42

# ADVANCES IN PARASITOLOGY

*Edited by*

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# *Advances in* PARASITOLOGY

VOLUME 42

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# PREFACE

This volume starts with a summary by Chris Schofield (London School of Hygiene and Tropical Medicine, UK) and João Carlos Pintas Dias (Centro de Pesquisas René Rachou, Belo Horizonte, Brazil) of current progress in what must be one of the largest-scale disease control programmes ever – the Southern Cone Project to control if not eliminate transmission of *Trypanosoma cruzi* to humans in the southern part of South America. Covering over $6 \times 10^9$ km$^2$, the project was initiated in 1991 as a 10-year collaborative scheme between the World Health Organization, the Pan American Health Organization and the governments of Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay. The emphasis of the project has been on the elimination of the major vector of *T. cruzi*, *Triatoma infestans*, and mass screening of blood donors. This example of disease control on the grand scale is now roughly at its midpoint and, as the authors point out, considerable progress has already been made. Well over 2 million houses have been sprayed with insecticide at a cost of about US$75 each, with massive reductions of the prevalence of domiciliary *T. infestans* (in many areas to zero). The authors stress that, in addition to the considerable financial benefits resulting from the programme in terms of the saving in medical and hospital expenditure and increased labour productivity, the benefit to the local population is very considerable and much appreciated. Hopefully, to this will be added, in the future, benefits resulting from the reduction in morbidity due to Chagas disease.

Erney Camargo (University of São Paulo, Brazil) then reviews the relatively little-known trypanosomatids that parasitize plants, *Phytomonas* species. Although these most commonly infect plants of the family Euphorbiaceae, many other plants are also infected, including the economically important coconut palms, coffee bushes, cassava, maize and tomatoes. Although, as Camargo points out, Koch's postulates have never been fulfilled, there is considerable evidence of pathogenicity in coconut palms, coffee and cassava. This review covers virtually all aspects of the biology of these interesting organisms, including morphology, distribution within the host (in latex, phloem, fruits or flowers), nutrition, biochemistry, molecular biology, taxonomy, phylogeny, pathogenesis, transmission, reservoir hosts, diagnosis and treatment. The history of their discovery in 1909 by the

laboratory technician Mr David, after whom the first species was named, is also described.

In the next chapter David Blair (James Cook University, Townsville, Australia), Zhi-Biao Xu (Beijing Tropical Medicine Research Institute, China) and Takeshi Agatsuma (Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan) discuss the many new species from various parts of the world of, and new findings on, the lung flukes, *Paragonimus*, since the reviews by Yokogawa in *Advances in Parasitology* in 1960 and 1965. There have been over 1400 papers published since then and the literature is very scattered, while some is inaccessible. This comprehensive and thorough review considers much new literature from China and Japan and reviews in Chinese and Russian. The authors estimate that about 20 million people are infected world-wide, about double the estimate 20 years ago. It is likely that many of the new animal species described are possible human parasites.

The rat tapeworm, *Hymenolepis diminuta*, is found in almost every parasitology teaching laboratory because it is simple to maintain and has a short and simple life cycle. Jørn Andreassen (University of Copenhagen) and Eva Bennet-Jenkins and Chris Bryant (Australian National University, Canberra) report on the wealth of new research on its immunology and biochemistry that has been carried out over the last few years. The rat and the parasite co-evolve and the tapeworm needs to survive in a host that has a complex adaptive immune system. *Hymenolepis diminuta* used to be regarded as a non-immunogenic commensal and not so very interesting from an immunological viewpoint, but actually there is a highly complex equilibrium between host and parasite; the tapeworm also has a metabolism similar to, but subtly different from, that of its host.

The penultimate chapter in this volume is an important contribution dealing with control strategies for human intestinal nematode infections. The three authors, Marco Albonico (Ivo de Carneri Foundation, Italy), David Crompton (University of Glasgow, UK) and Lorenzo Savioli (World Health Organization, Switzerland), have considerable experience in this area and have produced an authoritative overview of the current situation as well as highlighting advances that have been made in the past few years. Nematode control is a public health problem of enormous magnitude, with current estimates suggesting that at least one-quarter of the world's population may be chronically infected with intestinal helminths. The article deals with the main issues concerning strategies for control, stressing that there is a difference between infection and disease. Periodic anthelmintic chemotherapy remains the key intervention method and the major drugs are considered in detail, together with the need to monitor changes that might occur in nematode populations. The authors stress the importance of health education, sanitation and the possibility of adopting an integrated

approach for the control of filariasis, intestinal helminths and schistosomiasis. Consideration is given to planning intestinal helminth control programmes and a useful section provides an insight into experience gained in Zanzibar, Seychelles, Sri Lanka, South Africa and Mexico. The authors conclude that the challenge for the future will be to develop sustainable approaches to control intestinal helminth infections in line with the demographic changes taking place in developing countries.

In recent years considerable attention has been given to the introduction and use of DNA vaccines and remarkable progress has been made. No effective vaccine is available for the important parasites of humans and it is hoped that nucleic acids may provide an approach to the development of novel vaccines. We are extremely fortunate to have the final chapter in this volume by Jason Alarcon, Gary Waine and Don McManus from the Queensland Institute of Medical Research who present an up to the minute account of the technology behind the introduction of DNA vaccines and their possible application against parasites and microbial agents. The chapter is comprehensive, dealing with methods of delivery, the immune responses generated and the use of DNA vaccines against infectious and parasitic diseases. This is an authoritative account and provides an extremely useful synthesis of this area of research and development. Rapid progress has been made and it now remains to be seen whether these exciting ideas and promising results can be translated into commercially viable vaccines.

John Baker
Ralph Muller
David Rollinson

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# The Southern Cone Initiative against Chagas Disease

C.J. Schofield[1] and J.C.P. Dias[2]

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## ABSTRACT

*Chagas disease (also known as American trypanosomiasis) is now ranked as the most serious parasitic disease of the Americas, with an economic impact far outranking the combined effects of other parasitic diseases such as malaria, schistosomiasis and leishmaniasis. Although the chronic infection remains virtually incurable, transmission can be halted by eliminating the domestic insect vectors and screening blood donors to avoid transfusional transmission. In line with this strategy, governments of the six Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay) launched in 1991 an ambitious initiative to control Chagas disease through elimination*

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*of the main vector,* Triatoma infestans, *and large-scale screening of blood donors. Now at its mid-point, the programme has achieved remarkable success, with transmission halted over vast areas of the previously endemic regions. Well over 2 million rural houses have been sprayed to eliminate* T. infestans, *and the programme has already shown significant economic rates of return in addition to the medical and social benefits.*

## 1. INTRODUCTION

The Southern Cone Initiative against Chagas disease is one of the largest disease control programmes ever mounted, covering an area of over 6 million $km^2$ over most of the southern part of Latin America. It was planned as a 10-year programme to eliminate transmission of the causative agent, *Trypanosoma cruzi,* throughout the endemic areas of six countries: Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay. Over two-thirds of all cases of Chagas disease occur in these countries, where transmission is mainly due to domestic populations of the insect vectors, of which the most important is *Triatoma infestans.* In addition, about 10% of transmission is attributable to blood transfusion from infected donors. The programme has two interdependent objectives, reflecting the two primary interventions of vector control and control of transfusional transmission.

1. To interrupt vector-borne transmission, by eliminating all domestic and peridomestic populations of *Triatoma infestans* throughout the endemic areas, together with elimination of domestic populations of other vector species occurring in the same regions.
2. To interrupt transfusional transmission, mainly through improvements in the screening of blood donors.

The programme was formally launched on 30 July 1991, by resolution of the Ministers of Health of the Southern Cone countries meeting in Brasilia, to 'create an Intergovernment Commission for Chagas disease, charged with development of a programme and plan of action for the elimination of domestic *Triatoma infestans* and the interruption of transfusional transmission of *Trypanosoma cruzi*' (Acto de Brasilia, resolution 04-3-CS, 1991). Now at its mid-point, the programme has achieved remarkable success, with transmission virtually eliminated from Uruguay (WHO, 1994) and from large areas of Chile (WHO, 1995), Argentina (WHO, 1996) and Brazil (Southern Cone Commission, 1996, 1997; Schmunis *et al.*, 1996; WHO, 1997a). Paraguay and Bolivia have also made progress, and Peru has now joined the programme with the aim of eliminating *Triatoma infestans* from endemic regions in the southern departments (Southern Cone Commission, 1997). This review seeks to present the technical, economic and political background to the Southern Cone Initiative,

to serve as a model for regional co-operation in disease control in Latin America and, perhaps, elsewhere.

## 2. EPIDEMIOLOGY OF CHAGAS DISEASE

Chagas disease (American trypanosomiasis) is one of the most serious public health problems in Latin America. The disease takes its name from the Brazilian clinician Carlos Justiniano das Chagas, who described the first cases from the Brazilian state of Minas Gerais (Chagas, 1909). The causative parasite, *Trypanosoma cruzi,* is transmitted to humans mainly via the faecal droppings of large blood-sucking reduviid bugs of the subfamily Triatominae. Well over 100 species of Triatominae have been described, mainly from the Americas, but most of them occupy silvatic habitats associated with small nest-building mammals and birds. Species of Triatominae, often infected with *Trypanosoma cruzi,* can be found in silvatic habitats from northern USA to southern Argentina, but in parts of Latin America a few species have become closely adapted to the domestic environment where they are able to transmit *Trypanosoma cruzi* to humans. The domestic species live in cracks and crevices of traditional rural dwellings, emerging at night to suck the blood of the sleeping occupants. They can contribute to chronic iron deficiency anaemia in addition to their role as vectors of *Trypanosoma cruzi.* In recent years, especially as people have migrated from rural areas to cities, transmission of *Trypanosoma cruzi* by blood transfusion and organ transplant from infected donors has become more significant; *Trypanosoma cruzi* can also be transmitted orally (e.g. by eating food contaminated with infected bug faeces) and by transplacental transmission from infected mother to fetus. Nevertheless, vector-borne transmission remains numerically the most important route, accounting for almost 90% of all transmission to humans.

Current World Health Organization (WHO) estimates indicate 16–18 million people infected with *Trypanosoma cruzi*, with a further 100 million at risk (WHO, 1990, 1991). The infection is most prevalent in rural savannah-like regions of Latin America, such as the norte chico of Chile, the cerrados and caatinga of Brazil, and the chaco of northern Argentina, southern Bolivia and western Paraguay. Over two-thirds of these cases occur in the six Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay) and southern Peru, which show a combined prevalence of over 11 million people (Table 1). Because of the chronic nature of the infection, incidence is difficult to measure directly, but the prevalence estimates imply over 300 000 new infections per year in the Southern Cone countries in the absence of control measures (Hayes and Schofield, 1990).

*Table 1* Estimated prevalence of *Trypanosoma cruzi* infection in the Southern Cone region, 1992

| | 1990 Population[a] | Population at risk[b] | Seroprevalence of *Trypanosoma cruzi*[b] | Incidence (new cases/year)[c] |
|---|---|---|---|---|
| Argentina | 32 322 000 | 6 900 000 | 2 640 000 | 53 064 |
| Bolivia | 7 314 000 | 1 800 000 | 1 333 000 | 47 980 |
| Brazil | 150 368 000 | 41 054 000 | 6 340 000 | 169 270 |
| Chile | 13 173 000 | 1 000 000 | 187 000 | 4 095 |
| Paraguay | 4 277 000 | 1 475 000 | 397 000 | 14 053 |
| Peru | 21 550 000 | 1 907 700 | 643 000 | 19 161 |
| Uruguay | 3 094 000 | 975 000 | 37 000 | 614 |
| Total | 232 098 000 | 55 111 700 | 11 577 000 | 308 237 |

[a] Data from Interamerican Development Bank.

[b] WHO estimates, based on reports from Ministries of Health.

[c] Incidence calculated using the model of Hayes and Schofield (1990) assuming absence of control measures.

Chagas disease has a 5–15% mortality rate in the initial acute phase (especially amongst young children) but the prognosis for those surviving to the chronic phase is also very poor. Although many chronic-phase infections are asymptomatic, around 30–40% develop severely debilitating lesions of heart, nervous system and, in some cases, parts of the digestive tract. Cardiac arrhythmias are common in chronic infections, and may require pacemaker implants. Death in the chronic phase may be caused by progressive or acute organ failure. The social and economic costs of the disease are very high. Extrapolation from Argentinian, Brazilian and Chilean data suggests that direct medical costs associated with the disease (e.g. consultation, hospital admission and supportive treatment) add up to almost US$50 million per year per cohort of 100 000 infected people, although indirect costs due to chronic disability and lost production associated with the disease could reach much higher levels. Brazilian data, for example, indicate an average of nearly 2000 working years lost per year per 100 000 population (Pereira, 1984), suggesting overall economic losses of well over US$1000 million per year for the Southern Cone countries.

In spite of extensive research, vaccines against *Trypanosoma cruzi* are unavailable and curative treatment of the infection is still possible only during the early acute phase. However, the two drugs that can be used — nifurtimox and benznidazole — are not widely available because of their toxicity. Successful treatment requires early (or presumptive) diagnosis, followed by regular spaced doses over a period of 10–35 days depending on patient response, with careful monitoring for dangerous side-effects. As a result, the drugs cannot be used routinely outside a clinical setting and

are unsuitable for large-scale use in the affected rural communities. During the chronic phase of infection, treatment can only be supportive, generally involving long-term use of vasodilators and/or antiarrhythmics such as amiodarone. Severe cases may require implantation of a cardiac pacemaker, or corrective surgery for intestinal lesions.

On a global scale, Chagas disease is now ranked as the third most serious of the parasitic diseases (after malaria and schistosomiasis) in terms of disability adjusted life years (DALYs) lost per year (World Bank, 1993). In Latin America, however, Chagas disease is by far the most serious of all parasitic diseases on this measure, and ranks as the fourth most serious of all communicable diseases after respiratory and diarrhoeal infections and acquired immune deficiency syndrome.

## 3. CHAGAS DISEASE IN THE SOUTHERN CONE

The Southern Cone of Latin America is not a formal grouping tied by convention, such as the Andean Pact, but has been used since 1986 as a regional grouping by the Pan American Health Organization (PAHO) for discussion of regional problems. In all these six countries, the most important vector of Chagas disease is *Triatoma infestans*, which also occurs in parts of southern Peru. *Triatoma infestans* is considered to be responsible for around 80% of all transmission of *Trypanosoma cruzi* in this region, with other species of Triatominae, such as *Triatoma brasiliensis* in north-east Brazil and *Panstrongylus megistus* in humid coastal regions being responsible for about 10%. The remaining 10% of transmission is attributed to transfusional and congenital routes.

Historical records combined with modern genetic analysis indicate that *Triatoma infestans* probably originated from the Cochabamba and Sucre region of central Bolivia, which is the only locality where silvatic populations of this species have been encountered (Dujardin *et al.*, 1987, 1997a). However, recent population analysis, using RAPD (random amplified polymorphic DNA) and morphometry, suggests an absence of gene flow between silvatic and neighbouring domestic populations, indicating incipient separation between them (Carlier *et al.*, 1996; Dujardin *et al.*, 1997a,b). The spread of *Triatoma infestans* to its current wide distribution appears to have been relatively recent, associated mainly with human migration patterns (Schofield, 1988). In the Andean region of Bolivia, southern Peru and northern Chile, where *Triatoma infestans* is virtually the only known domestic vector, pre-Columbian mummies with signs of chronic Chagas disease have been dated at up to 3000 years before present (Rothhammer *et al.*, 1985; Fornaciari *et al.*, 1992) and linguistic evidence

also points to a pre-Colombian association between *Triatoma infestans* and human settlements. In contrast, *Triatoma infestans* was unknown from the Brazilian state of Minas Gerais (where Carlos Chagas originally encountered *Panstrongylus megistus* in the houses) until the 1930s, and reached its current limits in north-east Brazil only in the 1970s (Barrett *et al.*, 1979; Santos *et al.*, 1981). In Uruguay, *Triatoma infestans* appears to have entered in two main waves, from the south during the 1860s (Hudson, 1965) and from the north during the early 1900s (Talice *et al.*, 1940; Rodrigues da Silva *et al.*, 1979), and even today it is possible to detect morphometric, isoenzymatic and chromosomal differences between northern and southern populations of *Triatoma infestans* in Uruguay (Panzera *et al.*, 1995; Pereira *et al.*, 1996). Experimental studies and computer simulations based on field data from Brazil indicate that long-range dispersal of *Triatoma infestans* is mainly due to passive transport amongst the belongings of people travelling from infested dwellings (Schofield and Matthews, 1985; Jedwab, 1987), although under certain conditions, adult bugs can also disperse by active flight over distances up to 1 km (Schweigmann *et al.*, 1988; Schofield *et al.*, 1992). *Triatoma infestans* is extremely well adapted to the habitat offered by rural dwellings, and can build up domestic populations of several thousand bugs (Dias and Zeledón, 1955; Schofield, 1980). In areas with a climate similar to that of the Cochabamba region, such as parts of northern Argentina and southern Peru, *Triatoma infestans* can also be found in peridomestic animal enclosures such as goat corrals and chicken coops, but elsewhere it seems that the insect is entirely restricted to the domestic environment and does not survive outside the house structure.

The behavioural biology and population dynamics of *Triatoma infestans* have been intensively studied, and several comprehensive reviews have been published (e.g. Schofield, 1979, 1994; Brenner and Stoka, 1988; Gorla and Schofield, 1989). Of particular interest in relation to vector control interventions is the insect's relatively slow rate of population reproduction (one or two generations per year, depending on climate) and the susceptibility of nymphs and adult bugs to modern pyrethroid insecticides (cf. Oliveira Filho, 1988, 1989; Zerba, 1988). In addition, recent work has shown the bugs to have a very specialized genetic system with holocentric chromosomes and extremely low rates of genetic variability (Garcia *et al.*, 1995; Dujardin *et al.*, 1998a), which helps to explain the difficulty in selecting for insecticide resistance in this species (cf. Schofield, 1991).

A synthesis of the biological characteristics of *Triatoma infestans* thus shows four key factors favouring control.

1. Limited range of habitats, with all stages confined to houses and peridomestic animal enclosures.

2. Slow rate of population development, and consequent slow rate of genetic rearrangement.
3. Extreme specialization with a low rate of genetic variability and consequently limited genetic repertoire for further adaptation (e.g. for insecticide resistance).
4. Complete susceptibility to modern pyrethroid insecticides.

On the other hand, the cryptic habits of the bugs and their capacity for passive dispersal over long distances concealed amongst the belongings of travellers are factors that make localized control extremely difficult, because bugs from untreated houses can be readily transported to reinfest controlled areas. This means that large-scale control and vigilance is necessary in order to eliminate foci from which reinfestation can occur.

## 4. EXPERIENCE IN CHAGAS DISEASE VECTOR CONTROL

The earliest trials of Chagas disease vector control were carried out in Venezuela, Brazil and Argentina during the 1940s, using organochlorine insecticides. DDT was generally found to be ineffective, but trials in Brazil (Dias and Pellegrino, 1948) and Argentina (Romaña and Abalos, 1948) showed that γ-benzene hexachloride (BHC; also known as HCH, lindane or gammexane) could achieve good results when sprayed at high doses over all the walls and inner roof of infested houses. These trials tested various application methods and dose rates, including fumigation, and concluded that best results were obtained by spraying wettable powder (WP) formulations containing not less than 30% γ isomer at a target dose rate of 0.5–2.0 g $m^{-2}$ of the active ingredient (a.i.). For many years BHC spraying (and, in some cases, dieldrin) continued to be the main approach to Chagas disease vector control and a great deal of operational experience was accumulated, especially in Venezuela (e.g. Guevara de Sequeda *et al.*, 1986), Argentina (e.g. Soler, 1967) and Brazil, especially in the state of São Paulo where *Triatoma infestans* was virtually eliminated during the 1960s (cf. Correa, 1961; Rocha e Silva *et al.*, 1979; Wanderley, 1994).

During the 1960s and 1970s several other compounds were tested in laboratory and field trials, including several organophosphates and carbamates. Alternatives to insecticides were also studied, including traps (e.g. Tonn *et al.*, 1976), insect growth regulators (e.g. Pinchin *et al.*, 1978) and various types of biological control agents. All were abandoned as less effective and/or more costly than conventional insecticide spraying with BHC. During the late 1970s, however, a number of modern pyrethroids became available that showed extremely good activity against domestic Triatominae even when sprayed less frequently and at very low doses (e.g.

Pinchin *et al.*, 1980, 1982). These compounds were also more acceptable to householders and spraymen in terms of lack of odour and ease of application and, because of their biodegradability, posed little risk of environmental contamination. The new compounds were more expensive per kilogram than BHC but, because of the much lower doses and enhanced efficacy, they were found to be considerably more cost-effective (cf. Oliveira Filho, 1989).

By the late 1980s, all countries of the Southern Cone had accumulated operational experience in planning and implementing Chagas disease vector control trials using residual house spraying against domestic Triatominae. Without exception, the results followed a similar pattern, showing a sharp decline in domestic bug population densities and house infestation indices (e.g. proportion of houses with apparent bug infestations). Often, however, it proved difficult to consolidate the success due to restricted geographical coverage and discontinuities in surveillance, which allowed recrudescence of transmission by bugs brought in from untreated foci.

