confirmation of the infection. This is the only way medical personnel can handle the patients correctly. Special attention is given to the advantages and limitations of the different diagnostic methods as well as different examples on how these diagnostic tools may be applied by physicians in real-life situations.

28.2 History of Diagnosis in Chagas Disease

Four years after the discovery of the parasite *T. cruzi* by Carlos Chagas, as well as the disease it produces in humans and the epidemiology of the infection, the complement fixation (CF) or Bordet–Gengou test was adapted by Guerreiro and Machado (1913) to the serological diagnosis of what is now known as Chagas disease.

The CF test was the only one routinely used for diagnosis for more than 50 years, using as a source of antigen, organs of infected animals (Camargo and

*E mail: luquetti@hc.ufg.br

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Takeda, 1979). Later on, extracts of *in vitro* cultured epimastigotes were employed. These extracts were treated with benzene, ether, or acetone (Freitas and Almeida, 1949; Batista and Santos, 1959; Fife and Kent, 1960; Maekelt, 1960, 1964; Baracchini et al., 1965) in order to remove the lipids that could cross-react with serum samples positive for syphilis. The final reagent was suspended in water or methanol and kept with glycerol at 20°C or lyophilized at 4°C. Throughout this time, the whole crude extract of epimastigotes was replaced by proteins or polysaccharides derived from it (Fife and Kent, 1960; Goncalves and Yamaha, 1969; Bergendi et al., 1970; Gonzalez and Kagan, 1973; Camargo et al., 1976).

The CF test was cumbersome to perform. The amount of complement available, the temperature, and the time of incubation were all crucial for this test, and these variables were not always followed by the laboratories that performed the test. The diversity of antigens available also made the test difficult to standardize. In any case, it was the only test available and continuous efforts to improve standardization made the CF test reliable when in the hands of those laboratories that complied with the strict norms necessary to obtain the maximum reactivity (Almeida, 1956; Siqueira, 1964; Almeida and Fife, 1976). In these conditions, the CF had from 90% to 100% sensitivity (Freitas, 1951; Salgado et al., 1970), and cross-reactivity was mainly with visceral and tegumentary leishmaniasis. Another problem was the variable number of samples in which the serum was anticomplementary, which made the results inconclusive. Doing the CF and the indirect hemagglutination test (IH) on thousands of serum samples, agreement among the two tests was 96.5% to 98.5%. There were also 1.5−3.5% of samples that were doubtful because there was no agreement among the tests or because anticomplementary results were obtained with the CF test. (Cerisola, 1972).

The xenodiagnosis is another old diagnostic method described by Brumpt (1914). The principle is to reproduce the natural cycle in the vector host by feeding bugs in infected individuals. Several variables have been adjusted with time, which were standardized by Cerisola et al. (1974). It was used in the past mainly in Brazil, Argentina, and Chile and is now available in only a handful of laboratories in the world.

From the early 1940s until the end of the 1970s, other diagnostic tests appeared based on the detection of antibodies (Table 28.1). With exception of the indirect immunofluorescence antibody test (IIF) and the IH, none of the others survive as tests for routine use. The IIF was used as a test for many infectious diseases, but the first reports applied to Chagas disease using epimastigotes as antigen by Fife and Muschel (1959); it started to be widely used some years later after the standardization by Camargo (1966). The first publication on the IH was from Cerisola et al. (1962) in Argentina and Knierim and Saavedra (1966) from Chile. Because of its simplicity, it was quickly adopted as one of the methods of choice for diagnosis.

28.3 Diagnosis of *T. cruzi* Infection

Diagnosis of infection by *T. cruzi* should include, as in any infectious disease, clinical data, supported by the epidemiology and confirmed or not by laboratory tests.

Table 28.1 Tests for Diagnosis of Chagas Disease 1940–1971

Year	Test for Diagnosis
1913	Complement fixation
1914	Xenodiagnosis
1944	Agglutination
1944	Precipitation
1949	Conditioned hemolysis
1952	Immobilization test
1955	Indirect Coombs
1958	Immunofluorescence with epimastigotes
1959	Dye test
1962	Indirect hemagglutination test
1964	Immunofluorescence with amastigotes
1967	Trypanolisis
1970	Latex test
1971	Direct agglutination test

Source: Modified from Cerisola (1972).

Table 28.2 Relation among Positive Serology for Chagas Disease and Some Characteristic Alterations in Infected Patients

Alteration	Positive Serology	%	Negative Serology	Total
Complete right bundle branch block[a]	847	98.0	18	865
Megaesophagus	1575	93.3	114	1689
Megacolon	916	94.6	52	968

[a]As a single or combined ECG alteration. Patients were all younger than 61 years old.
Source: Laboratorio de doença de Chagas, Hospital das Clinicas, UFG and Rezende and Luquetti (1994).

This general statement has some pitfalls in Chagas disease, mainly because more than half of the infected individuals may have no complaints and clinical examination, as well as some exams such as electrocardiogram (ECG), chest X-ray, barium swallow, and barium enema may be normal (Rassi et al., 1992). In these cases, the suspicion may arouse from the epidemiological background of the patient. Past residence in endemic areas, infected mother or siblings, or work with this pathogen may be recorded and evaluated, and laboratory examinations used for exclusion or confirmation. The third aspect of diagnosis, laboratory tests, may be the only proof of disease and thus should be properly done.

On the other hand, some clinical alterations are so frequent in those patients with symptoms of Chagas disease that a negative laboratory result should be taken with caution and a request to repeat the exam, may be advisable. We refer to complete right bundle branch block, an ECG alteration which is typical (but not patognomonic) of Chagas disease (Table 28.2). Less than 20% of infected individuals

have digestive tract manifestations, as megaesophagus and/or megacolon. These alterations are also not frequent in the general population. If present, they strongly suggest Chagas disease. In fact more than 90% of the patients with any one of these disturbances, in an endemic area, have positive serological tests for *T. cruzi* (Rezende and Luquetti, 1994) (Table 28.2).

Laboratory diagnosis includes parasitological and serological tests. Infected individuals/patients may look for medical care during the acute phase or, more often, during the chronic phase. Laboratory tests to be ordered depend on the suspected phase of the disease. For those unusual cases during the acute phase, which lasts only for 2 months, parasitological tests are mandatory. On the other hand, in the chronic phase which lasts for life, serological tests are the diagnostic method of choice. These principles are explained by the natural history of Chagas disease, with easily detected parasites during the acute phase, which is defined because of the presence of them. Weeks after symptoms start, the immune response will destroy the majority (but not all) the parasites and they will be no longer easily detectable. By this time (4 weeks) IgG antibodies should be present, with higher affinity as time goes on, and they will hold for the rest of life, unless the patient would receive anti-*T. cruzi* treatment (Luquetti and Rassi, 2000).

In summary, to confirm or exclude an acute infection by *T. cruzi*, parasitological tests should be performed as the first choice. When the suspicion is of a chronic infection, serological tests must be performed.

28.4 Parasitological Tests

Overview

The natural course of Chagas infection starts with an acute phase, whose manifestations, if present, will arouse some days after the entrance of the parasites. Parasites multiply exponentially until the antibody response starts, which becomes evident 10−20 days later. As a consequence, the number of parasites begins to decline, and, at the end of the first month, they are scarce. This phase lasts for 2 months, is defined by the easy detection of the parasite, which becomes more difficult to find after the first weeks. For diagnosis of this phase, parasitological tests should be employed (Luquetti and Rassi, 2000).

At this stage, it is useful to include as acute cases all the following conditions, independent of the mechanism of transmission: acute phase by vector transmission, congenital infection, transfusion or by organ transplantation, oral route, accidental infection, and reactivation by immunosuppression. Even if the finding of parasites is the hallmark, clinical manifestations may be different. In those by vector contamination, most of them will not be recognized. Those infected through transfusion may also not have been diagnosed, and the manifestations may appear with a longer incubation period, the reasons for which have not been understood yet (Amato-Neto and Dias, 1969). Congenital transmission may also remain undiagnosed, because most of the cases are born with normal weight and without any other features, apart from the

infected mother. Oral route may have digestive manifestations, probably because of the large inoculum and the multiplication of parasites at the digestive tract. For these reasons, many acute cases are not diagnosed at this phase, but many years later by chance (i.e., during a blood donation) (Luquetti et al., 2005).

After the acute phase, which is estimated to be asymptomatic in more than 95% of the infected individuals, even without diagnosis or treatment, the infected person enters the chronic phase, usually in the indetermined form (no symptoms or signs, no ECG, or X-ray abnormalities) which lasts for the entire life. Spontaneous cure has been reported but is certainly highly unusual (Zeledon et al., 1988). Circulating parasites are scarce, never found by direct observation (see methods later) and may be absent for circulation for some periods. It has been said (Luquetti and Rassi, 2000) that parasitemia at the chronic phase is variable, not constant, and present at a given moment only in a few mL of the 5 L of blood that a human being has. This fact explains several observations made in the natural history of the infection: transmission by transfusion is successful only in 20% of the cases up to 50% in hyperendemic areas (Rohwedder, 1969; Cerisola et al., 1972; Zuna, 1983; Atias et al., 1984).

When performing several parasitological methods (see later) at the same time, it is normal to find parasites only with one of them; by using xenodiagnosis (40 bugs), positive bugs are only 1 to 3 of them, the others remain negative because they did not ingest a single parasite. Cerisola et al. (1974) observed that each bug is as a "single microhemoculture processed at the digestive tract of each bug," hence the chances to find positive bugs increase as more bugs are included.

Indications

For all the earlier reasons, parasitological methods should not be used for diagnosis of chronic phase of Chagas disease because most of the results will be negative. A negative parasitological result has no value in terms of diagnosis. On the other hand, a positive test has tremendous value, even when antibodies are present in low titers. For diagnosis of the chronic phase, parasitological examination should not be used.

Parasites are easily detected only in the acute phase or during the reactivation of the chronic phase because of immune suppression. Actually, this is the definition of acute phase. What means "easily detection"? This is when finding of parasites by direct observation or by concentration techniques (see later) is possible. These tests are easy to perform and cheap, but only available in specialized laboratories in the field. There is no need to buy them, because they use simple tools that any laboratory has (glass slides, tubes, centrifuges, microscopes). However, there is need to have personnel with expertise to search and diagnose *T. cruzi* and at the same time, avoid being infected by live parasites (see Section 28.9).

There are methods for the detection of *T. cruzi* when it is present in low numbers (i.e., the chronic phase). These methods are based on another principle: the multiplication of parasites that are not easily found in the sample (usually peripheral blood). For this multiplication, the conditions offered to the parasite should be optimal.

Methods most used are classic xenodiagnosis and hemoculture, and the relatively recent (Gonzalez et al., 1984; Ashall et al., 1988; Sturm et al., 1989) polymerase chain reaction (PCR), a molecular test, based on the amplification of DNA

or RNA present in *T. cruzi* through probes. Xenodiagnosis and hemoculture have common features; they are not commercially available, demand some time to get multiplication of the parasites (weeks, months), are time consuming, and need expertise from technicians. In addition, the former demands a colony for growing bugs, which is available in less than a handful of laboratories in the world; and the latter needs to be processed in absolute sterility and at 4°C, conditions difficult to find in the field. As both have been in use for decades, they are properly standardized (Cerisola et al., 1974; Chiari et al., 1989).

PCR may be performed in a few hours, but is more expensive, requires special skills, and attempts for standardization and validation have only recently been made. This technique has been applied for *T. cruzi* since the early 1990s, with high interest after the first publications indicating 100% positivity (Avila et al., 1993). Further studies revealed much lower figures, depending on the population group studied. When analyzing the first results, they were performed with patients who previously had positive xenodiagnosis, so a bias was introduced. The technique revealed variable sensitivity depending not only on the characteristics of the population analyzed but also from the test itself, the volume and conservation of samples, DNA extraction method, parasite sequences used, primers, reagents, and thermo-cycling conditions (Schijman, 2009).

The need for a standardization was recently stressed (2008) in a multicenter evaluation sponsored by TDR/WHO and performed in two steps. In the first, 29 research groups were invited to participate and received three sets of samples blind. The first set comprised five 10-serial dilutions of *T. cruzi* DNA from three reference stocks. The second were blood samples spiked with 10-fold dilution of culture parasites and the third was a panel of 40 clinical samples (10 from noninfected persons) from several countries. Each laboratory performed its own method with the same samples. From the 49 different strategies used by the 29 laboratories, only four methods were selected, on basis of their performance with a specificity of 100% and a sensitivity of 56−63%. In the second step, 18 participants were selected by their better performance, participated in a workshop, performing the analysis of the clinical samples with the same reagents and methods. Silica gel column extraction followed by satellite-based PCR was more specific and sensitive than other methods, and a standard operating procedure was defined for the test (Schijman, 2009).

There are two main questions regarding PCR. The first is because of the natural history of the parasitemia at the chronic phase. As written above, some infected individuals may be free of circulating parasites for long periods of time, so PCR could not find DNA of nonexisting parasites, and this fact may explain why several recent reports cannot reach the 100% sensitivity predicted years ago. The other main question relates to the reliability of PCR diagnosis, with a high frequency of false positives due to contamination observed in some studies, as well as false negatives due to inhibitory substances in the lysate (Schijman, 2009).

Conclusions

For all the reasons exposed, these multiplication methods have several limitations and a short range of applications like the follow-up of treated patients to check

treatment failure and, in research, for isolation of parasites. These situations are seen in very specialized laboratories devoted to the study of the disease, but up to now not for routine diagnosis.

Nevertheless, in the best conditions, parasitemia can be found in a number of individuals at the chronic phase. Statistics are different according to geographical area (i.e., lower in Piaui, Brazil; Junqueira et al., 1996), age of the patients (higher parasitemia before 11 and after 60 years old), pregnancy (increases), and use of specific treatment (decreases). Because of the lower parasitemia, the chance to find parasites increases with the number of examinations. All the studies show that figures are higher in the same individual, if the same method is performed two or more times (Rassi et al., 1991; Castro et al., 2002). Also, if more than one method is applied, the chance of obtaining parasites increases (Junqueira et al., 1996). Recent studies show that a single hemoculture or xenodiagnosis may be positive in 24−52% of the patients. The same sort of studies show that if more examinations are performed, the figures increase to a maximum of 65% (Luz et al., 1994). It is clear from these studies that some patients are always negative, irrespective of the number of tests applied. On the other side, some positives always turn out that way (around 8−12% depending on the study). These are classified as having high parasitemias (each test applied is positive). Another way to measure the degree of parasitemia is to record the number of tubes of hemocultures or the number of positive bugs, which allow you to classify the degree of parasites present. These studies are particularly useful when a new drug is tested (Rassi et al., 2007).

28.4.1 Acute Phase (Direct and Concentration Methods)

Several easy methods are available to search for parasites when they are present in large numbers, as in the acute phase. They could be divided in direct tests and concentration methods.

28.4.1.1 Direct Tests

The simplest and cheapest direct test is the fresh blood smear. A drop of peripheral blood from the patient is collected from the ear, fingertip, foot, or from a vein through a syringe. Ten μL of blood is immediately deposited on a smear and a cover slip (22 × 22 mm) covers the drop. The amount of 10 μL is ideal for a nice preparation (i.e., a very thin smear that allows seeing red blood cells separated from each other). The preparation should be mounted in a microscope with an objective of 40× and ocular of 10× (i.e., 400×). If *T. cruzi* is present, it will be seen as a refringent body with very quick movements, disturbing the quiet red blood cells. It is advisable to have some previous training, which may be just an observation for some minutes of such a preparation. As an exercise, this could be prepared from infected mouse blood in those laboratories that work with *T. cruzi*. This parasite easily contaminates humans, so all measures to avoid a laboratory accident should be taken, like the use of gloves, facial mask, etc. (Brener, 1984; Ministerio da Saude, 1997). More than 100 laboratory accidents have been

documented and published and probably a similar number of cases have not been recorded.

In cases of vector transmission, with less than 20 days from the start of symptoms, it is common to find one parasite every 10−50 fields. The smear should be looked at for 100−400 fields before being informed as negative. The test may be performed in several smears or on different days. If a single examination is negative, concentration methods may be applicable if the clinical suspicion persists. In some transfusional cases (Luquetti and Rassi, 2000) or immunosuppression, several parasites may be seen in one field.

If a motile, refringent flagellate is found, the diagnosis is made. No further analysis is necessary. Check if clinical data are available (i.e., fever of unknown origin, recent transplantation or transfusion). The preparation may be dried and stained, but for better visualization, a proper smear as for differential count of leukocytes is preferable.

28.4.1.2 Other Direct Tests

The dry smear as for differential count has a much lower sensitivity, and will be positive only when a large number of parasites are present, equivalent to more than one parasite per field with the fresh smear. It is not recommended because of that. The thick smear, as used for malaria diagnosis, also has lower sensitivity but is better than the dry smear. The morphology of the parasite is generally not well preserved, but is an excellent option when used in the field in malaria regions. If negative for hematozoan, the presence of a flagellate could be diagnostic. Many otherwise nondiagnosed cases were found by malaria control program personnel, after proper training, in Brazil and other countries.

28.4.1.3 Concentration Methods

Two are most used: the Strout technique and microhematocrit.

Strout (1962)

The Strout technique is very simple. Centrifuge tubes, a centrifuge, and a microscope will suffice. Blood (3−5 mL) is collected without anticoagulant and left to clot, at room temperature, or quicker, at 37°C. Once the clot is formed (15−60 min) the blood exudate is transferred with a pipette to a centrifugal tube and spun down at low speed (i.e., 50 g, 500 rpm according to the radius of the centrifuge) for 5 min. This will allow for the separation of most (but not all) red blood cells that remain at the bottom of the tube. Take all the supernatant (approximately 1 mL) and transfer to another centrifuge tube and spin hard (i.e., 500 g, around 2000 rpm) for 10 min. This will clear the suspension, having a clear supernatant. Take nearly all the supernatant and store for serology. Now, with the last drop remaining at the bottom of the tube, resuspend it and apply 10 μL to a glass slide and cover slip, with the same methodology as the fresh blood smear explained above. Look on the microscope. Parasites are there. The rationale of this method is that all the parasites escape from the clot to the serum. By separating most of the red blood cells with the first spin, we get rid of them. By the second spinning,

parasites are forced to remain at the bottom of the tube. Hint: Do not delay in preparing the smear after the centrifuge stops, otherwise the parasites will swim to the sera and will be lost.

Microhematocrit

Microhematocrit is very useful for congenital infection, because of the need of only 100 μL of blood for each test. Ideally collect from the plantar region of the baby's foot, four glass capillaries (heparinized, to avoid clot). Spin down as for hematocrit in a proper centrifuge. Look at the interface of the microscope. Hematocrit will have an upper layer of plasma, the interface where leukocytes and *T. cruzi* are, and a lower layer of packed red blood cells. It is not necessary to break the capillary tube, but if necessary it could be done. In this case, take strict care to avoid accidental contamination, as described before (Freilij et al., 1983).

28.4.1.4 Other Methods for Concentration

Several other methods have been described, as the separation by Ficoll-Hypaque (Budzko and Kierszenbaum, 1974), on the same layer as mononuclear cells, but in practical terms the two already described are the top choices.

28.4.2 Chronic Phase

28.4.2.1 Xenodiagnosis

The main variables are species of bug to be used; number of bugs to be applied; stage of bug; time for application on patient; number of examinations to be performed; days of fast before application; method of examination of bugs; temperature and humidity to preserve alive bugs; ideal time for examination after feeding; method of examination; individual or pool examination; number of readings; and expression of results. After many publications on the subject, a standardization was published by Cerisola et al. (1974). which has been followed until now. Some variables are still a matter of discussion, such as if it is better to use an autochthonous species of bug. After Cerisola et al. xenodiagnosis is performed as follows: for *Triatoma infestans*, 40 bugs, third instar, 15 days fast, divided in 4 boxes (10 bugs in each), each box in arms and/or legs, applied for 30 min, bugs stored in chambers at 25−30°C, humidity 70%, examined at 30 and 60 days. Results may be expressed as number of positive boxes (i.e., 1+/4). The number of examinations will depend on the investigator. Several improvements were developed later, mainly the use of artificial xenodiagnosis, because allergic reactions were observed in some patients, and the risk of contamination from the bugs, in immunocompromised host, mainly after the era of AIDS. This last variation allowed use of a large number of bugs, up to 360 with 10−30 mL of venous blood, increasing the chances of positivity (Santos et al., 1995; Franco et al., 2002).

There are several limitations of this technique. There is a need for a large insectary, with more than 100 female and male adult bugs, in order to provide enough eggs to perform the examination. Bugs (adults and nymphs) need to be fed on hens or other birds, every 15 days. Colonies of bugs may be contaminated with

Blastochritidiae triatoma, morphologically similar to *T. cruzi*, and if this happens all the bugs should be eliminated and the research started from scratch (Cerisola et al., 1971). The examination of bugs is time consuming and requires a proper infrastructure. Results will be ready after 60 days or more. The advantages of this technique are that it may be performed in the field (it is only necessary to transport bugs with 15 days on fast), does not require sterile handling, and allows for inoculation in animals. Very few laboratories in the world have the capacity to maintain such an infrastructure and those who need to isolate strains are moving to hemoculture whenever possible. The preferred method is artificial xenodiagnosis.

28.4.2.2 Hemoculture

Developed many years after xenodiagnosis, for a time hemoculture had a lower yield in terms of positivity (Chiari and Brener, 1966). Some improvements developed mainly by Chiari et al. (1989) helped make it the current method of choice. Variables, as with xenodiagnosis, include the amount of blood to be collected, anticoagulant to be used, separation/inclusion of plasma, medium to be used, relation inoculum/medium, number of tubes to be used, temperature for maintenance, timing and number of readings, and method of examination. After the standardization, the conditions were: 30 mL of peripheral blood with heparin handled quickly (less than 2 h) at 4°C, spin down at 4°C to get rid of plasma, wash in medium liver infusion tryptose (LIT), made six replicates by 1 mL washed blood and 3 mL of LIT each, maintain at 26°C, examine every 30 days for 5 consecutive months. Results may be expressed as number of positive tubes (i.e., 2+/6).

The main limitation is sterility, because blood needs to be processed in several steps. The medium (LIT) should be prepared (not commercially available) and has several variables which are out of the scope of this chapter.

28.4.2.3 Animal Inoculation

Rarely used nowadays, animal inoculation was once employed in the field. It is essential to use a susceptible animal, often mice (Balb C), which should be young (up to 30 days of age) and male, as they are more susceptible. Examination of the tail blood should be done daily or every 3 days, since parasites may be present for very short periods. It is time consuming, and animal house facilities should be available. It may be used in combination with xenodiagnosis by inoculation of positive bug feces in order to isolate a stock for further characterization. After the first passage, mice could be treated with immunosuppressors (i.e., cyclophosphamide) to increase parasitemia (Oliveira et al., 1993).

28.4.2.4 Antigenuria

This method was once more popular (Corral et al., 1996) but has several disadvantages, as the need for concentrating large volumes of urine.

28.4.2.5 Polymerase Chain Reaction

As discussed earlier (see "indications of parasitological tests"), PCR is the newest parasitological test in use. It has higher sensitivity than the other multiplication methods, but like the others is not 100%. The goal is now to assure that the specificity of this technique is 100% (Schijman, 2009). As it is an "in house" test, it is not commercially available.

28.5 Serological Tests

28.5.1 Antigenic Makeup

The kinetoplastid *T. cruzi* has many antigens distributed on its surface (cytoplasmic membrane and flagellum), and from the different organelles at the cytoplasm and nucleus. Some of these antigens are exposed when the parasite is circulating, as a trypomastigote, and others will be presented to the immune system after it is dead. A different set of antigens may be excreted. This parasite may also shed antigens and after some time internalize antigens that were previously exposed.

The antigenic makeup of *T. cruzi* from different geographical areas is rather similar, as demonstrated in several publications that used reagents made from *T. cruzi* circulating in the Southern Cone to diagnose serum from patients of Mexico (Luquetti et al., 2009). As known, *T. cruzi* is heterogeneous, and two main *T. cruzi* are recognized: *T. cruzi* I and *T. cruzi* II. The former has been isolated from silvatic regions in the USA and also from humans infected above the Amazon River. The second, now split into five groups, is found to the south of Amazon River. A recent classification (Zingales et al., 2009) expanded to six groups defined as discrete typing units, from TcI to TcVI. Antigenic differences among those six *T. cruzi* genotypes are currently under study. In some specific cases, studies performed with Colombian patients show that a few of them will have lower titers when assayed with reagents made with *T. cruzi* II, but further studies to ascertain the magnitude of this difference should be performed (Gutiérrez et al., 2004).

Nevertheless, *T. cruzi* belongs to the order Kinetoplastida, and may share some antigens with other protozoan of this order. The most important family, tripanosomatina, is divided into 11 genera, most of them parasites (Vickerman, 1976). The nearest to *T. cruzi* are other protozoa of the same genus: *Trypanosoma* (Salivaria and Stercoraria). The parasite that infects humans is *T. brucei* (*gambiense* and *rhodesiense*), but the geographical distribution is different so that no overlap of infections with both pathogenic trypanosomes are likely. There is another trypanosome, *T. rangeli*, which is not pathogenic for humans but may be present in the same geographical regions as *T. cruzi* and may share some antigens. Several studies show that infected humans with this parasite, diagnosed by hemoculture, do not have anti-*T. cruzi* antibodies, at least with the high titer seen in those with Chagas infection. The reasons are probably due to the short contact with humans in their brief life spans (D'Alessandro and Saravia, 1992; Vasquez et al., 1997).