The experience of Brazil amply demonstrates this point. Up to 1980, Chagas disease vector control had been successful in the state of São Paulo, implemented by the state organization SUCEN. For the rest of the country, however, control had been sporadic with some localized successes (for example in central Minas Gerais) but a general lack of continuity in the national operations. By the early 1980s (spurred by the 1979 international congress on Chagas disease in Rio de Janeiro) it was felt that the methods and operational experience of SUCAM (the executive arm of the Ministry of Health) could be used to good effect to eliminate most, if not all, of the vector-borne transmission due to *Triatoma infestans*. The national campaign was launched in 1983, and was the first to have the declared objective of eliminating *Triatoma infestans* from the national territory. It relied on three independent sources of finance (Ministry of Health, the Brazilian social security fund FINSOCIAL and the Brazilian National Development bank BNDES) used a proven method of residual spraying with BHC (progressively replaced with pyrethroids), and was ably administered by SUCAM staff at both national and regional levels (Dias, 1987, 1988). It was highly successful. By 1986, around 75% of the geographical objectives had been attained, in the sense that affected municipalities had been mapped, surveyed for house infestations and sprayed with residual formulations of BHC or pyrethroid insecticides. House infestation rates in treated areas had declined to less than 5%. However, 1986 saw the arrival in Brazil of the insect vector of another pathogen, *Aedes aegypti,* with concurrent outbreaks of dengue transmission in Rio de Janeiro, São Paulo and Recife. Although uncomplicated dengue is far from life-threatening, the political outcry forced diversion of funds and personnel from the rural Chagas disease programme to emergency control of *Aedes* in coastal cities.

A distillation of the Chagas disease vector control experience throughout the Southern Cone countries thus leads to the following operational conclusions.

1. Available techniques for Chagas disease vector control, using proven operational procedures with modern pyrethroid insecticides, can be highly effective.
2. Reinfestations of treated areas can be prevented by (i) extensive geographical coverage to eliminate sources of reinfestation, and (ii) community-based surveillance to detect and notify any apparent re-infestations so that they can be selectively retreated.
3. Elimination of domestic vector populations leads to elimination of seroconversion to Chagas positive.

On the other hand, any interruption in the continuity of surveillance and selective treatment would raise the risk of domestic reinfestations (by either *Triatoma infestans* or another vector species) with consequent recrudescence of transmission.

## 5. DEVELOPMENT OF THE SOUTHERN CONE PROGRAMME

By 1990, it was becoming clear that the problems of Chagas disease control rested less with biological, technical or operational aspects, and more with political and economic questions (cf. Dias, 1991). Few countries were giving priority to Chagas disease control, partly because the disease was endemic and rural (rather than an urban epidemic) and partly because there seemed no clear end-point to merit concerted action. The Brazilian campaign of 1983–1986 had changed this to some extent, by offering vector eradication as a principal objective, and the Uruguayan programme initiated in 1988 had achieved local success by virtually eliminating *Triatoma infestans* from areas of the northern departments of Artigas and Rivera. In general, however, the control services were underfunded and given insufficient priority.

1. In Argentina, the Servicio Nacional de Chagas had been decentralized in 1983. Some provinces, especially Chaco, Córdoba, Jujuy, La Rioja and Salta, had achieved good results with the resources available, but others had achieved little.
2. In Bolivia, no priority was given to Chagas disease control, and local activities depended entirely on the strength of individual scientists and/or international aid projects.
3. In Brazil, Chagas disease was given relatively low priority (below malaria, schistosomiasis, dengue and yellow fever). Control was the responsibility

of the executive arm of the Ministry of Health (SUCAM) (except in São Paulo, which has its own executive organization known as SUCEN). However, the operational objectives of SUCAM were decided at a political level and, at this time, the organization was being restructured to devolve resources and decision-making to state and/or regional authorities.

4. In Chile, Chagas disease control was underfunded, in the affected regions depending largely on the Intendents (the governors and highest political authority in each of Chile's 13 Regions). This resulted in a limited and heterogeneous (although often locally successful) approach to vector control.
5. In Paraguay, there was no formal activity against Chagas disease.
6. In Peru, there was also no formal activity against Chagas disease, although the regional authorities of Arequipa had been carrying out trials whenever resources became available.
7. In Uruguay, the programme was underfunded. In 1988, the national budget for operations against Chagas disease was US$15 000, so that activities depended entirely on the ability of the national co-ordinator to convince municipal authorities to invest in vector control campaigns.

In a sense, the Southern Cone Initiative can be seen as a scientific response to political uncertainty. Certainly the originators were all scientists with close working involvement with control activities, who could not only see the feasibility of large-scale vector control but also experienced some frustration that the accumulated experience was not being fully implemented. Assessment of practical experience indicated that the techniques for vector control, and for screening blood donors, could be highly effective in reducing *Trypanosoma cruzi* transmission. Moreover, analysis of the biological and epidemiological aspects indicated that large-scale vector control was both feasible and necessary in order for the initial impact to be sustained. There was a scientific consensus that without large-scale control there would always be a risk of reinvasion of treated areas; by contrast, the biological characteristics of *Triatoma infestans* that made it such a good domestic vector of Chagas disease also rendered it a uniquely vulnerable target for control. In addition, analysis of the recent spread of *Triatoma infestans,* especially into north-east Brazil, indicated that without effective control there could be a risk of its spreading into new areas such as the southern Amazon region, promoting new outbreaks of Chagas disease transmission similar to those seen in northern Bahia when *Triatoma infestans* first crossed the São Francisco river in the mid-1970s (cf. Barrett *et al.*, 1979).

Detailed analyses of operational costs were available from Brazil (Oliveira Filho, 1989) and Chile (Silvana Corona, unpublished report, University of Chile, 1990), and preliminary estimates of benefits suggested

that a large-scale programme of vector control would be economically attractive (Schofield and Dias, 1991). In March 1991, the ideas were aired during the twentieth congress of the Brazilian Society for Tropical Medicine meeting in Uberaba, close to where Carlos Chagas had made his first discoveries. In May the proposals were put to the Director of PAHO, and in June the Italian Consiglio Nazionale delle Ricercha hosted a discussion meeting in Rome to coincide with the launching of a new film on Chagas disease. The dream, a multinational programme for the eradication of all domestic and peridomestic populations of *Triatoma infestans*, was gathering scientific momentum.

The political framework was being developed by PAHO through periodic meetings of Ministers of Health of the Southern Cone countries. Already in 1986, during the first of these meetings, it had been recognized that Chagas disease control would require multinational action, at least along frontier regions, and by 1990, delegates to the twenty-third Pan American Sanitary Conference (PASC) had included Chagas disease in a list of ailments to be studied as possible targets for elimination in the Americas (PASC resolution XVI.4.b, September 1990). Heading this list, however, was rabies, which became the prime candidate considered for regional emphasis during the next historic meeting of the Southern Cone ministers in Brasilia in July 1991. A month earlier, however, President Carlos Ménem of Argentina had publicly endorsed the revised national plan of Chagas control, so that the Argentinian delegation, backed by that of Uruguay, was able successfully to propose Chagas disease as the priority target. The resulting resolution, part of which is reproduced here in its original form (Figure 1), formed the basis for the Southern Cone Programme against Chagas disease: the dream was becoming a reality.

By September that same year, the first planning meeting for the Southern Cone Programme was held under the auspices of the University of Chile in Santiago. This had been originally constructed to consider eradication of *Triatoma infestans* from Chile alone, but was quickly amplified with help from the Commission of the European Communities (CEC) to include delegates from a number of other Southern Cone countries. There was a clear scientific consensus, both for the feasibility of the proposals and for the operational methods to be used. The CEC continued its support, and a full planning meeting involving delegates from all Southern Cone countries was organized and held just 2 months later, in November 1991, under the auspices of the Ministry of Public Health in Montevideo, Uruguay. The Southern Cone Initiative was formalized through appointment of an Intergovernment Commission under the co-ordination of PAHO and, at the first of their annual meetings in 1992 in Buenos Aires, the delegates from each of the Southern Cone countries presented detailed operational plans for the vector eradication programme.

*RESOLUCIÓN 04-3-CS*

*CONTROL DE ENFERMEDADES (ZOONOSIS)*

*CONSIDERANDO QUE:*

Las zoonosis y otras enfermedades transmisibles causan importantes daños sanitarios, económicos y sociales por lo que se considera imprescindible el reforzamiento de los programas nacionales de prevención y control de las zoonosis y enfermedades transmisibles prioritarias para lo cual,

*SE RESUELVE:*

3. Crear una Comisión Intergubernamental para la enfermedad de Chagas, con la OPS como secretaria, para la elaboración de un programa y un plan de acción subregional para la eliminación del *Triatoma infestans* domiciliario y la interrupción de la transmisión del *Tripanosoma cruzi* por transfusión. Ese trabajo deberá considerar especialmente la situación y los planes macionales existentes y los mecanismos de cooperación técnica interpaíses, y deberá ser concluido en un plazo de seis meses.

*Figure 1* Paragraph 3 of Resolución 04-3-CS, Control de Enfermedades (Zoonosis). (Courtesy of G.A. Schmunis.)

## 6. IMPLEMENTATION OF THE SOUTHERN CONE PROGRAMME

Although during the planning meetings of 1991 and 1992 an idealized operational programme had been outlined (cf. PAHO, 1993), the real implementation of the Southern Cone Programme mainly involved extension of existing national programmes. Only in Paraguay, where there had been no prior programme, was it possible to contemplate the idealized approach, but even this involved extensive national planning and adaptation to the existing operational structures of the executive vector control service, SENEPA (Servicio Nacional de Eradicación del Paludismo) (Rojas de Arias, 1996). In Bolivia, too, commitment to the Southern Cone Initiative was associated with major reorganization of the executive services, leading to some delay in implementation of the programme.

To a large extent, screening of blood donors and the vector control programmes are implemented independently, although serological data from blood banks do provide useful epidemiological information in support of the vector control activities. However, screening blood donors is a relatively localized intervention, linked to their screening for other infectious agents such as syphilis, hepatitis and human immunodeficiency virus (HIV) (Table 2). At national and international levels it required development of appropriate legislative instruments, backed by a system of reference

*Table 2* National prevalence amongst candidate blood donors of transfusionally transmitted diseases, as reported by the Southern Cone countries in 1993

| Country | Number of donors examined | Prevalence (%) | | | | |
|---|---|---|---|---|---|---|
| | | Syphilis | HIV[a] | Hepatitis B | Hepatitis C | Chagas disease |
| Argentina | 498 380 | 0.9 | 0.2 | 0.7 | 1.0 | 5.6 |
| Brazil | 1 099 601 | 2.4 | 0.3 | 0.9 | 1.3 | 0.7 |
| Chile | 215 762 | 1.2 | 0.3 | 0.3 | 0.2 | 1.3 |
| Paraguay | 30 252 | 4.4 | 0.1 | 2.0 | –[b] | 5.3 |
| Uruguay | 57 205 | 0.8 | 0.04 | 0.4 | 0.5 | 0.8 |

[a] Human immunodeficiency virus infection.

[b] Not reported.

laboratories using well-characterized serological tests* (cf. Wendel *et al.*, 1992). By 1992, most countries of the Southern Cone (except Bolivia and Paraguay) already had relevant laws obliging national and private blood banks to screen for *Trypanosoma cruzi* infection, but it was necessary to extend and standardize the networks of reference laboratories and to develop systems for counselling and supporting those with positive serology. New legislation for the screening of blood banks was passed by Paraguay in 1994, although in Bolivia this was delayed until early 1996 (Dias and Schofield, 1998).

The vector control activities were largely based on existing protocols (e.g. SUCAM, 1980) but with operational modifications reflecting the eradication objective. In essence the strategy involves three phases: preparatory, attack, and vigilance (e.g. FNS, 1994). In the endemic area, each administrative unit (e.g. municipality) is declared infested if one or more houses are found to have *Triatoma infestans.* The unit is then targeted for the attack phase, which involves spraying all houses and peridomestic dependencies with residual pyrethroid formulations (Table 3), irrespective of whether each is known to be infested. In general, only a single application is made, although in some areas a repeat spraying is done after 6 months. The rationale for the mass coverage is, firstly, because the marginal cost of spraying a house is small compared with the cost of visiting it and checking for actual infestation (i.e. over 80% of the unit cost of spraying is expended on transport and salaries; insecticides generally form less than 20% of the

* Following a previous WHO and PAHO determination, the Southern Cone Initiative recommended phasing out the long-used complement fixation (CF) test because of its technical complexity and relatively high margin of error. CF was to be progressively replaced with at least two tests — indirect fluorescent antibody (IFA), indirect haemagglutination IHA and/or enzyme-linked immunosorbent assay (ELISA) — using defined antigens from a network of reference laboratories.

*Table 3* Pyrethroids used for the elimination of domestic Triatominae

| | Manufacturer | Trade name | Usual formulation[a] | Target dose rate (mg a.i. $m^{-2}$)[b] |
|---|---|---|---|---|
| Cyfluthrin | Bayer | Solfac | 10% WP | 50 |
| Beta-cyfluthrin | Bayer | Responsar | 12.5% SC | 25 |
| Cypermethrin | Various | Various | 20–40% WP | 125 |
| Deltamethrin | AgrEvo | K-othrine | 2.5% SC (or 5% WP) | 25 |
| Lambda-cyhalothrin | Zeneca | Icon[c] | 10% WP | 30 |

[a] SC, suspension concentrate; WP, wettable power.

[b] a.i., active ingredient.

[c] Commodore in Argentina.

unit spray cost). The second reason is that available sampling methods are imprecise, and may not detect low-density infestations. It is therefore more efficient to spray some non-infested houses, rather than risk not spraying houses that do in fact have bug infestations.

Concurrently with the preparatory and attack phases, discussions are held with the local community to explain the objectives and enlist their support. The role of the community is important during the attack phase itself, in the sense that householders should co-operate with the spray teams, but becomes vital in the subsequent vigilance phase. Once a municipality has been sprayed it is considered to be under vigilance until at least 3 years have passed with no report of the presence of *Triatoma infestans*. During this period, householders are organized to report the finding of any bug to their local information post. The area inspector visits periodically to check on these reports and, if confirmed (i.e. if the insect found is indeed a triatomine, although not necessarily *Triatoma infestans*), he will arrange for that house to be resprayed. In addition, he will check (and respray if necessary) all neighbouring houses within a 200 m radius, which is considered to be the mean effective range for active dispersal of *Triatoma infestans* adults (Schofield and Matthews, 1985).

## 7. RESULTS

Results of the Southern Cone Initiative are monitored in several ways. At the annual meetings of the Southern Cone Commission, co-ordinated by PAHO, progress is reviewed and operational targets are set for the year. The first progress indicator, therefore, is the degree to which operational targets have been met. Since initiation of the programme, about 2.2 million

*Table 4* Number of houses sprayed against *Triatoma infestans* in the Southern Cone[a]

| | 1992 | 1993 | 1994 | 1995 | 1996 |
|---|---|---|---|---|---|
| Argentina | 115 785 | 116 330 | 143 068 | 114 434 | Vigilance[b] |
| Bolivia | 1 358 | 12 271 | 18 616 | 26 863 | 25 442 |
| Brazil | 379 661 | 188 286 | 175 533 | 488 052[c] | 235 661[c] |
| Chile | 1 648 | 1 669 | 2 333 | 23 542 | 6 202 |
| Paraguay | – | – | 16 637 | 32 502 | 42 024 |
| Uruguay | 11 911 | 1 507 | 2 038 | 3 847 | Vigilance[b] |
| Total | 510 363 | 320 063 | 358 225 | 689 240 | 309 329 |
| Grand total | | | | | >2 187 220 |

[a] Attack phase only, excluding houses resprayed during vigilance phase.

[b] Attack phase complete, all areas now in vigilance phase.

[c] Includes houses sprayed against other triatomine species such as *T. brasiliensis* in north-east Brazil.

houses have been sprayed during the attack phase (Table 4), with a further 300 000 resprayed during the vigilance phase. The unit cost, averaged over all countries and including administration and training, has been about US$75 per house treated.

The second key indicator is the house infestation rate. Full evaluations are not yet available, and each country presents results in different forms. In Argentina, the attack phase was completed in 1995, when evaluation of 268 007 houses revealed *Triatoma infestans* in 20 022 of them, mainly in the provinces of Santiago del Estero and La Rioja. These houses are now being resprayed under the vigilance system. In Bolivia, it is estimated that about 600 000 houses remain to be sprayed, and the geographical distribution of *Triatoma infestans* remains little altered except for parts of Potosi, Tarija and Chuquisaca, where substantial areas have already been treated (Guillen *et al.*, 1997). In Brazil, *Triatoma infestans* can now be found only in isolated regions in the north of Minas Gerais, in parts of Rio Grande do Sul, and in a slightly more extensive region including parts of the states of Goias, Tocantins and Bahia. Elsewhere it appears to have been completely eliminated (Figure 2). In 1996, only 351 individual *Triatoma infestans* were captured during evaluation of 808 390 houses, which is a remarkable finding considering that, in 1983, over 84 000 *Triatoma infestans* were collected during routine house evaluations in Brazil. In Chile also, *Triatoma infestans* is now rarely captured, with the 1995 evaluation revealing bugs in only 334 houses, mainly in Region IV. In Paraguay, where full implementation of the programme was delayed due to budgetary restrictions, control has concentrated on the more heavily populated eastern part of the country (the departments


(a)
(b)
(c)

of Cordillera, Caazapá, Misiones and Paraguari), although substantial parts of the chaco region around the town of Filadelfia have also been treated. In Uruguay, *Triatoma infestans* is occasionally reported from houses in the departments of Salto Grande and Tacuarembo, but elsewhere only a few peridomestic specimens were collected during 1996. (After this chapter was written, on 25 March 1998 Uruguay was formally declared free of vector-borne Chagas disease transmission, although the evaluation committee emphasized that surveillance against *Triatoma infestans* should continue.)

In epidemiological terms, however, the key indicator is taken from serological surveys, especially of children born since the programme began although reductions in seroprevalence amongst other age-groups and blood donors can also provide useful information. During the 1980s, the WHO-TDR programme had supported a series of national seroprevalence surveys that provided the baseline against which current serological results can be assessed (cf. Tables 1 and 2). On the basis of partial results from more recent surveys, the incidence of new infections has clearly been substantially reduced in the programme area (Table 5). Data from blood donors also show a steady decline in seroprevalence, and a more marked decline in prevalence amongst younger age groups and women of childbearing age (Dias, 1997). The progress of infection control is also illustrated by data on hospital admissions for Chagas disease in Brazil, which show a progressive decline in accordance with the vector control interventions (Figure 3).

A further indicator involves estimates of the economic rate of return of the programme. At the outset, this was predicted to be at least 14% on a total investment of US$190–350 million (Schofield and Dias, 1991), although this estimate considered only savings in direct medical costs associated with chronic infections, and disregarded other important benefits such as those associated with improved labour productivity. A concurrent study in Chile, where these benefits were included, indicated benefits to the Chilean economy of around US$36 million per year, which can be contrasted with the annual expenditure on vector control of about US$300 000 (S. Corona and W. Apt, unpublished data) (Table 6). Since then, a detailed study from the province of Salta, in north-west Argentina,

*Figure 2* (opposite) Evolution of Chagas disease vector control in Brazil. The original endemic area (a) indicates the number of municipalities with active vector-borne transmission before 1983 when the first national control campaign was launched; (b) municipalities with *Triatoma infestans* in 1991 when the Southern Cone Programme was launched; (c) the results at the end of 1995. Of the 711 municipalities with *Triatoma infestans* in 1983, only 83 had this species in 1995. Note, however, that under the Brazilian reporting system, a municipality is shown to be infested even if only a single bug was encountered. (Data from FNS, Ministerio da Saúde, Brazilia, courtesy of A.C. Silveira and Vilma Feitosa.)

*Table 5* Seroprevalence of *Trypanosoma cruzi* infection in five Southern Cone countries from large-scale surveys before and after initiation of the Southern Cone Programme[a]

| | 1981–1985 | 1993–1995 | Reference |
|---|---|---|---|
| Argentina | 4.8 | 1.2 (18-year-old military recruits) | WHO (1996) |
| Brazil | 4.5 | 0.2 (7–14 years old) | WHO (1997a) |
| Chile | 5.4 | 1.4 (<10 years old) | Lorca *et al.* (1996) |
| Paraguay | 9.2 | 4.0 (18-year-old military recruits) | Southern Cone Commission (1997) |
| Uruguay | 2.4 | 0.2 (<12 years old) | WHO (1994) |

[a] Values are percentage seropositive in the age groups shown.

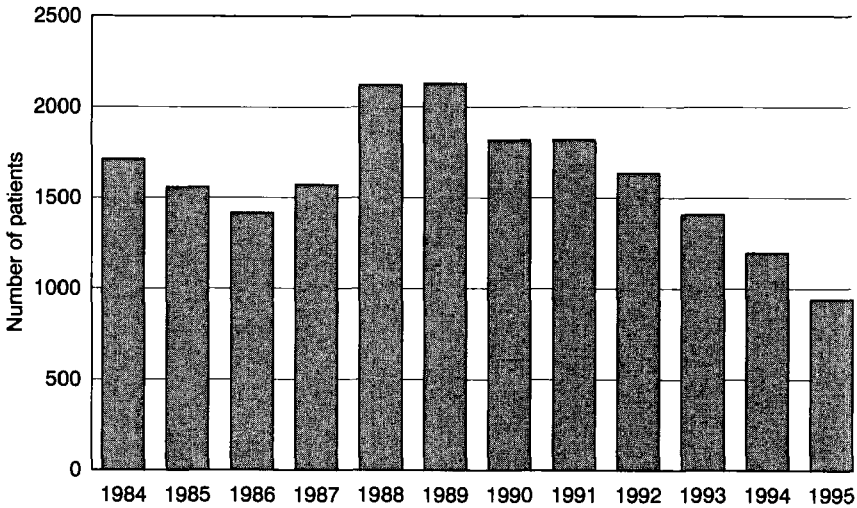


*Figure 3* Hospital admissions for Chagas disease in Brazil, 1984–1995. The number of admissions parallels the vector control interventions, with a decline during the first national campaign in 1983–1986, followed by an increase as Chagas disease control activities were interrupted. Since the start of the Southern Cone Programme in 1991, the number of admissions has declined steadily. (Data from FNS, Ministerio da Saude, Brazilia, courtesy of D. Akhavan.)

*Table 6* Government expenditure on Chagas disease vector control in the Southern Cone (US$)[a]

| | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 [b] |
|---|---|---|---|---|---|---|
| Argentina | 9 000 000 | 17 000 000 | 21 500 000 | 18 000 000 | 7 500 000 | 13 000 000 |
| Bolivia[c] | – | – | 83 000 | 717 035 | 706 943 | 2 300 000 |
| Brazil | – | 18 424 000 | – | 48 549 600 | 28 000 000 | 28 640 000 |
| Chile | 300 000 | 300 000 | 300 000 | 300 000 | 1 000 000 | 650 000 |
| Paraguay | – | 893 000 | 1 144 349 | 1 250 000 | 1 252 331 | 2 020 000 |
| Peru | – | – | – | – | – | 2 500 000 |
| Uruguay | 102 000 | 41 000 | 126 000 | 133 000 | 73 900 | 40 000 |

[a] Source: Intergovernment Commission for the elimination of vectorial transmission of Chagas disease and interruption of transfusional transmission of *Trypanosoma cruzi*.

[b] Provisionally budgeted figures.

[c] Excludes contributions from the United Nations World Food Programme.

indicated an economic rate of return of over 64%, although this study took account of interventions carried out since 1983 (Del Ray *et al.*, 1995; Basombrio *et al.*, 1998). Similarly, a study of the Brazilian programme, initiated by the World Bank, indicated average annual returns of over 35% on investments in Chagas disease control made since 1984 (Akhavan, 1997)*.

The indicator that is less frequently considered, however, derives from discussions with those living in houses previously infested with bugs. Their gratitude can be very moving.

## 8. REINFESTATION OF TREATED HOUSES

At the outset of the Initiative, it was recognized that reinfestation of treated houses could be an important technical limitation of the programme. Three

* The data given by Akhavan (1997) show that, in the 20 years up to 1995, the total national expenditure on Chagas disease control in Brazil was equivalent to just over US$530 million (in 1994 US$), of which 82% was expended on vector control activities and 18% on blood bank control and other measures. Avoided losses attributable to the vector control interventions amounted to just over US$3 billion (a return of US$7.16 for each dollar invested) compared with just US$78.9 million attributed to the blood screening component (a return of US$0.83 per dollar invested). The apparent poor return of the blood screening component was due partly to the high cost of screening (US$2.00–6.00 in state blood banks, rising to over US$10.00 in private clinics) but mainly because the death of patients requiring transfusion was generally due to their original condition, so that avoidance of death attributable to Chagas disease tended to be discounted.

scenarios were envisaged: (i) reinfestation from silvatic foci of *Triatoma infestans*; (ii) reinfestation from domestic foci of *Triatoma infestans*; and (iii) reinfestation by vector species other than *Triatoma infestans*. The first of these was applicable only to the Cochabamba–Sucre region of central Bolivia, where silvatic *Triatoma infestans* occur in isolated foci under rock piles, although genetic studies have now demonstrated incipient separation between silvatic and neighbouring domestic populations, with no evidence of active gene flow between them (Carlier *et al.*, 1996) nor of migration from silvatic foci to nearby houses from which the domestic bugs have been eliminated (Dujardin and Casini, 1996; Dujardin *et al.*, 1997a, b). The second could be applicable to any area where domestic *Triatoma infestans* populations remain untreated, and appears to have been a significant problem in parts of southern Brazil and neighbouring Uruguay during the early years of the programme. However, concerted action through a joint Uruguay–Brazil agreement now seems to have eliminated the remaining domestic foci of *Triatoma infestans* in this region (Southern Cone Commission, 1996). Elsewhere, apparent reinfestations with *Triatoma infestans* seem most frequently to represent local control difficulties where small residual populations have survived the initial treatment (e.g. Dujardin *et al.*, 1996). In some cases this was due to unusual construction features such as the dense roofs of brushwood and packed earth of some houses in the chaco region, or in one case a large clay oven, which provide deep shelter for domestic bugs, where insecticides cannot readily penetrate. In spite of this, community-based vigilance and selective re-treatment seem to eliminate such residual foci (e.g. Garcia Zapata, 1990).

The third reinfestation scenario, involving species other than *Triatoma infestans*, is more frequently reported. In central Brazil, for example, *Triatoma sordida* is now the most frequently collected species, primarily from peridomestic habitats. Several other species have also been reported, including *Panstrongylus megistus, Rhodnius neglectus, Triatoma vitticeps, Triatoma costalimai* and *Triatoma pseudomaculata*, although these too are almost invariably from peridomestic habitats. In southern Brazil and Uruguay, however, *Triatoma rubrovaria* has been reported invading dwellings as well as peridomestic habitats (R. Salvatella and R. Rosa, personal communication). In general, all these species were known from peridomestic habitats before interventions against *Triatoma infestans*, and their frequency seems to be increasing only in areas concurrently experiencing major land use changes. Moreover, even in areas such as northern Minas Gerais, where the peridomestic presence of *Triatoma sordida* has increased dramatically in recent years, there is no evidence of a recrudescence of *Trypanosoma cruzi* transmission (Diotaiuti *et al.*, 1994, 1995). Recent studies in a part of Bolivia where primary domestic infestations of *Triatoma sordida* have been found in houses where *Triatoma infestans* has never been recorded indicate that even

in these conditions *Triatoma sordida* is not a good colonizer of human dwellings and, although readily infected with *Trypanosoma cruzi*, is associated with very low human infection rates (Noireau *et al.*, 1997).

The potential importance of secondary vectors of *Trypanosoma cruzi* has been well recognized through the setting up of a WHO task force on 'non-domiciliary vectors of Chagas disease' and by the formation of the European Community–Latin America triatomine research network (ECLAT). The research need — for the whole of Latin America, not just the Southern Cone countries — is to develop genetic markers for monitoring bug populations, and to understand the mechanisms by which silvatic or peridomestic species of Triatominae may adapt to human dwellings and so assume greater epidemiological significance. Nevertheless, current evidence suggests that the basic strategy adopted by the Southern Cone countries, with its emphasis on community-based vigilance and selective intervention after the initial attack phase, is applicable to the control of other species of Triatominae wherever they assume local importance. It is to be hoped that additional measures can be implemented in the future, for example through programmes of economic development and rural housing improvement, in order to discourage further domestic colonization by other species of Triatominae, although it is recognized that such programmes are costly and difficult to implement on a large scale (e.g. Guillen *et al.*, 1997).

## 9. POLITICAL DEVELOPMENTS

A review of the history and progress of the Southern Cone Initiative would not be complete without mention of its important impact on countries outside the region. *Triatoma infestans* is certainly not the only vector of Chagas disease and the Southern Cone region is not the only region affected, but other Latin American countries (with the exception of Venezuela) had paid scant attention either to the disease or its vectors.

Based on the Southern Cone experience, WHO and PAHO have worked closely with the governments of these countries to develop legislation and operative systems for improved screening of blood banks. This not only reduces the transmission of *Trypanosoma cruzi* but also provides important indicators of areas most at risk and helps to demonstrate the epidemiological importance of the infection. The impetus has been maintained through research and a series of major workshops to demonstrate and discuss adaptation of the Southern Cone approach for new initiatives in other areas. In the last year, several new initiatives have been annnounced for Andean Pact countries and for Central America (WHO, 1997b). The

primary target in these regions is *Rhodnius prolixus*, which seems to be entirely domestic in Central America and may therefore be a candidate for local eradication (cf. Schofield *et al.*, 1996; Dujardin *et al.*, 1998b), although there is a series of other vectors in the region that may be less amenable to radical control because of their widespread silvatic ecotopes (Schofield and Dujardin, 1997). Nevertheless, the basic control strategy — large-scale attack followed by community-based vigilance and selective action — seems to offer a good chance that Chagas disease transmission can be substantially reduced throughout the Americas within the next few years.

## ACKNOWLEDGEMENTS

In this brief review of a continent-wide programme, it is impossible to include every aspect, or to acknowledge the roles played by all concerned. Those who contributed know who they are, and know also that the programme was not initiated to provide personal credit. The satisfaction comes from seeing scientific and operational expertise moulded into a remarkable initiative, and from knowing that one could now build any house in the previously endemic areas without risking colonization by *Triatoma infestans* and consequent transmission of Chagas disease.

We do, however, offer special tribute to those who have implemented the programme, especially the brigades of field personnel who patiently visit and check the houses, liaise with the local communities and carry out house spraying where necessary. We also formally acknowledge the important role of those organizations who contributed most to the initiation of the programme, particularly the Pan American Health Organization, the Commission of the European Communities (DGXII), Bayer AG, Roussel–Uclaf (now AgrEvo Environmental Health Ltd), Zeneca plc and Studio Maunoir (Geneva). We hope their support will continue.

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# *Phytomonas* and Other Trypanosomatid Parasites of Plants and Fruit*

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* This review is dedicated to the memory of Franklin G. Wallace, a pioneer in the modern taxonomy of trypanosomatids.

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## ABSTRACT

*Trypanosomatid parasites are fairly common in the latex, phloem, fruit sap, seed albumen, and even in the nectar, of many plant families. They are transmitted to the plants in the saliva of phytophagous hemipterous bugs (Insecta). Morphologically, plant trypanosomatids have no special characteristic, except perhaps a very twisted cell body. Most occur in plants as promastigotes and a few as choanomastigotes. It is still controversial whether or not they are pathogenic in lactiferous plants or fruit, but it is certain that the phloem parasites are pathogenic in coconut palms and coffee bushes. In these plants, they cause lethal diseases responsible for the destruction of many plantations in Central and South America, but fortunately nowhere else in the world. Probably more than one genus of Trypanosomatidae is represented among the plant parasites. The most important is certainly* Phytomonas, *but* Leptomonas, Crithidia *and* Herpetomonas *may also be present. The distinction between them is difficult and only recently have molecular markers become available to help in their identification. At present,* Phytomonas *can be identified by DNA hybridization with a specific probe (SL3') complementary to a sequence of the mini-exon or spliced leader gene. The development of a polymerase chain reaction coupled to SL3' hybridization has facilitated the detection of* Phytomonas *in plants. The phylogeny of* Phytomonas *is still being worked out. For the moment it can only be said that the genus is very close to* Herpetomonas.

## 1. INTRODUCTION

In the second half of the past century, although variously named, trypanosomatids were first described in worms, insects and vertebrates. At the beginning of this century, they were already well-known agents of human and animal diseases such as sleeping sickness, kala-azar, oriental sore, surra and nagana. However, only in 1909 were trypanosomes discovered in plants. This was when Alexandre Lafont was the Director of the *Laboratoire de Bacteriologie* of Mauritius, a French island off the east coast of Africa. Lafont was primarily concerned with surra, an equine trypanosomiasis, but was not deaf to rumours about the medicinal properties of local plants. In this respect, a colleague directed the attention of Lafont to the lactiferous Euphorbiaceae, particularly *Euphorbia pilulifera* (=*E. hirta*). This plant, popularly known as 'Jean Robert', was reputed to have anti-asmatique, emétique and many other curative properties. Out of curiosity, Lafont conceived a comparative study of the latex of various euphorbias. Years later, França (1920) and Holmes (1930) said that Lafont was considering latex as a possible culture medium for trypanosomes, but no historical record supports this assertion. Whatever his motives, since microscopy was the foremost scientific tool at the time, Lafont ordered his technician, known only as Mr David, to examine the latex of these plants. Studies on the starch grains of the latex might have turned out fruitless were it not for the keenness of Mr David, who promptly discovered fast-moving flagellates in the milky juice of the plants. After fixing, staining and microscopically examining the preparations, Lafont declared that the flagellates corresponded to a trypanosomatid of the genus *Leptomonas*. Most appropriately, Lafont named the flagellate after his technician as *Leptomonas davidi* (Lafont, 1909, 1910). The name 'David' was a well-deserved homage, but why *Leptomonas* and not *Herpetomonas*, *Crithidia* or something else?

Regardless of their generic name, plant trypanosomes became an instant 'hit'. Donovan immediately found them in the euphorbias of Madras. Shortly thereafter, French researchers confirmed the presence of trypanosomes in the euphorbias of almost all African and American French possessions, while researchers from all over the world quickly joined the dash to find plant trypanosomes in euphorbias. In 1916, in South America, another family of lactiferous plants (Asclepiadaceae) was found harbouring trypanosomes (Migone, 1916). Other plant families followed shortly. North America was late getting into the plant trypanosome hunt, which it did only in the 1920s (Noguchi and Tilden, 1926) but with increasing fervour thereafter. Oceania came last and never became too deeply involved with plant trypanosomatids (Bancroft, 1927; Holmes, 1931). In the space of 20 years, about four dozen different species of lactiferous

plants were found harbouring trypanosomatids on the five continents. In addition, there were repeated findings of flagellates in the same plant species from distinct geographical localities. It looked as if plant trypanosomes were omnipresent, which indeed they proved to be.

However, it is no credit to scientific creativity that scientists have, for almost 20 years, devoted themselves to searching in lactiferous plants only, ignoring the rest of the vegetable kingdom. Only in the 1930s did someone look for plant trypanosomes elsewhere. This was Gerold Stahel, a competent scientist working in Surinam who discovered flagellates in diseased coffee trees (Stahel, 1931a). It took scientists another 40 years to discover pathogenic trypanosomatids in diseased coconut and oil palms (Parthasarathy *et al.*, 1976; Dollet *et al.*, 1977) and in cassava (Kitajima *et al.*, 1986).

The most recent reports of the occurrence of trypanosomatids in plants are related to fruit. Although fruit flagellates were already known to Franchini (1922a) and Gibbs (1957), little attention was paid to them until the 1980s when reports of their occurrence grew exponentially (see Tables 1–3).

## 2. EARLY NOMENCLATURE

When Lafont examined stained preparations of the flagellates of *Euphorbia pilulifera* he had no difficulty in ascribing them to the family Trypanosomatidae, but he clearly had problems in defining their genus.

In 1909, the genera *Trypanosoma*, *Leishmania*, *Herpetomonas*, *Leptomonas* and *Crithidia* were already known. The first two genera comprised parasites of vertebrates and Lafont probably did not seriously consider them as possible genera for the new flagellates, although he recognized the similarity between plant flagellates and culture forms of *Leishmania* (see Lafont, 1910). Species of the genus *Crithidia* (Léger, 1902), as erroneously emended by Lühe (1906) and Patton and Strickland (1908), were said to display a diagnostic undulating membrane that was clearly missing from the *Euphorbia* flagellate. Thus, Lafont had but two choices: *Leptomonas* and *Herpetomonas*.

Both genera were created by Kent (1880), *Leptomonas* to accommodate a trypanosomatid discovered by Bütschli (1878) in the gut of a nematode and *Herpetomonas* for parasites of the intestinal tract of the housefly (Burnett, 1851; Leidy 1853). In a review, Bütschli (1884) considered the two genera to be synonymous, giving precedence to *Herpetomonas*. The problems might have ended there because, as far as it was possible to discern at the time, the two genera were actually identical. However, Prowazek (1904) reported that the housefly flagellate had two flagella, an erroneous observation that served to reinstate the distinction between the genera *Herpetomonas* and *Leptomonas*.

Not surprisingly, in the description of his flagellate, Lafont expressed doubts about its proper generic placement. After some thought, Lafont chose the genus *Leptomonas* on the following basis: 'one nucleus, a little corpuscle (centrosome or blepharoplast), one flagellum, the longitudinal reproduction together with the absence of an undulating membrane permit one to classify this flagellate in the genus *Leptomonas*' (translated from Lafont, 1910). Not very convincing, but the best Lafont could do at the time. One can easily understand the difficulties of Lafont by examining the drawings of the Portuguese Carlos França (1920), which purportedly distinguished *Leptomonas* from *Herpetomonas* (Figure 1).

Just after Lafont took the trouble to ascribe the plant flagellate to the genus *Leptomonas*, Donovan, out of the blue, proposed the genus *Phytomonas* for trypanosomatids of plants in general (Donovan, 1909).

Many authors refused to recognize the new genus *Phytomonas*. What França said about the proposal of Donovan can be translated as: 'I think

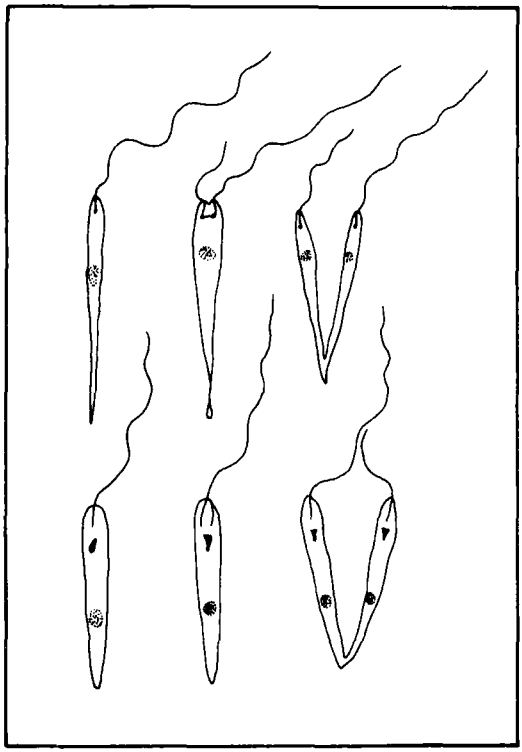

*Figure 1* *Leptomonas* (top) and *Herpetomonas* (bottom). This diagram, redrawn from França (1920), depicts the morphological characteristics which, according to the author, distinguished the two genera.

that this is going too far into generic cutting. The flagellate . . . has nothing in its morphology that separates it from the forms living in the gut and hemocele of insects' (França, 1914).

For the next 18 years the *Phytomonas* denomination was ignored by authors who erratically used either *Leptomonas* or *Herpetomonas*, and sometimes even *Trypanosoma* (for example, see Franchini, 1922b). Only in 1927 was the name *Phytomonas* officially given to a new species of plant trypanosomatid (Aragão, 1927). Ironically, this first *Phytomonas* received the name of *Phytomonas francai*, in honour of Carlos França, the foremost foe of the genus *Phytomonas*.

That the name *Phytomonas* was not very popular with earlier researchers is shown by the fact that, among the 18 isolates from plants that received a new species name (Table 4), only six were originally assigned to the genus *Phytomonas* and five of them after 1930.

It was only in the 1970s and 1980s that the name *Phytomonas* gained acceptance as the generic name for all flagellates of plants. As we shall see, this was also wrong because trypanosomatids of more than one genus turned out to occur in plants. Thus, precipitately naming flagellates recovered from plants as *Phytomonas* should be avoided.

At a recent meeting on *Phytomonas*, Keith Vickerman (1996, unpublished observations) suggested that plant flagellates could be divided into 'lacticola, phloemicola and fructicola' according to their origin from latex, phloem and fruit, respectively. Vickerman's nomenclature is used in this review in an informal, non-committal way, with the objective of avoiding premature taxonomic engagement whenever the generic placement of a flagellate under discussion is unclear. *Phytomonas* is used only when it has been ascertained that the flagellate actually belongs to this genus (see Section 7). 'Floricola' is added to Vickerman's categories, to designate trypanosomatids isolated from flowers.

## 3. MORPHOLOGY

### 3.1. General Morphology

Very few plant flagellates have been reported to be choanomastigotes (Noguchi and Tilden, 1926; McGhee and Hanson, 1971; Fiorini *et al.*, 1995b) or to develop opisthomastigotes in culture (Franchini, 1922b; McGhee and Postell, 1976; Attias and De Souza, 1985). The overwhelming majority of plant flagellates correspond to promastigotes according to the terminology of Hoare and Wallace (1966).

Morphology does not distinguish plant trypanosomatids from insect trypanosomatids. Perhaps for this reason the two groups informally con-

stitute 'the lower trypanosomatids', a denomination which, by the way, is apparently in disagreement with their phylogenetic position (see Section 8).

At the light microscope level, stained preparations of plant flagellates display the standard cellular architecture of the Trypanosomatidae: a nucleus, a kinetoplast, an oblong cell body and a flagellum. Twisted cellular bodies with many torsions along their longitudinal axis are a common feature of plant flagellates, as already noticed and drawn by Lafont (1910) (Figure 2). Twisted bodies are not very common in promastigotes of other trypanosomatid genera (*Leptomonas* and *Herpetomonas*) but are common in promastigotes of the gut of flies (Roubaud, 1911a,b). Thus, although suggestive of plant origin, long twisted bodies are not diagnostic of *Phytomonas*.