The other genus that has human pathogenic parasites is *Leishmania*, and again, some antigens may be shared. Several leishmanias are recognized to be present at the same geographical areas as *T. cruzi*, such as the agents of muco-cutaneous leishmaniasis (*Leishmania mexicana* and *Leishmania braziliensis*) and *Leishmania donovani chagasi*, the American agent of visceral leishmaniasis (kalazar). Both diseases are quite different: in the former, amastigotes remain inside macrophages mainly on the skin and mucosal surfaces. The immune response is mainly by cell-mediated immunity and antibody responses are poor, making the serological diagnosis of muco-cutaneous leishmaniasis difficult. On the contrary, visceral leishmaniasis is a severe disease, with a poor prognosis if not diagnosed and treated. As known, *L. donovani* parasites are widely distributed in bone marrow, liver, spleen, and in circulation. It is also well known that this parasite stimulates B lymphocytes in a polyclonal activation, with a huge increase in antibodies of several classes and subclasses, leading to a polyclonal hypergammaglobulinemia. As a consequence, in advanced stages, antibodies to an array of antigens are present. In relation with the diagnosis of Chagas disease, nearly any reagent used with sera from kalazar patients will be positive. It is our experience that, in laboratory terms, it is not possible to differentiate a kalazar patient, serologically, from a chagasic one. All the difference is on the clinical setup, which must be evaluated by the clinician. The kalazar patient who shows a positive serology for Chagas disease is severely ill, with fever, liver and spleen enlargement, edema, hemorrhagic lesions, and with a hemogram that shows pronounced anemia, leukopenia, and plaquetopenia. Not one of these alterations is present in the chronic phase of Chagas disease. The laboratorial diagnosis of kalazar is confirmed by bone marrow aspiration, which should be conducted if this diagnosis is suspected. So, in this case, diagnosis should be clinical, epidemiological, and supported by the laboratory.

28.5.2 Antibody Response

As part of the life of *T. cruzi* is in the circulation, the immune system is continuously stimulated by an array of antigens, and the production of antibodies of high affinity is the rule. Because of this, most of the infected individuals mount a strong antibody response against it. Some antibodies may kill trypomastigotes by a complement dependent mechanism, as shown *in vitro* by the antibody dependent complement-mediated lysis test (Krettli and Brener, 1982). This is probably one of the mechanisms that maintains the parasitemia at low levels *in vivo*, but not so efficiently, because some parasites evade it and may be found alive, as demonstrated in xenodiagnosis or hemocultures.

Other antibodies may be directed to shed antigens, such as shed acute phase antigen (SAPA) , present mainly during the acute phase of the disease, and evanish both antigens and antibodies after few months (Affranchino et al., 1989).

The more useful antibodies for diagnosis may be directed to antigens present in the alive *T. cruzi* or against internal antigens that will be exposed only after the disintegration of the parasite. These antibodies are present in nearly any infected

patient, usually in high concentrations, which allow a serological diagnosis in the vast majority of patients.

The antibody response at the acute phase is possible to be detected after 1−2 weeks, mainly by the presence of specific IgM class antibodies. It should be emphasized again that the diagnosis at this phase is by parasitological methods. Only when parasites are not found and the clinical suspicion persists should the search for IgM anti-*T. cruzi* begin. These antibodies may be detected by a few techniques, most commonly indirect immunofluorescence, and direct agglutination with 2-ME has been employed as well. There is no commercial kit available for IIF-IgM or ELISA-IgM. An additional difficulty is obtaining positive controls. There are several pitfalls with "in house" IIF, mainly the presence of rheumatoid factor, which may yield a false positive result (Cabral, 1982). Also, some chronic phase patients may have IgM anti-*T. cruzi* (Luquetti et al., 1996). It has not been recommended for diagnosis of congenital infection, since several infected children have shown negative results (Carlier and Torrico, 2003). Serological conversion should also be considered when a negative result with IgG is obtained followed, days after, by a positive one. In such cases the conversion indicates an acute phase. Shortly after IgM responses arouse, specific IgG will be detectable, with lower titers in the first month, increasing progressively after that. These IgG antibodies are first detected by IIF and IH and later by ELISA tests (Luquetti and Rassi, 2000). By the end of the second month after symptoms start, most infected individuals have a detectable IgG response, which will last for life, unless interventions (i.e., specific treatment) are performed.

In viral diseases transmitted through transfusion, there is a window period in which recently infected individuals are asymptomatic and serologically negative by the assays usually used in Latin America for the screening, but they may transmit the infection. This window period is 20−25 days for HIV, up to 84 days for HBV, and 51 days for HCV. The possibility that this may happen in *T. cruzi* infection is remote. Most recent infections occur in childhood or adolescence and in rural areas. In addition, those recently infected will have fever, so they should not be accepted as donors (Schmunis and Cruz, 2005).

28.5.3 Different Serological Tests

Serological tests may be divided into conventional and nonconventional methods. The first group comprises the classical methods used for diagnosis since the discovery of the disease. They use antigenic mixtures, most are commercialized and able to diagnose most infected individuals. Great experience in all endemic countries has been achieved with time and many groups of investigation published results comparing their performance. Nonconventional methods were introduced in the last 30 years (since 1980), most are not available for universal use (not commercially available) and experiences with their results are restricted to a few groups.

Progress has been made for both groups of methods. At the time of their introduction, all conventional tests were *in house*, and comparisons between different laboratories, by employing the same sera, were not consistent (Camargo et al.,

1986). Each group of investigators produced their own reagents, made their own readings, and their own interpretations.

By 1980s, the need to improve detection of infectious diseases to screen blood donors was stressed. A number of specialized manufacturers began to sell their products, and the quality of reagents improved fast. Some studies performed in academia with commercial IH and ELISA tests helped improve their quality and reproducibility (Oelemann et al., 1998; Saez-Alquezar et al., 1998). On the other hand, considering that the main customers were the blood banks, kits started to fulfill their requirements (i.e., the highest sensitivity) (Ministerio da Saude, Brazil, 2006). Of course good kits in wrong hands would still give poor results. Several countries aware of this fact published detailed guides for good laboratory practices (GLP) (Cura and Wendel, 1994; Ministerio da Saúde, Telelab, 1998). Nowadays, with kits of proven good quality and GLP, it is relatively easy to get the same result with the same sera, performed in different centers and countries.

Conventional serology, if properly done, will solve most of the diagnostic doubts, mainly if following WHO recommendations to employ at least two tests in parallel. When the problem is donor screening, as in blood banks, one ELISA test will suffice. Previous problems of specificity are minimal by now, with exception of kalazar, as was mentioned before.

At the same time that commercial kits were improving their quality, a need for a better specificity arouse, and several purified antigens were studied and their results published (Chapman et al., 1984; Scharfstein et al., 1985; Schechter et al., 1985). Very soon after, with the recombinant technology, several putative candidates were available. Also synthetic peptides were candidates. By the end of the 1980s, several dozen of candidates were available for a better diagnosis, and each claimed to be the better. To solve this array of new antigen candidates for diagnosis, TDR of WHO decided to launch a multicentric study. By July 1989, the antigens have been tested, with the participation of 10 laboratories around the world (Moncayo and Luquetti, 1990) (see later).

28.5.3.1 Conventional Methods

The Complement Fixation Test

This was the first test employed for diagnosis, very soon after the discovery of the disease (Guerreiro and Machado, 1913). Most of reagents were prepared *in house* and the test was time consuming when a large amount of samples were necessary to test, as in blood banks. By the time that standardization was ready, other simpler tests were progressively substituting for the CF (mainly IH and IIF). By 1985 most laboratories abandoned the CF, moving to simpler tests.

The Indirect Hemagglutination Test

This is one of the methods of choice for diagnosis. It has several advantages which hold up to now, such as few steps (dilution of sera and contact with sensibilized red blood cells) low cost, no equipment, and quick results (1−2 h). The substitution of tubes for microplates was a further advantage, allowing researchers to process more samples in a shorter time. For all these reasons, different manufacturers

started to produce and sell kits, containing microplates, sensibilized red blood cells, and dilution media. Several brands were on the market by 1985, mainly produced in South America (Argentina, Brazil, and Chile). Specificity was high (98−99%) and often higher than ELISA or IIF at that time, but it was perceived that sensitivity of tests from different manufacturers varied widely (Lorca et al., 1992, 1994; Saez-Alquezar et al., 1997). This made the test unacceptable in situations where sensitivity was essential, as in blood banks. Nevertheless, it is still considered a good test for confirmation of the infection.

Even with the advantage of simplicity, there are a few details that should be kept in mind: some sera have a nonspecific reaction with some kits (natural antibodies), and it is recommended in these cases that serum should be treated with 2-mercaptho-ethanol (2-ME) (incubation of 30 min at 37°C with a dilution of 1/100). Depending on the source of red blood cells, some sera have antibodies against some species of animals, and the kits should include nonsensitized red blood cells in case of a discrepancy with the other tests (positive only in IH).

There is also a discussion in relation with the better bottom of the microplates to facilitate the reading and manufacturers sell the kits with V or U bottom, but this last allows a better reading. The cut-off point depends on the kit, but positive results with dilutions higher than 1/40 are considered reactive. The common titers obtained in 95% of infected individuals is higher than 1/128−1/160 (Luquetti and Rassi, 2000).

The Direct Agglutination Test

The direct agglutination test (Vattuone and Yanovsky, 1971) is also a simple test which has been widely used in some countries, but now it is not commercially available. The principle is the agglutination of the parasite by antibodies, if present. It has advantages, as the IH, and the possibility to search for IgM anti-*T. cruzi* if samples are run in parallel with and without 2-ME. If differences of more than two titers occur, there is a strong possibility that they are due to IgM. This test has been applied with success during the acute phase (Schmuñis, 1991b). The disadvantages are mainly related to the reading because of the clear color of epimastigotes. Titers obtained with this test are higher than 1/32 in most infected people. Typical results of serum from acute phase are 1/256 without and 1/16 with 2-ME (Peralta et al., 1981).

The Indirect Immunofluorescence

Indirect immunofluorescence is used mainly in research laboratories or diagnostic centers that handle a limited amount of samples per day. One of the advantages for laboratories is that the same conjugate (antihuman IgG) may be used for the diagnosis of several diseases and the ability to use the same equipment (fluorescence microscope). This test is conducted by reacting serum with smear fixed epimastigotes and, after washing, incubating with conjugate. The smears are read in the fluorescence microscope. The key advantage of this test is very high sensitivity. It is quite hard to find a serum from an infected individual which does not react. However, a disadvantage is that this same extreme sensitivity may lead to cross-reactions with several diseases. These cross-reactions are observed mainly in lower titers (1/40−1/80) and a higher one is quite characteristic of Chagas disease, unless visceral leishmaniasis is present.

Table 28.3 Titers Obtained in 1280 Sera from Nontreated Chronic Phase Patients by Indirect Immunofluorescence[a]

Titer	Nr	%
1/40	1	0.1
1/80	4	0.3
1/160	13	1.0
1/320	85	6.6
1/640	223	17.4
1/1280	338	26.4
1/2560	361	28.2
1/5120	255	19.9
Total	1280	99.9

[a]All patients have had complete right bundle branch block, and/or megaesophagus and/or megacolon (Luquetti et al., 2008).

This test has several steps and each may be critical, so it should be performed by a skilled technician. Among the variables, the growing phase of the epimastigotes, the pH of phosphate buffered saline, the pH of glycerol buffer, the concentration of conjugate, and the life of the microscopic light need to be checked for top performance. The reading is subjective, and after examination of more than 20 smears (200 wells), results may not be reliable because of exhaustion of the technician. Nevertheless, in good hands this is an excellent test. In a recent search, in an analysis of 1280 sera from infected individuals with typical clinical alterations, 98.6% had a titer of 1/320 or higher. More than half of this group had high titers of 1/1280 or more (Luquetti et al., 2008). The cut-off region is between 1/20 and 1/40 and some individuals of the noninfected population may be reactive in these low titers (Table 28.3).

The Immunoenzymatic (EIA) Test of ELISA

This test was first applied for Chagas infection in 1975 in a study on filter paper (Voller et al., 1975). It is rather similar to IIF, because it has many steps, needs a well-trained technician, and has extreme sensitivity, which is one advantage, but carries the problem of a more limited specificity. On the other hand, the advantages over IIF are the objective readings (spectrophotometer) and the possibility of automatization of handling hundred of samples at a time. The classic technique described by Voller et al. (1975) had several steps and the time to run a plate was 6 h. Now kits have improved the incubation time and the same plate may be ready in 2 h. As with the IIF, it is difficult to have a sample of serum from an infected individual that yields a negative result. Cutoff should be calculated as per technical instructions, but a curve with negative, low-positive, and high-positive controls is useful for the range of responses. Once the cutoff has been calculated, it is accepted that positive samples should be considered as such when OD are at least 10% higher than the cutoff.

In a recent study performed by the Ministry of Health, Brazil, 12 different brands of commercially available ELISA kits, approved by the sanitary authorities,

were tested with 150 positive and negative sera, including sera of low reactivity, by four independent laboratories. Results showed that the sensitivity for all of them was high (97−100%) but specificity was low (60−98%) (Ministério da Saúde, 2006). The interpretation was that kits were tailored to fulfill the needs of the main client (blood banks) and acceptable for them, but not good enough for the serological diagnosis of an individual patient.

To summarize, the ELISA test is excellent for the purpose of screening blood donors, but in order to confirm the infection, other tests of higher specificity should be employed in parallel. We will come back in these issues when the indications of serological tests will be discussed.

28.5.3.2 Nonconventional Methods

Radioimmunoprecipitation Assay (RIPA)

This test was developed in the USA by Kirchhoff et al. (1987) and is claimed to be the gold standard for serology. It is not widely available as it is only performed in few research institutions, is expensive, time consuming, and uses radioisotopes, which make its application as a standard procedure remote. One study compared their results with conventional tests (Leiby et al., 2000) and, although sensitivity compared well with IIF, IH, and ELISA of several brands, the number of negative sera used in this study ($n = 19$) was not enough to evaluate specificity.

Western Blot (Including TESA-Blot)

These tests have been widely used in research institutions, and several publications are available (Stolf et al., 1990; Teixeira et al., 1994; Mendes et al., 1997). They are time consuming and costly (estimated at US$20.00 per sample). Several antigens have been employed, among them the TESA-blot, a trypomastigote excreted−secreted antigen (Umezawa et al., 1996). This test has been employed with eluates of filter paper in a serological survey of children less than 5 years old in Brazil to learn if insecticide spraying to kill the vector was effective to halt the transmission. More than 8000 samples were run in the laboratory that was the quality control of the study (105,000 samples were collected) and results were excellent in terms of specificity and sensitivity (A. W. Ferreira, unpublished results).

Lytic Assays Including Flow Cytometry

The complement-mediated lysis of trypomastigotes test, developed by Krettli in the 1980s (Krettli and Brener, 1982), was used as a diagnostic tool and evaluation of treated patients. The rationale of this test was to screen for lytic antibodies, present in infected individuals, but absent in those successfully treated. This antibody would disappear before those detected by conventional tests (Galvão et al., 1993). In practice is a rather simple test that reacts trypomastigotes from irradiated mice with sera. If lytic antibodies are present, the parasites will be killed after the addition of complement when counted in a cytometer (Neubauer chamber). This apparently simple test has several drawbacks that made it unsuitable for routine use. Among them, the use of irradiated mice poses a problem and the use of living

parasites makes it potentially dangerous. The use of complement adds more problems, similar to those described with the CF test.

More recently, the adaptation of this test with flow cytometric techniques shows good results with the group of treated patients, but the test has been employed only in one research institution and is not available in the market (Martins-Filho et al., 1995; Vitelli-Avelar et al., 2007).

Chemiluminescence

The combination of a purified antigen analyzed by chemiluminescence has been used by Almeida et al. (1994) and applied as a diagnostic tool for evaluation of treatment success on treated patients. Antibodies against this specific antigen disappear before the crude antigens employed in conventional tests (Andrade et al., 1996). The problem is that the preparation of the antigen is difficult and the need of a microplate chemiluminescent apparatus makes this test difficult to be used as a routine test. It is not commercially available.

28.5.4 Antigens

T. cruzi, as with most pathogens, has an array of antigens of different origins. In antibody detection systems, as with serology, the selection of a proper antigen is essential in order to get a reliable result. The selection of antigens also depends on what investigators want to know. If the point is to diagnose the disease, any antigen or group of antigens present in the parasite will suffice. Antigens selected should be exclusively from *T. cruzi* and not present in other pathogens (Stolf, 1992). Historically the easiest antigens to use were extracts of the full parasite. Obviously some of them may cross-react with other pathogens. This lead to purify antigens from different structures, obtaining better results in terms of specificity. A further step to gain specificity was the use of recombinant antigens and immediately after that, the use of synthetic peptides. Excreted antigens were also found and used. It was soon clear that as the antigens selected were more restricted, some infected people have no responses against some of them. Then, the idea to sum up several recombinants or peptides was considered now. So, the tendency now is to have a mixture of now-refined antigens.

28.5.4.1 Crude Preparations

Crude preparations were first employed in 1914 and remained popular until the 1980s. The extraction procedure varied in order to select antigens present mainly in this parasite (see historical section). The source of *T. cruzi* also should be considered. It may be present in cultures in epimastigote form (with 5% trypomastigotes; the percentage that increases in old cultures), with a relatively easy growth, which facilitates large amounts of antigen, making it the preferred option. Preferred medium is LIT and best temperature at 26°C. Cross-reactions may occur with other pathogens. One of the issues is that this form is not present in human tissues or blood. Another option is to employ trypomastigotes, which are more difficult to

grow, mainly because a lineage of cell culture should be used and maintained in a CO_2 incubator at 37°C to reproduce the cycle in humans. It has been shown that these preparations are more specific. Some authors tried to use amastigotes (Primavera et al., 1990), which are also difficult to prepare in large numbers, but again, results were better than with epimastigotes.

The strain employed may differ as well. Preferred strains are those that grow easily, as with the "Y" strain, Tulahuen, and others. Theoretically, the best strain to use would be the one isolated from the infected, which is not possible. In practice, some studies did show that responses may be better (high titers) with strains commonly used than with stocks recently isolated from the same patient (Borges-Pereira et al., 1987).

The use of crude preparations also depends on the support used and the type of test. For IH, extracts of epimastigotes grown in LIT are used to sensitize red blood cells, which may be from different animals (sheep, rabbit, hen, etc.). For IIF, the entire parasite is used, fixed in smears. For ELISA, an extract is prepared from the whole parasite by several cycles of freezing and thawing. Technical details such as the use of enzymes are out of the scope of this chapter, and readers are invited to consult specific reviews on each technique (Venkatesa and Wakelin, 1993).

28.5.4.2 Purified Antigens

As a need to have better tools for diagnosis grew, in the 1980s several purified antigens were tested with panels of well-characterized sera. The first attempts used surface glycoproteins, theoretically more exposed to the immune system. Several publications using antigens of 25 kDa (Scharfstein et al., 1985), 90 kDa (Schechter et al., 1985), and 72 kDa (Schechter et al., 1986) did show that results improved with higher specificity and without loss of sensitivity. The problem was that preparation of these antigens required special technical conditions and manufacturers were not interested. These purified antigens have been tested mainly in ELISA systems.

28.5.4.3 Recombinant Proteins

By the end of 1980s, recombinant protein technology had grown and several laboratories published an array of candidate antigens for use in diagnostic tests, claiming good specificity and sensitivity. Even if most of them were different proteins, some proved to be homologous. The real value of these recombinant proteins was tested with the support of TDR/WHO in 10 laboratories that received 50 coded sera (Moncayo and Luquetti, 1990). Results showed that a single recombinant have few chances to detect all the infected samples. Therefore the tendency was to mixture several recombinants (Silveira et al., 2001). The better mixture (FRA and CRA) was used in kits which have been distributed to all government laboratories in Brazil.

Other recombinant proteins have been produced by several manufacturers and are widely available (i.e., Wiener ELISA Recombinante®, ImmunoComb II Chagas®) with good results. Interestingly, some of these kits may have occasional false-positive results, probably due to reactions to remaining antigens in the process

of fabrication. These recombinant antigens have also been used in combination to make rapid tests, several of which are available in the market (see later).

28.5.4.4 Synthetic Peptides

These have been synthesized and used with panels of sera, and again, part of the infected population do not respond to a single antigen, so an array of them is necessary to get good sensitivity (Peralta et al., 1994; Silveira et al., 2001). They have been applied in different supports and some are available as rapid tests (PaGia®) (Rabelo et al., 1999).

28.5.5 Different Supports

The first serological tests were devised to be used in glass tubes where the sera would be mixed, in several dilutions, with the antigens in ideal proportions. Glass smears were used for agglutination and demarked smears for immunofluorescence. With the era of plastics, polypropylene tubes were used, with the advantage of a single use and discard, avoiding the time consuming and imperfect process of washing.

Very shortly after, plastic (polypropylene or other) microplates were applied in serology, with easy handling of hundreds of samples. Their use was routine by the time ELISA was described (1975). Antigens in ELISA were fixed in the wells by special buffers, and all the reaction faster. Later, for those laboratories that process few samples of sera a day, detached rows of the plate (strips) were available.

When purified antigens, recombinant proteins, and synthetic peptides were available, spots in filter papers (or different materials) were used, substituted in blots by nitrocellulose membranes.

28.5.5.1 Membranes

Nitrocellulose membranes are commonly used for Western blot tests, but none are commercially available. Tests are expensive and time consuming, requiring several steps to complete and with a few samples used in each run.

28.5.5.2 Gels

The use of gels in microtubes separated the mixture of antigens and reagents by centrifugation. If reaction occurs, the complex does not come down and in negative samples, the reagent (generally colored) passes through to the bottom (PaGia®) (Rabelo et al., 1999).

28.5.6 Rapid Tests

A new generation of rapid tests became available at the end of the 1990s. They had several applications (pregnancy, diabetes, etc.) and several advantages apart from speed. You do not need a skilled worker to perform them. They are very important in rural areas devoid of specialized laboratories when a diagnosis of *T. cruzi* infection may be

crucial (i.e., in an emergency for a blood transfusion). Also, in serological surveys, and in some remote places that require such costly displacement that the possibility to give a result at once, solve problems of public health. Some laboratories have so few samples per day to test, that the possibility to use a rapid test may, at the end, reduce costs.

The industry quickly became aware of this niche and responded in kind. Devices were produced with recombinant antigens and in a quick run (10−20 min) after a drop of serum and buffer are placed, results were obtained. A positive control band should be seen in negative and positive sera, indicating that the system did work. Otherwise, the result is not validated. In a few years, several rapid tests were available at the market and became extensively used in some areas, such as Central America and Bolivia. Several papers indicated good sensitivity and specificity, but they should not be used as a single test for a case confirmation (Luquetti et al., 2003; Ponce et al., 2005). Main brands available are Stat-Pack® and Inbios®.

28.6 Blood Banks, Serology, and Quality Control for Chagas Disease

Transfusion of blood and blood products is an essential part of health care for patients deficient in one or more blood components. In order to avoid transfusion of tainted blood, every country should have an organized blood transfusion service based on a national blood policy, including relevant legislation, rules, and regulations, which in turn must be an integral part of any national health policy. For practical purposes, the status of the blood supply may depend on: (i) the existence of a pool of healthy donors, making the supply sufficient to cover the country's needs; (ii) mandatory screening of blood donors for infectious diseases, following quality assurance procedures, and (iii) appropriate use of blood. This is especially important for Chagas disease, as the second most common way of transmission of *T. cruzi* was considered to be, for many years, transfusion of blood or blood products. In fact, the minimum screening of blood donors in Latin America includes HIV, HVB, HVC, syphilis, and *T. cruzi* (World Health Organization, 1991, 2002; Organizacion Panamericana de la Salud, 2009).

In the 1980s and early 1990s transfusion infection was also considered a public health problem. At that time there were a high number of blood transfusions as well as few countries mandating serology for *T. cruzi* for screening blood donors. Danger may come not only from whole blood, but also from packed red cells, platelets, white cells, fresh frozen plasma, and cryoprecipitate. On the other hand, the use of lyophilized products seemed to be safe (Schlemper, 1978; Schmunis 1991a; Contreras et al., 1992).

In the 1970s, with 4 million transfusions yearly, an annual incidence of 10,000−20,000 cases was thought to occur in Brazil (Amato-Neto, cited by Dias and Brener, 1984; Wendel and Pinto Dias, 1992). At that time it was also assumed that 10,000 cases occurred in the Sao Paulo metropolitan area yearly (Camargo, 1977, cited in Dias, 1979).

These numbers were revised downward in the early 1990s. The number of donors with positive serology for *T. cruzi* in that country was estimated at 55,000, of whom 11,000 were not screened. Taking into account that the probability of getting a *T. cruzi* infection after receiving an infected unit was considered to be 20% (Rohwedder, 1969), the number of infected cases by blood transfusion would be 1500−3000 individuals (Amato Neto, 1993). In fact, the real incidence of *T. cruzi* acquired through blood was unknown, because most cases are unapparent or *T. cruzi* is not recognized as the etiological agent (Bergoglio, 1984).

The risk of receiving *T. cruzi*-infected blood will increase in proportion to the prevalence of the infection in the donor population and the number of transfusions received. Therefore, polytransfused individuals like hemophiliac patients, those with other hematologic disorders, or patients undergoing dialysis are at greater risk. Fifty percent of hemophiliacs became infected after receiving 30 or more transfusions from a blood bank with a 2% prevalence of positive serology for *T. cruzi* (Cerisola et al., 1972). Another study showed that 15% of individuals who had multiple transfusions had positive serology for *T. cruzi*, while the general population was 2% positive (Lorca et al., 1988). Polytransfused individuals from a blood bank with 2% positive serology for *T. cruzi* were 8.7 times more likely to be positive than individuals who did not receive transfusions (Atias et al., 1984).