Like insect trypanosomatids, plant flagellates multiply by binary longitudinal fission, although an unusual form of multiple transverse division has been decribed in the flagellate from the latex of *Ficus* by Franchini (1931) and Holmes (1935). Recently, M.A. Desouza (1997) described in a flagellate from latex 'pairs of apposed cells attached by their posterior ends' and migration of the corresponding nuclei, which she interpreted as indicative of sexual fusion.

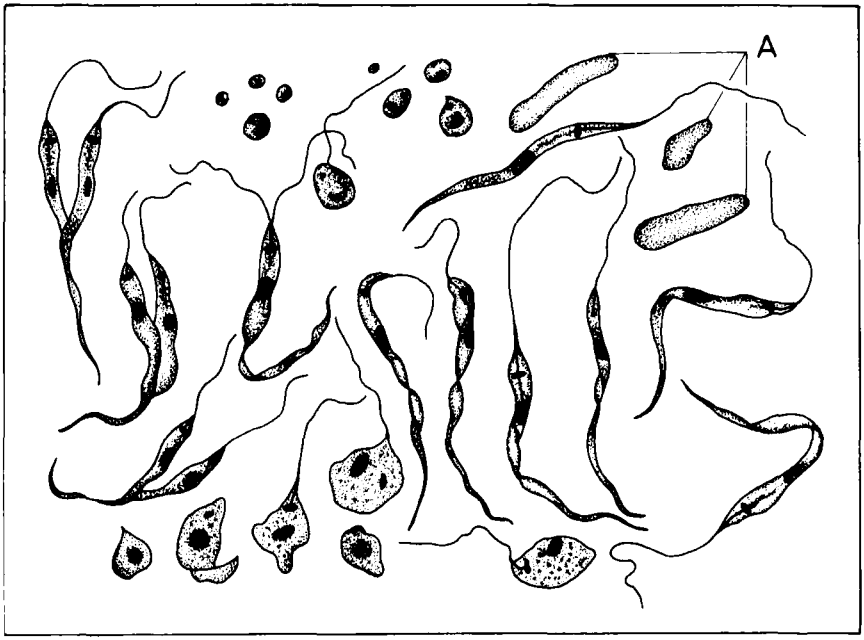


*Figure 2* Flagellates from the latex of *Euphorbia*. 'A' shows starch granules. (Redrawn from Lafont, 1910.)

The facts that almost all plant flagellates reproduce by binary fission and that they are promastigotes and remain as such throughout their life cycles may give the false idea of a morphologically monotonous group, whereas, in fact, the hallmark of plant flagellates is their extreme polymorphism. The same organism may have different sizes and shapes not only in different hosts or culture media but also in the same host. A few examples suffice to illustrate this point.

França (1920) encountered, in the latex of euphorbias, 'elongated parasites twisted 2–3 times'; 'sturdy forms without torsions'; and 'round and oval forms without flagellum' (translated from França, 1920). In the flagellate of *Asclepias*, França (1921) noted that 'Ce que la culture de *L. elmassiani* a de plus remarquable, c'est l'extraordinaire polymorphisme'.

The polymorphism of plant flagellates is also noticeable in the organs of their vectors. Vickerman (1962) recorded promastigotes with a five-fold difference in length in the digestive tract of the vector of the *Pergularia* flagellate. Jankevicius *et al.* (1989) recorded an even greater polymorphism in the cell body length of promastigotes of the gut (4.1 and 16.6 μm), Malpighian tubes (5.6 μm) and salivary glands (5.7, 24 and 80 μm) of the vector of *Phytomonas serpens*, the tomato flagellate (Figure 3).

In culture, plant flagellates are also polymorphic. The same flagellate in different culture media shows considerable morphological differences.

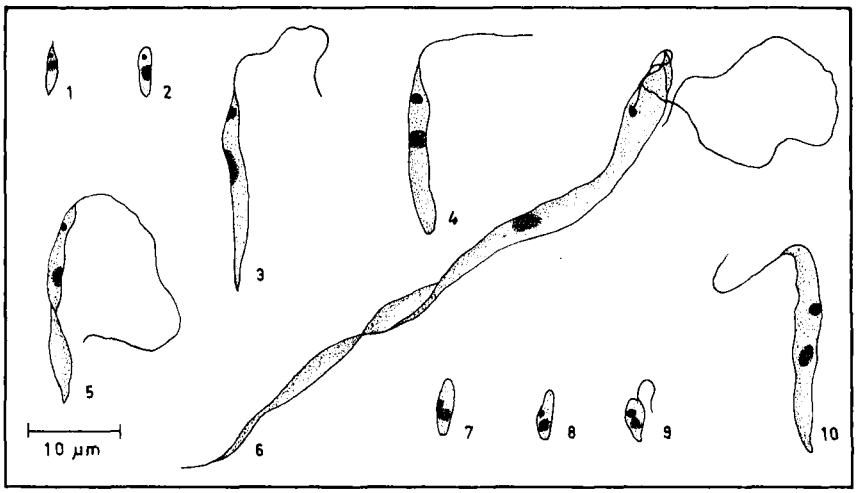


*Figure 3* Polymorphism of *Phytomonas serpens*, the tomato parasite: 1 and 2, forms from the fruit sap; 3 and 4, flagellates from biphasic blood agar/LIT medium; 5 and 6, large flagellates from old stationary LIT medium cultures; 7 and 8, non-flagellated forms from the urine of the hemipteran vector; 9 and 10, flagellates from the digestive tract of the vector.

Franchini was the first to notice this: 'we have to point out that in different media the shape of the parasite has greatly changed' (translated from Franchini, 1923a). Noguchi and Tilden (1926) also remarked on the morphological changes in different media: 'the morphological features vary greatly according to the medium on which the organism was grown'. Extensive polymorphism has also been observed by different authors at different times (França, 1921; Jankevicius *et al.*, 1989; Romeiro *et al.*, 1994).

The experienced Robert McGhee added evidence of the morphological plasticity of plant flagellates by showing that promastigotes of an asclepiad turned into choanomastigote-like flagellates when inoculated into a member of the Apocynaceae (McGhee and Hanson, 1971).

Promastigotes also may undergo morphological changes in the same culture medium. Jankevicius *et al.* (1989) reported that 10-μm long, non-twisted promastigotes predominated in exponentially growing cultures of *Phytomonas serpens* in liver infusion tryptose (LIT) medium. In old cultures there appeared gigantic, twisted promastigotes 60 μm long, excluding the flagellum (see Figure 3).

Examples of polymorphism and morphological plasticity in plant flagellates are numerous. Some may be artefacts resulting from non-physiological pH, osmotic and nutritional changes. Others are clearly not.

I consider that the polymorphism of plant flagellates has two important implications.

1. Morphologically distinct promastigotes may correspond to unrecognized developmental stages in the life cycles of the flagellates. In each of their hosts, plant flagellates undergo morphological and, presumably, physiological changes. These changes may be sequentially programmed and may correspond to steps in the flagellate's life cycle, as in the other two heteroxenic genera *Trypanosoma* and *Leishmania*. What may have hindered the recognition of specialized forms in the life cycle of *Phytomonas* is that flagellates remain as promastigotes all the time. Although in many instances life cycles have been reproduced in the laboratory, so far no specific function has been assigned to a specific form. This subject is entirely open to investigation.
2. The marked polymorphism of plant flagellates renders morphology a poor criterion for species indentification. Due to intraspecific polymorphism, promastigotes of one species may incidentally resemble more closely developmental forms of another species. This impairs the use of morphology to distinguish species of plant flagellates. Measurements of body and flagellum length as well as distance between nucleus and kinetoplast (of considerable taxonomic use in the past) are equally useless for this purpose. That is why they are intentionally omitted from this review.

### 3.2. Ultrastructure

The ultrastructure of Trypanosomatidae is well known and its general features have been described in comprehensive reviews (Vickerman, 1974; Vickerman and Preston, 1976; Molyneux, 1977; De Souza, 1984). Electron microscopic studies on the genus *Phytomonas* in particular were first carried out by Paulin and McGhee (1971). These authors studied the flagellate of *Asclepias curassavica*, *Phytomonas elmassiani*, comparing its ultrastructure with that of other trypanosomatids.

Many plant flagellates have been examined by electron microscopy since then (Thomas *et al.*, 1979; Postell and McGhee, 1981; Attias and De Souza, 1985, 1986, 1992, 1995; Kitajima *et al.*, 1986; Attias *et al.*, 1988, 1992, 1993; Jankevicius *et al.*, 1989; Almeida *et al.*, 1990; Freymuller *et al.*, 1990; De Souza and Attias, 1991; Sanchez-Moreno *et al.*, 1992). As interesting as their twisted bodies can be, yielding star-like images in cross-sections (Figure 4A,B), nothing has been found in plant flagellates that might signify a departure from the general organization of trypanosomatids. The only trait that seems to be a little more pronounced in plant flagellates is the large quantity of glycosomes, whose three-dimensional organization has been studied in detail by Attias and De Souza (1995). Sometimes, a cell may resemble a sac of glycosomes, whereas in other cells these organelles are arranged as rows of dense granules (Attias *et al.*, 1988; Freymuller *et al.*, 1990). Whatever their arrangement, glycosomes are always present in larger quantities than in any other genus of Trypanosomatidae (Figure 4C).

In summary, there is no electron microscopic characteristic that could be said to be diagnostic of plant flagellates in general or of *Phytomonas* in particular. However, this is not to say that electron microscopy cannot be used as a tool for distinguishing flagellates. Attias *et al.* (1988), while comparing plant flagellates, have disclosed many distinctive and 'personalized' characteristics that could be used for the identification of a particular flagellate. Thus, in *Phytomonas francai* the cisternae of the endoplasmic reticulum (ER) are organized in parallel rows along the longitudinal axis of the cell just below the subpellicular tubules and a large number of lipid droplets crowd around these ER cisternae. The flagellate isolated from *Euphorbia pinea* has a very peculiar ER organization, the cisternae apparently originating somewhere in the middle of the cellular body and branching out in all directions. The flagellate from *Euphorbia hyssopifolia* has a conspicuous contractile vacuole close to the flagellar pocket. One of the strains of flagellate isolated from maize possessed a noticeable surface coat, whereas others did not (Jankevicius *et al.*, 1993). Some *Phytomonas* isolates had an unusual intracellular flagellum that encircled the nucleus before emerging at the anterior end (Milder *et al.*, 1990). Most strains of *Phytomonas staheli*, the coconut parasite, were found to contain virus-like par-

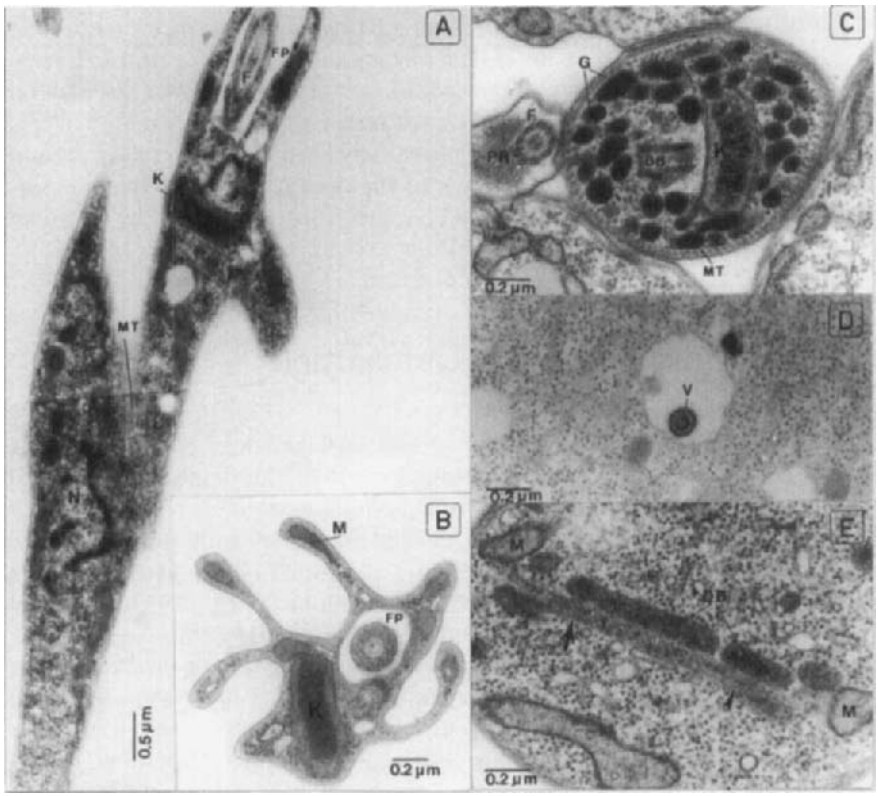


*Figure 4* Electron microscopy of plant flagellates. A, *Phytomonas serpens*: longitudinal section showing an apparent cleft in the flagellate which actually corresponds to one of the cell body twists. B, transverse section through body twists of *P. serpens* in the salivary gland of its vector, producing a star-like image. C, large numbers of glycosomes (G) in the cytoplasm of a plant isolate of *P. serpens*. D, a virus-like particle inside a large vacuole in *P. serpens*. E, a novel structure consisting of a double string of vesicles (arrow) in a mesh of fibrils (arrowhead), parallel to an array of unidentified dense bodies. BB, basal body; DB, dense bodies; F, flagellum; FP, flagellar pocket; K, kinetoplast; M, mitochondrion; MT, microtubules; N, nucleus; PR, paraxial rod; V, virus-like particle. (Courtesy of Dr Regina Milder.)

ticles (Marché *et al.*, 1993), which can also be seen in the cytoplasm of *Phytomonas serpens*, the tomato parasite (Figure 4D). Rhabdovirus-like particles were also seen in the cytoplasm of the flagellates from *Asclepias curassavica* (see Dollet and Gargani, 1989).

These few examples suffice to demonstrate that, by providing a kind of visual 'fingerprint', detailed electron microscopic documentation of plant

flagellates may be a powerful tool in at least two circumstances: (i) to help certify the authenticity of an isolate in possible cases of cultures having been mixed; and (ii) to serve as an additional argument in the description of new species and in the comparison of isolates.

However, and most importantly, there may exist in plant trypanosomatids unidentified structures, as shown in Figure 4E, that may be of importance for the understanding of cell architecture in general or of plant flagellates in particular.

## 4. OCCURRENCE AND WORLD DISTRIBUTION

Tables 1–3 are intended to list plant hosts of flagellates known up to July 1997. They are based on a critical assessment of the data of the original papers cited in the tables and of the following reviews, some of which deal preferentially, whereas others deal incidentally, with the occurrence of flagellates in plants: França (1914); Teodoro (1920); Mesnil (1921); Migone (1922); Ponta-Delgada (1922); Franchini (1922c, 1923b); Aubertot (1925, 1927); Bensaude (1925); Gaschen (1926); Wenyon (1926); Holmes (1930); Lipa (1958, 1963); Wallace *et al.* (1983, 1992); Dollet (1984, 1991); Camargo *et al.* (1990); Podlipaev (1990); Camargo and Wallace (1994).

Tables 1–3 record only the first report of a new host species. The same host species may have been recorded later as harbouring flagellates in other geographical regions. Although relevant for emphasizing the ubiquity of plant flagellates, these tautological reports (Bouet and Roubaud, 1911; Lafont, 1911a,b; Léger, 1911; Noc and Stevenel, 1911; Rodhain and Bequaert, 1911; Iturbe, 1918; Laveran and Franchini, 1921; Franchini, 1922a,b, 1923a,c,d; Aubertot, 1923, 1925; Holmes, 1925a, 1935; Noguchi and Tilden, 1926; Rodhain, 1926; Hewitt, 1940; Harvey and Lee, 1943; Ruiz, 1958a, 1961; Lipa, 1963; Vitteta and Guttman, 1967; McGhee and McGhee, 1971; Ayala *et al.*, 1975; Bezerra and Figueiredo, 1982; Oliveira and Bezerra, 1982; Kastelein and Parsadi, 1984; Kastelein *et al.*, 1984a; Kastelein, 1985; Kastelein and Camargo, 1990; Fiorini *et al.*, 1993a; Sanchez-Moreno *et al.*, 1993; Podlipaev, 1995) are not included in the tables.

Duplicate findings are included in the tables only when a new species name was given to the flagellate or a culture was obtained which is currently available. In the latter case I also provide the name of the depositary of the culture.

The tables record only naturally infected species. Species infected experimentally, like those described by Laveran and Franchini (1920), Franchini

(1922d, 1923e), Aragão (1931), Hanson *et al.* (1966), McGhee and Hanson (1971) and Vermeulen (1968), are not listed.

Host species names are those given in the original publication in spite of the fact that some genera and species have been altered in later taxonomic revisions.

Table 4 lists the plant flagellates that have been given new species names and their respective hosts. In fact, before the redefining criteria recently proposed for the genus *Phytomonas* (see Nunes *et al.*, 1995; Teixeira *et al.*, 1996; this review, Section 7), all flagellates listed in Table 4 should have been placed in this genus according to the then prevailing host origin criterion (Donovan, 1909). If they were not, the cause was not the authors' ignorance of Donovan's proposal but, more probably, resistance to it. Reviewers have tried to enforce the rules of nomenclature by renaming all species of plant flagellates as *Phytomonas* (e.g. Wenyon, 1926; Camargo *et al.*, 1990; Wallace *et al.*, 1992). However, at least four species passed unnoticed by them and still bear generic names distinct from *Phytomonas*: *Herpetomonas funtumiae*, *Trypanosoma euphorbiae*, *Herpetomonas euphorbiae* and *Crithidia oncopelti* (see Appendix).

## 4.1. Lacticola (Latex Flagellates)

Lacticolous flagellates, or latex flagellates for short, occur in many families of lactiferous plants (Table 1). Dubious or somewhat confused reports have been omitted from the table. After Lafont's initial finding, there was a rush to detect flagellates in plants. In many cases reports have not been thoroughly documented, if documented at all. This was particularly the case with some of the papers by Giuseppe Franchini, a proficient discoverer of flagellates, amoebas and spirochaetes in lactiferous plants. Mathilde Bensaude (1925) considered 'hasty the series of preliminary notes of Dr Franchini' and Francis Holmes (1930) warned that 'nuclei of latex cells of the plants stream out with the latex . . . they have been taken for micro-organisms'. Some of Franchini's notes were indeed hasty. For instance, when referring, in French, to an undetermined and an unknown species of *Euphorbia*, he did not hesitate to italicize the words in pseudo-Linnean notation as, respectively, *Euphorbia indéterminée* (Franchini, 1922e) and *Euphorbia inconnue* (Franchini, 1922f). However, hastiness was not unique to Franchini and not a few of his contemporaries produced papers that left something to be desired in terms of scientific rigour. Reports that did not present convincing evidence of the real presence and nature of the flagellates, such as those by Franchini (1922a,e,g,h), Nelson (1922) and Ioff (1927), are not listed in Table 1.