Serology for *T. cruzi* was mandatory for blood donors in a few countries by 1980s (Argentina, Brazil, Honduras, Uruguay, and Venezuela). In others, serology for *T. cruzi* was done by decision of the transfusion services like the Red Cross in Ecuador, or the blood banks from the endemic area, as happens in Chile. Some governments did not have the will for implementing prophylactic measures or to enforce them. On the other hand, while the number of transfusions was unjustifiably high, the medical profession at large and even those devoted to blood banking did not pay enough attention to the possibility that *T. cruzi* could be transmitted by this way (Dias, 1979, 1992; Dias and Brener, 1984; Schmunis, 1991a). Therefore, health personnel in particular, and the public in general, must be educated to the possibility that *T. cruzi* could be transmitted by blood (Schmunis and Cruz, 2005).

Thousands of immigrants from Latin American endemic countries living in Europe created a Chagas disease problem there. In France, blood donors born in *T. cruzi* endemic areas or from a mother coming from an endemic area must be screened. Donors returning from endemic areas are deferred from blood donation for 4 months, and blood used in French Guyana comes directly from France (Assal and Aznar, 2007). In Spain, the country in Western Europe that received the most Latin American immigrants, a Royal Decree (Decreto Real, 2005) deferred permanently as donors those individuals with positive serology for *T. cruzi*. If tests for screening of blood donors are not available, individuals born in endemic areas, newborns from mothers coming from endemic areas, and those who received transfusions in endemic areas need to be excluded from donating blood for labile components. These individuals may donate blood only when having a negative serological test for *T. cruzi*.

The regulatory system in the USA is based on private initiatives, either the Red Cross or community-based nonprofit organizations. They are responsible for obtaining and processing the blood and blood products, under strong government and

professional society supervision (Goodman et al., 2003). In Canada, implementation of activities related to blood and blood products belonged to the Canadian Red Cross until the responsibility switched to the central Canadian Blood Services (CBS) and one provincial government (Hema-Quebec) after a scandal in the 1990s led to safety concerns (Health Canada, 1998). In the USA, the three main organizations dealing with blood (American Red Cross, Council of Community Blood Centers, and America Blood Centers) for many years reported blood-related accidents and incidents. The Red Cross alone covers 50% of the hospitals in USA and collects information for hemovigilance in those hospitals. The Food and Drug Administration (FDA) also has a thorough collection of data related to the use of blood and blood products. In addition, information from other sources, like the Retrovirus Epidemiology Donor Survey, provides data used for establishing residual risk (Goodman et al., 2003).

The FDA introduced a concept of "zero risk blood supply" as the industry goal and regulatory agencies require blood donor screening laboratories, blood banks, and transfusion services to establish and follow a quality control and quality assurance program for their licensing, certification, and accreditation (Du, 2002). All errors and accidents must be reported to the FDA promptly (Manitove, 1999).

In order to prevent transfusion transmission of *T. cruzi* infection, it is recommended (but not mandatory) that the donor pool be screened for *T. cruzi*. Positive units must be removed from distribution and the donor not allowed to make donations (Centers for Disease Control and Prevention, 2007). The US Food and Drug Administration (2006) recommended testing of blood donors with an approved EIA assay. It is estimated that about 65% of the blood supply is being screened. The AABB (formerly known as the American Association of Blood Banks) recommends that blood donations that have repeatedly tested reactive by ELISA should be quarantined and removed from distribution, and the donor be kept from making donations (CDC, 2007). Recipients of blood components from donors who tested positive should be identified and tested for *T. cruzi* infection. Those who tested positive for *T. cruzi*, their at-risk family members, children from infected mothers, and potentially infected recipients should receive a comprehensive clinical assessment (CDC, 2007).

Countries in continental Latin America have a regulatory framework that mandates the safe use of blood and blood products through the adequate selection of donors, screening for infectious diseases, and the use of the blood or blood products according to good clinical practices (Rios, 1992; Organizacion Panamericana de la Salud, 1993; Bolis, 2009). Blood transfusion activities are government regulated, while implementation of these activities is the responsibility of either a government central blood bank, the Social Security Institute, hospital blood banks, nongovernmental institutions such as the Red Cross, or a combination of all. Professional societies may have an advisory role.

Laws, decrees, norms, and/or regulations related to blood transfusion began to appear in the 1960s (Argentina, Brazil, Chile, Costa Rica); 1970s (Bolivia, Colombia, Ecuador, Paraguay, Uruguay, Venezuela); 1980s (Honduras, Mexico, Nicaragua); and 1990s (Guatemala, Panama, Peru) (Rios, 1992; Organizacion Panamericana de la Salud, 1993; Bolis, 2009). They began because of concerns about transmission of infectious diseases like syphilis and Chagas disease. They were followed by worries

about hepatitis in the 1970s, and then HIV in the 1980s. They have evolved through time, from focusing at the beginning only on disease screening to later mandates on voluntary donations and quality assurance. Enforcement varied from stringent to weak, and most countries do not have a well-trained group of inspectors to make mandatory visits to independent laboratories or blood banks that do the serological screening. In addition, in some countries there are so many blood banks that process a small number of blood donors that visiting all of them annually or even every 2 years is a daunting (and expensive) task.

The situation is further complicated by decentralization, especially in countries such as Argentina, Brazil, and Mexico, where states or provinces have their own local authorities, including those dealing with health issues. With few exceptions, the Ministries of Health lack the human and material resources needed to oversee the organization, functioning and performance of all existing blood banks, independent of their administrative association. In other instances, such as Ecuador, Honduras, and Nicaragua, the Ministry of Health has the collaboration of the local Red Cross for running the national blood program, in addition to collecting, processing, and distributing blood (Schmunis and Cruz, 2005).

The first Expert Committee on Chagas disease from WHO (1991) recommended that screening for *T. cruzi* in blood donors should be conducted in all countries where *T. cruzi* was endemic using at least two serological tests based on different principles (e.g., an IH and ELISA, or an IIF and IH, or an ELISA and IIF). There should be a ban on paid blood donors and a program of quality control for serology must be implemented (WHO, 1991) At that time, only two countries, Argentina and Brazil, had mandatory tests for *T. cruzi* screening, and several of the other countries did not screen for *T. cruzi* in all donors, and a few did not screen blood donors at all. Even for the two countries in which two tests were mandatory, both tests were not always employed. A report from Brazil showed that in spite of the serological screening, 12 of 1513 samples tested were false negative. Data from Argentina indicated that 50% of 423 centers carried one serological technique for screening (Perez and Segura, 1989). A similar survey done in Brazil covering 850 counties in 1988−1989 indicated that there were 1525 health services that provided some sort of blood transfusion services. Eight hundred and eighty-two did serology for *T. cruzi*. Of them, 55% did one serological test for diagnosis, while 26.8% and 12.2%, respectively, did two or three tests (Dias, 1992). In addition, from the 61 services that did *T. cruzi* serology in 1990, 11.5% did only one test, 55.7% two, and 32.8% three or more tests (Valerio-Wanderley et al., 1992).

Before 1993 there was no nationwide official information system in any Latin American country on indicators of the status of blood supply, including the prevalence of infectious diseases markers. Since 1993, countries began to provide nationwide data of the total number of donors, percentage of donors screened (screening coverage), and prevalence of infectious diseases markers for HIV, HBV (HBsAg), HCV, and *T. cruzi*. Those diseases and syphilis were selected for surveillance by the Regional Standards for Blood Banks (OPS, 2009).

Data became available from nine countries in 1993, four more in 1994, one each in 1995 and 1997, and two more in 1999. This allows an analysis of the overall

situation: how safe was the donor pool? The answer is based on the percentage of different categories of blood donors from the country; repeat voluntary donors are the safest, replacement donors should be avoided, and paid donors must be banned (OPS, 2009). Information available on: (i) the total number of donors; (ii) the total number of donors screened; and (iii) the prevalence of *T. cruzi* infection made it possible to estimate the risk of blood receptors of receiving an infected unit or potentially acquire a *T. cruzi* infection (Schmunis et al., 1998, 2000, 2001; Schmunis and Cruz, 2005).

From 1993 to 2005, it was assumed that reagents used for routine screening for *T. cruzi* antibodies, either ELISA or IH, had a sensitivity of 90% and a specificity of 95% (Schmunis et al., 1998). Later, commercial reagents improved, and some but not all of those used for the ELISA or IH test had sensitivity and specificity equal or higher than 90% (Oelemann et al., 1998; Saez-Alquezar et al., 1998). This fact stressed the need that government agencies, or any technical group in which the government delegates this activity, test the different diagnostic reagents and vouch for their usefulness. Tables 28.4, 28.5, and 28.6 show the sensitivity and specificity of different commercial reagents and the differences in sensitivity and specificity.

Tables 28.7 and 28.8 show the number of donors, the percentage of donors screened, and the prevalence of *T. cruzi* infection in donors from Latin American countries from 1993 to 2007 (Schmunis et al., 1998, 2000, 2001; Schmunis and Cruz, 2005; OPS, 2005 and 2007).

The second WHO Expert Committee (2002) faced the reality that 9 years after the first Expert Committee, only in Argentina and Brazil were two tests for the screening of blood donors mandatory for *T. cruzi* antibodies. The fact that several EIASA in the market had high sensitivity and specificity and could be used in an automated system, as well as that the cost of two tests were not affordable for several countries, weight in the decision that mandatory screening for Chagas disease in blood donors could be made using a single test and this should be an ELISA test of high sensitivity (WHO, 2002).

The simplest way to follow up progress in this area is to establish the number of countries with 100% screening for *T. cruzi* in blood donors. Table 28.8 shows how these figures were improved from 1993 to 2005. In 1993, 2 out of 9 countries reported 100% screening coverage for *T. cruzi*; 7 out of 16 in 1999; and 9 out of 17 in 2005. In any case, 100% screening in blood donors alone may not be enough. It is necessary also to have reagents of excellent quality; to follow manufacturer's guidelines; and that laboratories follow good laboratory practices (Organizacion Panamericana de la Salud, 1999) (see Section 28.7).

Early information in the region on results on performance evaluation schemes has been somewhat distressing. A comparison of results from six blood banks with a reference laboratory in the central region of Brazil showed that sensitivity of the screening for *T. cruzi* ranged from 50% to 100%, thus suggesting that transmission of *T. cruzi* could be occurring, despite serological screening (Andrade et al., 1992). A blind panel containing positive samples for blood-borne diseases distributed to 57 major public blood banks in four sequential programs showed 64 (3.7%) false-negative results for *T. cruzi* (Saez-Alquezar et al., 1998). Another similar

Table 28.4 Sensitivity and Specificity of Different Commercial ELISA Tests for Screening for *T. cruzi* Infection in Blood Donors, 2006

Kits	Kappa	% Sensitivity	CI 95%	% Specificity	CI 95%
Adaltis	0.71	100	94.0–100.0	60	46.0–73.2
Bio manguinhos[a]	0.95	100	94.0–100.0	93	82.2–97.7
Bio manguinhos[b]	0.97	97	89.7–99.5	98	89.7–99.9
Biomerieux	0.97	100	94.0–100.0	95	85.4–98.7
Biochile	0.98	99	91.9–99.9	98	89.9–99.9
Biozima Chagas	0.98	100	93.9–100.0	97	87.3–99.4
Ebram	0.97	99	91.5–99.9	97	87.5–99.4
Hemagen	0.98	100	93.9–100.0	97	87.5–99.4
Patozime Chagas	0.97	99	91.3–99.9	97	87.5–99.4
REM Gold	0.97	99	91.8–99.9	97	87.0–99.4
Wama diagnostica	0.98	99	91.5–99.9	98	89.9–99.9
Wiener	0.97	100	94.0–100.0	95	85.4–98.7

[a]Recombinant
[b]Conventional. CI = Confidence interval.
Source: Ministerio da Saude, Brazil (2006).

Table 28.5 Sensitivity and Specificity of Hemagglutination and Agglutination Tests

KIT	% Sensitivity	% Specificity
Indirect Hemagglutination, 1997*		
Imunoserum	94.64	95.42
Ebran	88.69	59.92
Wama	100.00	95.80
Hemagen	93.45	87.79
Biolab	99.40	97.33
Particle Agglutination, 2006		
Serodia	100	97.71
ID PaGIA	98.81	98.85

**Source*: Saez-Alquezar et al. (1997).

program in Brazil (1999–2001) with participation of 116 institutions showed that 1.6% of 5406 positive samples for *T. cruzi* were reported as negative in 58 laboratories. On the other hand, 0.32% of 32,855 negative samples had been reported false positive (Saez-Alquezar et al., 2003). In 2002, 131 institutions each received a panel of six unknown samples of HIV, HTLV, and Chagas disease. From the 123 participants that responded, 17.9% had discordant results for Chagas disease (Oficina Sanitaria Panamericana, 2003). The percentage of accurate results detected by the participants was 96% for Chagas; but the highest numbers of false-negative results were for Chagas disease and syphilis (Oficina Sanitaria Panamericana, 2003). Another program was conducted in Colombia in 1998, with blind positive

Table 28.6 Anti *T. cruzi* Antibodies in 1455 Blood Donors from the Province of Chaco, Argentina. 2006−2007

Tests	% Reactive	% Concordance
Chagatek (EIA BioM)	25.2	–
Chagas BiosChile (EIA BiosCh)	23.5	98.1
BioZima Chagas (EIA BioZ)	24.4	98.4
Chagatest rec. (EIA Wrec)	24.7	97.0
Chagas Serodia (PA)	20.6	95.2
Chagas HAI (IHA)	18.9	92.1
HAI LC (IHA LC)	18.8	88.0

Source: Remezar et al. (2009).

and negative samples sent to 46 blood banks, of which 43 responded. There were two false-negative results for Chagas disease (Beltran and Ayala, 2003). In Argentina, 30% of participating institutions (52 in 1999, 102 in 2000, and 118 in 2001) to which a panel similar to the one in Colombia plus *Brucella* was sent detected at least one false-negative result (Oknaian et al., 2003). It was clear that improvement was needed.

An international performance evaluation program on serology for infectious diseases with participation, depending on the year, of 13 to 21 national reference centers from 11 to 16 Latin American countries was active from 1997 to 2008 (Otani, 2003; Saez-Alquezar et al., 2003). The program sent out panels with positive or negative samples for HIV, HBV, HCV, syphilis, and *T. cruzi*. Five panels with 24 unknown samples each, 2−3 of which were negative samples, were sent to participating institutions in 1997−2000; 81% of them sent the results of the five panels back; and 87−100% of the institutions responded on time (within 60 days of receiving the panel) (Otani, 2003). Results regarding *T. cruzi* serology showed false-negative results in 3.2% of 527 positive tests for *T. cruzi*, and 0.73% of 2329 samples positives for *T. cruzi* were false positive (Otani, 2003; Saez-Alquezar et al., 2003).

In 2007, 28 institutions in 16 countries participated in the international performance evaluation on serology for infectious diseases in blood banks. They received 24 positive samples reactive for the diseases transmitted for transfusion (5 were positive and 1 was negative for *T. cruzi*). Only one of the laboratories did not send the results back and only one had four results that did not agree with those of the organizer laboratory when testing the samples with *T. cruzi* (two false positives and two false negatives) (Programa de evaluacion externa del desempeño en serología, 2008). In 2008, 24 institutions from 16 countries received a 44-sample panel, 4 of them were positive for *T. cruzi*. None of the 44 samples were negative for all markers. Fourteen laboratories did not make any mistake but seven laboratories diagnosed false-positive or false-negative results. One laboratory was able to identify the two false-positive samples for *T. cruzi* (Programa de evaluacion externa del desempeño en serología, 2009).

Up to 2001, 11 Latin American countries had programs of performance evaluation for the serology of infectious diseases in blood banks: Argentina, Bolivia, Brazil,

Table 28.7 Number of Donors, Screening Coverage, and Prevalence (in Thousands) of Serology for *T. cruzi* in Blood Donors, 1993−1999

Year	1993			1995			1997			1999		
Country	# Donors	% Screen*	Serologic Prevalence *T. cruzi*	# Donors	% Screen[a]	Serologic Prevalence *T. cruzi*	# Donors	% Screen[a]	Serologic Prevalence *T. cruzi*	# Donors	% Screen[a]	Serologic Prevalence *T. cruzi*
ARG				811,850	96.0	49.0	742,330	100	44.0	810,259	100.00	55.0
BOL	37,948	29.40	147.9	22,146	66.0	137.0	40,056	44.0	172.0	20,628	23.18	454.6
BRA										1,663,857	100.00	7.6
CHI	217,312	76.70	12.0				228,801	77.0	9.7	218,371	87.00	1.0
COL	352,316	1.40	12.0	370,815	46.0	13.0	425,359	99.2	11.0	353,991	99.89	10.0
COR	50,692	0.00	8.0	45,311	13.0	8.0	58,436	7.0	25.8			
ECU				100,774	75.0	1.0	110,619	72.0	1.3	103,448	90.30	1.3
ELS	48,048	42.50	14.7	52,365	99.0	23.0	55,069	100	22.0	67,224	100.00	25.0
GUT	45,426	75.00	14.0				40,732	100	3.1	31,939	100.00	8.1
HON	27,885	100.00	12.4	31,937	90.0	17.0	32,670	99.0	11.9	40,933	99.36	20.5
MEX							936,662			1,092,741	13.18	3.8
NIC	46,001	58.40	2.4	48,030	51.0	1.0	46,539	62.1	39.0	45,000	100.00	3.5
PAN				40,325	11.0	12.0	42,342	0.7	17.0	43,921	17.00	14.0
PAR				34,216	83.0	58.0	40,721	97.99	37.7	45,597	99.80	47.0
PER				82,656	4.0	0.0	205,826	60	2.0	311,550	99.80	1.4
URU				111,518	100	6.0	115,490	100	6.5	116,626	100.00	4.5
VEN	204,316	100.00	13.2	202,515	100	8.0	262,295	100	7.8	30,2100	100.0	6.0

Cell blank: No information or incomplete information.

[a]Screening coverage. Argentina (ARG); Bolivia (BOL); Brazil (BRA); Chile (CHI); Colombia (COL); Costa Rica (COR); Ecuador (ECU); El Salvador (ELS); Guatemala (GUT); México (MEX); Nicaragua (NIC); Panamá (PAN); Paraguay (PAR); Perú (PER); Uruguay (URU); Venezuela (VEN).

Table 28.8 Number of Donors, Screening Coverage, and Prevalence (in Thousands) of Serology for *T. cruzi* in Blood Donors, 2001−2007[a]

Country	2001			2003			2005			2007		
	# Donors	% Screen	Serologic Prevalence	# Donors	% Screen	Serologic Prevalence	# Donors	% Screen	Serologic Prevalence	# Donors	% Screen	Serologic Prevalence
ARG	804,018	100	45.0	780,440	100	45.0						
BOL	24,747	86.09	99.1	38,621	79.58	76.5	46,764	99.26	86.10	54,951	99.84	25.3
BRA	1,763,130	100	6.5	2,931,813	100	6	3,738,580	100	6.10			
CHI	210,403	81.42	6.1	173,814	67	5.1	178,079	68.7	2.70	238,124	72.28	3.4
COL	399,171	99.8	6.7	495,004	99.9	4.1	527,711	99.99	4.10			
COR	55,737	6.17	5.8	48,625	93	2.4	54,170	100	0.90			
ECU	65,496	94.05	0.8	79,204	100	3.6	124,724	100	0.10	144,600	100	4.3
ELS	72,545	100	37.0	76,142	100	33	80,142	100	24.00	81,246	100	2.9
GUT	43,622	92.27	14.8	68,626	99.82	12.3	77,290	100	14.00	76,485	100	6.9
HON	36,781	100	14.0	48,783	100	13	52,317	100	14.70	52,497	99.31	4.3
MEX	1,135,397	13.3	0.5	1,136,047	32.67	4.4	1,351,204	36.34	5.00	149,3674	53.31	6.6
NIC	49,346	94	5.6	46,558	94.2	6.3	54,117	100	9.00	59,755	94.5	6.2
PAN	42,867	33	9.0	46,176	95.5	1.3	42,771	97.64	1.20	46,947	99.5	9.0
PAR	48,406	99.15	44.6	29,718	96.1	41.4	47,060	99.83	33.00	54,538	100	7.2
PER	347,250	100	2.9	145,665	96.36	8.4	179,721	76.46	5.70	177,215	100	7.7
URU				99,675	100	3.6	95,686	100	2.60			
VEN	345,953	100	6.7	342,526	100	6.5	465,653	100	6.10			

Cell blank. No information or incomplete information.
[a]Screening coverage. Argentina (ARG); Bolivia (BOL); Brazil (BRA); Chile (CHI); Colombia (COL); Costa Rica (COR); Ecuador (ECU); El Salvador (ELS); Guatemala (GUT); México (MEX); Nicaragua (NIC); Panamá (PAN); Paraguay (PAR); Perú (PER); Uruguay (URU); Venezuela (VEN).
Source: Organización Panamericana de la Salud (2009).

Colombia, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Paraguay, and Venezuela (Otani, 2003; Saez-Alquezar et al., 2003). Another was later implemented in Ecuador. In Chile, all blood banks participate in a proficiency testing program for clinical laboratories (Otani, 2003). It is unfortunate, however, that not all blood banks in most of the countries participate less than 50% do in Argentina, Colombia, and Venezuela, up to 90% or more in El Salvador and Paraguay (Otani, 2003).

These results show the usefulness of a program for performance evaluation as well as that the performance of some laboratories should improve. Another aspect that should improve is the management of the program. In order to properly evaluate performance, the samples should be tested in the same time period used in the routine of the participating laboratories. Usually, it takes less than 18 h for the routine serology to provide results that allow the blood unit to be liberated. So, to give the participating laboratories in the performance evaluation 30 days or more to reply is too much. Even if they respond without errors, there is no relation to what happens in the daily routine, so it gives a false sense of security.

28.7 Application of Diagnostic Tests in Different Contexts

As explained in the beginning of this chapter, Chagas disease has two main phases, acute and chronic, and diagnostic tests should be applied according to the suspected phase. During acute phase, parasitological tests are preferred and serological tests are used only if the former were negative and suspicion persists. For the chronic phase, serological tests are the option, and parasitological only used in special circumstances. Even if major groups of tests are parasitological and serological, other tests which are not routinely used have been employed. Search for antigen during the acute and chronic phase has been the subject of a few investigations. Skin tests have been tried without success (Zeledon and Ponce, 1974; Teixeira et al., 1995) and, as an invasive procedure, may have ethical consequences, mainly when there are other tests that are easier to perform.

Furthermore, there is the context of the clinical situation to consider. For example, there is the confirmation of a suspected case against exclusion of a donor. A responsible diagnostic laboratory should concentrate all efforts to demonstrate or exclude the presence of the infection. The hematologists need to be sure that the blood is not infected. If there is any suspicion, even if not confirmed after, blood should be discarded. There are other situations in which a special array of tests or timing is necessary, such as epidemiological surveys and congenital transmission. Each one of these situations needs to be analyzed.

28.7.1 Parasitological and Serological Tests in the Acute Phase and Reactivation (Immunosuppression)

a. Clinical situation: person with fever, of some days/weeks of evolution. Common causes of fever discarded. Not severely ill. Few nonspecific findings on examination. Sometimes a portal of entry (one eye, both eyelids) or skin.
b. Epidemiology: living or has been recently in endemic area for Chagas disease.

c. Laboratory: ask for fresh blood smear, looking for motile flagellates. Ask for Strout or microhematocrit technique.
d. Action: if positive, the diagnosis is acute phase of Chagas disease, probably by vector contamination. Start treatment at once.

If negative and the clinical suspicion persists, try to collect further blood mainly during febrile peaks. If negative persist, ask for IgM antibodies, by IIF. If positive, decide with the clinician if the clinical picture, evolution and this result may justify etiological treatment.

Hints: Romaña sign and other eventual portal of entry, will remain for several weeks. Conjunctivitis will disappear in few days. Differential diagnosis with visceral leishmaniasis should be made if negative parasitological results are obtained, mainly if the evolution of the patient is to a progressive worsening.

a. Clinical picture: generally at hospital, with severe disease (or not), started with fever of unknown origin. Frequent causes have not been found.
b. Epidemiological fact: received transfusion recently (last 2 months) or transplantation (bone marrow, other organs) and has fever after, unknown causes.
c. Perhaps some laboratory technician saw strange pathogens in the smear, during a routine differential count. Try to identify the parasite. Ask for a fresh blood smear. If negative, ask for Strout or microhematocrit technique. Transfusional transmitted cases have easily detected parasitemias.
d. Action: if positive, the diagnosis is acute phase of Chagas disease, *by transfusion or transplantation* in a patient with other disease, which motivates the transfusion/transplan tation. Start specific treatment.
a. Clinical picture: several people living at the same house or neighbors who participate recently in some festivity, and used the same food, became febrile. A disease transmitted by foods is suspected. Some have digestive manifestations, even with hematemesis (blood vomiting). Some patients may look severely ill. Liver tests may show alterations (biliru bins, transaminases).
b. Epidemiological fact: endemic region or not endemic, that uses fresh foods from endemic regions.
c. Ask for a fresh blood smear. If negative, ask for Strout or microhematocrit technique.
d. Action: if positive, the diagnosis is acute phase of Chagas disease, by oral route. Start treatment and diagnose the other similarly affected. Direct diagnosis should be made in all the persons that eat or drink the identified food (if any identified).
a. Clinical picture: normal person working in a health service, laboratory, or research insti tution start with fever of unknown origin. Main causes have been discarded.
b. Epidemiological fact: works with reduviid bugs, or with *T. cruzi*, in cultures, mice or other animals. May be is working in a nonendemic region.
c. Ask for a fresh blood smear. If negative, ask for Strout or microhematocrit technique.
d. Action: if positive, the diagnosis is acute phase of Chagas disease, *by laboratory acci dent*. Start treatment and collect a sample of blood at once. Perhaps in endemic regions, the worker is already infected.
a. Clinical picture: patient with known HIV infection or with diagnosed cancer or other dis ease (autoimmune) which requires, as part of the treatment, large doses of corticoid or other immunosuppressors. During the course of the disease or the treatment, not expected skin lesions and/or nervous system involvement, which was not present, mainly with loss of consciousness. Any other not predicted clinical manifestation.

b. Epidemiological fact: has had Chagas disease diagnosed years ago, or was born/lived in endemic areas for Chagas disease.
c. Ask for a fresh blood smear. If negative, ask for Strout or microhematocrit technique.
d. Action: if positive, the diagnosis is reactivation of chronic phase of Chagas disease, by immune suppression, which is in fact an acute phase, because the criteria of having easily detectable parasites is fulfilled. Start treatment and collect a sample of blood at once for serology. This serology should be positive, because the patient was in the chronic phase.

The congenital route is a special situation that behaves as an acute phase and will be described below.

28.7.2 Parasitological and Serological Tests for Confirmation of Congenital Transmission

Two of the main routes of transmission of Chagas disease are in retreat in the endemic countries of Latin America. Vector transmission by the main household vector, *T. infestans*, was interrupted in Uruguay and Chile in the late 1990s (Anonymous, 2000), and Brazil in 2006 (WHO, 2006), and partially in the geographic endemic area of Argentina (OPS IV, 2000). In Central America, the main intradomicile vector was *Rhodnius prolixus.* Transmission by this vector was interrupted in Guatemala in 2008 (Ministerio de Salud, 2008). In addition, increase in screening coverage for *T. cruzi* in blood banks of the continental western hemisphere has made congenital Chagas disease a more important route of transmission than before in relative terms.

Transplacental infection may occur in different pregnancies in the same woman, while siblings of congenitally infected children may not be infected. In newborn twins, infection with *T. cruzi* has been described in one or both (WHO, 1991). It was estimated that there were 12,000−15,903 cases of vertical transmission in 1985 (Schmunis, 1994, 2000), and 14,385 in 2005 (Organizacion Panamericana de la Salud, 2006).

The reported rate of transmission of transplacental infection varies from country to country (Table 28.9).

The origin of these differences is not clear. Potential explanations have been the parasitemia level in the mother, parasite strain, techniques used for diagnosis (microhematocrit, PCR, histopathology, and/or serology), the time the sample for testing is obtained after delivery, and/or the target population in which the study was made (e.g., underweight newborns). The only Latin American countries in which vertical transmission has not been reported are Costa Rica, Ecuador, El Salvador, Nicaragua, and Panama. In fact, congenital Chagas disease was found in every endemic country examined. Any newborn from an infected mother is at risk.

In Europe, there has also been reports of congenital *T. cruzi* infection in newborns delivered by women who were Latin American immigrants; one case from Sweden (Pehrson et al., 1981), four from Spain (Riera et al., 2006; Guarro et al., 2007; Muñoz et al., 2007; Flores-Chavez et al., 2008), and one case from Switzerland (Jackson et al., 2009). In the USA, there seems to be two autochtonous cases (Leiby et al., 1999).

It has been reported that congenital Chagas disease may cause abortion or premature birth, stillbirth, and/or low-birth-weight babies; however, there is consensus

Table 28.9 Reported Rate of Transmission of Transplacental Infection

Country	From (%)	Up to (%)	Source
Argentina	0.5	7.0	Schmunis and Szarfman (1977); WHO (1991); Arcavi et al. (1993); Freilij et al. (1994); Streiger et al. (1995); Zaidenberg (1999); Blanco et al. (2000); Gurtler et al. (2003); Mora et al. (2005); Sosa Estani et al. (2005).
Bolivia	5.0	9.5	Azogue et al. (1985); WHO (1991); Freilij et al. (1994); Torrico et al. (2004); Brutus et al. (2007, 2008); Salas et al. (2007); Bern et al. (2009).
Brazil	2.0	10.5	WHO (1991, 2002); Bittencourt and Barbosa (1972); Bittencourt (1976); Nisida et al. (2005).
Chile	0.5	19.0	Howard (1962); WHO (1991); Freilij et al. (1994); Garcia et al. (2001); Lorca et al. (2005).
Paraguay	3.6	10.0	Freilij et al. (1994); Russomando et al. (1998, 2005)
Uruguay	0.13	5.6	WHO (1991); Sarasúa (1993).

that most cases are asymptomatic or olygosymptomatic (WHO, 1991, 2002; Carlier and Torrico, 2003). The exception is infected newborns in Bolivia, where around 50% were symptomatic, with a mortality rate of 2−13% (Carlier and Torrico, 2003; Torrico et al., 2004). When symptoms in newborn babies are present, they appear soon after delivery, with hepatosplenomegaly being the most common finding (WHO, 1991; Carlier and Torrico, 2003; Torrico et al., 2004).

For diagnosis, there is no specific clinical marker of congenital Chagas disease except finding parasites (trypomastigotes) in the blood (Carlier and Torrico, 2003) of a newborn delivered by an infected mother. Most cases must be considered as an acute *T. cruzi* infection. As such, diagnosis may be made by finding parasites by direct microscopy in a drop of blood (e.g., from the umbilical cord). If negative, an enrichment method for identifying parasites in cord blood should be used, by the microhematocrit concentration method or a variation of the Strout method (the micro Strout) (WHO, 1991; Carlier and Torrico, 2003; Freilij et al., 1994). Both are more sensitive than direct microscopy and a fresh smear, but manipulation of live trypomastigotes requires special training to avoid becoming infected (WHO, 1991). The use of the Strout method is not advisable, because of the amount of blood required.

Another method for detecting a possible congenital infection is the pathology of the placenta. Those infected are bigger and heavier than the noninfected (Lisboa, 1960; Bittencourt, 1963; Howard and Rubio, 1968). Microscopically infiltrates of lymphocytes and hystiocites, edema, foci of necrosis, vascular alterations like thrombosis and vascularitis, and proliferation of Hofbauer cells may be seen. The finding of amastigotes confirms the diagnosis. However, it is not always possible to correlate the presence of amastigotes in the placenta with an infected newborn (Rassi et al., 1958), and not every infected newborn has amastigotes in the placenta (Rassi et al., 1958; Bittencourt, 1963, 1967).

The finding of specific IgG antibodies by EIASA or any other conventional test (IIF or IH) in the newborn at delivery is of no use because the antibodies may have been transmitted from the mother. However, follow-up of noninfected newborns from infected mothers has shown that IgG antibodies from the mother usually do not stay in circulation more than 5 months after delivery (Grill et al., 1976). The obvious conclusion is that if IgG antibodies to *T. cruzi* are present after 5 months of delivery, they belong to the infant (Schmunis and Szarfman, 1977).

Therefore, conventional serology like an EIASA allows the diagnosis of congenital infection after the disappearance of maternally transmitted antibodies. There is consensus that antibodies passively transmitted from the mother should have disappeared in all infants 6−9 months after delivery (Schmunis and Szarfman, 1977; Freilij et al., 1994; Carlier and Torrico, 2003; Russomando et al., 2005). Thus, a positive serology at that time with any combination of two accepted diagnostic tests would confirm a congenital Chagas case that requires immediate treatment (WHO, 1991, 2002; Carlier and Torrico, 2003).

Of course, delay in confirming the diagnosis means that a potentially infected child would not comply with a needed visit to the hospital with 6−9 months after delivery, so a *T. cruzi* infection will remain undetected and untreated.

In theory, detection of specific IgM antibodies in the newborn may be another way to confirm the infection either by IIF (Stagno and Hurtado, 1970, 1971; Lorca et al., 1995) or by the direct agglutination test with or without 2-ME (Szarfman et al., 1973). However, it has been shown that *T. cruzi* chronically infected mothers may have IgM antibodies, so if there is a placental linkage, the newborn would have a false-positive result. As a consequence, an unnecessary treatment of a noninfected newborn may happen. The opposite has also been true, a proved congenital case with a negative IgM. This fact has been attributed to a very late infection during pregnancy or infection at delivery (Szarfman et al., 1975). In any case, if the mother does not have IgM antibodies against the parasite but the newborn does, the newborn is infected (Schmunis and Szarman, 1977). Nevertheless, detection of IgM antibodies has been somewhat discredited because *T. cruzi* IgM testing has shown false-positive as well as false-negative results (Carlier and Torrico, 2003).

Still another possibility is to confirm the presence of parasites with PCR (Russomando et al., 1992; WHO, 2002; Schijman et al., 2003). PCR reagents have recently been standardized and validated (Anonymous, 2008), but they are not yet commercially available. Two (3%) infected newborns among 65 newborns delivered by *T. cruzi*-infected mothers in Paraguay were detected with the microhematocrit. However, another infected infant was detected by PCR. In another location in the same country, only PCR detected 3 (10%) congenital cases at birth among 30 newborns from infected mothers. Overall, six babies from both locations were positive by PCR and two were also positive by direct microscopy. None of the newborns were found to have IgM specific antibodies against *T. cruzi* at birth (Russomando et al., 1998). Also, in Paraguay, 20 (1.44%) of 1381 babies were found to be infected by the microhematocrit technique, but this technique missed 77 cases that were later detected by other techniques. The PCR was positive in 60 (7.4%) of 815 babies, and conventional serology by an EIASA was positive in 89 (7%) of 1248 babies 6 months

after delivery. At the end, PCR and serology failed to diagnose 7 and 2 cases, respectively, of the 104 infected babies (Russomando et al., 2005).

In the infected child, cure is almost 100% if the treatment is performed up to 1 year of age with either of the two drugs currently available: nifurtimox or benznidazole (Freilij et al., 1994; WHO, 2002; Carlier and Torrico, 2003).

Having different diagnostic possibilities opens the question of which is best for the patient and the available health services. The question is not academic. Secondary prevention means detection of infection in the mother with conventional serology, and detection of infected infants and their treatment as soon as diagnosis is made. For operational programs it is recommended that every woman be screened by serology by two tests of different principles (ELISA, IH, IIF, etc.). Infants of those identified as positive will be advised to return 6–9 months after delivery to repeat the serology with conventional techniques. Of course, the delay in confirming the diagnosis has the risk that a potentially infected child would not comply with a needed visit to the hospital 6–9 months after delivery, so a *T. cruzi* infection will remain undetected and untreated. Strong efforts from the hospital social services would be necessary to avoid this outcome.

28.7.3 Tests in the Chronic Phase

Application of tests in the situations that follow are always serological for routine purposes. Parasitological tests are performed only for research.

28.7.3.1 Confirmation of a Clinical Case

The most common situation is the confirmation or exclusion in a particular case. This case may arrive at the clinician by several channels: (i) an out-patient consultation as a routine checkup; (ii) from a blood bank because of exclusion as a donor; (iii) during a selection for a job in a country in which screening for *T. cruzi* is mandatory and an infection was found; (iv) patient has relatives with Chagas and wants to exclude/confirm the infection; (v) a gynecologist may send the pregnant patient for evaluation of risk to have the infection and then to investigate for congenital transmission.

In any case, the physician should look for symptoms and signs common in Chagas disease, ask for epidemiological background (an infected mother is important), and ask for laboratory tests for confirmation or exclusion. These serological tests should be very specific and performed in parallel, with at least two tests of different principles (i.e., ELISA and IH; IIF and IH, recombinant ELISA and IH). The results should be expressed in titers for IIF, IH, and OD of the sample and of the cutoff of the plate for ELISA, together with a table with the normal values for the population of the region. If results demonstrate anti-*T. cruzi* antibodies in high titers in both tests, the chances of error are minimized.

An error at this stage may have legal consequences. A false-negative result may lead the physician to look for other diseases or believe the patient is noninfected. Perhaps a false positive may be even worse, because he/she will have the stigma of a severe disease that may be fatal. This person may even have had relatives dead from Chagas disease.

28.7.3.2 Epidemiological Surveys

Serology is used in epidemiological surveys for prevalence purposes and as a tool to measure efficacy of control. In order to know the prevalence of Chagas disease in different endemic areas, governments and research groups in some countries launched serological surveys. Results obtained would help to focus prophylaxis on the affected areas, which is basically application of insecticides to kill domiciliated bugs.

These surveys involve a large number of samples, on the order of thousands, after a proper statistical selection of the regions to be studied. It is not possible to obtain venous blood from thousands of individuals in a short period of time in rural areas. The need of centrifugation on the same day, as well as the possibility of misrotulations, the need for a cold chain, and problems of space made this common way to obtain clinical samples nonpractical for these purposes. The alternative in this situation is blood collection by finger prick, collecting the blood on filter paper, which is easier and quicker. It allows collection of hundreds of samples per day that may be stored at room temperature in small containers, to be tested in central laboratories weeks or laboratories weeks later. It could even be transported by postal services, avoiding the expensive costs of transportation.

Once at the laboratory, filter papers should be cut to a proper size and eluted with saline buffer overnight. The eluate obtained may be then tested with the reagents, mainly conventional tests. Even if results are not so clear cut as those obtained with sera, positive samples are detected and the confirmation may be done later by a proper venous blood collection. The number of tests applied may vary according to the question to be solved, but there is consensus to employ tests of high sensitivity.

The most well-known example was a survey conducted in Brazil in 1975−1980. A single IIF was used, collecting more than 1,200,000 samples covering all the states of Brazil but Sao Paulo, which has been previously studied. To the best of our knowledge, this has been the largest survey in the world for Chagas disease. A map was built with the prevalence of the infection all over the country to delineate endemic areas (Camargo et al., 1984).

In other surveys, it was possible to estimate the prevalence in urban workers (Zicker et al., 1990) and compare filter paper and venous blood results. In another example, now in rural communities, in order to establish the efficacy of specific treatment in children, the population was visited in their schools, and it was possible to select and treat those infected, confirming results with venous blood (Andrade et al., 1996).

Other uses of epidemiological surveys in Chagas disease are to check if prophylactic measures (insecticide spraying of houses) were really effective. In this approach, the goal will be to detect infected people born after the colonization of bugs in homes was controlled. To this end, another national survey was launched, also in Brazil, from 2001 until 2008, in all states but Rio de Janeiro, from children born after the insecticide spraying (i.e., less than 5 years old). More than 105,000 samples were collected and processed with two tests of recognized high sensitivity, IIF and ELISA. Quality control was performed in another laboratory with 10% of the samples. This survey detected 110 positive samples, from which 45 were confirmed as passive transmission from the infected mother, 20 were considered cases of vertical transmission,

and 11 originated from vectors other than *T. infestans* which was the target of the elimination campaign (unpublished results from Prata et al., 2009).

28.7.3.3 Follow Up of Specifically Treated Patients

Tests that follow up of specifically treated patients (serology for cure and parasitological tests for failure) have revealed that specific treatment is effective in nearly all infected by congenital route, in 60−80% of acute phase patients, children, and early chronic phase, and in 25−30% of those treated during the chronic phase (Rassi and Luquetti, 1992, 2003). Treatment is mandatory for first two groups and optional for the last. The follow-up of treated patients to know if they were cured is performed through serological and parasitological tests, though the latter are not essential. Cure is attained when antibodies, formerly present, disappear. Parasitological tests are useful only when positive and indicate treatment failure (Rassi et al., 2007).

In Chagas disease, antibodies demand different amounts of time to disappear, related to the phase of the disease and to the type of *T. cruzi*. In congenital infection, nearly all treated are cured, and one year follow-up is enough. Acute and chronically infected individuals with less than 10 years of evolution (mainly children) are cured at a lower rate (60−80%) and the antibodies may take 2−10 years to disappear (Rassi and Luquetti, 2003). It has been recently demonstrated that the type of parasite present is of utmost importance (Yun et al., 2009). In those countries where *T. cruzi* I predominates (Mexico, Central America, Colombia, and Venezuela), the antibody responses vanish by 16 months in children. Where *T. cruzi* II predominates, as in Brazil and Bolivia, the same group of patients takes more than 5 and often 10 years to be free of antibodies (Yun et al., 2009).

Those treated during the chronic phase usually take longer: no studies with large series of adults are available from *T. cruzi* I region but below the equator line (*T. cruzi* II) it usually takes 15 to 30 years to obtain a negative serology and the number cured is no higher than 30%. This long time is probably due to the time the immune memory would forget a parasite which was in circulation for decades (Luquetti and Rassi, 1998).

Antibody response should be measured by conventional tests as described. The use of recombinant antigens may yield equivocal results. In some already cured, the responses to recombinant antigens is still high when there are no more anti-*T. cruzi* antibodies found by ELISA, IIF, and IH. A number of nonconventional tests show that antibodies measured by these systems could indeed have a quicker shift and decrease after shorter periods of time (Almeida et al., 1997; Sosa Estani et al., 1998).

28.8 Quality Control in Serology

Although serological diagnosis is relatively simple to perform considering the complex tasks that involve the activities of the modern diagnostic laboratory, some principles should be followed in order to guarantee either a correct screening or a final diagnosis:

- use of diagnostic reagents of proven quality. Validation of the reagents available in the market should be performed by a competent authority or an institution in which this

validation is delegated. For *T. cruzi* screening a sensitivity of at least 99.8% and a speci ficity of 99.5% are appropriate, but there is still room for improvement;
- internal quality assurance of the equipment, procedures, and diagnostic reagents;
- complete records of all activities verified through periodic audit (at least every 2 years);
- continuous training of staff; and
- mandatory participation in performance evaluation schemes for which unknown samples are tested together with the routine work and results informed with the same celerity.

28.9 Prophylaxis to Avoid Accidental Contamination

As this chapter is intended to be useful to personnel from diagnostic laboratories, some guidelines for prophylaxis of accidental contamination are included. Accidental contamination with *T. cruzi* has been reported in more than 100 cases (Brener, 1984; Ministério da Saúde, 1997). Contamination may be of laboratory staff who works with infected bugs, with inoculated animals, cultures for antigen production, cryopreserved samples, and blood from acute phase patients. However, there are guidelines to help avoid this danger. It is advisable that all personnel working with this parasite have a sample of blood collected and serum stored each year. This is useful to know if someone was infected before arrival at the laboratory, and to compare antibody levels in the case of an accident or recognize an otherwise unnoticed contamination. The main measures to avoid an accident are, in order of importance: (i) eye and mouth protection, by using a mask; (ii) gloves and covered shoes (no sandals allowed); (iii) do not work alone; (iv) concentration on the task. Some of these are obvious and a part of GLP.

28.10 Future Perspectives

The serological diagnosis has had profound advances in the last decade. Most infected individuals are correctly diagnosed. The main problems are due to poor control in two main variables: quality kits and laboratory practices. In any case a few sera (up to 2%) are at one or at the other end of the Gauss curve. Diagnosis ought to be handled by a clinician who decides based on clinical findings and serology results.

Although recent research showed that with the tests we have, children and adolescents will have negative serology in less than 15 months after treatment, proving that conventional tests are good enough, and that the long time required for negativation is more dependent on the type of *T. cruzi* than the tests, new tests capable of measuring antibodies that assess cure instead of antibodies measured by conventional tests would help.

Progress also could be made in diagnosis in rural areas, and rapid tests should help with this goal. Some efforts were performed to have several recombinants at the same device, but this will increase costs, and it seems that companies were not commercially interested. Another advantage would be development of a PCR kit that could be commercially available and is able to compete with serology in sensitivity and specificity.

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29 AIDS and Chagas Disease

Marcelo Corti and María F. Villafañe*

HIV/AIDS Division, Infectious Diseases F.J. Muñiz Hospital, Buenos Aires, Argentina

29.1 Introduction

Chagas disease, or American trypanosomiasis, is a zoonotic disease caused by the flagellated protozoan *Trypanosoma cruzi*, which was first identified by Carlos Chagas in 1909 in Minas Gerais, Brazil. Generally, this zoonotic disease is transmitted to humans and animals by *Triatoma infestans* (vectors) known as "vinchucas" in Argentina and "barbeiros" in Brazil. Less frequently, *T. cruzi* can be transmitted by transfusions of infected blood, transplacental route, and organ transplantation from an infected donor. Sharing intravenous needles with an infected person with parasitemia is another possible source of infection (Cohen et al., 1998). Rarely, the oral route, including breast milk and laboratory accidents is an alternative source of infection (Coura, 2006).

Several authors have observed severe forms of Chagas disease, such as meningoencephalitis and myocarditis in patients with advanced immunodepression. The majority of cases have been described in Brazil, in Argentina and in the other countries in which this parasitosis is endemic. Generally, the clinical manifestations of the reactivation of *Trypanosoma cruzi* infection in immunosupressed patients is the result of the reactivation of chronic and previously asymptomatic or oligosymptomatic disease (Ferreira et al., 2002). Chagasic patients infected with the human immunodeficiency virus (HIV) present episodes of reactivation of the *T. cruzi* infection. Acute exacerbation of chronic Chagas disease is more frequent in AIDS than in other immunocompromised patients. Reactivation of Chagas disease in AIDS is possible in those patients with CD4 T-cell counts less than 200 cells/μL and the greatest risk occurs with CD4 T-cell counts less than 50 cell/μL.

29.2 Epidemiology of Chagas Disease in Latin America and Argentina

Chagas disease is a vital health problem in these areas of the world. Chagas disease is observed from the southern USA to southern Argentina. According to the World

*E mail: marcelocorti@fibertel.com.ar

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Health Organization (WHO) there are 100 million people at the risk of infection with 10−12 million people infected and 50,000 deaths per year, the majority due to dilated myocarditis (Cordova et al., 2008). Urban migration transformed Chagas disease into a health problem in nonendemic countries. In the USA, approximately 50,000−100,000 Latin American residents have evidence of chronic *T. cruzi* infection (Maguire, 2006), and in recent years many multifactorial associated cases were diagnosed in the USA, Canada, and Europe (Schmunis, 2007). According to estimates, the prevalence of *T. cruzi* in Latin American immigrants is 16/1000 in Australia, 9/1000 in Canada, 25/1000 in Spain, and 8−50/1000 in the USA (CDC-P, 2007). Rural and urban migration, contamination of donated blood products, intravenous drug abuse, laboratory accidents, and congenital transmission increase the diagnosis of this parasitosis in large cities. Since 2007, blood transfusions have been screened for Chagas disease in the USA (Leiby et al., 1997).

In Argentina, there are 7 million people exposed to the risk of *T. cruzi* infection with approximately 2.5−3 million infected and 400,000 patients with different grades of cardiomyopathy (Corti, 2000). Cardiac involvement becomes evident 20−30 years after the first infection but 5−10% of the patients can develop symptomatic myocarditis in the acute phase of the disease (Punukollu et al., 2007).

According to the Centers for Diseases Control and Prevention (CDC) of the USA, only 3 of the 12 disease-defining AIDS opportunistic infections are parasitoses: toxoplasmosis, cryptosporidiosis, and isosporidiosis. In endemic areas, reactivation of *T. cruzi* infection should be included among the potential opportunistic pathogens indicative of AIDS (Corti, 2000).

29.3 Natural History of Chagas Disease

T. cruzi populations include multiclonal strains with different biological properties such as replication rates, drug susceptibility, virulence, and tissue tropism, which may be implicated in the clinical forms of the disease (Macedo and Pena, 1998; Macedo et al., 2004). *T. cruzi* is an obligate intracellular parasite. Among immunocompetent individuals the clinical course of Chagas disease is usually divided into three stages: acute, indeterminate, and chronic. The indeterminate phase usually extends 10−20 years, but in the majority of the patients it lasts a lifetime (Texeira et al., 2006; Marin-Nieto et al., 2007). As with some immunocompromised patients, they are likely to show signs and symptoms associated with parasitemia during the acute stage of the disease (Figure 29.1). These patients can present clinical manifestations of acute myocarditis or meningoencephalitis (Shikanai-Yasuda et al., 1990; Bern et al., 2007). When *T. cruzi* invade the myocardial cells, it induces a diffuse and severe neutrophilic and monocytic inflammatory infiltrate and myofibrillar lesions with or without fibrosis that can be seen in endomyocardial biopsies. Also, during the acute phase of the disease, cardiac parasympathetic neurons are damaged by the parasite (Rassi et al., 2006). Meningoencephalitis occurs mainly in acute Chagas disease in children under 2 years. After the acute phase, the majority of cases progress to a subclinical and latent period named as

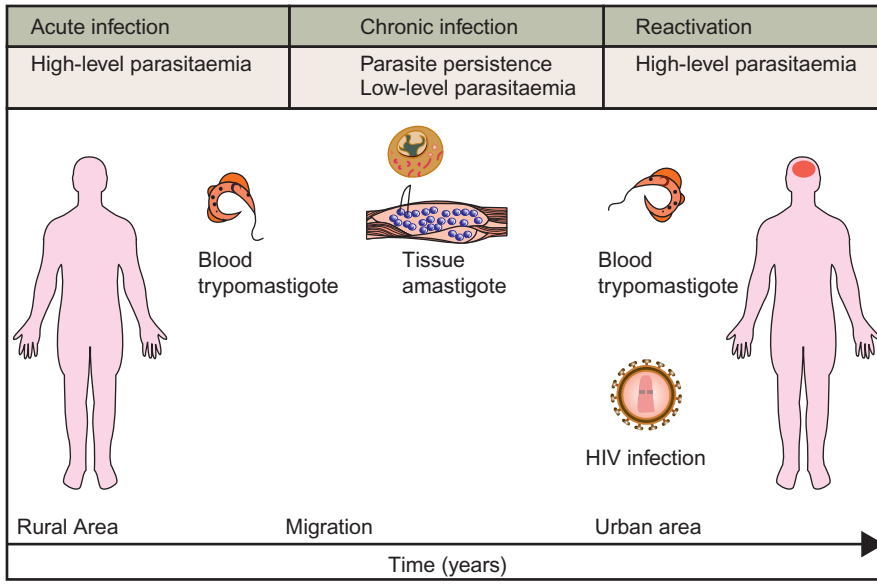

Figure 29.1 Natural history of HIV and Chagas disease coinfection.
Source: Taken from DiazGranados et al. (2009).

indeterminate phase (Rossi and Bestetti, 1995; Pinto Dias, 2006). Finally, 10−30% of seropositive individuals developed various syndromes in the context of symptomatic chronic Chagas disease: chronic cardiomyopathy with frequent and marked cardiac arrhythmias, especially sinus bradycardia, with right bundle brunch block and other electrocardiographic abnormalities. In other patients, the destruction of visceral autonomic neurons of the digestive tract leads to progressive enlargement of visceral organs (especially megaesophagus and megacolon) named as megasyndromes or chronic gastrointestinal Chagas disease. Megaesophagus and megacolon may or may not be associated with cardiac involvement and occur in 10−20% of cases according to the area under consideration (Prata, 2001; Barret et al., 2003). A small number of patients develop different grades of neuropsychiatric compromise.