*Table 1* Host plants of latex trypanosomatids

| Host plants | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| **Euphorbiaceae** | | | | |
| *Euphorbia pilulifera* | Africa | *Leptomonas davidi* | Lafont, 1909 | – |
| *Eu. pilulifera* | Asia | *Phytomonas davidi* | Donovan, 1909 | – |
| *Eu. thymifolia* | Africa | None | Lafont, 1910 | – |
| *Eu. hypericifolia* | Africa | None | Lafont, 1910 | – |
| *Eu. peplus* | Europe | None | França, 1911 | – |
| *Eu. segetalis* | Europe | None | França, 1911 | – |
| *Eu. indica* | Africa | None | Rodhain and Bequaert, 1911 | – |
| *Eu. schimperiana* | Europe | None | Visentini, 1914 | – |
| *Eu. cupani* | Europe | None | Visentini, 1914 | – |
| *Eu. dulcis* | Europe | None | Laveran and Franchini, 1920 | – |
| *Eu. falcata* | Europe | None | Laveran and Franchini, 1920 | – |
| *Eu. nereifolia* | Europe | None | Laveran and Franchini, 1920 | – |
| *Eu. virosa* | Europe | None | Laveran and Franchini, 1920 | – |
| *Eu. grandis* | Europe | None | Laveran and Franchini, 1920 | – |
| *Eu. peploides* | Africa | None | Sergent, 1921 | – |
| *Eu. helioscopica* | Europe | None | Zotta, 1921 | – |
| *Eu. esula* | Europe | None | Zotta, 1921 | – |
| *Eu. gerardiana* | Europe | None | Galli-Valerio, 1921 | – |
| *Eu. coerulescens* | Europe | *Trypanosoma euphorbiae* | Franchini, 1922b | – |
| *Elaeophorbia drupifera* | Europe | None | Franchini, 1922h | – |
| *Exoecaria emmarginata* | Europe | None | Franchini, 1922h | – |
| *Euphorbia antiquorum* | Europe | None | Franchini, 1922f | – |

| | | | | |
|---|---|---|---|---|
| *Eu. laro* | Europe | None | Franchini, 1922e | – |
| *Eu. cereiformis* | Europe | None | Franchini, 1923a | – |
| *Eu. splendens* | Europe | None | Franchini, 1923g | – |
| *Eu. officinarum* | Europe | None | Franchini, 1923g | – |
| *Eu. cereiformis* | Europe | *Herpetomonas euphorbiae* | Nieschulz, 1924 | – |
| *Eu. callitrichoides* | Americas | None | Strong, 1924 | – |
| *Eu. striata* | Africa | None | Fantham, 1925 | – |
| *Manihot palmata* | Americas | *Phytomonas francai* | Aragão, 1927 | – |
| *Euphorbia uralensis* | Asia | None | Ioff, 1927 | – |
| *Eu. characias* | Europe | None | Poisson, 1930 | – |
| *Eu. tirucalli* | Africa | *Phytomonas tirucalli* | Reichenow, 1940 | – |
| *Eu. heterophylla* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. cyathophora* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. pinetorum* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. adenoptera* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. buxifolia* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. deltoidea* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. hirta* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. hyssopifolia* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. maculata* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. mathewsii* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. oligonifolia* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. tracyi* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. conferta* | Americas | None | Harvey and Lee, 1943 | – |
| *Eu. braziliensis* | Americas | None | Siqueira-Jaccoud, 1955 | – |

*Table 1* Host plants of latex trypanosomatids (*continued*)

| Host plants | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| *Jatropha macrantha* | Americas | None | Burstein and Romero, 1956 | MD, TCC/USP[c] |
| *Euphorbia gemella* | Americas | None | McGhee and Postell, 1976 | – |
| *Eu. heterophylla* | Americas | *Phytomonas davidi* | McGhee and Postell, 1976 | ATCC 30287, TCC/USP[d] |
| *Eu. foliolosa* | Americas | None | Dollet *et al.*, 1979 | – |
| *Eu. prostrata* | Americas | None | Dollet *et al.*, 1979 | – |
| *Eu. bacteriana* | Americas | None | Dollet *et al.*, 1979 | – |
| *Eu. hirtella* | Americas | None | Dollet *et al.*, 1979 | – |
| *Eu. pinea* | Europe | *Phytomonas* sp. | Dollet *et al.*, 1982 | MD, TCC/USP[c] |
| *Eu. lasiocarpa* | Americas | None | Dollet *et al.*, 1983 | – |
| *Eu. hirta* | Europe | *Phytomonas* sp. | Kastelein *et al.*, 1984b | TCC/USP[c] |
| *Eu. characias* | Europe | *Phytomonas* sp. | Dollet, 1984 | MD, TCC/USP[c] |
| *Manihot esculenta* | Americas | *Phytomonas francai* | Vainstein and Roitman, 1986 | TCC/USP[c] |
| *Eu. hyssopifolia* | Americas | *Phytomonas* sp. | Attias and De Souza, 1986 | WS, ATCC 50166, TCC/USP[d] |
| *Chamaesyce thymifolia* | Americas | *Phytomonas* sp. | Santos *et al.*, 1994 | WS |
| **Asclepiadaceae** | | | | |
| *Araujia angustifolia* | Americas | *Leptomonas elmassiani* | Migone, 1916 | – |
| *Morrenia odorata* | Americas | *Leptomonas bordasi* | Migone, in França, 1921 | – |
| *Cynanchum acutum* | Europe | None | Zotta, 1924 | – |
| *Asclepias syriaca* | Americas | *Herpetomonas elmassiani* | Holmes, 1924 | – |
| *As. curassavica* | Americas | None | Holmes, 1925b | – |
| *Daemia extensa* | Africa | None | Rodhain, 1926 | – |

| | | | | |
|---|---|---|---|---|
| *Asclepias nivea* | Americas | *Herpetomonas oncopelti* | Noguchi and Tilden, 1926 | – |
| *As. syriaca* | Americas | *Herpetomonas oncopelti* | Noguchi and Tilden, 1926 | ATCC 12982, TCC/USP[d] |
| *Sarcostemma australe* | Oceania | None | Bancroft, 1927 | – |
| *Hoya australis* | Oceania | None | Bancroft, 1927 | – |
| *Secamone elliptica* | Oceania | None | Bancroft, 1927 | – |
| *Asclepias glauscescens* | Americas | None | Hewitt, 1940 | – |
| *Funastrum clausum* | Americas | None | Harvey and Lee, 1943 | – |
| *Asclepias curassavica* | Americas | *Phytomonas elmassiani* | Ruiz, 1958a | – |
| *Pergularia extensa* | Africa | None | Vickerman, 1962 | – |
| *Sarcostemma clausum* | Americas | None | McGhee and McGhee, 1971 | – |
| *Asclepias incarnata* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. obovata* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. michauxii* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. humistrata* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. amplexicaulis* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. tuberosa* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. viridiflora* | Americas | None | McGhee and McGhee, 1971 | – |
| *As. exaltata* | Americas | None | McGhee and McGhee, 1971 | – |
| *Cynanchum laeve* | Americas | None | McGhee and McGhee, 1971 | – |
| *Asclepias fruticosa* | Americas | None | Ayala *et al.*, 1975 | – |
| *Blepharodon nitidus* | Americas | *Phytomonas* sp. | Kastelein *et al.*, 1984b | TCC/USP[c] |
| *Cynanchum sibiricum* | Asia | None | Podlipaev, 1986 | – |
| **Apocynaceae** | | | | |
| *Cerbera odollan* | Europe | None | Franchini, 1922h | – |
| *Funtumia elastica* | Europe | *Herpetomonas funtumiae* | Franchini, 1922c | – |
| *Thevetia nereifolia* | Europe | None | Franchini, 1922c | – |
| *Allamanda cathartica* | Americas | *Phytomonas* sp. | Kastelein *et al.*, 1984b | TCC/USP[c] |
| *Bonafousia tetrastachya* | Americas | None | Kastelein *et al.*, 1984b | – |
| *Mandevilla scabra* | Americas | *Phytomonas* sp. | Kastelein *et al.*, 1984b | TCC/USP[c] |
| *Rhabdadenia biflora* | Americas | *Phytomonas* sp. | Kastelein *et al.*, 1984b | TCC/USP[c] |

*Table 1* Host plants of latex trypanosomatids (*continued*)

| Host plants | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| **Moraceae** | | | | |
| *Ficus benjamina* | Europe | None | Franchini, 1922h | – |
| *Fi. parietalis* | Europe | None | Franchini, 1922a | – |
| *Fi. carica* | Europe | None | Franchini, 1922e | – |
| *Fi. edulis* | Africa | *Herpetomonas ficuum* | Fantham, 1925 | – |
| *Fi. scabra* | Oceania | None | Bancroft, 1927 | – |
| *Fi. scabra* | Oceania | *Herpetomonas bancrofti* | Holmes, 1931 | – |
| *Fi. hochstetteri* | Africa | *Herpetomonas ganorae* | Franchini, 1931 | – |
| *Fi. costarricana* | Americas | *Phytomonas tortuosa* | Ruiz, 1958b | – |
| **Sapotaceae** | | | | |
| *Sideroxylon inerme* | Europe | None | Franchini, 1922h | – |
| **Cecropiaceae** | | | | |
| *Cecropia surinamensis* | Americas | None | Kastelein *et al.*, 1984b | – |
| *Ce. palmata* | Americas | None | De Leeuw and Kastelein, 1987 | – |

[a]None, no specific name was given by the original describer.

[b]Depositaries are identified in the Appendix. They correspond to (i) the experimenter who isolated the flagellate, if he still keeps the cultures or (ii) the Trypanosomatid Culture Collection of the University of São Paulo (TCC/USP) in cases in which it received first-hand cultures from the researcher who isolated the flagellate. Three species deposited at the American Type Culture Collection (ATCC) also are listed.

[c]Culture flagellates that have been tested by several methods and found to correspond to *Phytomonas* (see Section 7).

[d]The flagellate is not *Phytomonas*.

### 4.2. Phloemicola (Phloem Flagellates)

Host plants of phloemicolous, or simply phloem, flagellates are listed in Table 2. Although coffee trees and other plants harbour phloem flagellates, palms are their preferred hosts. Some of the flagellates of palms are also encountered in the xylem. I have not distinguished between palms with infected phloem and xylem and palms with infected phloem alone. Citations that did not record the presence of flagellates, although recording symptoms or signs of flagellate infection (Vermeulen, 1968; Slobbe, 1977; Parthasarathy and Slobbe, 1978; Slobbe *et al.*, 1978), are also not included in Table 2.

### 4.3. Fructicola (Fruit Flagellates)

Fructicolous flagellates (fruit flagellates) have been found in fleshy fruits such as berries (tomato, mulberry, grape) and hesperidia (orange, bergamot) as well as in dry fruits such as legumes (bean, soybean) and caryopses (corn [maize]). However, Table 3 does not record the type of host fruit nor the part in which the flagellates were encountered. This is because most reports do not provide this kind of information.

### 4.4. Floricola (Flower Flagellates)

The search for flagellates in flowers (floricolous or simply flower flagellates) has been a neglected field. The first record of flagellates in a flower was that by Galli-Valerio (1920), in the nectar of a Swiss *Colchicus autumnalis*. The next record was 75 years later when Fiorini *et al.* (1995a) saw flagellates in the flowers of a squash, *Cucurbita moschata*, in Brazil. Cultures of this flagellate are being maintained by two depositaries (JF and TCC/USP; see Appendix). It seems unreasonable to assume that flower flagellates are restricted to these two flowers. More likely, many reports on flower hosts will appear in the future.

## 5. CULTURE AND NUTRITION

### 5.1. Culture

Lafont attempted to culture the latex flagellates as soon as he found them, but they survived for only a few days in coverslip-sealed latex preparations

*Table 2* Host plants of phloem trypanosomatids

| Host plant | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| **Rubiaceae** | | | | |
| *Coffea liberica* | Americas | *Phytomonas leptovasorum* | Stahel, 1931a | – |
| *Co. arabica* | Americas | None | Stahel, 1931b | – |
| **Palmae** | | | | |
| *Cocos nucifera* | Americas | None | Parthasarathy *et al.*, 1976 | – |
| *Elaeis guineensis* | Americas | None | Dollet *et al.*, 1977 | – |
| *Maximiliana maripa* | Americas | None | Slobbe *et al.*, 1978 | – |
| *Cocos nucifera* | Americas | *Phytomonas staheli* | McGhee and McGhee, 1979 | – |
| *Elaeis guineensis* | Americas | *Phytomonas staheli* | McGhee and McGhee, 1979 | – |
| *Attalea funifera* | Americas | None | Bezerra *et al.*, 1983 | – |
| *Betinckia nicobarica* | Americas | None | Kastelein, 1987 | – |
| *Roystonea regia* | Americas | None | Attias *et al.*, 1987b | – |
| *Cocos nucifera* | Americas | None | Menara *et al.*, 1988 | MD |
| *Elaeis guineensis* | Americas | None | Gargani *et al.*, 1992 | MD |
| **Euphorbiaceae** | | | | |
| *Euphorbia lasiocarpa* | Americas | None | Dollet *et al.*, 1983 | – |
| **Zingiberaceae** | | | | |
| *Alpinia purpurata* | Americas | None | P. Hunt, in Muller *et al.*, 1994 | MD |
| **Amaranthaceae** | | | | |
| *Amaranthus* sp. | Europe | None | M. Sanchez-Moreno, in Muller *et al.*, 1994 | SM, TCC/USP[c] |
| **Leguminosae** | | | | |
| *Trifolium* sp. | Europe | None | M. Sanchez-Moreno, in Muller *et al., 1994* | SM, TCC/USP[c] |

[a] None, no specific name was given by the original describer.

[b] Depositaries are identified in the Appendix. They correspond to (i) the experimenter who isolated the flagellate, if he still keeps the cultures or (ii) the Trypanosomatid Culture Collection of the University of São Paulo (TCC/USP) in cases in which it received first-hand cultures from the researcher who isolated the flagellate.

[c] Culture flagellates that have been tested by several methods and found to correspond to *Phytomonas* (see Section 7).

*Table 3* Host plants of fruit trypanosomatids

| Host plant | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| **Moraceae** | | | | |
| *Ficus parietalis* | Europe | None | Franchini, 1922a | – |
| *Morus* sp. | Americas | None | Cavazzana *et al.*, 1993 | JJ |
| *Morus* sp. | Americas | None | Fiorini *et al.*, 1995b | JF, TCC/USP[d] |
| **Solanaceae** | | | | |
| *Lycopersicon esculentum* | Africa | *Leptomonas serpens* | Gibbs, 1957 | – |
| *Ly. esculentum* | Americas | None | Fiorini *et al.*, 1986 | JF, TCC/USP[c] |
| *Solanum gilo* | Americas | None | Fiorini *et al.*, 1986 | – |
| *Lycopersicon esculentum* | Americas | *Phytomonas serpens* | Jankevicius *et al.*, 1989 | JJ, TCC/USP[c] |
| *Solanum americanum* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *So. concinnum* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *So. diflorum* | Americas | None | Kastelein and Camargo, 1990 | – |
| *So. erianthum* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *So. robustum* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *So. variabile* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *So. viarum* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *Capsicum annuum* | Americas | None | Kastelein and Camargo, 1990 | – |
| *Ca. praetermissum* | Americas | None | Kastelein and Camargo, 1990 | – |
| *Nicandra physaloides* | Americas | None | Kastelein and Camargo, 1990 | TCC/USP[c] |
| *Physalis angulata* | Americas | None | Kastelein and Camargo, 1990 | – |
| *Lycopersicon esculentum* | Europe | None | Sanchez-Moreno *et al.*, 1995b | SM, TCC/USP[c] |
| **Leguminosae** | | | | |
| *Phaseolus vulgaris* | Americas | None | Jankevicius *et al.*, 1987 | JJ |
| *Glycine max* | Americas | None | Jankevicius *et al.*, 1987 | JJ |
| *Cajanus flavus* | Americas | None | Cavazzana *et al.*, 1993 | – |

| | | | | |
|---|---|---|---|---|
| **Rosaceae** | | | | |
| *Prunus persica* | Americas | None | Conchon *et al.*, 1989 | – |
| *Malus* sp. | Americas | None | Cavazzana *et al.*, 1995 | JJ |
| **Anacardiaceae** | | | | |
| *Anacardium occidentale* | Americas | None | Conchon *et al.*, 1989 | – |
| **Oxalidaceae** | | | | |
| *Averrhoa carambola* | Americas | None | Conchon *et al.*, 1989 | – |
| **Rutaceae** | | | | |
| *Citrus bergamia* | Americas | None | Conchon *et al.*, 1989 | TCC/USP[c] |
| *Ci. reticulata* | Americas | None | Conchon *et al.*, 1989 | TCC/USP[d] |
| *Ci. aurantium* | Americas | None | Fiorini *et al.*, 1990 | JF |
| *Ci. aurantium* | Americas | None | Carrara *et al.*, 1992 | JJ |
| **Punicaceae** | | | | |
| *Punica granatum* | Americas | None | Conchon *et al.*, 1989 | – |
| *Punica* sp. | Americas | None | Catarino *et al.*, 1991 | JJ |
| **Passifloraceae** | | | | |
| *Passiflora edulis* | Americas | None | Conchon *et al.*, 1989 | – |
| **Bixaceae** | | | | |
| *Bixa orellana* | Americas | None | Almeida *et al.*, 1990 | JJ |
| **Vitaceae** | | | | |
| *Vitis vinifera* | Americas | None | Carrara *et al.*, 1992 | JJ |
| **Gramineae** | | | | |
| *Zea mays* | Americas | *Herpetomonas mcgheei* | Jankevicius *et al.*, 1993 | JJ, TCC/USP |
| *Zea mays* | Americas | *Phytomonas mcgheei* | Teixeira *et al.*, 1997 | JJ, TCC/USP[c] |

*Table 3* Host plants of fruit trypanosomatids (*continued*)

| Host plant | Place | Original name[a] | Reference | Depositaries[b] |
|---|---|---|---|---|
| **Anonaceae** | | | | |
| *Anona cerimolia* | Europe | None | Sanchez-Moreno *et al.*, 1995b | SM, TCC/USP[c] |
| **Myrtaceae** | | | | |
| *Eugenia uniflora* | Americas | None | Cavazzana *et al.*, 1996 | JJ |

[a] None, no specific name was given by the original describer.

[b] Depositaries are identified in the Appendix. They correspond to (i) the experimenter who isolated the flagellate, if he still keeps the cultures or (ii) the Trypanosomatid Culture Collection of the University of São Paulo (TCC/USP) in cases in which it received first-hand cultures from the researcher who isolated the flagellate.

[c] Culture flagellates that have been tested by several methods and found to correspond to *Phytomonas* (see Section 7).

[d] The flagellate is not *Phytomonas.*

*Table 4* Named species of plant flagellates

| Current name | Original genus[a] | Host species (first report) |
|---|---|---|
| *Phytomonas davidi* (Lafont, 1909) Donovan, 1909 | *Leptomonas* | *Euphorbia pilulifera* |
| *Phytomonas elmassiani* (Migone, 1916; Holmes, 1924) Ruiz, 1958 | *Leptomonas* | *Araujia angustifolia* |
| *Phytomonas bordasi* (França, 1921) Wenyon, 1926 | *Leptomonas* | *Morrenia odorata* |
| *Herpetomonas funtumiae* Franchini, 1922 | *Herpetomonas* | *Funtumia elastica* |
| *Trypanosoma euphorbiae* Franchini, 1922 | *Trypanosoma* | *Euphorbia coerulescens* |
| *Herpetomonas euphorbiae* Nieschulz, 1924 | *Herpetomonas* | *Euphorbia cereiformis* |
| *Phytomonas ficuum* (Fantham, 1925) Camargo *et al.*, 1990 | *Herpetomonas* | *Ficus edulis* |
| *Crithidia oncopelti* (Noguchi and Tilden, 1926) Gill and Vogel, 1963 | *Herpetomonas* | *Asclepias syriaca* |
| *Phytomonas francai* Aragão, 1927 | *Phytomonas* | *Manihot palmata* |
| *Phytomonas bancrofti* (Holmes, 1931) McGhee and McGhee, 1979 | *Herpetomonas* | *Ficus scabra* |
| *Phytomonas ganorae* (Franchini, 1931) Camargo *et al.*, 1990 | *Herpetomonas* | *Ficus hochstetteri* |
| *Phytomonas leptovasorum* Stahel, 1931 | *Phytomonas* | *Coffea liberica* |
| *Phytomonas tirucalli* Reichenow, 1940 | *Phytomonas* | *Euphorbia tirucalli* |
| *Phytomonas tortuosa* Ruiz, 1958 | *Phytomonas* | *Ficus costaricana* |
| *Phytomonas serpens* (Gibbs, 1957) Podlipaev, 1986 | *Leptomonas* | *Lycopersicon esculentum* |
| *Herpetomonas davidi*[b] (McGhee and Postell, 1976) Teixeira *et al.*, 1997 | *Phytomonas* | *Euphorbia heterophylla* |
| *Phytomonas staheli* McGhee and McGhee, 1979 | *Phytomonas* | *Cocos nucifera* |
| *Phytomonas mcgheei*[b] (Jankevicius *el al.*, 1993) Teixeira *et al.*, 1997 | *Herpetomonas* | *Zea mays* |

[a] According to the rules of zoological nomenclature, all these species should have been placed in the genus *Phytomonas* when first described.

[b] These species have been renamed according to the new criteria for defining the genus *Phytomonas* (see Section 7).

(Lafont, 1911a). França (1914) also failed in his attempts to culture *Leptomonas davidi*. Eventually he succeeded in culturing *L. elmassiani* in blood–agar medium, as had Migone (1922). In both cases, the cultures survived for only a few passages.

In the early 1920s, Franchini reported culturing plant flagellates in broth–blood medium, blood–gelatine, Nöller's agar medium, latex, and other media (Franchini, 1922a,e,h,i, 1923a,f). However, his papers were rather confused and included the culturing of amoebas, which occasionally Franchini claimed to be part of the life cycle of plant trypanosomes. Moreover, from his papers we cannot figure out exactly the composition of the various media. Therefore Franchini's papers are not very informative, although we know that he obtained at least one pure trypanosomatid culture from *Euphorbia cereiformis*, which Nieschulz (1922, 1924) maintained for some time.

Hydeo Noguchi and Evelyn Tilden were the first to obtain a stable culture of plant trypanosomatids (Noguchi and Tilden, 1926). These authors succeeded in isolating and culturing, from *Asclepias syriaca*, the flagellate *Herpetomonas oncopelti*, now *Crithidia oncopelti*, a very well known and much studied symbiont-harbouring flagellate (Newton and Horne, 1957; Roitman and Camargo, 1985).

Noguchi and Tilden (1926) reported that the flagellate was hard to obtain in culture and that, at the beginning, it grew exclusively in 'leptospira medium'. They reported that the flagellate grew as well at 37°C as at 25°C, although they did not mention whether the cultures survived many passages at the higher temperature. Later, Fish *et al.* (1978) reported that *Crithidia oncopelti* did not resist more than 10 passages at 37°C.

As a rule, since the work of Noguchi and Tilden, trypanosomatids have been cultured at 25–28°C, although it is known that some can grow well at 35–37°C (Roitman, 1970; Ellenbogen *et al.*, 1972; Roitman *et al.*, 1976, 1977; Fish *et al.*, 1978).

From the start, experimenters have faced the same problem: after a few passages, cultures were lost. This, in a pre-antibiotic era, was usually due to bacterial contamination. Nowadays, antibiotics are always included in culture media.