29.4 Pathogenic Mechanisms of Chagas Disease Reactivation in AIDS Patients

The diversity of clinical manifestations of Chagas disease in humans has been attributed to the host's immune response and to the genetic heterogeneity of the parasite, which may be formed by a multiclonal population with various biological profiles (Macedo et al., 1992; Lages-Silva et al., 2001). The preferential location of *T. cruzi* in the central nervous system (CNS) may be associated with the presence of subpopulations with neurotropic characteristics that may be reactivated during the immunosuppression of the host. Patients with HIV infection do not lead to new

T. cruzi genotypes because *T. cruzi* stocks isolated from these patients were closely related to clonal genotypes previously identified (genotypes 30 or 32) in 89% and 94% of the stocks isolated from HIV-positive and HIV-negative patients, respectively (Perez-Ramírez et al., 1999). Reactivation of Chagas disease does not occur spontaneously and is more strongly associated with immunosuppression in patients who have oncohematologic neoplasms (Fontes Rezende et al., 2006), those chronically treated with high doses of corticosteroids, and those with advanced HIV/AIDS (Lages-Silva et al., 2002). In these groups of immunocompromised patients chronically infected by *T. cruzi*, it is possible that the parasite load can increase and the detection of parasitemia is more frequent (Rocha et al., 1994; Karp and Auwaerter, 2007).

In this context, and especially in AIDS patients, the high level of parasitemia is related to the development of acute CNS or heart disease. However, the relation between *T. cruzi* parasitemia and organ damage is not absolute; sometimes we can see that severe disease is associated without detectable parasitemia. In these patients, it is necessary to obtain biopsy smears to achieve a definitive diagnosis (Shikanai-Yasuda et al., 1990; Lazo et al., 1998; DiazGranados et al., 2009).

29.5 AIDS and Chagas Disease

Reactivation of the chronic stage of Chagas disease is uncommon; acute exacerbations of a latent or chronic infection due to *T. cruzi* can occur in individuals with involvement of cellular immunity, especially in those with advanced HIV/AIDS disease, prolonged corticosteroid use, or other immunosuppressive therapies and transplant-associated immunosuppression (Ferreira et al., 1997; Ferreira, 1999).

In a prospective study that included patients in the pre− and post−highly active antiretroviral therapy (HAART) era, the frequency of reactivation of *T. cruzi* infection was approximately 20% (Ministerio Da Saude, 2006).

In patients coinfected with HIV, the reactivation of Chagas disease may be related to the selected cellular immune depletion or to the characteristics of the parasite. This situation has been demonstrated by the presence of trypomastigotes in blood by microhematocrit of quantitative buffy-coat (QBC) and by the invasion of the CNS or the heart (Ferreira et al., 1991; Labarca et al., 1992; Sartori et al., 1995).

29.5.1 Clinical Aspects

The majority of the patients present with CNS or myocardial involvement; less frequent clinical manifestations include the digestive tract, especially the esophagus and the colon (Ferreira et al., 1991). Skin lesions (Sartori et al., 1999), peritoneum involvement (Iliovich et al., 1998), and cervix uteri compromise (Concetti et al., 2000) should be described infrequently.

Generally, the majority of AIDS patients with reactivated Chagas disease have CD4 T-cell counts below 200 cells/μL and, especially, less than 50 cells/μL.

In contrast with heart transplant recipients with reactivated chronic Chagas disease in whom myocarditis is the predominant clinical form of presentation, in

AIDS patients, CNS manifestations occur frequently and can include meningoencephalitis or brain masses (named as chagomas). CNS involvement represents 80–90% of total episodes of reactivation of chronic Chagas disease in AIDS patients (Ferreira et al., 1991). In patients with cerebral masses, neurological symptoms and signs include headaches, fever, cognitive changes, seizures, or neurological focal signs depending on the number, size, and location of these lesions. On the other hand, patients who develop diffuse meningoencephalitis present with fever, headaches, meningism, and altered mental status.

CNS tumor-like lesions are, in our experience, the most common clinical manifestation, and these are indistinguishable from other opportunistic infections such as toxoplasmosis or the neoplastic process, such as the primary CNS lymphoma (PCNSL) that can involve the CNS in AIDS patients (Gluckstein et al., 1992).

Cerebral chagomas are contrast-enhancing single or multiple focal brain lesions with hypodense areas of perilesional edema and a mass effect on the middle-line structures (Figure 29.2).

Generally, lesions due to *T. cruzi* are located in the white matter and occasionally involve the cerebral cortex. In a study by Cordova et al. (2008), which included 15 patients with reactivation of Chagas disease with CNS involvement, the most frequent findings were a single supratentorial hypodense lesion compatible with abscess and involving predominately the white matter of brain lobes (Figure 29.3).

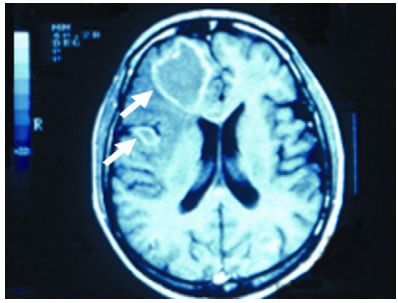

Figure 29.2 Axial T1 weighted MRI image shows two large lesions in the right parietal and frontal lobes (arrows), with contrast enhancement, perilesional edema, and mass effect on the middle line structures.

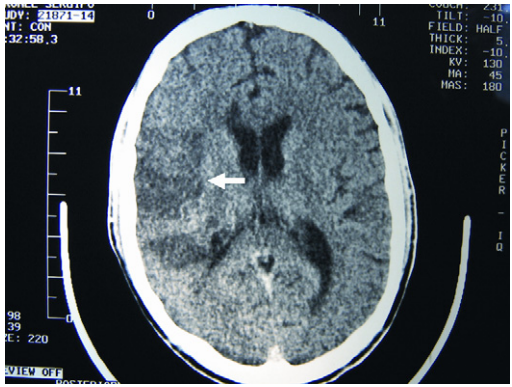

Figure 29.3 CT of the brain showing a single hypodense lesion in the right temporoparietal lobe, with minimum contrast enhancement, no displacement of normal structures, and edema (arrow).

To the contrary, the lesions caused by *Toxoplasma gondii* frequently compromise the brain cortex, thalami, and the basal ganglia. In both cases, histopathological examination reveals necrotic encephalitis; however, the multifocal identification of parasites is more frequent in the reactivation of Chagas disease than in *Toxoplasma* encephalitis.

The second most frequent expression of the reactivation of *T. cruzi* infection in the CNS is diffuse meningoencephalitis. In these patients, the diagnosis is easily confirmed by a lumbar puncture and the centrifugation of cerebrospinal fluid (CSF) with the detection of trypomastigotes with Giemsa smear. Lumbar puncture has a high sensitivity for the diagnosis of chagasic meningoencephalitis and should always be performed in the absence of contraindications. In the Cordova et al. (2008) series, the sensitivity of this technique was 85%. CSF examination showed typical parasitic meningitis with leptomeningeal involvement and lymphomononuclear pleocytosis (Livramento et al., 1989).

Pagano et al. (1999) studied 10 patients with AIDS and Chagas disease. All patients presented cerebral tumor-like lesions and six of them developed intracranial hypertension syndrome. In addition, Cordova et al. (2008) showed only 2 patients out of 15 with meningoencephalitis as a unique clinical expression of the disease.

In Argentina, the myocardium is, after the CNS, the second target organ in patients with reactivation of chronic Chagas disease and AIDS. Acute myocarditis with arrhythmias and refractory congestive heart failure with rapid and generally fatal evolution represents the most frequent clinical presentation (Labarca et al., 1992; Rocha et al., 1993; Sartori et al., 1998a).

Diagnosis of this complication should be suspected in AIDS patients with dilated cardiomyopathy, epidemiological antecedents, and positive serologic tests for *T. cruzi* antibodies. Symptoms and signs are similar to other dilated cardiomyopathies (Sartori et al., 1998b; Dobarro et al., 2008).

Less frequently, medical literature describes the existence of patients with chronic diarrhea and amastigotes of *T. cruzi* in duodenal biopsy smears, prolonged fever, and parasitemia.

29.5.2 Laboratory Diagnosis

Laboratory diagnosis of Chagas disease in immunocompromised patients is based on the same methods used in the immunocompetent population. Diagnostic methods include serological tests and the direct detection of the parasite (Figure 29.4).

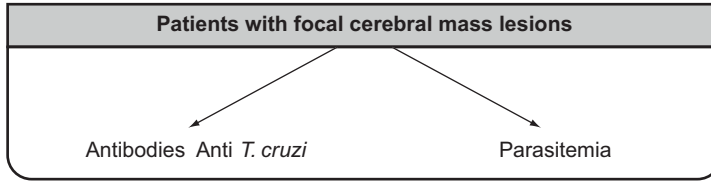

Figure 29.4 Diagnosis algorithm of focal brain lesions in AIDS patients.

However, knowledge of the natural history of Chagas disease is important to determine the stage and to interpret the laboratory findings (Figure 29.1). The reactivation of Chagas disease in an AIDS patient with chronic infection due to *T. cruzi* includes the following laboratory findings: (i) serological tests to detect specific antibodies to *T. cruzi*; (ii) detection of the parasite in fresh blood by QBC, Strout method, or microhematocrit (Luquetti and Rassi, 2000); (iii) in patients with meningoencephalitis, the detection of the parasite by microscopic examination of CSF with Giemsa smear; and (iv) the detection of *T. cruzi* amastigotes with an acute and necrotic inflammatory infiltration in tissue by biopsies obtained in patients with chagomas or myocarditis (Sartori et al., 2007).

Reactivation of Chagas disease in AIDS patients is usually associated with the detection of *T. cruzi* trypomastigote forms by direct microscopic examination of blood. High levels of *T. cruzi* parasitemia can precede the clinical manifestations or may be found later, during the reactivation. The dynamics of *T. cruzi* parasitemia in HIV-coinfected patients may play an important role in the reactivation of the disease. *T. cruzi* parasitemia was detected more frequently in HIV-seropositive patients with chronic Chagas disease than in HIV-negative individuals. HIV-coinfected patients also had higher levels of parasitemia (Sartori et al., 2002). According to the number of triatomines fed, Sartori et al. (2002) classified the parasitemia level in three categories: (i) very high parasitemia; (ii) high parasitemia; and (iii) low parasitemia when trypomastigotes of *T. cruzi* were detected by direct examination of blood or CSF.

More recently, different studies have reported a higher sensitivity of polymerase chain reaction (PCR) to detect the DNA of *T. cruzi* in laboratory samples. PCR appears to be a specific and highly sensitive laboratory method to inform the number of DNA copies present in the sample examination. In order to investigate the reactivation of *T. cruzi* infection in immunosupressed patients, in 2005, the Brazil Consensus of Chagas disease proposed that PCR should be performed directly from the patient's fresh blood (Ministério Da Saúde, 2005).

In patients with AIDS, the differential diagnosis between acute encephalitis due to *T. gondii* and *T. cruzi* may be difficult; in this context, PCR in peripheral blood and brain tissue obtained by stereotactic biopsy provided a rapid differential and sensitive diagnosis method of *T. cruzi* reactivation. This allows the prompt administration of specific therapy, which can modify the poor prognosis of this kind of patient (Burgos et al., 2005).

Qualitative parasitological techniques such as xenodiagnosis and hemoculture (the gold standard of laboratory Chagas disease diagnosis) have a low value in AIDS patients with suspected Chagas disease reactivation (Figure 29.5) (Shikanai-Yasuda et al., 2006).

29.5.2.1 Diagnosis of Chagasic Meningoencephalitis

CNS compromise is the most frequent manifestation of the acute reactivation of chronic infection due to *T. cruzi* in AIDS patients and can include meningoencephalitis or brain mass. The CSF may be normal or can be associated with mild to

Suspicion of Chagas disease reactivation in immunosuppressed patients Techniques proposed for the laboratory diagnosis
1. Direct microscopic examination, buffy-coat examination (alternatively Strout) and PCR of fresh blood.
2. Depending on the laboratory availability A-xenodiagnosis couple to microscopic examination and to PCR of triatomines feces, performed on days 1–5 (two or three tests), 10 and/or 15 (one or two tests), 30 and 60 days after blood meal and/or B-hemoculture couple to microscopic examination and to PCR of hemoculture aliquots, performed on days 1–5 (two or three tests), 10 and/or 15 (one or two tests), 30 and 60 after seeded.

Model for the investigation of Chagas disease reactivation in immunosuppressed patients.

Figure 29.5 Laboratory diagnosis of suspected Chagas disease reactivation in AIDS patients.
Source: Taken from Braz et al. (2008).

moderate pleocytosis with prevalence of mononuclear cells and low to mild protein levels. For this reason, in our opinion, all CSF samples from AIDS patients should be routinely sent to the parasitology laboratory for the detection of trypomastigotes. Diagnosis of meningoencephalitis is confirmed by the direct observation of trypomastigotes in CSF by Giemsa smear. Centrifugation of the CSF enhances the sensitivity of this test (Ferreira et al., 1991).

Current tests for diagnosis of CNS invasion in Chagas disease have a low sensitivity, and in some situations do not allow establishment of the etiology. In these cases, when there exists a strong diagnosis suspect, rapid and specific techniques such as PCR, which can detect minimal quantities of the parasite DNA, may be useful for early diagnosis and for monitoring treatment (Avila et al., 1991; Gomes et al., 1998; Lages-Silva et al., 2002; Barret et al., 2003).

Recently, several studies have reported a higher sensitivity of PCR to the laboratory diagnosis of Chagas disease (Braz et al., 2008). Anatomopathological findings associated with Chagas disease meningoencephalitis in AIDS patients include the presence of lymphomonocytic meningitis with the presence of *T. cruzi* amastigotes in the meninges and generalized cerebral edema with hemorrhagic necrosis (Sartori et al., 1999).

29.5.2.2 Diagnosis of Chagasic Tumor-Like Lesions

In patients with cerebral mass lesions, neuroimages typically show one or more ring-enhancing lesions involving both gray and white matter, the cerebellum, and the brain stem (Di Lorenzo et al., 1996; Walker and Zunt, 2005; Corti and Yampolsky, 2006). Magnetic resonance imaging (MRI) and cranial computed tomography (CT) reveals single or multiple tumor-like lesions with hypodense or

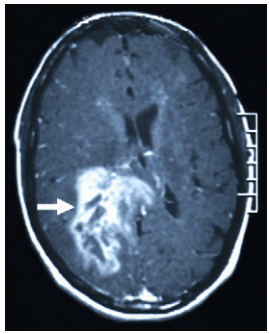

Figure 29.6 Axial T1 weighted MRI showing a heterogeneous right parieto occipital lesion with irregular gadolinium enhancement, mass effect on the middle line structures, and perilesional edema (arrow).

hypointense centers, with or without contrast enhancement, areas of perilesional edema, or effect on the middle-line structures (Figure 29.6).

This imaging pattern of brain chagoma is similar and indistinguishable from that of cerebral toxoplasmosis (Hoff et al., 1978; Livramento et al., 1989; Montero et al., 1998). MRI spectroscopy showed a significant increase in choline/creatine ratios (Cho/cr) associated with an increased membrane synthesis and the pathological evidence of lipid or lactate signal related to the presence of anaerobiosis or necrosis in the central area of the abscess (Figure 29.7).

In endemic areas for *T. cruzi* infection, all HIV patients presenting with cerebral brain lesions should be evaluated for specific anti−*T. cruzi* antibodies and for parasitemia (see Figure 29.4) (Brito et al., 2001).

When *T. cruzi* trypomastigotes cannot be demonstrated in the CSF, a cerebral biopsy of focal brain lesions may be necessary. Histopathological findings include a granulomatous abscess with necrosis, cerebral edema with focal necrotizing, and hemorrhagic encephalitis with uni- or multifocal lesions of undefined limits containing amastigotes of *T. cruzi* in Giemsa smears (Figure 29.8) (Karp and Neva, 1999). The most striking finding is the presence of many small organisms within the macrophages (amastigotes). Electron microscopic features of the organism included parallel microtubules under the cell membrane, a kinetoplast, and a flagellar pocket containing rudimentary flagellae. These lesions are generally localized at the brain periphery, affecting the gray and, especially, the white matter. Lesions can also occur in the brain stem and in the cerebellum (Nijjar and Del Bigio, 2007).

29.5.2.3 Diagnosis of Heart Compromise

The diagnosis of Chagas cardiomyopathy in AIDS patients should be suspected in those with epidemiologic and positive serologic tests and signs and symptoms of myocardial involvement. The frequency of cardiomyopathy is 30−40%. In AIDS patients, the most common clinical presentation is acute dilated myocarditis characterized by symptoms and signs of heart failure or severe arrhythmias. In some cases, the only clinical expression is the electrocardiographic alterations or the histopathological findings of myocarditis. The most frequent electrocardiographic pattern is the right bundle branch block with or without left anterior fascicular block,

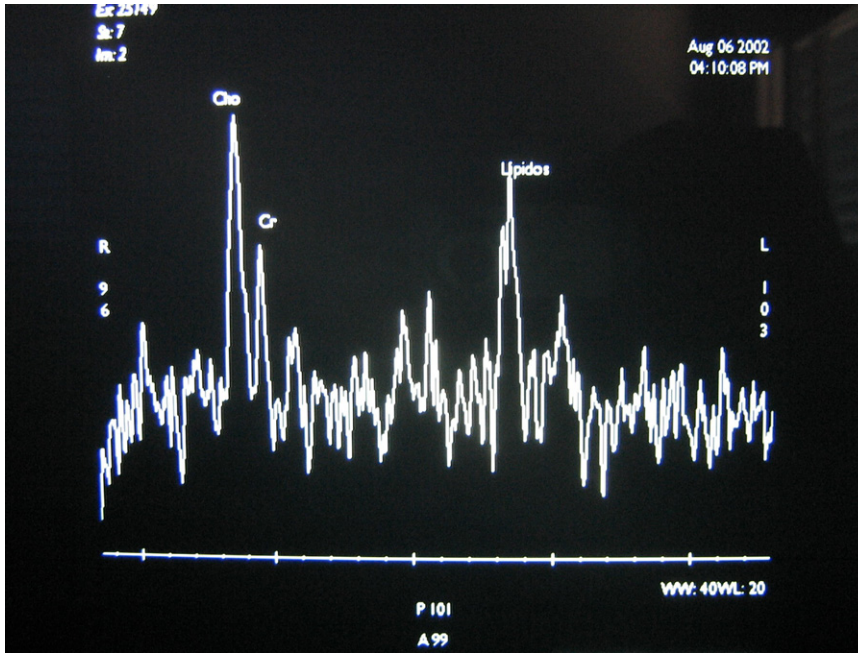

Figure 29.7 MRI spectroscopy in axial T1 weighted with a single voxel localized at the lesion and corresponding to the patient of Figure 29.6. There is a significant increase in choline/creatine ratios (Cho/cr) associated with an increased membrane synthesis and the pathological evidence of lipid signal related to the presence of anaerobiosis or necrosis.

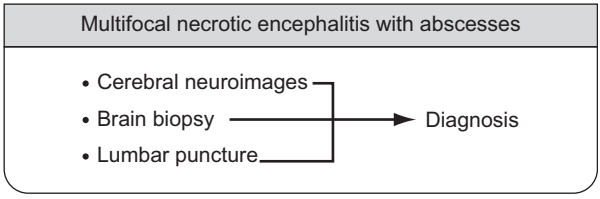

Figure 29.8 Diagnosis algorithm of cerebral abscesses in AIDS patients.

sinus bradycardia, or malignant arrhythmias. Bidimensional echocardiogram shows a dilated cardiomyopathy, segmental contractile abnormalities, apical aneurysms, which are frequently associated with mural thrombus, and pericardial effusion. MRI can detect myocardial fibrosis, wall motion abnormalities, and compromise of the left ventricular function (Rochitte et al., 2005). Diagnosis is confirmed by the histopathological examination, which reveals cardiomegaly with a dilated myocarditis associated with the ruptures of myofibrils and inflammatory infiltration with plasma cells, lymphocytes, and macrophages in myocardial tissue obtained by endomyocardial biopsies or autopsy studies. Also, it is possible to observe an intense parasitism of cardiac fiber cells with numerous amastigotes of *T. cruzi*. Epicarditis and

endocarditis can also be observed in these patients (Oddo' et al., 1992). Using light and electron microscopy, abundant amastigotes of *T. cruzi* with the nucleus, the kinetoplast, and a rudimentary flagella were seen in the cytoplasm of the macrophages in muscle fibers (Oddo' et al., 1992; Nijjar and Del Bigio, 2007).

29.5.2.4 Differential Diagnosis

Diffuse chagasic meningoencephalitis should be included in the differential diagnosis of other opportunistic infections that can cause CNS encephalitis in AIDS patients. This syndrome may be caused by various opportunistic pathogens including *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, neuro herpesvirus, and *Treponema pallidum*.

The focal neurological disease or chagomas should be included in the differential diagnosis of toxoplasmosis, CNS primary lymphoma, tuberculomas, pyogenic abscesses due to *Nocardia* species and *M. tuberculosis*, and, finally, the progressive multifocal leukoencephalopathy.

Differential diagnosis of myocarditis includes HIV, *T. gondii*, cytomegalovirus, and other herpesvirus.

29.5.3 Treatment

At this moment, there are only two effective drugs for the treatment of Chagas disease: nifurtimox and benznidazole. These two drugs are particularly useful in the acute and early phase of the infection and in the reactivation observed in immunocompromised patients (Skiest, 2002). Nifurtimox and benznidazole have great limitations in use related to their frequent and occasionally severe side effects and the necessary long-term treatment.

Other drugs such as allopurinol, ketoconazole, fluconazole, and itraconazole have been used for the reactivation treatment by some authors with apparent success, but they are not recommended as first-line therapy (Nishioka et al., 1993).

In consequence, drugs usually used for the therapy of Chagas disease in AIDS patients are identical to those that we use in immunocompetent patients. Nifurtimox and benznidazole are the only two drugs effective in killing this parasite in blood and tissues. In the majority of South American countries, these drugs are not commercially available for patients in pharmacies. Nifurtimox is unavailable in Argentina, and the distribution of benznidazole depends on the Health Ministry.

The majority of the patients with CNS reactivation of Chagas disease associated with AIDS have a poor prognosis and a high mortality related to delay in the diagnosis and specific therapy. However, the mortality rate diminishes to approximately 20% in patients with at least 30 days of specific treatment (Lazo et al., 1998). Both drugs induce significant side effects and some strains of *T. cruzi* are resistant to treatment (Coura, 2009).

Nifurtimox is a nitrofuran derivate that is used at the dose of 8−10 mg/kg per day divided into two or three doses daily, orally, for 60−90 days. The mechanism of action of nifurtimox involves the production of nitroanion radicals, which, in the

presence of oxygen, leave *T. cruzi* incapable of detoxifying free radicals (Do Campo and Moreno, 1986).

In countries where nifurtimox is commercialized, the drug has a similar efficacy to that of benznidazole.

The most frequent adverse side effects observed include anorexia, weight loss, psychological and psychiatric changes (excitability, muscle tremors, somnolence, and hallucinations), and digestive manifestations such as nausea, vomiting, abdominal pain, and diarrhea. The side effects can be controlled by administrating diazepam, metoclopramide, antihistamines, and other symptomatic medications.

Benznidazole is a nitroimidazole derivate that is administrated orally at the dose of 5 mg/kg/day in two doses for a period of 60 days. The action of benznidazole is related to the introduction of components of the DNA and K-DNA of *T. cruzi* and the lipids and proteins of the parasite (Polak and Richie, 1978). Side effects of benznidazole generally occur in the first days of therapy and include cutaneous eruption (including dermatitis, generalized angioedema, and Stevens−Johnson syndrome), peripheral neuropathy, and bone marrow depletion (granulocytopenia, thrombocytopenia, with purpura). The side effects can be controlled with antihistamines, corticosteroids, and, in severe cases, with the necessary suspension of the treatment (Figure 29.9) (Ferreira and Borges, 2002).

After remission of the clinical manifestations, a second prophylaxis is recommended, because further reactivations can occur later. In this aspect, an expert committee recommended the use of benznidazole at the dose of 5 mg/kg/day three times a week up to immune reconstitution associated with antiretroviral therapy (Figure 29.10).

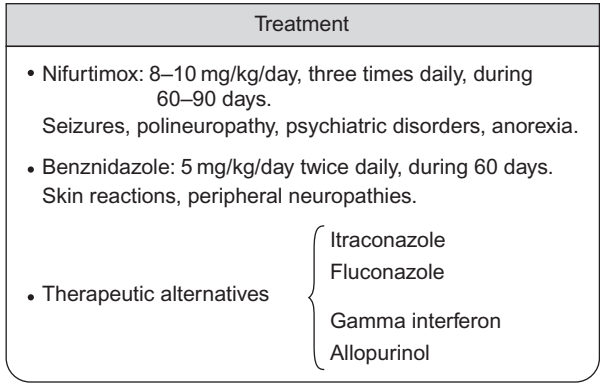

Figure 29.9 Therapeutic alternatives for reactivation of Chagas disease.

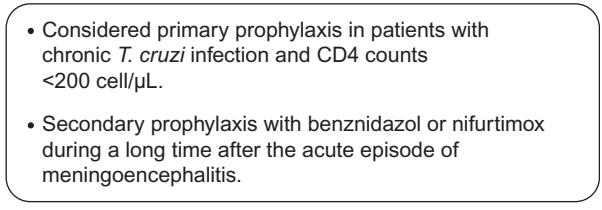

Figure 29.10 Primary and secondary prophylaxis in patients with AIDS and *T. cruzi* infection.

Highly active antiretroviral therapy should be systematically indicated in these patients in order to achieve the immune reconstitution (Jannin and Villa, 2007).

Primary prophylaxis in chagasic patients infected with HIV is not recommended.

29.6 Conclusion

The identification of latent *T. cruzi* infection in HIV/AIDS patients is highly important. Continued attempts to do so should be considered both in endemic and nonendemic areas of Chagas disease.

Early diagnosis of reactivation of chronic Chagas disease with CNS involvement followed by specific antiparasitic therapy may modify the poor prognosis of this kind of patient.

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30 Treatment of Chagas Disease

*Werner Apt**

Biological Science Institute, University of Chile, Santiago, Chile

30.1 Introduction

Chagas disease has existed for at least 9000 years. Of the desiccated human mummies from coastal valley sites in northern Chile and Peru, 41% were found to be positive by polymerase chain reaction (PCR) and hybridization probes for kDNA of *Trypanosoma cruzi*. These tissue extracts correspond to the cultural groups that lived from 7000 BC to 1500 AD. These findings confirm that the sylvatic animal cycle of Chagas disease was well established by that time (Aufderheide et al., 2004).

Although Chagas disease is an old zoonosis, its treatment is recent. The present human treatment with nifurtimox (NF) and benznidazol (BZN) dates from the 1970s and is based on an empirical therapy (Pinto Dias, 2004; Coura, 2005; Steverding and Tyler, 2005).

There are several drugs which act *in vitro* on *Trypanosma cruzi*, in cultures of epimastigotes and trypomastigotes, tissue cultures of amastigote forms and *in vivo* in different species of infected animals with diverse strains (subpopulations) of the parasite. It is important to point out that the drugs used in Chagas disease therapy must have an effect on the intracellular amastigote forms, which are the reproductive forms in the vertebrate host. The epimastigote and trypomastigote forms of these hosts derive from the amastigotes, and for this reason their response to different drugs has less importance (Andrade and Andrade, 2000; Teixeira et al., 2006). In Table 30.1 several drugs are described that have been empirically applied to *T. cruzi* in experiments on animals and humans between 1940 and 1975. With the majority of these drugs a decrease of the parasitemia and lethality may be obtained, but not a parasitological cure (Brener, 2000).

30.2 Drugs Which Inhibit Protein or Purine Synthesis

A rational therapy of *T. cruzi* should be based on drugs that inhibit protein or purine synthesis. One of these drugs is allopurinol. *T. cruzi* is not able to synthesize purines *de novo* as humans do. Allopurinol (4-hydroxypyrazol (3,4-*d*) pyrimidine,

* E mail: wapt@med.uchile.cl, wernerapt@hotmail.com

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Table 30.1 Drugs Administered from 1940 to 1975 to Laboratory Animals and Chagas Disease Patients Which Produced a Reduction of the Parasitemias But Not a Parasitological Cure

8 Aminoquinolines (Primaquine)
Bisquinaldines (Bayer 7602)
Arsenebenzenes (Spirotrypan)
Fenantridines (Cardibium)
Emetine and derivatives
Nitrofurans and derivatives
Nitroimidazole and derivatives
Piperazine and derivatives
Triphenylmethane
Triaminoquinazolines
2 Acetamide 5 nitrodiazole

Source: Adapted from Brener (2000).

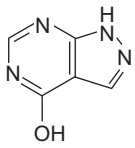


Figure 30.1 Chemical structure of allopurinol (4 hydroxypyrazol (3,4*d*) pyrimidine).

HPP) (Figure 30.1) is an analog of hypoxanthine, which decreases uric acid and the conversion of hypoxanthine to xanthine.

For this reason it is used to treat gout, which is characterized by the deposit of uric acid in the joints. HPP inhibits the epimastigote forms in culture. In mice infected with *T. cruzi* and treated with allopurinol, an important reduction of the parasitemia is obtained, although some parasite strains are resistant to the drug (Avila and Avila, 1981). *T. cruzi* changes HPP to APP (4-aminopyrazol (3,4-*d*) pyrimidine), which is 15 times more powerful against epimastigotes than HPP. If APP is administered to infected *T. cruzi* mice a suppression of the parasitemia is obtained with a dose 400 times lower than allopurinol (Avila et al., 1983). In patients with acute Chagas disease treated with allopurinol at high doses (20−30 mg/day) for 60 days, no reduction of the parasite burden was obtained. In a multinational study performed in Argentina, Brazil, and Bolivia in patients with chronic Chagas disease treated with 900 mg/day for 60 days, no parasitological cure was obtained. This drug was well tolerated in a number of studies performed in patients with chronic Chagas disease, and in some of these an improvement of the electrocardiographical alterations in Chagas cardiopathy (CCC) was demonstrated (Apt et al., 2005). It has been used in heart transplant in Chagas patients with good results (Tomimori-Yamashita et al., 1997). In exceptional cases it has been necessary to suspend the treatment due to its secondary effects (Apt et al., 1988).

Megazole (CL 641855), 2 amino 5(methyl-5-nitro-2-inidazole)-1,3,4 thiazole is a nitroimidazole-thiadiazol derivative that inhibits the protein synthesis of *T. cruzi*. With this drug a cure of mice inoculated with Y^{20} strains and Colombian *T. cruzi*

was obtained. The parasitological cure was demonstrated by hemocultures, reinoculations, and an indirect immunofluorescence (IF) test. The drug has not been used in human clinical studies, since the experimental survey in animals demonstrated that it was mutagenic (Enanga et al., 2003; Nesslany et al., 2004).

MK-436 is a compound 3(methyl-5-nitroimidazole-2-il) 3 α 4,5,6,7,7α hexahydro-1,2-benzisoxazole that is a substitute for 5-nitroinidazole and its derivative dihydro (L - 634, 549). It affects amastigotes in culture tissue, and in acute and chronic infections in mice. With this drug a parasitological cure of the treated animals is obtained. To date it has not been used in human patients with Chagas disease.

30.3 Inhibitors of Ergoesterol

Diverse azolic products have been used with success in human and veterinary medicine. These drugs interface in sterol synthesis, and together with other heterocyclic nitrogenated compounds belong to the group of drugs that inhibit ergosterol synthesis. *T. cruzi* has ergosterol; the antimycotic prevents its synthesis without affecting the human host, who has cholesterol. Cholesterol differs from ergosterol by the presence of a 24 methyl group and double bonds in 7A and 22A. The three enzymes that produce the methylation and the double bonds of ergosterol do not have counterparts in mammalian cholesterol synthesis. Several of these azolic products have been studied for Chagas disease treatment: miconazole, econazole, ketoconazole, itraconazole, fluconazole, and posaconazole (Figure 30.2).

With these drugs a parasitological cure has been obtained in mice with acute and chronic Chagas disease (Urbina, 2002). Ketoconazole, itraconazole, and DO 870 inhibit cytochrome P450-dependent lenosterol C14 demethylase, thus reducing ergoesterol synthesis. Although mammals have this enzyme, it is much less sensitive to the drugs than those of fungi or of *T. cruzi*. Itraconazole has been applied in the treatment of chronic indeterminate Chagas disease and CCC. The drug prevents cardiopathy, compared to controls without therapy, and improves 50% of the electrocardiographic alterations of patients with CCC. Twenty percent of these cases are "cured" of parasites, determined by xenodiagnosis, PCR in blood, hybridization probes in blood, PCR in dejections, and hybridization probes in dejections of triatomines applied to these patients. However, none of the "cured" cases presented a negative conventional serology (indirect hemagglutination (HAI), indirect IF, or ELISA) for *T. cruzi* (Apt et al., 2005; Zulantay et al., 2007). When the drug DO 870 was administered to mice with acute infection (10^5 *T. cruzi* Y strain), the treated animals lived longer than the controls without treatment or treated with NF or ketoconazole, 105 days survival versus 21. A 60% cure was obtained by control of parasitemia, hemocultures, and PCR. When this therapy was applied to mice with chronic infection (10^4 *T. cruzi* Bertoldo strain), after 40−50 days 50% of the controls survived and 30% of these had negative PCR, while the parasitological cure was 80−90% in the treated group (Urbina, 2002). Today DO 870 has been discontinued, but posaconazole (SCH 56592), BMS-207, 147 (Ravuconazole), VR-9825,

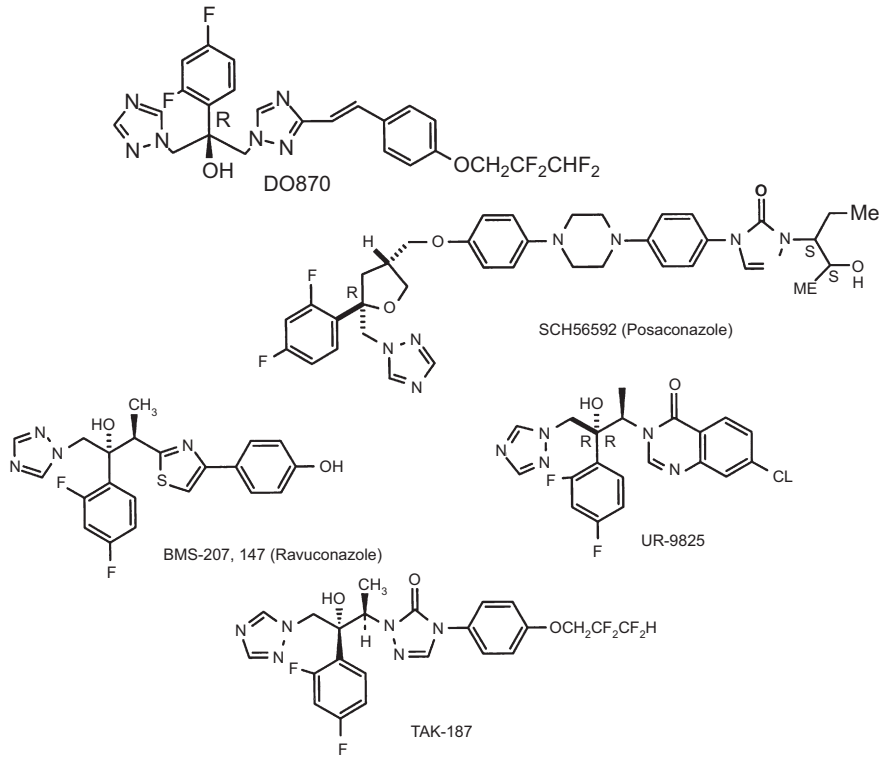


Figure 30.2 Chemical structure of triazole derivatives, inhibitors of *Trypanosoma cruzi* sterol C14α sterol demethylase.

and TAK-187 have demonstrated activity against *T. cruzi in vitro* and *in vivo*. Of these compounds, posaconazole has proven to be efficient, with very good tolerance in studies performed in patients with oropharingeal candidiasis (Skies et al., 2007). It has been observed that the majority of these compounds (Posaconazole, Ravuconazole, VR-9825, and TAK-187) have activity against *T. cruzi* strains partially resistant to NF, benznidazole (BNZ), and in which ketaconazole does not function (Urbina, 2003). Posaconazole with amiodarona, an antiarrhytmic drug, has a synergistic activity against *T. cruzi in vitro* and *in vivo* (Benaim et al., 2006).

30.4 Ofloxacine

Ofloxacine is an inhibitor of topoisomerase II, an essential enzyme for bacteria. This drug blocks the differentiation of amastigotes in tissue cultures. Ultramicroscopy shows morphological alterations of the kinetoplast of *T. cruzi*, suggesting that ofloxacine destroys the parasites. *In vivo* experiments did not confirm the utility of this enzymatic inhibitor.

30.5 Inhibitors of Trypanothione Metabolism

Trypanothione (N^1, N^8-*bis*-(glutatyonil)-spermidine) (Figure 30.3) and trypanothione reductase are unique systems in the kinetoplastid protozoa which replace intracellular glutathion and glutathion reductase, the principal mechanism of the thyol−redux system.

Although trypanothione reductase is an essential enzyme in *Leishmania donovani* and *L. mayor*, the overexpression of the enzyme in *L. donovani* and *L. mayor* does not alter its sensitivity *in vitro* to agents that induce oxidative stress such as NF, nitrofurazone, and gentian violet. Inhibitors of trypanothione metabolism such as buthionine sulfoximine (BS0) are ideal potential candidates as drugs against *T. cruzi* alone, or jointly with free radical−producing drugs such as NF and BNZ (Maya et al., 2006) (Figure 30.4).

30.6 Inhibitors of Cysteine Protease (CPI)

Cruzipain (cruzain, gp 51/57) is a simile of catepsine L-cysteine protease responsible for the proteolytic activity in all the life stages of *T. cruzi*. The genes which code for this protein have been cloned and expressed. A recombinant enzyme has been elaborated and different drugs have been studied that specifically inhibit the CPI protease *in vitro*, blocking the proliferation of epimastigotes and amastigotes and arresting metacyclogenesis. It has been demonstrated that these drugs block the development of cruzipain and its transport by lysosomes. These facts indicate that cruzipain is an ideal target; but to date, although CPI has been applied in murine models with acute and chronic infection, obtaining parasitological cure with minimal toxicity, the short half life of the drug, which requires high and increasing doses, inhibits its use in clinical practice (Cazzulo, 2002).

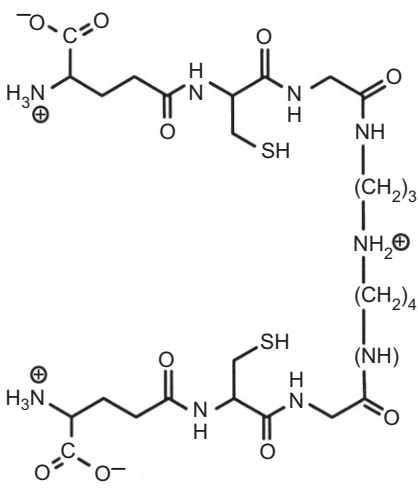


Figure 30.3 Chemical structure of trypanothione (N^1, N^8 *bis* (glutationyl) spermidine).

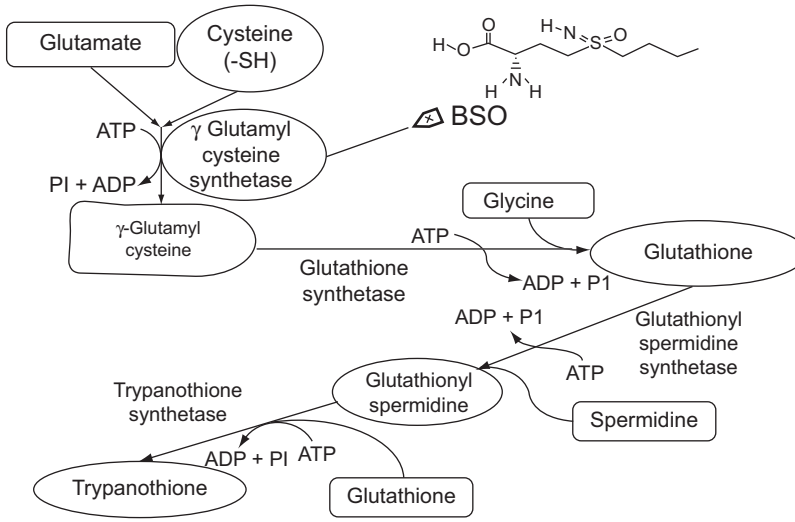


Figure 30.4 Biosynthesis of glutathione and trypanothione in *Trypanosoma cruzi*: glutathione is synthesized by the consecutive action of γ glutamylcysteine synthetase and glutathione synthetase in an ATP dependent reaction. In *T. cruzi*, two molecules of glutathione are conjugated with spermidine to synthetize trypanothione (N^1, N^8 *bis* glutathionyl spermidine, $T(SH)_2$). The host is unable to synthesize $T(SH)_2$.
γ Glutamylcysteine synthetase is the step limiting enzyme in this process and can be inhibited by buthionine sulfoximine (BSO).
Source: Adapted from Maya et al. (2006).

30.7 Inhibitors of Phospholipids

Alkyl-lysophospholipids (ALP) are synthetic analogs of lysophospholipids that have been shown to be effective *in vitro* and *in vivo* on *T. cruzi* and trypanosomatides. Miltefosine, one of its representatives, has been used orally in visceral leishmaniasis with good results. ALP blocks selectively the biosynthesis of phosphatydil-coline (PC) of *T. cruzi* through the transmethylation of the Greenberg pathway, in contrast to the vertebrate host where the Kennedy pathway of CDP choline is predominant (Urbina, 2003).

30.8 Inhibitors of Pyrophosphate Metabolism

The inorganic pyrophosphates ($P_2\ O_7^{4}$; PPi) and other short chain, tri and tetra polyphosphates are those that have the greatest energy of phosphate compounds in trypanosomatides (*T. cruzi*, *T. brucei*, and *L. mexicana*) and apicomplexa parasites (*Toxoplasma gondii*). They have 10−15 times more energy than ATP. PPi is distributed throughout the cell, but is concentrated in the acidocalcisomes, specialized

acid vacuoles with large quantities of Ca^{++}. PPi enzymes of *T. cruzi*, such as a proton-translocating pyrophosphose in acidocalcisomes and pyruvate phosphate dikinase in glycosomes, suggest that PPi has an important role in parasite survival. This has been confirmed by the observation that pamidronate, alendronate, and risedronate, which contain biphosphonates, nonmetabolizable pyrophosphate analogs currently used in human medicine in alterations of bone reabsorption and selectively inhibit the proliferation of intracellular amastigotes and tachyzoites of *T. gondii*. It has been demonstrated that residronate (Ris) acts *in vitro* on epimastigotes and cell cultures of amastigotes of *T. cruzi*, also reducing the infection in mice with acute infection, eliminating almost completely the parasitemias and intracellular amastigote forms. This drug inhibits farnesyl pyrophosphate synthase of the parasite, blocking the biosynthesis of polyisoprenoids (Garzoni et al., 2004). It has not yet been used in humans. One inhibitor of farnesyltransferase, Tipifarnib (R 115777), which inhibits cytochrome P450 sterol demethylase (CYP_{51}), is a potential target against *T. cruzi*, but in spite of its success in experimental animals it has not been applied to humans (Hucke et al., 2005).

The sterol 14 demethylase of *T. cruzi* has been studied (TCCYP51). It is related catalytically to the CYP51 of animal fungi (Lepesheva et al., 2006). Inhibition by obtusifoliol and its analogs reduces enormously enzyme activity. TCCYP51 constitutes a potential target against *T. cruzi*; however, to date no experimental surveys have been performed to establish its efficacy in animals.

30.9 Natural Drugs

A great spectrum of natural products has been used against *T. cruzi*, but very few are useful at a concentration of 10 μg/mL, considering that the IC_{50} for NF and BNZ is less than 3 μg/mL. Some products block the respiratory chain of the parasite, such as boldo (*Peumos boldus*) alkaloids, and naphthoquinone, extracted from *Calceolara sessilis* (Morello et al., 1994). Other alkaloids extracted from Brazilian plants that have isoquinoline have an effect on *T. cruzi* (Tempone et al., 2005). Some natural drugs inhibit the response of *T. cruzi* to oxidative stress by producing superoxide radicals (Maya et al., 2006). The triterpenes of *Arrabidae triplinervia* and their derivatives, such as diterpene, komaroviquinone, and terpenoids isolated from *Pinus oocarpa*, have action on epimastigotes and trypomastigotes of *T. cruzi* (Rubio et al., 2005; Uchiyama et al., 2005; Leite et al., 2006). In the majority of the natural drugs the exact mechanism of action is not known. The great majority of them have effects on epimastigote forms and some on culture amastigotes. Very few have been used in experimental studies in murines, and none have been used in clinical surveys. To date no natural product that acts on *T. cruzi* transialidase has been studied, although this enzyme is an optimal target. Only one natural drug has been used on cysteine protease (CPI), inhibiting cruzipain synthesis; this product is a 164 residue amino acid protein extracted from seeds of *Bahuinia balviniodes*. No experimental investigations have been performed with this product (de Oliveira et al., 2001).

30.10 Other Drugs

Recently it has been demonstrated that MCPs, methylcarboxypeptidases that belong to the M32 peptidase family, are present in the cytosol of *T. cruzi*. Previously it was thought that these enzymes only existed in bacteria and prokaryotes. *T. cruzi* has two MCPs: TcMCP-1 and TcMCP-2. The former is found in all stages of the parasite, while TcMCP-2 only exists in epimastigotes and trypomastigotes. Because this enzyme does not exist in humans, its inhibition could represent an effective therapy against *T. cruzi* (Niemirowicz et al., 2007; Rawlings, 2007).

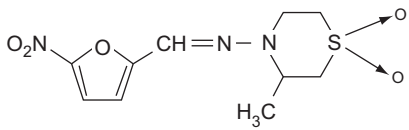
30.11 Treatment of Human Infection

Treatment with NF and BNZ began in the 1970s and is based on an empirical treatment (Prata, 1997; Rassi et al., 1997; Pinto Dias, 2006). NF is 4-([5-nitrofurfurylidene] amino) 3 methylthiomorpholine-1,1-dioxide (Figure 30.5).

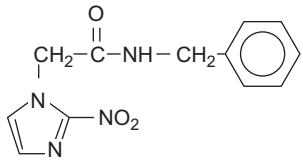
It acts by the production of free radicals, superoxide anions, hydrogen peroxide, and electrophilic metabolites. It has been demonstrated that in addition to the metabolic action of the drug on *T. cruzi*, its incorporation and transport by the parasite is of great importance. There are strains with some resistance to NF that differ due to a lower intake and transportation of the drug, rather than by the amount of free-radical production. *T. cruzi* in the presence of NF increases oxygen consumption, H_2O_2 and superoxide radical production.

The drug BNZ (*N*-benzyl-2 nitroimidazole-1-acetamide) was introduced in human clinical use in 1978. The drug inhibits protein synthesis, originating a degradation of macromolecule biosynthesis. Reduced metabolites of BNZ in covalent unions with macromolecules interact with the DNA of the parasite (Maya et al., 2006). The drug inhibits the respiratory chain. The free-radical production is lower than with NF.

Figure 30.6 describes the mechanisms of action of NF and BNZ. Both drugs produce important collateral effects, especially in adults, because newborns, infants, and small children tolerate the drugs better (Viotti et al., 2009). The secondary effects of NF and BNZ are described in Table 30.2.



Nifurtimox



Benznidazole

Figure 30.5 Chemical structure of nifurtimox and benznidazole.

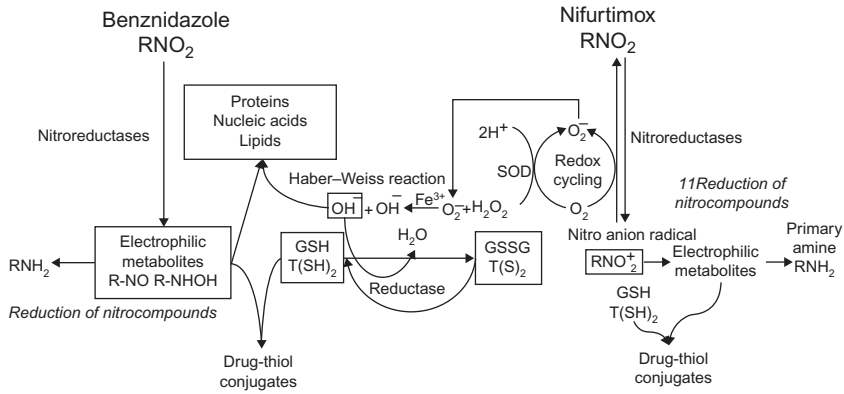


Figure 30.6 Role of glutathione and trypanothione in the action and metabolism of the antichagasic drugs nifurtimox and benznidazole. The nitro group of both antichagasic drugs is reduced to free radicals or electrophilic metabolites by *T. cruzi* cytochrome P450 related nitroreductases. The nifurtimox derived free radicals may undergo redox cycling with oxygen and H_2O_2 is produced by the further action of superoxide dismutase (SOD). The produced oxygen derived free radicals and electrophilic metabolites bind to intracellular macromolecules and damage them. In the parasite, trypanothione ($T(SH)_2$) and glutathione (GSH) neutralize the nifurtimox and benznidazole derived metabolites by conjugation, producing drug thiol conjugates that will be further metabolized to mercapturates in the mammal host. Free radicals are neutralized by oxidation of reduced GSH or $T(SH)_2$. Trypanothione reductase reduces oxidized trypanothione ($T(S)_2$).
Source: Adapted from Maya et al. (2006).

Based on this information, we will discuss the treatment of human Chagas disease in its different periods with the drugs currently in use.

30.11.1 Current Drug Therapy

Chagas disease must always be treated in the acute period, as well as in the initial, middle, and indeterminate chronic periods. The only exceptions from the etiological treatment are for those patients with chronic infections with Core Bovis and terminal cardiac insufficiency. The indication to apply specific therapy in chronic cases is the demonstration of parasites by PCR when they are not detected by optical microscopy. In these cases, therapy is effective. Today it is accepted that a precocious treatment is able to modify the natural evolution of the disease. This is why, due to the number of patients in countries where infection is prevalent, its treatment is a public health problem (Apt et al., 2008).