Regarding growth media, authors have resorted to variations of the Novy, MacNeal and Nicolle (NNN) blood–agar medium, of which 'leptospira medium' was one among many. Departing from the usual, Harvey and Lee (1943) inoculated flagellates into live coconuts and added several components to the 'milk'; they succeeded in keeping cultures alive for 30 days. Nevertheless, biphasic media remained the preferred choice and even today variations of NNN are used for the isolation of trypanosomatids from fruit, latex, flowers and insects. In addition to NNN, other culture media have been used successfully for trypanosomatids. Liver infusion

tryptose (LIT) medium was initially developed for *Trypanosoma cruzi* by Camargo (1964) but proved to be an excellent growth medium for most plant isolates. Besides LIT, two other culture media developed by Isaac Roitman are used either as overlay on blood–agar slants or as straight liquid media. They are a complex medium (folic acid, yeast extract, tryptic-case and sucrose: FYTS) and a defined medium (Roitman's defined medium: RDM), the composition of which is given by Roitman *et al.* (1972). Other overlay media, such as Grace's medium, have also been used (Kastelein *et al.*, 1984b, 1988; Dollet, 1992).

To circumvent bacterial contamination workers use antibiotics; antibiotics that do not interfere with eukaryotic metabolism should be preferred, but some authors have not adhered to this rule (Dollet *et al.*, 1982; Kastelein and Parsadi, 1988; Alves *et al.*, 1991; Jankevicius *et al.*, 1993). Antibiotics usually inhibit contaminating bacteria but not yeasts. Some authors use fungicides to control fungal growth, but most prefer to deal with the problem in a different way. To eliminate fungi, cultures are serially diluted and plated on blood–agar plates. Single flagellate colonies are then picked up and seeded in the desired medium. This is one of the procedures used for cloning trypanosomatids, which can thus serve a dual purpose.

When yeast contamination is very heavy, culturing the flagellates in U tubes may also be an efficient resource. Cultures are inoculated into one arm of the tube and, while the yeasts are deposited at the bottom, swimming flagellates can be collected from the top of the opposite arm after a few days of growth (Carvalho and Deane, 1974; Sbravate *et al.*, 1989; Jankevicius *et al.*, 1993).

With the use of antibiotics and an 'arsenal' of culture media, many authors have successfully isolated plant trypanosomatids, although obtaining pure cultures of these flagellates is not easy. The success rate may vary from laboratory to laboratory, and also depends on the flagellates' origin. Noguchi and Tilden succeeded only three times after 'many attempts' and, in the 75 years following Lafont's discovery, latex flagellates were isolated and cultured in only three instances (Noguchi and Tilden, 1926; McGhee and Postell, 1976; Dollet *et al.*, 1982). It is somewhat easier to isolate flagellates from fruits and from phytophagous insects than from latex or phloem.

The experience of Sbravate *et al.* (1989), in a survey of the occurrence of *Phytomonas* and other trypanosomatids in phytophagous insects, is representative of the methods used to isolate and culture flagellates from insects.

Smeared or cut portions of insects were seeded in test tubes containing a blood–agar base and an overlay of LIT. The blood–agar slants were standard ones consisting of a 2% agar base and rabbit red blood cells. The antibiotic was ampicillin at 20 $\mu g\ ml^{-1}$ of LIT. Sbravate *et al.* (1989)

obtained only 38 cultures from 133 infected insects, fewer than one in three. After being cleared of yeast and bacteria, cultures were transferred to liquid media (LIT or FYTS) for mass production. Cell densities of $50 \times 10^6$/ml and more were easily obtained.

The experience of Michel Dollet, Danielle Cambrony and Daniel Gargani is typical of the culturing of latex flagellates. These authors used a serum-containing but otherwise chemically defined medium to isolate and culture a flagellate from the latex of *Euphorbia pinea* (see Dollet *et al.*, 1982). After the parasites had become adapted to the culture medium, they were successfully transferred to either biphasic media overlaid with LIT, FYTS or RDM or monophasic cultures of one of these liquid media (Camargo *et al.*, 1987; Vainstein *et al.*, 1987; Attias *et al.*, 1988).

From the 1980s on, many new isolates have been obtained from the latex of various plants (see Table 1). The components of the isolation media have varied slightly (Dollet *et al.*, 1982; Attias and De Souza, 1986; Vainstein and Roitman, 1986; Kastelein and Parsadi, 1988; Fiorini *et al.*, 1993a,b; Santos *et al.*, 1994) but none has represented a fundamental departure from the traditional methods.

The same general features of isolation and culture of flagellates from insects and lactiferous plants apply also to fruit flagellates, which usually grow well in liquid media such as LIT and FYTS (Conchon *et al.*, 1989; Kastelein and Camargo, 1990; Carrara *et al.*, 1992; Cavazzana *et al.*, 1993; Fiorini *et al.*, 1993a,b; Jankevicius *et al.*, 1993; Catarino *et al.*, 1995; Sanchez-Moreno *et al.*, 1995a,b). *Phytomonas serpens* even grew in RDM supplemented only with serine, glutamine, inositol, glucose and haemin (Silva and Roitman, 1989).

To summarize, biphasic media (consisting of blood–agar slants and a liquid overlay of varying composition) seem to be the media of choice for obtaining first cultures of latex and fruit flagellates. They are also preferred for isolating flagellates from phytophagous bugs, some of which may also be of plant origin. After isolation, decontamination and a few passages in biphasic medium, flagellates can be kept in either biphasic or liquid media such as LIT, FYTS, RDM or Grace's medium. In order to obtain flagellates in large quantities, workers resort to one of these four liquid media. However, each flagellate seems to have its preferences and one has to find out empirically which medium is the best for a particular organism. Once this is done, flagellates can be grown to cell densities in the range $1 \times 10^7$ to $1 \times 10^8$ /ml and can be kept indefinitely by periodical passages.

Culture is not so easy with phloem trypanosomatids. Michel Dollet's group was the first to isolate and culture a flagellate from the phloem of the coconut palm (Menara *et al.*, 1988). They did so by introducing infected fragments of palm tissue into cultures containing feeder layers of haemocytes of the insect *Mamestra brassicae* in supplemented Grace's medium.

Dollet's collaborators encountered the problem noted by Noguchi and Tilden: primary cultures were slow to grow but once they got started they could easily be continued. Using feeder cell layer cultures, Dollet's laboratory has been isolating and maintaining isolates of palms of varied origin (Muller *et al.*, 1995). Dollet also reported (Dollet, 1994 and personal communication) that, after some passages in the presence of insect cells, flagellates can be grown as axenic cultures in supplemented Grace's medium alone, with no need for feeder layer cells.

Today, the isolation and culture of plant flagellates is not the problem it used to be. The big problem at present is the mixing and/or misidentification of cultures. Trypanosomatids are useful experimental models in many studies, and the keeping of their cultures spread accordingly to many laboratories. However, the handling of cultures is not always done with the care required to avoid their mixing and/or mislabelling (Shaw and Camargo, 1995). Presently, many cultures are not what their labels say they are. This is a serious potential source of misinformation and error, and to avoid it researchers are strongly recommended to obtain cultures from primary depositaries only and to refrain from passing them on to other laboratories.

Samples of original cultures should be stored in liquid nitrogen as a safeguard against mixing and mislabelling. Plant and insect trypanosomatids can be frozen in several ways. Each laboratory may have its own routine, but usually cultures are simply mixed with glycerol at 10% final concentration, kept at −70°C overnight and then transferred to liquid nitrogen. Alternatively, cultures can be centrifuged (3000 rpm, 10 min), resuspended in cold LIT containing 20% dimethyl sulphoxide and left overnight at −70°C before storing in liquid nitrogen. For some flagellates, undiluted fetal calf serum should be used instead of LIT. There is no rule other than trial and error for choosing between one of these three procedures or any other variation on this theme.

## 5.2. Nutrition

Published information about the nutritional requirements of plant flagellates is scarce, except for *Crithidia oncopelti*. This flagellate harbours an endosymbiont that enhances its biosynthetic capabilities (Gill and Vogel, 1962, 1963; Roitman and Camargo, 1985). Thus, the flagellate is able to grow in a very simple minimal defined medium containing methionine, thiamine, nicotinamide, glucose and *p*-aminobenzoic acid (Newton, 1956, 1957). It should be pointed out that this medium does not contain haemin, which is supposed to be a universal requirement for trypanosomatids (Galliard, 1931; Lwoff, 1940, 1951; Chang *et al.*, 1975; Shapiro *et al.*,

1978). However, information about the nutritional requirements of *Crithidia oncopelti* cannot be generalized to plant flagellates. This species is atypical and can be compared only with other symbiont-harbouring flagellates such as *Crithidia deanei* or *Herpetomonas roitmani* (see Camargo and Freymuller, 1977; Roitman and Camargo, 1985; Silva *et al.*, 1991).

There is no information on the nutritional requirements of other plant trypanosomatids besides *Phytomonas serpens*, which grows in defined RDM medium. Although RDM medium contains haemin (Silva and Roitman, 1989), it has not been ascertained whether *Phytomonas* requires it. Phloem flagellates growing in Grace's medium apparently do not need added haemin (Menara *et al.*, 1988).

## 6. BIOCHEMISTRY AND MOLECULAR BIOLOGY

Most of the knowledge about the biochemistry of plant flagellates has resulted from experiments in which the flagellates were used as instruments in biochemical investigations rather than from studies investigating the biochemistry of the plant flagellates themselves. This is probably why these studies are scattered through a variety of subjects and, in spite of the amount of information gained in recent years, our knowledge of the biochemistry of *Phytomonas* remains fragmentary.

### 6.1. Carbohydrate Metabolism

André Lwoff (1934) was the first to study glucose consumption by the plant flagellate *Crithidia oncopelti*; he showed that 90% of the flagellate's respiration could be inhibited by cyanide, a highly effective inhibitor of mitochondrial respiration. Clausen (1955) and, after her, Ryley (1955) studied additional aspects of the respiration of this flagellate, showing that it had a well-developed cytochrome system and that glucose fermentation under aerobic or anaerobic conditions produced succinic and pyruvic acids in addition to ethanol and glycerol. Monohexoses, certain disaccharides and some amino acids stimulated respiration. Enzymes of the glycolytic and oxidative phosphorylation pathways were detected in cell homogenates of this flagellate. However, *Crithidia oncopelti*, besides having its actual plant origin questioned, is an atypical trypanosomatid harbouring an endosymbiont that actively participates in its metabolism (Gill and Vogel, 1962, 1963; Camargo and Freymuller, 1977).

Working with flagellates of unquestionable plant origin, Silva and Roitman (1988) reported that the consumption of oxygen by flagellates

from latex (four isolates) and fruit (one isolate) was only partially inhibited by $10^{-3}$ M cyanide. Dimitri Maslov (personal communication) also verified that in *Phytomonas serpens* the uptake of oxygen was not sensitive to cyanide or azide, but was totally inhibited by salicylhydroxamic acid (SHAM), an inhibitor of the glycolytic pathway.

In a very informative study, Manuel Sanchez-Moreno *et al.* (1992) investigated the carbohydrate metabolism of a latex flagellate isolated by Dollet (1984) from *Euphorbia characias*. Several interesting facts emerged from this study.

1. Sugar-digesting enzymes such as amylase, invertase and methylcellulase were excreted by the flagellate.
2. The monohexoses fructose, glucose and mannose served as major energy substrates.
3. Hexose utilization occurred via the glycolytic pathway.
4. Accordingly, all the enzymes of the Embden–Meyerhof pathway were detected in homogenates of the flagellate.
5. Also in accordance, the major respiration end-products were acetate, pyruvate, glycerol, succinate and ethanol.
6. Cytochromes were not detected and, consistently, mitochondria were not functional in the oxidation of substrates.
7. Antimycin, azide or cyanide failed to inhibit respiration, which was, however, totally inhibited by SHAM.

These studies were later extended and confirmed by $^{1}H$ nuclear magnetic resonance (Sanchez-Moreno *et al.*, 1995a).

In a related paper from the laboratory of Fred Opperdoes, it was shown that the flagellate from *Euphorbia characias* processed glucose under aerobic or anaerobic conditions and that the major end-products were acetate/$CO_2$ and ethanol/glycerol/$CO_2$, respectively. These major end-products, with additional minor ones, were also detected in cultures grown in different media (Chaumont *et al.*, 1994).

Thus, it seems that different plant flagellates may be differently equipped for respiration: *Crithidia oncopelti* exhibits fully functional mitochondria, whereas the flagellates from *Euphorbia characias* and *Phytomonas serpens* do not, relying solely on the glycolytic pathway. Unfortunately, these studies have been conducted on a limited sample of flagellates, precluding generalizations. Nevertheless, we know for certain from the above examples that culture forms of the flagellate of *Euphorbia characias* and *Phytomonas serpens* have non-functional mitochondria. This finds support in electron microscopic studies that revealed only scanty mitochondrial cristae in the kinetoplast of some latex and fruit flagellates. Moreover, DNA maxicircles are rare and seem to be 'present in a low proportion' in the kinetoplast DNA (kDNA) network of the *Phytomonas* of *Euphorbia*

*pinea* (see Riou *et al.*, 1987). These facts concur with the non-functionality of the mitochondria of culture forms. However, we know nothing about their functionality in the insect vector or the host plant. Could the cytochrome genes and/or other maxicircle genes have been lost after years of subculturing in complex media? We know that some maxicircle genes are present in *Phytomonas serpens*, like the genes for 12S and 9S RNA and the genes *ND8* and *ND9* (D. Maslov, personal communication). However, the presence of these maxicircle genes does not necessarily mean that cytochrome genes are also present and/or functional.

Recently, Uttaro *et al.* (1996) and Uttaro and Opperdoes (1997) described the occurrence in *Phytomonas* spp. of a mitochondrial enzyme, isopropanol dehydrogenase (iPDH), which exhibits a low affinity for ethanol. This may explain the production of ethanol as an end-product of fermentative respiration in *Phytomonas*. Among several trypanosomatids examined, the enzyme was found only in flagellates from phloem and fruit. From these results the authors concluded that the presence of the enzyme may serve as a taxonomic marker for the genus *Phytomonas*. However, the sample examined is still limited.

Another interesting observation by Sanchez-Moreno *et al.* (1992) was that the glycolytic enzymes were clustered in cytoplasmic organelles. These organelles, christened glycosomes by Opperdoes and Borst (1977), are known to be present in many trypanosomatid genera (Opperdoes, 1987). In addition to glycolytic enzymes, the glycosomes of the flagellate of *Euphorbia characias* also possess catalase, like peroxisomes. Glycolytic enzymes were also found in glycosomes of the latex flagellates from *Euphorbia indica* and *Euphorbia hyssopifolia*, phloem flagellates from coconut, and fruit flagellates from tomato and annona (Sanchez-Moreno *et al.*, 1993). A malate dehydrogenase, reported to be of glycosomal origin, was also detected in various trypanosomatids, including some of plant origin (Uttaro *et al.*, 1996).

Concerning the utilization of carbohydrates, Almeida *et al.* (1992) reported that 19 strains of fruit flagellates isolated from grains and seeds grew well on carbon sources such as the monohexoses glucose, fructose and mannose but failed to grow with many disaccharides (sucrose, lactose, maltose, cellobiose, melibiose, trehalose) or pentoses (arabinose, xylose, ribose). This was confirmed for the flagellate of apples (Catarino *et al.*, 1995) but not for isolates from grapes, mulberries, beans, oranges and tomatoes, which could grow at least on sucrose (Ono *et al.*, 1993). Shiduca Itow-Jankevicius (personal communication) re-examined the problem and found that all fruit flagellates, although capable of utilizing sucrose, were unable to use other disaccharides.

The latex flagellates from *Euphorbia characias*, thanks to the amylase and invertase they excrete (Sanchez-Moreno *et al.*, 1992), seem to be well

equipped to digest the starch they swim in or to deal with the disaccharides they may encounter in the latex of the plant. They need no external help to obtain glucose from starch. This is not so for some of the fruit flagellates, particularly those from seeds, which seem to be unable to grow on most disaccharides (Almeida *et al.*, 1992; Catarino *et al.*, 1995). Thus, they require external help and this may be fully or partially provided by their vectors, phytophagous insects. Most of the bugs that live on legume or caryopsis (corn) fruits are seed-suckers (Camargo and Wallace, 1994). The saliva they inject while piercing the seeds contains digestive enzymes that transform the starches and proteins of the endosperm into a fluid that can be sucked up. Thus, seed flagellates may thrive in these minute pits filled with a 'soup' of digested starches and other sugars. The soup may be made richer by the activity of proteases secreted by the flagellates themselves (Branquinha *et al.*, 1994, 1996; Ueno *et al.*, 1994; Almeida *et al.*, 1996).

However, it must be pointed out that more than one type of fruit flagellate may exist with respect to sugar fermentation. The flagellates isolated from apples, corn (maize), annatos and pomegranates did not utilize the disaccharides maltose, melibiose or trehalose, whereas the flagellates isolated from grapes and mulberries easily did so (Almeida *et al.*, 1992; Ono *et al.*, 1993; S. Itow-Jankevicius, personal communication). In this context, one should remember that flagellates of berries, drupes or pomes may face an environment completely distinct from those thriving on seeds.

## 6.2. Arginine–Ornithine Metabolism

Another aspect of the intermediary metabolism of plant flagellates that has received some attention from researchers is ornithine–arginine metabolism. Trypanosomatid genera are differently equipped to promote the interconversion of ornithine←→citrulline←→arginine, a metabolic cycle equivalent to the urea or Krebs–Henseleit cycle of vertebrates.

Trypanosomatids cultivable in defined media require one of these amino acids to grow, and excrete either urea or ammonia depending on their enzymatic repertoire. The enzymes involved in this cycle in trypanosomatids are (i) arginase, which converts arginine to ornithine, $CO_2$ and urea; (ii) arginine deiminase, which converts arginine to citrulline and ammonia; (iii) citrulline hydrolase, which hydrolyses citrulline to ornithine, $CO_2$ and ammonia; (iv) ornithine carbamoyltransferase, which synthesizes citrulline from ornithine and carbamoyl phosphate; and (v) argininosuccinate synthetase and argininosuccinate lyase, which convert citrulline plus aspartate to arginine plus fumarate.

The enzymes of the urea cycle (except citrulline hydrolase) do not occur randomly in trypanosomatids. On the contrary, their occurrence is genus-

related (Camargo and Freymuller, 1977; Camargo *et al.*, 1978, 1987; Figueiredo *et al.*, 1978; Galinari and Camargo, 1978; Yoshida and Camargo, 1978; Yoshida *et al.*, 1978; Wallace *et al.*, 1992). Thus, among the genera of insect trypanosomatids, arginase occurs in *Crithidia* and *Leptomonas*, whereas arginine deiminase occurs in *Herpetomonas*. Arginase and arginine deiminase never coexist.

Studies on the urea cycle enzymes of latex and fruit flagellates have disclosed that the enzymic make-up of *Phytomonas* spp. is similar to that of *Herpetomonas* spp., i.e. they lack arginase and have the arginine deiminase–citrulline hydrolase system for converting arginine to ornithine (Camargo *et al.*, 1987, 1992; Sbravate *et al.*, 1989; Teixeira *et al.*, 1996, 1997).

An interesting fact emerged recently concerning arginase. Silva and Floeter-Winter (1996) have shown (through hybridization with a probe derived from the arginase gene of *Leishmania amazonensis*) that the gene for arginase, or at least part of it, is present in all trypanosomatid genera, including *Herpetomonas* and *Phytomonas* which do not exhibit arginase activity. The factors involved in the still unknown mechanism of repression or inactivation of the arginase gene may prove to be an exciting field of research.

## 6.3. Fatty Acids

Early in the 1980s, Fish *et al.* (1982) studied fatty acid biosynthesis and composition in several species of the various trypanosomatid genera. They found that three species of *Herpetomonas* isolated from flies and *Phytomonas davidi*, a latex flagellate from *Euphorbia heterophylla* (see McGhee and Postell, 1976), possessed 'a unique assemblage' of iso-branched polyunsaturated fatty acids, with two to five double bonds interrupted by methylene. The fatty acids were chains of 18, 20 and 22 carbons. The unusual fatty acids, which the authors thought could 'influence membrane barrier properties', were absent from all other trypanosomatids examined (Fish *et al.*, 1982). Today we know that *Phytomonas davidi* is not a species of *Phytomonas* but of *Herpetomonas* (see Camargo *et al.*, 1992; Nunes *et al.*, 1994; Teixeira *et al.*, 1997). Nevertheless, it is a plant flagellate and it would be interesting to know whether other plant flagellates, including true *Phytomonas* spp., also have a similar fatty acid composition, so uncommon among eukaryotes. Apparently this is not the case, if a preliminary report by Nakamura *et al.* (1988) is confirmed by additional data. These authors found that *Phytomonas serpens*, the tomato parasite, exhibits a fatty acid profile quite distinct from that of *Herpetomonas davidi*, with the predominance of a long-chain fatty acid ($>C_{24}$). Thus, it may be that the unusual fatty acid composition reported by Fish *et al.* (1982) is a characteristic of

the genus *Herpetomonas*, having no relationship whatsoever with plant parasitism.

## 6.4. Cell Surface Components

In a continuation of their studies on surface G proteins of *Trypanosoma cruzi* (see Eisenchlos *et al.*, 1986; Coso *et al.*, 1992), Mirtha Flawiá and collaborators found $G_{\alpha s}$ proteins on the surface of the latex flagellate of *Euphorbia characias* (Farber *et al.*, 1995). The surface G protein was found to be coupled to an adenylyl cyclase on the cytoplasmic side of the cell membrane, but the ligand(s) of the external domain remain(s) unknown.