30.11.2 Acute Cases

Patients with clinical manifestations must receive treatment. This includes those with an infection of less than 4 months, as well as the acute cases with easy detection of

Table 30.2 Adverse Reactions to Nifurtimox and Benznidazole

Digestive alterations
Gastric upset
Nausea
Vomiting
Hematologic alterations (by hypersensitivity)
Leukopenia
Thrombocytopenia
Agranulocytosis
Dermatological alterations
Erythematus, light sensitive rash
Atopic dermatitis (mild or severe)
Occasionally Stevens−Johnson syndrome, which requires the suspension of therapy
Neurological alterations
Polyneuropathy, dose dependent
In general it appears in schedules with high dosages
In the usual dose of 5 mg/kg/day of benznidazole, 10−30% of patients present neuropathies, especially at the end of treatment.

parasites in fresh samples and smears, and those with positive conventional serology: IHA, CF, IF, ELISA, and immunoblotting (IB) with positive IgM. The idea is to treat these cases with NF 8 mg/kg/day for 30−60 days in adults and 10 mg/kg/day for the same period in children. This daily quantity must be divided into three doses taken after meals (every 8 h). In Brazil, where NF is not available, BNZ is used: 5 mg/kg/day for 60 days in adults, and 5−10 mg/kg/day (7.5 mg/kg/day) for 60 days in children, divided into two or three doses (every 8 or 12 h) after meals. In an investigation performed in Santiago del Estero (Argentina) in 470 cases, the majority (84.4%) of children aged 1−9 years with acute Chagas disease who presented an ophthalmic lymph node complex, 367 were treated with 25 mg/kg of NF for 15 days and continuing with 15 mg/kg for another 77 days; 40 received placebo and another 20 other antichagasic drugs. Drug tolerance was greater in small children and the 15-mg dose was better tolerated than the 25 mg. After 60 days of treatment the direct parasitological tests were negative in both groups who received therapy; however, in the placebo group there were 28.6% positive cases. After 18 months there was 69% seroconversion of the treated patients; in other words, they passed from positive to negative. However, the placebo group maintained positive serology (IHA, CF, and IF) (Cerisola, 1969). With BNZ in acute acquired cases, a 76% cure is obtained (Cançado, 2000).

30.11.3 Congenital Infection

Treatment must begin as soon as the diagnosis is performed, when the clinical suspicion is confirmed by observation of the parasite in fresh samples of blood smears, microstraut, etc. Sometimes the diagnosis is confirmed when the child is in the chronic period (8 or more months) by persistent positive serology after this period.

Better therapeutic results are obtained when the diagnosis is more precocious. It is important to perform a clinical, serological, and parasitological follow-up of the treated newborn. Recently the utility of PCR in the precocious diagnosis of congenital Chagas disease in neonates has been confirmed. Its effectiveness is greater than that of xenodiagnosis (Virreira et al., 2003; Sánchez et al., 2005). This technique has great utility as has been demonstrated by Paraguayan investigators and others in the follow-up of treated cases. In an investigation performed in the maternity ward of the Clinical Hospital of Asunción and in the Regional Hospital of San Pedro, Paraguay, the newborns of chagasic mothers were studied. Three percent were positive by microscopy, which increased to 10% when they added the cases with persistent positive serology at 6 months. Of 58 newborns, in two cases *T. cruzi* was observed at birth and four presented positive PCR with negative microscopic investigation. All the positive cases were treated with BNZ and followed for 4 years by conventional serology and PCR.

In another study performed in an endemic area of Paraguay of 1865 neonates with chagasic mothers, of the 104 cases congenital infection was demonstrated by direct microscopic observation, PCR, and serology: ELISA, ELISA AIDS, and IF. PCR was the most sensitive test (Russomando et al., 2005).

All congenital cases must be treated, since up to 98% of such treatments may produce negative serology and parasitemia; the earlier treatment is begun, the better the response obtained. NF must be administered in doses of 8−10 mg/kg/day for 60 days, taken every 8 or 12 h, or BNZ 5−7 mg/kg/day for 60 days. To avoid secondary effects (convulsions), it is recommended to associate phenobarbital in therapeutic doses during the first 15 days of treatment. In case of secondary dermatological reactions, it is suggested to add antihistaminics. Adverse reactions in neonates are fewer than in adults.

It is important to perform a precocious diagnosis of congenital cases to treat them as quickly as possible. In pregnant women, conduct a serological test jointly with other tests such as VDRL during the first trimester of pregnancy and conduct follow-up of the positive cases until the diagnosis of congenital infection is confirmed or discarded. This activity must be performed in all women of fertile age, in pregnant women from endemic areas, and in women with a history of having lived in these zones.

In newborns, run a serological study for *T. cruzi* infection, together with VDRL and other tests. In the positive cases a follow-up must be realized until confirmation or discard the diagnosis.

30.11.4 Accidental Chagas Disease

All accidental cases must be treated with the same drugs as the acute infections acquired from the vector, for 15 days. In this group the transfusion by error from a chagasic donor must be considered. In persons who work in laboratories and have a puncture accident with contaminated samples with infective *T. cruzi* forms, the confirmation of the contamination of the object with the parasite and the posterior infection of the patient (serology and PCR) must be performed. If there are positive

results, immediate treatment must be undertaken with BNZ 7−10 mg/kg/day for 15 days, depending on the immunological state of the person. A serological study must be done at 15−30 and 60 days.

30.11.5 Organ Transplants

A transplant where the donor or recipient has Chagas disease must always be treated with NF 8 mg/kg/day in adults and 10 mg/kg/day in children for 60 days, or BNZ 5 mg/kg/day in adults and 5−8 mg/kg/day in children for 60 days. In bone marrow transplants with *T. cruzi* infections (which receive it in 40% of the cases) the treatment must be maintained for 2 years and in solid organ receptors treatment must be given for the period in that immunosuppressors with insufficient CD4 lymphocytes for an adequate immune response are used. In these patients the most commonly prescribed drugs are the traditional NF and BNZ. Patients exposed by organ transplant might face primoinfections and reactivations; thus it is important to perform a good screening of donor and recipient before the transplant. The clinical manifestations of reactivation usually differ from those of the acute phase (primoinfection); for this reason the monitoring of patients after the transplant is relevant. In both situations, the receptor who receives an organ from a chagasic donor or a chagasic reactivated receptor, therapy must be initiated with NF or BNZ.

30.11.6 Reactivations of Chronic Chagas Disease and Treatment of Chagas Disease in Immunosuppressed Patients

Patients with chronic Chagas disease who acquire AIDS or in whom immunosuppressor therapy is administered must receive treatment at the same dose as the group mentioned above for 5 or more months. In these cases the most suitable strategy is prevention, performing serology for Chagas disease in all AIDS patients. In the primoinfection by *T. cruzi* in AIDS patients, the same treatment schedule must be prescribed as in reactivations. The same classic antiparasitic drugs are used in the standard doses until the immune response of the host is reconstituted (in some cases 60 or more days). Once the alteration of the immune system is normalized, including the relation CD4/CD8, the antiparasitic schedule is changed to every 3 days, balancing the parasiticide effects with the adverse effects. The patients with AIDS without retroviral treatment are the most severely affected. In these patients, once the CD4 levels are normalized with specific antiviral treatment, maintenance schedules may be used.

30.12 Evaluation and Follow-Up of Specific Therapy

The principal objection to the treatment of Chagas disease is its long duration. The treatment must be maintained at least for 60 days and the cure criteria depend on several factors (WHO, 2002; Viotti et al., 2006). Some authors consider serological conversion as necessary, but this sometimes happens 20 years or more after the end

of treatment of chronic Chagas disease, and there are cases in which former patients die without seroconversion. Recently it has been published that in treated cured mice experimentally infected with *T. cruzi* in which no parasites and no *T. cruzi* antigens could be demonstrated, CD8 central memory cells maintain a positive serology for more than a year (Bustamante et al., 2008). We do not know if this process occurs in humans, but if it does, we can explain why cures in chronic cases could be reached without seroconversion.

Others consider the following parameters as cure criteria of the chronic period: the conversion of the xenodiagnosis from positive to negative; conversion of qualitative PCR from positive to negative, and in cardiopaths, the elimination of electrocardiographic alterations. These changes must always be permanent and must persist for 12 or more years independent of the conventional serological results. There must be at least two parasitological and one or more clinical parameters to confirm the cure (Apt et al., 2005). In the acute indeterminate and determinate chronic periods a follow-up must be performed with hemocultures, quantitative PCR for *T. cruzi* (Duffy et al., 2009), hemogram, biochemical profile, and/or xenodiagnosis. It must keep in mind that serological tests in severe immunosuppressed patients usually have negative results and for this reason do not serve to follow up the treatment. A prolonged persistent negativity of PCR with these characteristics for *T. cruzi* is considered as cure criterion. Some authors give value to the disappearance of lytic antibodies as a complement to improve the criterion.

30.13 Resistance of *T. cruzi* to Drugs

In vitro and *in vivo* it has been demonstrated that certain *T. cruzi* strains are resistant to NF and BNZ. This is valid especially for *T. cruzi* clones isolated from sylvatic animals or vectors. It is important to emphasize that we do not know if what we observe in murine models happens in humans. Furthermore, in many publications the strains of *T. cruzi* have not been well characterized by isozymes (zymodemes), nuclear restriction enzymes (schizodemes), genetic composition, etc. (Solari et al., 1992). No consensus exists on the relation between the sensitive or resistant strains and virulence. Andrade et al. (1985) claimed that there is a relationship and the resistant strains are more virulent, while Filardi and Brener (1987) did not find this association. In relation to the genetic composition of *T. cruzi* and resistance to drugs, it has been demonstrated *in vitro* that a relation exists between genetic distances and biological differences, among the latter the resistance to NF and BNZ. This is true for epimastigotes and amastigotes. Trypomastigotes are the exception since in them a relation between genetic distances and sensitivity to drugs does not exist. It is necessary to make better genotypic studies of *T. cruzi* in relation to resistance to drugs. Recently it has been demonstrated that patients with chronic Chagas disease treated with itraconazole or allopurinol who did not respond to the specific therapy (there is no parasitological cure) were infected with the TCI lineage of *T. cruzi*, while those that responded to the therapy with

itraconazole had the lineage TCIIb, suggesting that TCI is resistant to these drugs and TCIIb is sensitive (Coronado et al., 2006).

30.14 Critical Comments

At present there is no effective therapy for the majority of the patients who have chronic Chagas disease in the indeterminate (70–80%) and determinate (10–20%) periods. In the acquired acute period, 70% of the cases are cured, and in newborn and suckling children with congenital Chagas disease a 98% cure is obtained. In the chronic acquired cases cures reach 20% and improvement of cardiopathy 50% (with itraconazole). New drugs are needed with high efficacy and without secondary effects, especially to treat chronic cases. In the last few years no new drugs have been developed against *T. cruzi* that can be applied to humans. This is due to the lack of interest of the big pharmaceutical companies to develop new drugs with low returns. The people with Chagas disease have low economic resources. Chagas disease is one of the "neglected diseases" and for this reason without interest. What will happen when the current supply of NF is exhausted? Bayer is not interested in continuing its production. Since ROCHE did not want to continue production of BNZ, it assigned the process to a Brazilian laboratory, which to date has not been able to produce the amount that Brazil needs. How will chronic chagasic patients be treated if no new drugs are produced? They may have to be treated only with itraconazole until a better drug is found.

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31 Vaccine Development for Chagas Disease

Angel Marcelo Padilla[1,2], *Cecilia Perez Brandan*[1] *and Miguel Angel Basombrío*[1,*]

[1]Instituto de Patología Experimental, Universidad Nacional de Salta, Salta, Argentina, [2]Center for Tropical and Emerging Global Diseases, University of Georgia, Georgia, USA

31.1 Introduction

Vaccines have had an indisputable impact on the control of many important human and veterinary diseases and unquestionably have shaped the health landscape of recent generations. In spite of the crucial benefits obtained with vaccines for diseases like smallpox and poliomyelitis, many tropical diseases, commonly referred to as "neglected diseases," suffer from the lack of an effective vaccine. Chagas disease is one of these neglected diseases, and it is a major health problem in Latin American countries, especially the poorest ones. The advantages of a Chagas disease vaccine would be significant not just in terms of public health but also economic and social development (Hotez and Ferris, 2006). Although some important advances on the comprehension of human immune response to *Trypanosoma cruzi* infection have occurred, most of our knowledge about immune mechanisms and protective response comes from experimental animal models.

31.2 Immune Mechanisms Associated with Protection Against *T. cruzi* Infection

In recent years, increasing knowledge about the immune response associated with Chagas disease has been invaluable for the design and testing of vaccination approaches, although fundamental questions still remain. We need to better define the immune response against *T. cruzi* to improve the understanding of the protective mechanisms involved, in order to strengthen them as well as to identify the weaknesses in the response that allow parasite persistence in the chronic infection.

* E mail: basombri@unsa.edu.ar

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T. cruzi infection is naturally initiated by the invasion of the parasite through mucosal or skin lesions contaminated with the parasite-containing feces deposited by the insect vector. Once the parasite has crossed the skin barrier, it encounters the host tissue cells at the site of entry, and the normal immune cells that populate in that tissue or are recruited at the lesion. Neutrophils are rapidly summoned to the site of *Leishmania* infection, where they phagocytize parasites and may be involved in facilitating the spreading of Leishmania infection (Ritter et al., 2009). In *T. cruzi* infection, it is possible that the first immune cells to be recruited at the site of entry are also neutrophils, but probably the majority of the parasites directly infect tissue cells rather than recruited immune cells. This notion is supported by the poor parasite migration to surrounding tissues or draining lymph nodes and the evidence of parasite proliferation at the site of infection (Padilla et al., 2009b). Therefore, infecting trypomastigotes probably invade host tissue cells (e.g., fibroblasts) at the site of infection, transform to cytoplasmic amastigote forms and proliferate, with very scarce parasites spread to adjacent tissues or draining lymph nodes (Giddings et al., 2006). It is not well established if the few parasites that gain access to the draining lymph nodes right after infection make their way by themselves or are passively transported by immune cells, like dendritic cells that migrate to the lymph nodes after acquiring antigen in the periphery. Ultimately, after several rounds of replication inside tissue cells, parasites would be released to gain access to other distant organs through the bloodstream. Parasites that arrive at the draining lymph node right after the initial infection do not seem to be effectively presented to trigger an adaptive immune response, which is developed only after the first week postinfection, coincident with the release of parasites that replicate at the site of infection (Tzelepis et al., 2007; Padilla et al., 2009b).

Several factors seem to be involved in this delay in the onset of the adaptive immune response during the first days postinfection, including the parasite number and the activation signals provided to the antigen-presenting cells that will initiate the adaptive response. This early period seems to be a rather immunologically "silent" one with very few immune mechanisms that reveal the infectious process. A vaccination approach that shortens this response time could have a favorable impact controlling the first parasite proliferation at the site of infection. However, if during this "silent" period parasites are not "visible" to the immune system due to an insufficient activation of the antigen-presenting cells that display the relevant antigens, the presence of already-generated memory cells from a previous vaccine may not drastically modify this initial response time. Some evidence suggests that reinfecting parasites may be rapidly controlled by a fully activated immune response maintained by an ongoing infection (premunition), but this parasite control at the site of infection may be delayed when the previous infection has resolved, leaving resting memory cells that need to be reactivated by new antigen presentation. It has been suggested that the delay in the generation of the adaptive response would allow the parasites to reach other tissues like muscle or adipose tissue where immunological or metabolic factors could allow them to chronically persist (Leavey and Tarleton, 2003; Combs et al., 2005). However, the effects on the chronic parasite load of an earlier generated adaptive response have not been fully

investigated. Based on the hypothesis that an insufficient activation of the antigen-presenting cells during *T. cruzi* infection could influence the speed of the origination of the immune response, vaccines based on live-attenuated parasites should incorporate additional immune activating molecules to those originally provided by the parasites. So far, some *T. cruzi* molecules have been shown to activate Toll-like receptors (TLR), TLR2 and TLR9 (Bafica et al., 2006), although these TLR ligands, GPI anchors, and DNA may not be freely available in live parasites initiating the infection, which would reduce their activating effect on antigen-presenting cells (Tarleton, 2007).

Once generated, the adaptive immune response is highly efficient in controlling the parasite level, even though this strong response does not totally remove the parasites. This adaptive immune response is mainly characterized by the presence of specific antibodies, some of which have the capacity to lyse trypomastigote forms and are generically called lytic antibodies (Krautz et al., 2000). The cellular branch of this adaptive immune response is characterized by $CD4^+$ and $CD8^+$ T cells, both of them crucial for parasite control (Kumar and Tarleton, 2001; Padilla et al., 2009a). Our knowledge about the specific $CD8^+$ T-cell response has recently increased due to the identification of specific parasite epitopes recognized by these immune cells and the application of new immunological techniques (Martin et al., 2006; Tzelepis et al., 2006). Among these specific epitopes, the TSKB20 peptide (ANYKFTLV) present in some proteins of the trans-sialidase (TS) superfamily has been successfully used to follow the kinetics of the $CD8^+$ T-cell response by staining with MHC class I complexes containing this peptide. The $CD8^+$ T-cell response against TSKB20 is one of the highest responses described so far, involving approximately 20−30% of the total $CD8^+$ T-cell population at its peak (Martin et al., 2006). After the contraction, these $CD8^+$ T cells persist in low levels with characteristics of a predominantly effector memory population (Martin and Tarleton, 2005) and a smaller subset of cells displaying central memory markers (Bixby and Tarleton, 2008). Despite the strong humoral and cellular response mounted against *T. cruzi* in the acute phase, parasites manage to avoid clearance and persist chronically. The reasons for the incapability of the immune system to completely eliminate parasites are not fully understood and represent an important aspect to be covered to develop an effective vaccine.

In spite of the strong $CD8^+$ T-cell response elicited against *T. cruzi* and the importance of this lymphocyte population for host survival, the precise mechanisms by which these cells control the infection are not completely understood. Depletion or lack of $CD8^+$ T cells leads to high susceptibility and mortality of infected mice (Tarleton et al., 1992), and cytotoxic $CD8^+$ T cells from infected animals have been shown to be able to identify and destroy cells loaded with parasite peptides or parasite-infected ones (Low et al., 1998). However, the importance of these cells to recognize and destroy parasite-infected cells *in vivo*, especially cells known to be chronically infected like muscle fibers or adipocytes, has not been clearly demonstrated. It is also uncertain if cytotoxic lysis of infected cells is an effective mechanism to control parasite load *in vivo*. Even more, experiments with perforin-deficient mice yielded contradictory results in terms of susceptibility to

the infection (Kumar and Tarleton, 1998; Tzelepis et al., 2006). Therefore, the development of a vaccine that stimulates a strong $CD8^+$ T-cell response against intracellular parasites is desirable, even when the mechanistic bases of the protection conferred by those cells is still not fully defined.

Other important effector function exerted by $CD8^+$ T cells is interferon gamma (IFN-γ) production. This cytokine is also produced by $CD4^+$ T cells and NK cells, among others. IFN-γ has been shown to have a crucial role in directing the development of naïve $CD4^+$ T cells toward a Th-1 phenotype as well as in activating macrophages. IFN-γ is considered a key cytokine involved in the control of *T. cruzi* because mice deficient in this cytokine are highly susceptible to the infection and succumb in the acute phase. Therefore, it is a generally accepted notion that a vaccine against *T. cruzi* should induce a response with a Th-1 cytokine profile (Hoft and Eickhoff, 2005). Regardless of its demonstrated involvement in the resistance to infection and its activation effect on macrophages, there is no clear mechanism linking IFN-γ production and parasite control *in vivo*.

Although there is some contradiction regarding the importance of the $CD4^+$ T cells in the development of the $CD8^+$ T-cell response during *T. cruzi* infection (Padilla et al., 2007; Tzelepis et al., 2007), their role in the control of the parasite is clearly demonstrated by the high susceptibility of mice defective in $CD4^+$ T cells, which do not survive the acute phase of the infection (Tarleton et al., 1996). The $CD4^+$ lymphocyte subset's role in the maturation of the antibody-producing B lymphocytes and orchestrating the cytokine profile makes them a fundamental branch of the immune response. However, little is known about either their priming characteristics during *T. cruzi* infection or their other antiparasitic features that would be advantageous for a vaccination protocol.

As mentioned earlier, a strong immune response is mounted against *T. cruzi* in the acute phase, yet parasites survive in the form of a latent, chronic infection. How do parasites avoid complete clearance by the immune system? How can we boost or modify this response by prophylactic vaccination to block the progression of the infection and prevent the persistence of the parasites?

One of the mechanisms suggested to participate in the chronic persistence of parasites is the apparent dysfunction of the $CD8^+$ T cells infiltrating the infected muscle tissue. As demonstrated by Leavey and Tarleton (2003), these cells have a lower capacity to produce IFN-γ after restimulation *in vitro* than their counterparts isolated from spleen. However, this dysfunction does not seem to be induced by T regulatory cells or TGF-β (Kotner and Tarleton, 2007; Martin et al., 2007). Although chronic infecting parasites cannot be efficiently removed by the immune system, they can successfully be eliminated by the administration of the trypanomicidal drug benznidazole (Bustamante et al., 2008). After clearance of the parasites, the $CD8^+$ T-cell populations specific against parasite epitopes change their phenotype from effector to central memory cells.

This change in the phenotype agrees with the current opinion that pathogen persistence continuously stimulates the cells, turning them antigen addictive, and preventing the development of a central memory population. Therefore, the expression of central memory markers (e.g., CD62L and CD127) in the specific $CD8^+$ T-cell

population has been proposed as an indirect indicator of the efficacy of the drug treatment in this mouse model of *T. cruzi* infection. Unfortunately, the cure by drug treatment does not provide sterile immunity; cured mice displaying specific central memory $CD8^+$ T cells are able to better control a subsequent infection but are unable to completely eliminate the totality of the reinfecting parasites. This poses a considerable challenge for the development of a vaccine against *T. cruzi* infection that would provide sterile immunity; such a vaccine should stimulate and maintain protective mechanisms that are not obtained after the cure of a natural infection. In this regard, the immune response elicited and maintained by a current infection is usually strong enough to provide protection against a second infection. Therefore, an ideal vaccine should elicit and keep a response as strong as the one produced by a virulent infection but lack the pathogenic effects produced by persistent parasites. So far, some experimental vaccines with attenuated live parasites have been shown to provide strong protection against a subsequent reinfection with more virulent parasites, although the parasites from the secondary infection are rarely completely cleared. Probably the maintenance of the protective effect is associated with the persistence of the vaccinating parasites. Moreover, commonly the strength of the immune response originated by the attenuated live parasites used as a vaccine is lower than a response induced by fully virulent parasites.

Here is another paradox of the immune response in *T. cruzi* infection: How is it maintained in the chronic phase so strongly as to provide protection against a reinfection but too inefficiently to clear the first infection? How can we boost or redirect the chronic immune response to recognize and eliminate the persistent parasites? Can we design a therapeutic vaccine to modify the already-established immune response?

These specific responses persist during the chronic phase, and the $CD8^+$ T cells specific against TSKB20 do not seem to suffer exhaustion a common phenomenon seen in other chronic infections that results in the loss of the effector functions of the cells and their final deletion (Shin and Wherry, 2007). This characteristic of the chronic $CD8^+$ T-cell response in *T. cruzi* opens the possibility for a therapeutic vaccine that could boost the immune response to achieve the complete clearance of the chronically infecting parasites. However, choosing the right antigens to restimulate the response and turn the chronic parasites "targetable" (and modify the already-established immunodominance hierarchy) could be a complicated task.

31.3 DNA Vaccination in Experimental Models of *T. cruzi* Infection

In the past two decades, the development of recombinant techniques allowed the production of different immunogens ranging from recombinant proteins to DNA and adenovirus vaccines for experimental *T. cruzi* infection (Garg and Bhatia, 2005; Cazorla et al., 2009). Recombinant proteins allowed testing several well-defined antigens, but the main immune feature induced by these antigens is the

production of specific antibodies. Unfortunately, antibodies are not as effective in controlling *T. cruzi* infection as they are in other infections. Parasites can persist as amastigotes inside host cells, avoiding direct contact with antibodies. Even after release from infected cells, parasites can survive and be readily detected in the bloodstream of chronically infected animals and patients despite the high level of specific antibodies circulating. In this context, a cellular response able to detect and eliminate infected cells seems more suitable for *T. cruzi* control. However, vaccination with native or recombinant proteins generally elicits a considerable antibody response but a limited cellular immunity. This is mainly due to the limited efficacy of exogenous antigens to be directed to the MHC class I pathway that activates cytotoxic T lymphocytes. Therefore, in recent years, vaccines that induce a cellular response as DNA vaccines have become more important against *T. cruzi* infection.

During DNA vaccination, a eukaryotic expression plasmid containing the gene of interest is delivered either by intramuscular injection or subcutaneously using a gene gun. This plasmid is incorporated by antigen-presenting cells that produce the protein codified in the gene and direct it to the MHC class I pathway for antigen presentation. Besides developing a strong cellular response, DNA vaccines have the potential for easy manufacturing and broad administration as well as noncold-chain requirements, key features for vaccines intended for the poor countries where tropical diseases are endemic. Also, the cytokine profile of the immune response generated by DNA vaccination can be directed toward a strong cellular Th-1 response, which is considered to be protective against *T. cruzi* infection, by adding in the vaccine formulation genes codifying for costimulatory proteins like cytokine IL12 (Katae et al., 2002; Miyahira et al., 2003).

Although sterile immunity after challenge has not been reported in DNA vaccine experiments, vaccination protocols have been successful at decreasing parasitemia, tissue damage, and mortality in mouse models immunized with different *T. cruzi* genes (Rodrigues et al., 2009). Primarily $CD8^+$ and $CD4^+$ T cells have been described as the effector mechanisms responsible for protective immunity in mice vaccinated with *T. cruzi* genes, since the depletion of these cell subpopulations resulted in the abrogation of the protection (Katae et al., 2002; Vasconcelos et al., 2004). The genes tested in DNA vaccines belong mainly to the large TS superfamily of surface proteins expressed in amastigotes or trypomastigotes, with a few examples of other unrelated genes. Immunization experiments carried out by different laboratories demonstrated the efficacy of DNA vaccines containing genes encoding amastigote surface proteins and TS proteins to confer protection against *T. cruzi* infection (Garg and Tarleton, 2002; Fralish and Tarleton, 2003; Vasconcelos et al., 2004). However, broad vaccination approaches based on antigens belonging to superfamilies with several different genes displaying high variability among parasite strains could be difficult due to the elevated chances of mutation on the antigen structure that could render the immune response against them ineffective (Haolla et al., 2009).