Michael Ferguson and collaborators (Redman *et al.*, 1995) have made a thorough study of the surface glycoinositol-phospholipids (GIPLs) of the *Phytomonas* of *Euphorbia characias*, comparing them with the known GIPLs of other trypanosomatids. They found four distinct GIPLs in cell extracts of *Phytomonas*, all of them of type 1. The GIPLs of *Phytomonas* exhibited unique features such as the presence of one or two α-glucose residues at the glycosidic end, a fact that makes them a novel subset of type 1 GIPLs. The presence in these GIPLs of a core very similar to that of the glycosylphosphatidylinositol (GPI) protein anchors, ubiquitous in eukaryotes, indicates that these GIPLs 'could serve as GPI anchor precursors for *Phytomonas* surface proteins'. However, the authors considered this possibility unlikely and preferred to think that, due to their abundance, the GIPLs may have a function of their own, possibly a protective one 'by providing a dense negatively charged glycocalyx' on the cell surface. Accordingly, Vommaro *et al.* (1989) found a 'net negative charge' on the cell surface of four latex flagellates, as indicated by cell electrophoretic mobility and surface binding of cationic particles.

An interesting observation was that the GIPLs of the *Phytomonas* from *Euphorbia characias* did not have galactose residues (Redman *et al.*, 1995). This would permit the flagellates to live in the latex of their host, which contains a galactose-specific lectin. From this observation the authors hypothesized that the 'host range of different *Phytomonas*' may depend on the compatibility between the surface glycoconjugates of the flagellates and the lectins present in the juices of their hosts. This seems to be a reasonable supposition which, however, cannot be formulated on the basis of GIPL composition alone and is somewhat in conflict with the data of Sanchez-Moreno *et al.* (1995b). These authors found that flagellates of the very same strain as that studied by Redman *et al.* (1995), which does not have galactose residues in its GIPLs, were nevertheless agglutinated by the galactose-specific lectin RCA 1 from the lactiferous plant *Ricinus communis*.

The only other available reference to the GIPLs of *Phytomonas* concerns *P. serpens*, the tomato parasite. In a preliminary note, Silveira *et al.* (1992) reported on the sugar and lipid composition of the GIPLs of this flagellate. They disclosed the presence of a unique 17:0 alkyl chain, in contrast with the 16:0 chain of the flagellate of *Euphorbia characias* and the varied length of the alkyl chains of other trypanosomatids.

An unexpected observation concerning cell surface components was that of Nakamura *et al.* (1993), who found by gas–liquid chromatography that *Phytomonas francai*, the latex flagellate of cassava, contains chitin. It is not known whether this *N*-acetylglucosamine polymer is present in other plant flagellates. Nakamura *et al.* (1992) had also previously shown that flagellates isolated from different plants possessed distinct surface saccharides. Finally, there were the studies of De Souza (1989) and Attias *et al.* (1992) on the chemical nature and localization of many surface components of flagellates of plants.

Sanchez-Moreno *et al.* (1995b) comparatively studied the agglutination of latex, phloem and fruit flagellates using seven distinct lectins. Interestingly, WGA (wheat germ agglutinin), which recognizes *N*-acetylglucosamine oligomers, agglutinated only the latex flagellates, largely ignoring the fructicolous and phloemicolous species. These observations might indirectly suggest the presence of chitin exclusively in latex flagellates. However, this remains to be demonstrated.

The report by Sanchez-Moreno *et al.* (1995b) also showed that the agglutination profiles of fruit flagellates were similar to those of phloem flagellates but quite distinct from those of latex flagellates. This raised the hope that lectin agglutination profiles could help in distinguishing groups of plant flagellates. Unfortunately, this hope did not materialize (see Section 7).

### 6.5. Cytoskeleton Proteins

Baqui *et al.* (1996) have recently shown the presence of a giant protein (Ps 2500) associated with the cytoskeleton of *Phytomonas serpens*. As demonstrated by immunogold labelling and electron microscopy, the megadalton protein (2500–3000 kDa) is located exclusively at the anterior end of the cell body. It apparently belongs to a meshwork of microfibrils located in the flagellar pocket that may serve as a link between the flagellar membrane and the cytoskeleton. It is interesting to note that the polyclonal serum raised against Ps 2500 failed to recognize the equivalent proteins of various trypanosomatids. This suggests the existence of species-specific epitopes in Ps 2500, which may eventually be of use for species identification and taxonomy.

## 6.6. Kinetoplast DNA

There are numerous reports and many comprehensive reviews encompassing various aspects of the kinetoplast of trypanosomatids in general, including those by Simpson (1972, 1986, 1987) Ryan *et al.* (1988), Landweber (1992), Perez-Morga and Englund (1993a,b), Zaitseva (1993), Simpson and Maslov (1994), Benne (1994) and Chen *et al.* (1995). However, few reports deal with plant flagellates and, among these, most are primarily related to the use of kDNA sequences for taxonomic purposes (see Section 7).

Information on the kinetoplast of plant flagellates is available only for the following species: *Herpetomonas davidi* (see Cheng and Simpson, 1978), *Crithidia oncopelti* (see Pestov *et al.*, 1990), *Phytomonas serpens* (see Jankevicius *et al.*, 1989; Sá-Carvalho *et al.*, 1993; Sá-Carvalho and Traub-Cseko, 1995), and various latex and phloem flagellates (see Riou *et al.*, 1987; Ahomadegbe *et al.*, 1990, 1992; Muller *et al.*, 1995).

Cheng and Simpson (1978) first studied the kDNA of *Herpetomonas davidi*, a latex flagellate then classified as *Phytomonas davidi*. They found that the minicircles corresponded to 1064 base pairs (bp), whereas maxicircles were about 25 times longer. Minicircles were readily cleaved by the restriction endonucleases *Hpa*II, *Hae*III, *Hpa*I and *Bam*HI, but not by *Eco*RI. A certain degree of base sequence heterogeneity was evident among minicircles, which prompted the separation and characterization of *Hpa*II-resistant kDNA.

Several authors have studied the minicircles of *Crithidia oncopelti*, a latex flagellate (Maslov *et al.*, 1982; Ntambi *et al.*, 1984; Rezepkina *et al.*, 1984; Pestov *et al.*, 1990). Minicircles were found to consist of 1848 bp, were digested by four- as well as six-cutter enzymes, presented marked base sequence heterogeneity and a long region rich in guanine (G) and thymine (T). A so-called universal minicircle sequence, later proposed to be identical in all trypanosomatids, was found to differ in two bases in *Crithidia oncopelti*.

Guy Riou and collaborators have shown that, in the *Phytomonas* sp. isolated from *Euphorbia pinea*, the kDNA corresponded to 30% of the total cell DNA; its buoyant density value was 1.694 g $ml^{-1}$; it had 65% adenine (A) and T residues; after digestion with topoisomerase II the network resolved into about 7000 minicircles; each minicircle consisted of approximately 2900 bp, lacking sites for the six-cutter restriction enzymes *Eco*RI, *Hae*II, *Bam*HI and *Hin*dII; *Hpa*II was the only enzyme capable of cleaving the minicircles; maxicircles were scarce (Riou *et al.*, 1987).

The unusually large size of the minicircles and their resistance to six-cutter enzymes were briefly thought to be diagnostic characteristics. However, Riou soon showed that the length of the minicircles of various

lacticolous and phloemicolous *Phytomonas* spp. could range from 1300 to 2900 bp and that resistance to six-cutter enzymes was not an absolute rule (Ahomadegbe *et al.*, 1990). In fact, *Phytomonas* spp. had varied susceptibility to different restriction enzymes.

Sá-Carvalho *et al.* (1993) examined the kinetoplast of isolate 30T from tomatoes, presumably *Phytomonas serpens*. The size of the minicircles was estimated to be about 1450 bp. No site was detected for the six-cutter enzymes *Acc*I and *Kpn*I, whereas digestion with the four-cutter *Rsa*I was extensive. The authors also sequenced the minicircle of the isolate and found the same overall organization of conserved and variable regions as in other trypanosomatids. However, they found that the conserved sequence blocks (CSBs) 1, 2 and 3 differed by one nucleotide each from the consensus of the corresponding sequence of trypanosomatids. The authors also disclosed long regions composed almost exclusively of G and T residues. On the basis of the existence of a polymerase arrest site in one strand and the absence of inverted repeats in the complementary one, they concluded that it corresponded to a type II arrest site. As a consequence of its base composition, this GT region had a high probability of forming G quartets like those commonly found in telomeric sequences. Using as model an oligonucleotide constructed after the last 35-nucleotide sequence of the GT region, the authors demonstrated that, *in vitro*, the oligonucleotide indeed formed four-stranded structures (Sá-Carvalho and Traub-Cseko, 1995).

Despite the many differences between the kDNA of *Phytomonas* spp., one thing seems to be common to them all: a relative scarcity of maxicircles.

## 6.7. Nuclear DNA

In *Phytomonas*, only two sets of genes have received attention, mainly because of their taxonomic utility, i.e. the ribosomal and the spliced leader of mini-exon genes (see Section 7).

### 6.7.1. *Ribosomal Genes*

In trypanosomatids, ribosomal genes are arranged in tandem repeats consisting of subunits, as illustrated in Figure 5. No study has been done with the intention of demonstrating the same organization in *Phytomonas*. However, from papers on restriction mapping and sequencing of ribosomal genes of *Phytomonas* spp. (Camargo *et al.*, 1992; Marché *et al.*, 1995) it can be inferred that their organization is, in general, similar to that of other trypanosomatids.

Camargo *et al.* (1992) constructed a restriction map of the ribosomal genes of trypanosomatids based on a comparative study of 54 organisms,

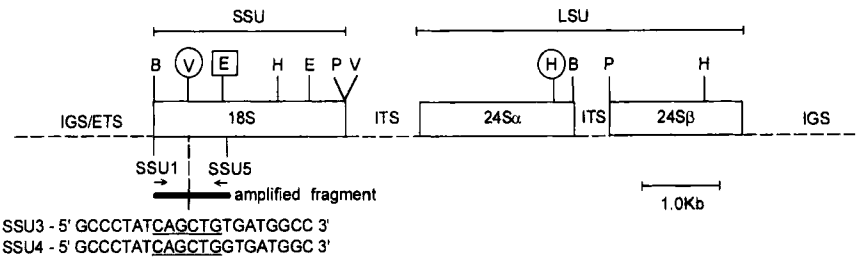


*Figure 5* Diagrammatic representation of subunits of ribosomal genes showing the universal restriction sites (capital letters) present in all trypanosomatids and the variable sites (within circles or squares) that are present in some genera only. Restriction enzyme sites: B, *Bgl*II; V, *Pvu*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. Gene segments: SSU, rDNA small subunit; LSU, rDNA large subunit; IGS, intergenic spacer; ETS, external transcribed spacer; ITS internal transcribed spacer. The synthetic oligonucleotides SSU1 and SSU5 are primers flanking the amplification target sequence, which lacks a *Pvu*II site in *Phytomonas*. Oligonucleotides SSU3 and SSU4 are complementary to alternative sequences that contain the *Pvu*II variable site.

including 12 isolates from plants, 10 of which were presumably *Phytomonas* spp. As depicted in Figure 5, conserved sites were present in all flagellates as well as sites that occurred selectively in genus-related patterns. Notoriously missing from *Phytomonas* spp. was a site for *Pvu*II located 360 bp downstream from the 5′ end of the small subunit (SSU).

Marché *et al.* (1995) also studied the ribosomal genes of latex and phloem flagellates. The authors produced the complete SSU sequence of the flagellates from coconut and *Euphorbia hirta* plus partial sequences of other flagellates from latex and phloem. They found an *Eco*RI site at position 782 in the SSU that occurred in phloem, but not in latex or fruit, flagellates. They also confirmed the absence of the *Pvu*II site in the SSU 360 bp downstream from the 5′ end.

Maslov *et al.* (1996) determined the SSU sequence of the fructicolous *Phytomonas serpens* (GenBank, accession U39577). Their sequence also lacked both the 360 *Pvu*II site and 782 *Eco*RI site, in agreement with the data of Camargo *et al.* (1992) and Marché *et al.* (1995).

### 6.7.2. *Spliced Leader of Mini-exon Genes*

All mature messenger RNAs (mRNAs) of trypanosomatids are capped at the 5′ end with a conserved sequence of 39 nucleotides. The mRNA maturation process involves *trans*-splicing of the 39-nucleotide sequence from a precursor, the medRNA. This precursor RNA is coded by the spliced leader or mini-exon genes, which occur in the genome of trypanosomatids as variable numbers (100–200) of tandem repeats, according to

the organism. Each repeat consists of a transcribed and a non-transcribed region. The transcribed region includes an exon that codes for the medRNA leading sequence of 39 nucleotides and an intron of 50–110 bp (Figure 6). The exon sequence is highly conserved throughout the family Trypanosomatidae with minimal differences between taxa. The transcribed intron sequences show some variability between taxa, whereas the non-transcribed region shows a high degree of variability between organisms (De Lange *et al.*, 1984; Bellofatto *et al.*, 1988; McCarthy-Burke *et al.*, 1989; Agabian, 1990; Murthy *et al.*, 1992; Fernandes, O. *et al.*, 1993, 1994, 1995, 1997; Hassan *et al.*, 1993; Glodring *et al.*, 1995; Nunes *et al.*, 1995; Sturm *et al.*, 1995). Various authors have taken advantage of both sequence conservation and variability for taxonomic purposes (see Section 7).

Mini-exon sequences of seven *Phytomonas* spp. were determined by two different groups, Nunes *et al.* (1995) and Sturm *et al.* (1995). The sequence of the 39-nucleotide exon was found to be identical in all seven *Phytomonas* by both groups. However, there were discrepancies in other gene segments. For instance, the intron sequence from positions 40 to 58 of the three flagellates studied by Sturm *et al.* (1995) differed from that of the five flagellates studied by Nunes *et al.* (1995) in the presence of an extra T at position 52 in the latter sequence. Accordingly, from this position onwards the sequences were out of phase. Since both groups sequenced *Phytomonas serpens* as a control, this discrepancy is puzzling.

It is known that *trans*-splicing involves the participation of small ribonucleoprotein particles (Bruzik *et al.*, 1988). A recent analysis of the sequence of the U6 small nuclear (sn)RNA of a *Phytomonas* sp., presumably

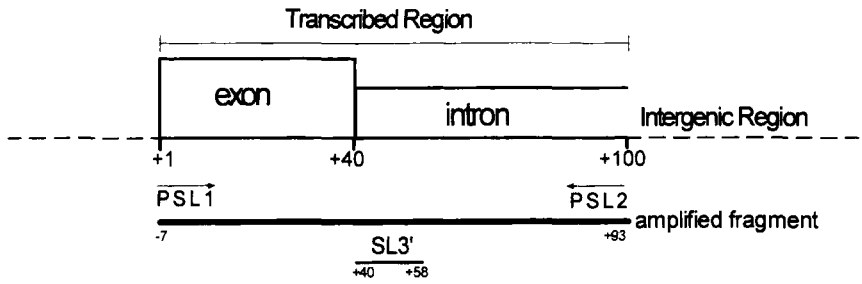


*Figure 6* Spliced leader or mini-exon gene of *Phytomonas*. PSL1 and PSL2 are the primers used to amplify the segment that contains the sequence hybridizing with SL3', a 19-nucleotide synthetic oligonucleotide specific for the spliced leader sequences of *Phytomonas* spp.

from *Euphorbia characias*, has disclosed four base substitutions at positions that are otherwise conserved in all trypanosomatids studied so far. One of these, a G→C (cytosine) change, occurs in the last position of the highly conserved hexanucleotide ACAGAG at the central domain of U6 (Wieland and Bindereif, 1995), which is considered to play an essential role in the catalysis of splicing. This may suggest alternative splicing mechanisms in trypanosomatids. However, it is premature to generalize data from a single organism to all plant flagellates.

## 7. TAXONOMY AND IDENTIFICATION

There are many problems concerning the taxonomy, and consequently the proper identification, of *Phytomonas*. Many of these problems have deep roots in the untidy systematics of the so-called lower trypanosomatids, which, in addition to *Phytomonas*, include the genera that parasitize non-haematophagous insects: *Crithidia*, *Blastocrithidia*, *Leptomonas* and *Herpetomonas*.

By the time Lafont described flagellates in the latex of euphorbias, great confusion prevailed about these genera, leading Lafont somewhat reluctantly to place the plant trypanosomatids in the genus *Leptomonas* (see Lafont, 1909, 1910). Donovan, after confirming the findings of Lafont on the *Euphorbia pilulifera* of Madras, suggested a new genus engulfing all plant trypanosomatids, the genus *Phytomonas* (Donovan, 1909). This was a precipitous decision which gave to a single behavioural characteristic, the plant origin, the power of defining the new genus. This was like proposing that all trypanosomatids of mammals should belong to the genus *Trypanosoma*, thus ignoring *Leishmania* and *Endotrypanum*. Contemporaries of Donovan apparently did not appreciate his arbitrariness and never accepted the new genus.

The taxonomy of the lower trypanosomatids remained more or less confused until the 1960s when Franklin Wallace tried to instil some order into the subject. Wallace proposed morphodevelopmental criteria for defining trypanosomatid genera (Wallace, 1963) and adopted a new terminology for the developmental forms of their life cycles (Hoare and Wallace, 1966). Unfortunately, even with the new criteria, *Phytomonas* remained in limbo because the host origin criterion continued to be central to its definition. This is apparent in the definition of *Phytomonas* as reworded by Vickerman (1976): 'flagellates with digenetic life-cycle in plants and insects, retaining the promastigote form throughout'.

Soon a conflict erupted between the host origin and the morphodevelopmental criteria. Researchers began to isolate from plants trypanosomatids

that clearly had morphodevelopmental characteristics attributable to other genera of trypanosomatids (Noguchi and Tilden, 1926; Conchon *et al.*, 1989; Fiorini *et al.*, 1996). These findings discredited the host origin criterion and at the same time posed two new questions: (i) Is there a consistent group of plant flagellates, distinguishable from other trypanosomatid genera, that we can call *Phytomonas*? (ii) If the answer is yes, what characters define it?

These are not simple questions and the overall problem is of a circular nature, the answer to one question depending on the answer to the other. However, researchers had no better choice and started comparative studies on the lower trypanosomatids. The aim was eventually to unveil characteristics shared by a group of plant trypanosomatids (*Phytomonas*) that served to distinguish them from the genera *Leptomonas*, *Herpetomonas* and *Crithidia*.

Morphology was not much help because plant trypanosomatids are generally of the promastigote type, with the exception of the choanomastigotes of the mulberry parasite (Fiorini *et al.*, 1996) and *Crithidia oncopelti* (Noguchi and Tilden, 1926) and the opisthomastigotes of *Herpetomonas davidi* (Attias and De Souza, 1985). Moreover, comparative electron microscopic studies of various plant isolates failed to reveal any specific characteristic that would distinguish them as a group from other trypanosomatid genera (Attias *et al.*, 1988).

The search for biochemical characteristics started only in the 1980s after cultures of latex flagellates were obtained by Dollet *et al.* (1982). The first comparative study involved the enzymes of the ornithine–arginine cycle, which were well studied in insect trypanosomatids (see Section 6.2). Essentially, unlike *Crithidia* and most *Leptomonas* spp., latex flagellates did not contain arginase, a trait they shared with *Herpetomonas* (see Camargo *et al.*, 1987). Afterwards, several plant trypanosomatids, particularly fruit flagellates, were found to possess arginase (Almeida *et al.*, 1992).

Monoclonal antibodies were produced against a flagellate from tomatoes, *Phytomonas serpens*, and *Phytomonas francai*, a flagellate from cassava. After extensive testing for cross-reactivity with representatives of the various trypanosomatid genera, monoclonal antibodies were obtained that reacted exclusively with a select group of latex flagellates. These antibodies were totally non-reactive against other latex flagellates such as *Herpetomonas davidi*, *Crithidia oncopelti* and a dozen other species of the various trypanosomatid genera (Teixeira and Camargo, 1989). The target antigens of these putatively specific anti-*Phytomonas* monoclonal antibodies were identified as glycoconjugates located on the surface of the flagellate's cell body, flagellum and flagellar pocket (Attias *et al.*, 1993; Teixeira *et al.*, 1995). These glycoconjugates apparently are restricted to *Phytomonas* spp., but their precise chemical composition is unknown.

In subsequent studies involving the testing of isolates from plants and insects, it was found that reactivity with *Phytomonas*-specific monoclonal antibodies was never associated with the presence of arginase. Thus far, not a single arginase-positive flagellate has been recognized by these antibodies. However, flagellates lacking arginase do not necessarily react with the monoclonal antibodies, because *Herpetomonas* and some species of *Leptomonas* also lack arginase.

Lectin agglutination was also assayed in an effort to distinguish *Phytomonas* from other trypanosomatids (Petry *et al.*, 1987; Sanchez-Moreno *et al.*, 1995b). Differences were found in earlier studies, although these were done on a small sample of flagellates. Subsequent studies failed to show any specific association between lectin profile and the genus *Phytomonas.* Therefore, lectin agglutination never became of use to characterize the taxon, although it may be useful for the identification of isolates (see below).