Several *T. cruzi* genes have been tested and shown to provide protection against the infection, opening the possibility of a multivalent vaccine composed of different parasite genes and costimulatory molecules (Bryan and Norris, 2010).

Heterologous priming and boosting protocols combining DNA vaccines with recombinant proteins or viral vectors have been successfully explored. Usually the immunogenic and protective characteristics of the combined protocols were improved over the single individual methodologies. In this sense, the recombinant proteins reinforced the $CD4^+$ and B cell activation of DNA vaccines while the boost with viral vectors strengthened the $CD8^+$ response in a more efficient way than a homologous DNA vaccine boost (de Alencar et al., 2009). This last combined immunization approach is highly interesting due to the induction of $CD4^+$ and $CD8^+$ T cell−mediated protection, which was demonstrated to be dependent on IFN-γ and perforin.

DNA vaccines have been used not only as preventive vaccines before the challenge infection but also as therapeutic vaccines administered to chronically infected animals. These experiments demonstrate the capacity of DNA vaccines to protect against a posterior virulent challenge and also to reduce the immunopathology associated with an existing infection (Dumonteil et al., 2004). Zapata Estrella et al. (2006) were able to dramatically reduce the parasitemia and the cardiac tissue damage as well as to rapidly increase the number of $CD4^+$ and $CD8^+$ T cells of mice injected in the acute phase with a DNA vaccine encoding two different *T. cruzi* antigens. Even though DNA vaccines with therapeutic properties are promising, not all the antigens used are equally efficient (Sanchez-Burgos et al., 2007). Recently, the therapeutic effect of DNA vaccines has been evaluated in experimentally infected dogs (Quijano-Hernandez et al., 2008). This study suggests that therapeutic DNA vaccines may be a promising novel therapy for Chagas disease in dogs, which are the principal domestic reservoirs for *T. cruzi* in endemic areas. Further experiments need to be done to elucidate the mechanisms involved in the reduction of pathology obtained with this therapeutic approach.

Many of the genes tested in DNA vaccines have been chosen for being present in the infective amastigote or trypomastigote forms (Silveira et al., 2008). The currently accepted opinion is that the immune response should be directed against the parasite stages present in the mammalian host, rather than against epimastigote extracts as used earlier, containing proteins expressed in the insect vector that could not be relevant for protection. Current availability of the *T. cruzi* genome, transcriptome, and proteome (Atwood et al., 2005; El-Sayed et al., 2005; Minning et al., 2009) allows for screening for genes in the entire genome and for choosing specific genes that codify for proteins with peptides that are predicted to bind specific MHC molecules or those identified as specific for a parasite stage. This detailed information about possible vaccine candidates and new technologies opens an unprecedented opportunity for the rational development of new vaccination strategies (Dumonteil, 2009).

One important advantage over other vaccination approaches used in experimental *T. cruzi* infection is the safety of DNA vaccines. They do not integrate into the genome as viral vector vaccines do, and they have been demonstrated to be safe in clinical trials for other diseases (Ledgerwood and Graham, 2009). Also, unlike the attenuated or genetically modified live parasites, they are not autoreplicative organisms that could revert to a virulent phenotype or trigger the pathology associated

with Chagas disease. Considering these aspects, DNA vaccines may be the most promising approach for a vaccine against *T. cruzi* infection applicable to humans. However, the protection level obtained with these vaccines is still very limited, and more research is needed in order to improve their efficacy.

A broader and stronger immune response than the one obtained with DNA vaccines can be achieved by immunization with live attenuated or genetically modified parasites. Nevertheless, the inoculation of live parasites in humans entails obvious ethical and practical problems, making human application very unlikely. Still, vaccination with genetically modified parasites could be eventually implemented for domestic animals involved in the transmission cycle of *T. cruzi*. The rationale for this intervention is to target domestic animals that are reservoirs of the parasite and can disseminate infection by feeding on insect vectors. Dogs have been pointed out as the best candidates to receive a vaccine intended to block the transmission to humans, mainly for their preponderant role as sources of parasites in the domestic environment.

31.4 Basic Laboratory Studies on Premunition Against *T. cruzi*

Pizzi and Prager (1952) reported that mice that had been inoculated with a *T. cruzi* epimastigote culture presenting low virulence became resistant to reinoculations of virulent parasites. From 1965 to 1990, Menezes published a series of papers based on the vaccination of mice against virulent *T. cruzi* infection by means of preinoculations with an avirulent strain (PF) derived from the virulent Y strain (Menezes, 1968a,b, 1969b). Animals inoculated with cultures in which epimastigote forms predominated became resistant and survived lethal *T. cruzi* inoculations (Menezes, 1972). The experiments were replicated in dogs, and levels of parasitemia and mortality rates were significantly reduced. Moreover, electrocardiographic determinations and histopathological studies of the myocardium showed a clear prevention of functional and anatomic lesions of the heart (Menezes, 1969a). Dr. Menezes' preclinical and phase I clinical studies were completed with inoculations into *Callithrix* monkeys and humans, including himself (Menezes, 1990). Most inoculations used by Menezes for experimental vaccination contained mostly epimastigotes, with a low proportion of trypomastigotes. The term "avirulent" was challenged by Chiari (1974), who demonstrated infections by means of hemoculture in mice inoculated with doses as low as 5000 metacyclic trypomastigotes from PF cultures, although direct blood examination was negative. Since Menezes himself had acknowledged exceptional cases of demonstrated infection in PF strain-inoculated animals, the term "attenuated" seemed more adequate to describe *T. cruzi* strains of very low infectivity.

Some other attenuated *T. cruzi* strains with protective activity have been described. Cultures of the "Corpus Christi" strain were unable to establish apparent infections when 10^7 live culture forms were inoculated into C3H(He) mice. Mice preinoculated with this strain developed resistance against further infection with

the virulent Brazil strain. Moreover, this resistance could also be obtained in naïve mice by transfer of spleen cells from immunized mice to nonimmunized receptor ones. Depletion of the B-cell population but not of the T-cell components abrogated the adoptive transfer of resistance (Rowland and Ritter, 1984).

The group of Gattas made a series of presentations to the Brazilian Meetings of Basic Research in Chagas Disease, reporting the apparent lack of infectivity of a *T. cruzi* clone derived from the CL strain of *T. cruzi* and named CL14. Inoculations of 2×10^6 metacyclic forms into highly susceptible, newborn BALB mice produced no detectable parasitemias and protected against virulent challenge in a time and dose-dependent manner, as shown by blood parasite counts and detailed histological observations (Lima et al., 1986). Further studies with more sensitive methods, such as hemoculture and xenodiagnosis, also failed to reveal infection with metacyclic forms of this clone, whether they were derived from vectors or from axenic cultures (Lima et al., 1992). The specific antibody levels were much lower in mice inoculated with clone CL14 than in those inoculated with the parental strain CL. Furthermore CL14-infected mice did not produce the polyclonal response characteristic of *T. cruzi* infection (Pyrrho et al., 1994).

Some partial insights into the mechanisms of attenuation of the CL14 clone were obtained by Yoshida et al. (Atayde et al., 2004). They carried out a series of comparisons between the attenuated CL14 and the "wild-type" CL strain dominant surface glycoproteins. The ability of this attenuated clone to infect HeLa cells *in vitro* was reduced fourfold, and no intracellular replication was observed. The expression of GP82, a dominant surface glycoprotein known to play an important role in infectivity, was shown to be close to 10-fold lower, as measured by fluorescent antibody-labeled parasites analyzed by flow cytometry. The genomic organization of the *GP82* gene family, as shown by hybridization patterns of *GP82*-labeled probes, did not show remarkable differences in the attenuated clone when compared with the original CL parental strain. However, chromosomal mapping of the gene family, which displayed a wide distribution across several pulse-field electrophoretically separated chromosomes, revealed some distinct patterns in CL14 as compared with CL.

Other metacyclic trypomastigote surface molecules known to interact with host cells, such as cruzipain (GP57-51), TS, and the mucin-like GP35-50, were examined in the CL14 clone. The expression of cruzipain was higher, a finding consistent with determinations by Paiva et al. (1998) for clone CL14. However, in three other attenuated strains (TCC, Tul 0, and Y-null), cruzipain gelatinolytic activity was considerably lower than that detected in three virulent strains (Duschak et al., 2001). Regarding TS, its activity in CL14 was 1.6-fold higher than in the CL wild-type strain (Atayde et al., 2004), confirming previous studies by Gattas et al. (1994). Similarly, a remarkable increase in TS activity was detected in the TCC-attenuated strain. The relative increase, in strains of low infectivity, of proteins known to play a role in infection may seem, in principle, paradoxical. However, the existence of multiple isoforms of these proteins, many of which may be inactive or irrelevant for infection, may explain their overexpression or rebound production in parasites of low infectivity.

A long-term culture, named TCC (*Trypanosoma Cruzi* de Cultivo) was shown to be unable to persistently infect mice or rabbits (Basombrío et al., 1982). It was cloned twice, in 1980 and 1990, and characterized after 2000 as belonging to *T. cruzi* I lineage. The attenuated phenotype was stable, since no virulent sublines could be derived by either mouse culture or insect vector passage, tested repeatedly until lines were extinguished. Since strains of very low virulence but unexpectedly displaying high pathogenicity have been described (Romaña and Borel, 1989), this possibility was examined for the TCC strain. Mice inoculated with cultures were subjected to long-term histopathological studies. This strain was unable to trigger immunopathological responses or to induce histopathological alterations in immunocompetent mice (Basombrío et al., 1982). Experiments involving laboratory conditions to enforce infection (high trypomastigote inocula into newborn or immunosuppressed animals) allowed the demonstration of infections with extremely low density; however, attempts to propagate these infections indefinitely from mouse to mouse always failed.

Leguizamon et al. (1993) examined the process of attenuation by prolonged passage in culture in an originally virulent *T. cruzi* strain named RA. They demonstrated a correlation between the attenuation of infectivity and the time the parasites were propagated in axenic culture (up to 2 years). Even though the original line was highly virulent, after 2 years in culture, inocula of 10^7 epimastigotes were unable to produce detectable parasitemia in immunocompetent, susceptible BALB mice. However, reactivation of virulence was shown by serial passage in athymic mice. Subsequently, similar serial passages were attempted with the attenuated TCC strain. Inocula of 10^5 TCC trypomastigotes into *nu/nu* athymic mice rarely caused detectable blood parasitemia, although parasite tissue nests were found more often in urinary bladder and heart atria. Homogenates of these tissues, where the presence of parasites was verified, were thus used routinely for serial passage and weakly infective TCC sublines could be recovered, but not serially propagated in immunodeficient mice.

Serological studies in mice (Basombrío and Arredes, 1987; Basombrío et al., 2002a) and dogs (Basombrío et al., 1993) indicated antibody titer regression to negative values in most animals, although some mice remained seropositive after 1 year. Inoculation of live TCC epimastigotes afforded protection, not only against acute infection but also against late development of chronic pathology induced either by the Tulahuen laboratory strain or by 17 wild isolates obtained in a broad endemic area of Argentina (Basombrío et al., 1986). Electrocardiographic studies in mice showed that TCC preinoculations before virulent infection significantly reduced functional alterations of the heart, such as sinus bradycardia, supraventricular tachycardia, and atrioventricular blocks (Cuneo et al., 1989).

31.5 Field Studies on Premunition in Guinea Pigs and Dogs

In contrast with the short-term resistance induced by nonreplicating immunogens, the TCC culture induced long-lasting resistance over a year after inoculation

(Basombrío et al., 1987). TCC-induced protection was not restricted to laboratory mice and was also demonstrated in an ecologic field model of *T. cruzi* transmission consisting of standard corrals of loose bricks surrounded by mosquito net tents, where guinea pigs and *Triatoma infestans* shared an entomologically isolated, semi-natural environment. Experiments involving large numbers of guinea pigs showed that TCC parasites did not propagate in a natural vector−host cycle. Moreover, TCC inoculations protected these domestic reservoir animals against naturally transmitted infection and the trafficking of parasites from hosts to vectors was significantly reduced, acting as a transmission-blocking vaccine (Basombrío et al., 1987, 1997).

Measurement of the efficiency of insect vectors to transmit *T. cruzi* infection to mammalian hosts is cumbersome since such variables as parasite load and frequency of bites, and also behavioral and physiological factors, are involved. Nevertheless, the systematic measurement of several transmission variables in guinea pig yards, such as number of hosts, number of vectors, days of exposure, proportion of fed vectors, and proportion of infected vectors allowed an estimate the "number of bites necessary for infection" (NBNI) (Basombrío et al., 1996). This estimation showed that vaccination with TCC parasites produced an average 4.28-fold increase in NBNI in several independent experiments.

Experiments with naturally infected dogs (Basombrío et al., 1993) also indicated a transmission-blocking effect of the TCC live-attenuated vaccine. The population of TCC-immunized dogs displayed a lower proportion of infected animals after 1 year of exposure to natural infection in an endemic area, as shown by serologic−xenodiagnostic parameters. A different parameter, the average percentage of bugs that became infected after feeding on vaccinated or control dogs exposed for 2 years to natural infection, also showed lower values in vaccinated dogs. Vaccination apparently exerted a transmission-blocking effect: lower parasite load and reduced capacity to disseminate the parasite through vectors. A similar effect of vaccination has been confirmed by Basso et al. (2007) in dogs immunized with killed *Trypanosoma rangeli* and challenged with *T. cruzi*.

31.6 Generation of Attenuated Parasites by Genetic Manipulation and Their Use as Potential Vaccines Against Chagas Disease

In spite of the good short-term immunization results achieved with nonreplicating experimental immunogens against *T. cruzi* (Fralish and Tarleton, 2003; Cazorla et al., 2008a,b; Fontanella et al., 2008; Duan et al., 2009), the inoculation and infection by live-attenuated parasites seem to confer so far the best vaccine effect, based on the quality of the immune response obtained and the length of the protection achieved. These attenuated strains have yielded substantial protection in murine models against a virulent challenge. However, the genetic background and the potential of reversion to a virulent phenotype cannot be foretold, making them

unsuitable for use in human vaccination trials. Nevertheless, gene transfer experiments and new molecular techniques for endogenous gene manipulation have offered the possibility of a better understanding of trypanosomatid genetics and biology. We are now able to identify specific genes *in silico*, and then, by gene target deletion through homologous recombination, remove them from the parasite genome. This alternative is tempting and promising, since genes related to virulence or persistence could be specifically and permanently deleted or altered.

In malaria, the use of genetically modified parasites as experimental vaccines has been well studied (Mueller et al., 2005; VanBuskirk et al., 2009; Vaughan et al., 2010). In different *Plasmodium* mouse models, it has been shown that infections with genetically manipulated malarial parasites conferred sterile protection against lethal challenge (Mueller et al., 2007; Aly et al., 2010). Vaccination approaches based on genetically altered live parasites are also currently under study for Leishmaniasis (Silvestre et al., 2008; Selvapandiyan et al., 2009). However, in comparison to what has been published for other parasitic organisms, there are relatively few genes that have been genetically manipulated in *T. cruzi*. The first report of stable transfection of a plasmid vector by homologous recombination in *T. cruzi* was done in 1993 (Hariharan et al., 1993). Since then and to our knowledge, not many proteins have been studied in *T. cruzi* through gene-targeted deletion (Cooper et al., 1993; Ajioka and Swindle, 1996; Caler et al., 1998; Allaoui et al., 1999; Manning-Cela et al., 2001; Conte et al., 2003; Annoura et al., 2005; MacRae et al., 2006; Gluenz et al., 2007; Wilkinson et al., 2007; Xu et al., 2009), mainly due to the lack of more straightforward methods for gene manipulation needed for reverse genetic studies in this organism. Thus far, homologous recombination is the only method available for gene suppression or downregulation because RNA interference has, to date, failed in *T. cruzi* (DaRocha et al., 2004). Most mutants were studied *in vitro* to elucidate metabolic pathways, resistance, or susceptibility to different anti−*T. cruzi* compounds, and differentiate life cycle stages. Only a few *T. cruzi* mutants have been evaluated in *in vivo* models, and even fewer have been evaluated as potential vaccines.

One of the *T. cruzi* proteins which has been well characterized by reverse genetic studies is the surface glycoprotein GP72. It is thought that this protein is involved in the differentiation of life cycle stages of the parasite (Sher and Snary, 1982), though its precise role in this process is not completely understood. Parasites carrying a targeted deletion of the gene coding for the GP72 protein were generated (Y-null), and the deletion unexpectedly altered the general shape of the parasite (Cooper et al., 1993). Contrary to observations for Y wild-type parasites, Y-null parasites could not easily be detected when injected in highly susceptible mouse models (Basombrío et al., 2002b). Polymerase chain reaction (PCR) assays to determine *T. cruzi* presence, as well as serological reactions for specific antibodies, indicated that Y-null infections were no longer detectable after 90 days postinfection, suggesting that the GP72 protein is essential for sustaining latent infections in immunocompetent animals. Inoculation of Y-null mutant parasites strongly protected adult Swiss mice against a challenge from virulent blood trypomastigotes, as shown by a decrease in parasite load in mice preinoculated with the

mutant parasites. Even though the protective effect was detectable and significant up to 14 months, the longest interval tested after priming, a weakening of the protection was observed (Basombrío et al., 2002c). Consequently, the duration of the protective effect is theorized to be directly related to the antigenic offer presentation to which the immune system is constantly exposed.

In another approach, a monoallelic mutant clone for the calmodulin−ubiquitin (*cub*) gene (TulCub8) was obtained from the highly virulent Tulahuen strain of *T. cruzi*. Genetic manipulation of the *cub* gene resulted in a remarkable reduction in parasite virulence as shown in a murine model, since the mutant clone could only be propagated in mice by means of highly sensitive hemoculture recovery. Swiss mice were inoculated subcutaneously with doses of TulCub8 epimastigotes and later challenged with virulent wild-type Tulahuen blood trypomastigotes. In this case, strong protection, based on a reduction in parasite burden in mice preinoculated with TulCub8 epimastigotes, was observed (Barrio et al., 2007).

The third *T. cruzi* mutant tested in protection assays was the *T. cruzi* clone L16, a *Lyt1* $^{-/-}$ null mutant carrying a biallelic deletion of the gene. The infective and protective behavior of the biallelic knockout clone L16 in a murine model was analyzed by Zago et al. (2008). A significant reduction in the infective capacity of clone L16 was observed in adult Swiss mice, determined by fresh blood mounts, spleen index, and tissue parasite load. Furthermore, the muscle inflammatory response elicited by the L16 clone was considerably less than that detected in wild-type parasites. However, a latent and persistent infection with this mutant clone was shown by positive *T. cruzi* DNA detection in blood samples until 12 months postinfection. Long-term protection was also observed in Swiss mice inoculated with L16 epimastigotes. On evaluation 14 months later, these animals were still strongly protected against a challenge with Tulahuen wild-type blood trypomastigotes, as shown by a reduction in the parasite load in blood (Zago et al., 2008).

To our knowledge, the three aforementioned studies are the only ones involving knockout *T. cruzi* clones in protection assays. These studies included control groups in which mice preinoculated with the "wild-type" nonengineered *T. cruzi* strain were challenged with virulent parasites. Inhibition of acute phase parasitemia was as strong as with the mutants, but this "protection" (or premunition) was obtained at the expense of a previous infection with heavy parasite load and development of disease. These control groups thus showed that the mutated parasites retained the immunogenic effects of a mild infection, sparing the untoward effects of a previous acute- and chronic-phase disease. However, the immunologic basis for the protection elicited by these genetically modified parasites was not always fully evaluated, nor was the presence of inflammatory infiltrates or amastigote nests in target organs.

Since the molecular basis of attenuation in the TCC strain of *T. cruzi* is still unknown, researchers have attempted to introduce targeted gene deletions into this clone as a safety mechanism against eventual reversion to the virulent phenotype. Recently, a TCC monoallelic mutant clone for the dihidrofolate reductase−thymidylate synthase gene (*dhfr-ts*) was generated (Pérez Brandán et al., unpublished data). Besides showing a remarkable attenuation in *in vivo* models, TCC

dhfr-ts$^{+/}$ parasites also showed some delay in growth rate in axenic cultures. Remarkably low *T. cruzi*-specific CD8^{+} T-cell counts were reported in mice inoculated with TCC *dhfr-ts*$^{+/}$ parasites. This is not ideal, since the generation of genetically attenuated parasites capable of inducing a strong CD8^{+} T-cell response is desirable. However, these mutant parasites retained their protective effect against a virulent *T. cruzi* challenge even 1 year after inoculation (Pérez Brandán et al., unpublished data).

Also attractive is the generation of mutant parasites expressing foreign genes, such as specific immune factors. CD40 is a cell surface receptor belonging to the TNF family, and its ligand, CD40L, is a costimulatory protein belonging to the same family. The CD40−CD40L complex not only presents immunomodulatory properties but is also involved in the humoral and cellular response (van Kooten and Banchereau, 2000). It has been previously shown that *T. cruzi* parasitemia and mortality rate could significantly be reduced in infected mice by the inoculation of CD40L-transfected fibroblasts together with *T. cruzi* parasites (Chaussabel et al., 1999). Mice infected with transgenic *T. cruzi* parasites carrying a plasmid encoding the gene *CD40L* were able to better control infection than those infected with wild-type *T. cruzi* ones. IFN-γ production and lymphocyte proliferation was observed by immunization with CD40L parasites. Even more important is the protective capacity conferred by these parasites. A very low or even null level of parasites was detected in the group of mice first infected with the transgenic parasites, suggesting that the infection by these parasites is able to induce protection against a subsequent virulent infection (Chamekh et al., 2005).

In summary, *T. cruzi* is an organism in which gene-targeted deletion and gene silencing have so far not been as successfully applied as in bacteria or even as in other trypanosomatids, such as *Trypanosoma brucei* and *Leishmania*. Studies in which *T. cruzi* mutants were compared with wild-type parasites using infectivity measurements almost invariably revealed a change toward attenuation. Sometimes this change was profound and sometimes partial. Therefore, targeted deletion of specific genes can be conceived as a potential procedure to generate clones that are able to develop in culture but less efficient to invade and persist *in vivo*; this strategy provides the potential for mass production coupled with a built-in safety device against reversion to the virulent phenotype. The use of *T. cruzi*−attenuated parasites is very appealing since the infection induced is much like a natural infection and may consequently lead to the establishment of a protective immune response. However, genome manipulation could lead to a loss of the protective immunity either because such genetically modified parasites do not express antigen epitopes essential for triggering a good immune response or because they cannot survive long enough to fully activate the immune system. In this sense, a wide spectrum of specific individual genes or a combination of them (as well as different *T. cruzi* strains) should be evaluated.

Considering the last improvements in genetic manipulation, the potential exists for a live, genetically attenuated parasite vaccine that would be applicable to dogs and other mammals that act as natural reservoirs and would be able to reduce the intensity and spread of the disease. An important factor to be considered is the

presence of antibiotic-resistance genes in the genome of these "vaccine" parasites and the possible implications of this. Drug-resistance genes are the common mechanism to select genetically modified parasites; nonetheless, the release of drug-resistant parasites to the environment implies an undesirable and potential risk. Hence, one of the main objectives for these mutant parasites is to turn them incapable of persistence in the host and completely unable to be spread or transmitted to the insect vectors. Another approach to tackle the drug-resistance problem is a new strategy exploited in the generation of *Leishmania* mutant parasites in which, after the target deletion, the drug-resistance gene is removed from the parasite genome (Denise et al., 2004). Furthermore, the generation of *T. cruzi* parasites genetically modified to "commit suicide" in response to external stimuli is also an appealing alternative. In this approach, genetically modified parasites could be inoculated and allowed to infect the mice until a fully immune response is developed before being induced to die. This technology has been well documented in other parasitic organisms, such as *Leishmania* (Ghedin et al., 1998; Muyombwe et al., 1998). However, attempts of this kind were not reported so far for *T. cruzi*. If this technology is successfully applied to *T. cruzi*, genetically modified parasites could be achieved and safely used as live vaccines.

The recent elucidation of the trypanosomatid genomes and the identification of new targets for genetic manipulation open the possibility of generating a wide variety of mutant parasites that could be evaluated as potential vaccines against Chagas disease. The generation of parasites carrying a combination of gene deletions or an expression of foreign genes could also be feasible. The strategy of superimposing attenuating mutations into already naturally attenuated and protective strains might provide additional mechanisms against reversion to virulent phenotypes.

31.7 Final Considerations

In the last few decades, repeated insecticide spraying in several endemic regions in South America has considerably controlled the insect vectors for Chagas disease and reduced the natural transmission of the parasite to humans. It has also become more evident that Chagas disease (as well as other neglected diseases) is a "poverty disease" and that basic improvement in the housing conditions and economic development of the endemic areas would have a huge impact on its control. Considering this, we might wonder: do we really need a vaccine against Chagas disease? The answer to this question seems to be yes, even more now than before. Unfortunately, the economic and social conditions of the rural areas in South America where the disease is endemic are unlikely to remarkably improve within the next 10 years. Additionally, the current occurrence of insecticide-resistant populations of vectors jeopardizes the achievements of the massive spraying and active control successfully implemented in some regions. Clearly, a human vaccine or a veterinary one intended to block the transmission of *T. cruzi* will not solve the problem of Chagas disease by itself. But unquestionably, the availability of a

vaccine in addition to effective insecticide spraying and dwelling improvements will have a remarkable impact on the health and social development of the people living in endemic areas.

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