Analysis of kDNA initially raised hopes that the large size of the minicircles and some of their biochemical properties could help separate *Phytomonas* from other trypanosomatids (Riou *et al.*, 1987). These hopes were not sustained but kDNA analysis proved useful in distinguishing between isolates of *Phytomonas* (see Section 8).

Restriction mapping of the ribosomal genes of trypanosomatids (Section 6.7.1) by Camargo *et al.* (1992) revealed that a restriction site for *Pvu*II, located 360 bp downstream of the 5′ end of the SSU, was lacking in 9 of 10 plant isolates examined, the sole exception being *Herpetomonas davidi*, which is not a *Phytomonas.* In contrast, the site was present in all species of *Crithidia*, *Leptomonas* and *Herpetomonas* examined. Interestingly enough, the flagellates lacking the 360 *Pvu*II site also lacked arginase and reacted positively with the *Phytomonas*-specific monoclonal antibodies. In a subsequent study, the association of the three characteristics held true for all presumed *Phytomonas* isolates, among a total of 27 plant flagellates (Teixeira *et al.*, 1996). On the other hand, reactivity with the monoclonal antibodies was never accompanied by the presence of arginase or of the *Pvu*II site in more than 100 trypanosomatids examined (Camargo *et al.*, 1992; Teixeira *et al.*, 1996, 1997).

In an effort further to characterize plant flagellates, Teixeira *et al.* (1996) took advantage of the fact that a certain sequence segment of the intron (+40 to +58) of the spliced leader gene (see Section 6.7.2 and Figure 6) was found to be highly conserved in seven *Phytomonas* isolates but distinct from that in all other trypanosomatid genera (Nunes *et al.*, 1995; Sturm *et al.*, 1995). They designed an oligonucleotide (SL3′) complementary to this region (Figure 6) and hybridized it with the DNA of 84 trypanosomatids of various genera and origins. The results were negative with 31 species of *Blastocrithidia*, *Crithidia*, *Endotrypanum*, *Herpetomonas*, *Leptomonas*,

*Leishmania* and *Trypanosoma*. In contrast, hybridizations were positive with all isolates from plants, except those which clearly were not *Phytomonas*, such as *Herpetomonas davidi* (see Attias and De Souza, 1985; Teixeira *et al.*, 1997) and the isolate from bergamot (Conchon *et al.*, 1989). What was absolutely consistent was that all flagellates hybridizing with SL3′ were promastigotes that also reacted with *Phytomonas*-specific monoclonal antibodies and did not have either arginase or the 360 *Pvu*II site.

These results indicate that there does indeed seem to be a group of trypanosomatids with characters in common that we can call *Phytomonas*. The members of this taxon have the following characteristics: (i) promastigote morphology; (ii) lack of arginase; (iii) lack of the 360 *Pvu*II site in their ribosomal SSU; (iv) specific surface antigens detectable by monoclonal antibodies; and (v) a specific base sequence at positions +40 to +58 of the spliced leader gene that is recognized by the probe SL3′. Since hybridization with SL3′ is always indicative of the presence of the other four characteristics, in practice it suffices to hybridize with SL3′ to verify whether a new isolate is or is not a *Phytomonas*, as defined by these characteristics. In Tables 1–3, cultures of plant flagellates proved to be *Phytomonas* by all the above criteria are marked with a superscript *c*.

Another question that emerges from these findings concerns the hosts of *Phytomonas*. On the one hand, it is well documented that not all plant trypanosomatids are *Phytomonas*. Some are *Crithidia* (see Noguchi and Tilden, 1926; Fiorini *et al.*, 1995b), *Herpetomonas* (see Postell and McGhee, 1981; Attias and De Souza, 1985; Teixeira *et al.*, 1997) or *Leptomonas* (see Conchon *et al.*, 1989). In addition, some fruit flagellates possessing arginase (Almeida *et al.*, 1992) most probably are not *Phytomonas*. On the other hand, it is not certain that all *Phytomonas* spp. are plant parasites. Some may be strict insect parasites. Teixeira *et al.* (1996) reported that 15 of 33 trypanosomatids isolated from phytophagous insects possessed the five key characters of the genus *Phytomonas* and, although of insect origin, should be classified as *Phytomonas*. It is possible, though, that these flagellates were ultimately of plant origin and were by chance collected while developing within their insect vectors. However, although quite possible, this is not obligatory since there is no natural law that forbids members of the taxon *Phytomonas* to parasitize exclusively insects (see Sections 10 and 11).

Regardless of the fact that this and other points need clarifying, at least we have a provisional definition for the genus *Phytomonas* that, as a valid scientific hypothesis, can be disproved or confirmed by new experimental evidence. This definition, which delimits a coherent group of trypanosomatids that we can call *Phytomonas*, is the best answer we have at the moment for the two questions posed at the beginning of this section.

In contrast, for defining or separating species of *Phytomonas* we are at a complete loss. The work of Guerrini *et al.* (1992) proved that, like

trypanosomatids in general (Tibayrenc, 1995), the taxon *Phytomonas* does not constitute a panmictic population but consists of populations with clonal propagation that exhibit obvious differences amongst themselves. These differences can be detected by a variety of methods, such as isoenzyme analysis, lectin agglutination, kDNA 'fingerprinting', random amplified polymorphic DNA (RAPD) studies, etc.

Vainstein *et al.* (1987) reported that the first four cultured isolates from *Euphorbia* could be easily distinguished from each other by isoenzyme profiling. Guerrini *et al.* (1992) comparatively examined by isoenzyme electrophoresis 14 gene loci of six phloem and 25 latex flagellates of varied origins. Their ensuing population genetics analysis demonstrated that '*Phytomonas* zymodemes behave as natural clones' and should be considered as taxa in their own right. Muller *et al.* (1994) also studied by isoenzyme electrophoresis 11 gene loci of 34 *Phytomonas* isolates. They found isolate-specific patterns for most flagellates examined, but also found identical patterns for two isolates from the phloem of coconut trees from distinct geographical areas. This suffices to show that the method is a powerful one to unveil differences or identities between isolates. In addition, isoenzyme data can be used to determine phylogenetic relationships (see Section 8).

Although lectin agglutination is useless for the characterization of the genus *Phytomonas*, Nakamura *et al.* (1989) showed that it is capable of distinguishing isolates from plants. After testing 16 lectins on three isolates, these authors found specific patterns for each isolate. Thus, lectin agglutination may be useful for characterizing and identifying isolates. However, much care is required because the testing methods are somewhat tricky, as shown by the conflicting results obtained by Petry *et al.* (1987) and Sanchez-Moreno *et al.* (1995b) using the same lectin, RCA I, and the same strain of flagellate from *Euphorbia characias.*

Digestion of kDNA with restriction enzymes followed by electrophoresis was initially used to separate strains (schizodemes) of *Trypanosoma cruzi* (see Morel *et al.*, 1980) and to separate species of lower trypanosomatids (Camargo *et al.*, 1982). When tested on *Phytomonas*, the method proved adequate for the same purposes, frustrating the initial hopes that 'minicircle cleavage pattern could be used to identify the genus' (Riou *et al.*, 1987). Thus, Ahomadegbe *et al.* (1990) found isolate-specific digestion patterns for 12 isolates from latex or phloem of plants from distinct geographical areas. The existence of isolate-specific patterns can also be inferred from the work of Muller *et al.* (1995), Ahomadegbe *et al.* (1992) and Fernandes-Becerra *et al.* (1996). In addition, Ahomadegbe *et al.* (1992) have shown that restriction enzyme cleavage is a useful means of determining whether two isolates are identical, irrespective of their geographical origin.

Monoclonal antibodies have also been used on a limited scale in attempts to distinguish isolates of *Phytomonas* (Petry *et al.*, 1989). The results were not conclusive, but in principle it remains possible to raise a monoclonal antibody specific for each isolate since, among other components, they seem to have distinct cell surface carbohydrate epitopes (Nakamura *et al.*, 1992; Teixeira *et al.*, 1995).

Size and sequence differences of the transcribed and non-transcribed segments of the spliced leader gene make it potentially useful for distinguishing isolates (Sturm *et al.*, 1995; Nunes *et al.*, 1995). Analysing the size of the intergenic region, Sturm *et al.* (1995) distinguished three flagellates from fruit, phloem and latex. Attempts to use the mini-exon gene to separate isolates stopped there.

Restriction enzyme cleavage of ribosomal DNA (rDNA) also yields isolate-specific profiles, although the method has not been used specifically for the identification of isolates of *Phytomonas* (see Camargo *et al.*, 1992).

More recently, in a preliminary assay Kaneshima *et al.* (1994) have succeeded in distinguishing several isolates from plants and phytophagous insects by means of RAPD.

All the methods mentioned above can disclose differences or identities between *Phytomonas* isolates and therefore are reliable identification methods. However, there are some problems. Two isolates found to be identical by one method may prove different by another. Moreover, even the same method may unveil differences between apparently identical isolates if more variables are introduced. This can be achieved, for instance, by testing a larger number of isoenzymes or by using a larger number of restriction enzymes in kDNA or rDNA digestion or, yet again, by using more random primers in RAPD. Thus the finer the analysis, the higher the diversity, i.e. any population can be split almost infinitely into subpopulations. This is not surprising, but raises a serious problem for identifying species.

I am not referring to the more complex and far-reaching question of what constitutes a species in asexual organisms (Dobzhansky, 1972; Wiley, 1978; Thompson and Lymbery, 1990; Poljanski, 1992). The question is a practical one: How many and which characteristics should be chosen to delimit a population that could be called a species among plant flagellates? There are still no objective criteria for that.

The tradition with 'lower' trypanosomatids is that flagellates from different hosts should be considered to be distinct species. Wallace *et al.* (1983) have already warned against this practice, which has also proved inadequate for *Phytomonas* spp. This is because quite distinct flagellates can be isolated from the same host species (Conchon *et al.*, 1989; Jankevicius *et al.*, 1993), whereas the same flagellate may colonize distinct hosts (Campaner *et al.*, 1996).

At present, in the absence of defined and objective criteria, the consensus of experienced specialists seems to be the most reliable criterion for defining an asexual species. Specialists on *Phytomonas*, since their first informal meeting in 1987, have considered that the state of knowledge of plant flagellates does not yet permit a decision on what criteria to use to define a species (Dollet and Wallace, 1987). Accordingly, they have recommended that naming a new species should be avoided and suggested ways for labelling and referring to isolates (see Appendix). Workers seem to have held to that because, although many plant flagellates have been isolated after 1987, only one new species has been named, *Herpetomonas mcgheei* (Jankevicius *et al.*, 1993), later reclassified as *Phytomonas mcgheei* by Teixeira *et al.* (1997). Although the recommendation was a cautious and opportune one, it created unforeseen problems. The *Phytomonas* field is growing and many more researchers are using *Phytomonas* isolates in their studies. For lack of an easy identification system, cultures have been passed along with faulty identification while references to isolates became vague and obscure. This, in addition to culture mixing, is seriously endangering the utilization of data for comparative purposes (Shaw and Camargo, 1995). All this resulting confusion may render it preferable to name a species (by specialist consensus), even at the risk of having to review or rename it later. It is time for the specialists to begin considering this aspect of the problem.

Still concerning taxonomy, attempts were or are being made to identify subgroups within the taxon *Phytomonas* by different methods. Muller *et al.* (1994), using isoenzyme electrophoresis, could distinguish two groups of phloemicolous, three groups of lacticolous and one group of fructicolous flagellates among 22 primary isolates and 12 clones of flagellates of differing plant origin. These observations were later confirmed by kDNA analysis (Muller *et al.*, 1995). I also know from personal information that *Phytomonas* isolates are being grouped by restriction enzyme mapping, spliced leader sizes and sequences, and RAPD (M.M.G. Teixeira, M. Serrano and S.I. Jankevicius, respectively; personal communications).

These efforts may be of importance in unveiling the phylogenetic relationships within the taxon *Phytomonas* and, in addition, may lead to practical applications. This was the case with the observation by Marché *et al.* (1995) of the presence of an *Eco*RI site at position 752 of the SSU of the rDNA gene, which appears to be exclusive to the flagellates of coconut and oil palms. This site does not occur in any other trypanosomatid group (Camargo *et al.*, 1992), and may thus be diagnostic of palm *Phytomonas* (Marché *et al.*, 1995).

## 8. PHYLOGENY

Interest in the phylogeny of the Trypanosomatidae is not recent (Hoare, 1925, 1967; Baker, 1963; Nicoli and Quilici, 1964; Lipa, 1965; Molyneux, 1986), but in recent years, thanks to molecular data, there has been a revival of the subject (see Vickerman, 1994; Maslov and Simpson, 1995). These data reversed earlier views based on morphology and life cycles, which considered the genus *Trypanosoma* to be the most evolved organisms and insect trypanosomatids the most primitive ones, hence the phrase 'lower trypanosomatids'. In addition, trypanosomatids are no longer considered monophyletic, and the adoption of vertebrate parasitism or its reversal is thought to have occurred more than once.

In spite of these advances, information about the phylogenetic relationships of the taxon *Phytomonas* is limited. This is because either some phylogenetic studies did not include *Phytomonas* (see Lake *et al.*, 1988; Briones *et al.*, 1992; Landweber and Gilbert, 1994) or the *Phytomonas* isolate examined was not an authentic representative of the genus (Fernandes, A.P. *et al.*, 1993; Santos *et al.*, 1994). Studies including genuine *Phytomonas* spp. involved either rDNA sequencing (Marché *et al.*, 1995; Maslov *et al.*, 1996; Lukes *et al.*, 1997) or isoenzyme electrophoresis (Muller *et al.*, 1994). These studies produced, respectively, phylogenetic trees and dendrograms, which have shown that *Phytomonas* is much closer to *Herpetomonas* than to any other genera and that both genera nest in the family tree somewhere below *Leishmania*, *Crithidia* and *Leptomonas* and above *Trypanosoma*.

In addition, Marché *et al.* (1995) examined several representatives of *Phytomonas* and *Herpetomonas* and showed that *Herpetomonas* is closer to phloemicola than to lacticola, a fact which can also be inferred from the dendrograms of Muller *et al.* (1994).

Sequence and isoenzyme data validate results of a different nature, which also suggested the close relationship between *Herpetomonas* and *Phytomonas*. Camargo *et al.* (1992) reported that, among 54 trypanosomatids examined, *Herpetomonas* and *Phytomonas* were the only ones to lack a site for *Hin*dIII at position 1500 of the 24α large subunit of the ribosomal gene. Nunes *et al.* (1994), comparatively studying kinetoplast sequences, also reported on the close relationship between *Herpetomonas* and *Phytomonas*. In this context it is worth mentioning that at least one species of *Herpetomonas*, *Herpetomonas davidi* (originally *Phytomonas davidi*), was recovered from lactiferous plants.

## 9. PATHOGENESIS

### 9.1. Diseases of Lactiferous Plants

A. Lafont lived in an exciting scientific atmosphere populated with evil-doing trypanosomes of humans and other animals. Thus, nothing was more natural for him than to ask a question which is still controversial today: are trypanosomatids pathogenic in euphorbias? If this question remains unanswered for euphorbias, there seems to be no doubt that trypanosomatids are pathogenic in palms, cassava and coffee.

#### 9.1.1, *Euphorbias and Asclepiads*

Lafont was convinced that flagellates were pathogenic in euphorbias due to their abundance in the lactiferous ducts of infected plants. In his description of the disease, he said that 'the leaves desiccated and corrugated before falling down whereas the stem would turn brownish and scrawny before drying out' (translated from Lafont, 1910).

Lafont argued with some of his contemporaries who did not consider *la flagellose des euphorbes* an actual plant disease (Lafont, 1911a). The cause of the dispute was the appearance of reports from different parts of the world emphasizing the dissociation between the presence of flagellates and symptoms of disease (Bouet and Roubaud, 1911; Léger, 1911; Noc and Stevenel, 1911; Rodhain and Bequaert, 1911).

On the side of Lafont was Carlos França, an author who studied the flagellosis of euphorbias more seriously (França, 1914, 1920). He was convinced that *Leptomonas davidi* was indeed pathogenic in *Euphorbia segetalis* in Portugal. However, he described the disease as restricted to a few branches of the plant, i.e. a localized rather than a systemic disease. França attributed the localized nature of the disease to the peculiar organization of the lactiferous tubes of the Euphorbiaceae. These ducts are of the non-articulated type and, although they branch all over the plant, they never establish anastomoses amongst themselves or with vessels of other types. Therefore, infection of one duct would remain restricted to its own territory only. Moreover, França claimed that coagulated latex plus the mass of flagellates clogged the ducts, a fact that also helped to circumscribe the disease. Nevertheless, to França, flagellate infection was equal to disease, limited as it might be. He also considered the flagellosis of the euphorbias to be a nutritional disease resulting from dilution of the latex and the disappearance of its starch granules, a fact easily verifiable by microscopy.

Holmes (1925a), in North America, held opposite views. Examining infected and uninfected plants of *Asclepias* spp. throughout the year, he

found no difference between them. He attributed the signs of decay presented by both infected and non-infected plants to climatic variations. Dollet *et al.* (1979) reported finding, in the same area of Ecuador, species of *Euphorbia* 100% infected but without symptoms of disease whereas other species had naked branches or brownish leaves in which flagellates were easily found.

Attempts to reproduce the disease by inoculating flagellates into plants have also yielded conflicting results (Noc and Stevenel, 1911; França, 1914, 1920; Laveran and Franchini, 1920; Franchini, 1923e). França always obtained only localized infections whereas Noc and Stevenel obtained generalized and devastating infections within 48 hours of inoculation.

Reflection about these observations clearly shows that the authors were talking about different things. Their experiments and observations were not comparable because plant hosts (obviously) and flagellate strains (probably) varied from observation to observation. This is because, as demonstrated by Guerrini *et al.* (1992), different species of *Euphorbia*, sometimes from the same geographical area, may harbour different flagellate populations (zymodemes). Thus, it is conceivable that different strains might exhibit different degrees of virulence.

However, praise is due to França, who seemed to be aware of these variables. He once said that 'the day will come when we will prove that the parasites described under the name of *Leptomonas davidi* belong to many species' (translated from França, 1922b).

Unfortunately, for the last 60 years no research has been performed on the pathogenicity of flagellates in euphorbias, which remains an unsolved question.

### 9.1.2. Phytomonas francai *and the disease of cassava*

*Manihot* is a genus of Euphorbiaceae originally from the Americas, presently cultivated in many tropical countries because its roots are an important food source known as manioc or cassava. There are large plantations all over the world in addition to small backyard plots for family consumption, particularly in undeveloped regions.

Henrique B. Aragão was the first to describe the presence of flagellates in the latex of *Manihot palmata* (=*M. esculenta*). He named the flagellates *Phytomonas francai* but in his reports did not mention any symptom of plant disease (Aragão, 1927, 1931). Siqueira-Jaccoud (1955) found flagellates in manioc in the Amazon region of Brazil, also without symptoms of disease.

In 1979, cassava plantations in the state of Espirito Santo, Brazil started to present marked chlorosis of the aerial parts and considerable atrophy of

the roots, leading to high economic losses. A variety of *Manihot esculenta*, the Unha cultivar, was particularly vulnerable to the disease, which became locally known as *chochamento das raizes* (shrinking of the roots) (Silveira and Pereira, 1983). In 1986, Elliot Kitajima reported finding flagellates in the latex of the diseased plants. As observed by França in other lactiferous plants, the infection was limited. Not all lactiferous ducts of the plants were infected. The flagellates were later cultured by Vainstein and Roitman (1986).

Although flagellates were present in large numbers in diseased plants (Figure 7A), it has not been demonstrated experimentally that they were the aetiological agents of the disease. Nevertheless, the search for viruses, bacteria, fungi and phytoplasms has been extensive but always negative (Kitajima *et al.*, 1986).

Fortunately, the Unha cultivar of cassava is not widely grown in Brazil and, although some plantations were decimated in parts of Espirito Santo in the 1980s, there has been no recent report of large-scale disease in Brazil or in any other part of the world.

Absolutely nothing is known about the transmission of the disease. The only suspect, the hemipteran tingid *Vastiga* sp., was acquitted by Kitajima *et al.* (1986) on the basis of the absence of trypanosomatids in its digestive tract.

## 9.2. *Phytomonas leptovasorum* and the Diseases of Coffee Trees

In 1906, several authors started reporting a new disease of coffee trees in the relatively small plantations of Surinam (reviewed by Stahel, 1931a; Vermeulen, 1963, 1968). The disease occurred in two major forms. In the acute form, trees withered and died within 2 months, a few black leaves left hanging from the bare branches to mourn their sad fate. In the chronic form the disease was insidious. Patches of leaves slowly turned yellowish and fell from the limp branches within a year. Death was inevitable whenever the disease appeared. This 'coffee wilt' had various names and various suspected aetiological agents, ranging from viruses to fungi, the most popular of which was the fungus *Ceratocystis fimbriata*.

Gerold Stahel devoted his time to the study of the disease from 1916 to the 1930s. After systematic examination of all plant parts by sectioning and microscopy, Stahel described what became the histopathological hallmark of the disease: necrosis of the root phloem accompanied by characteristic multiple division of the sieve tubes. Phloem necrosis became the accepted name for the coffee wilt. Until 1930 there was no known fact, only speculation, about the aetiology of the disease. Then Stahel found flagellates in the sieve tubes of wilted trees. It must have been an exciting moment when

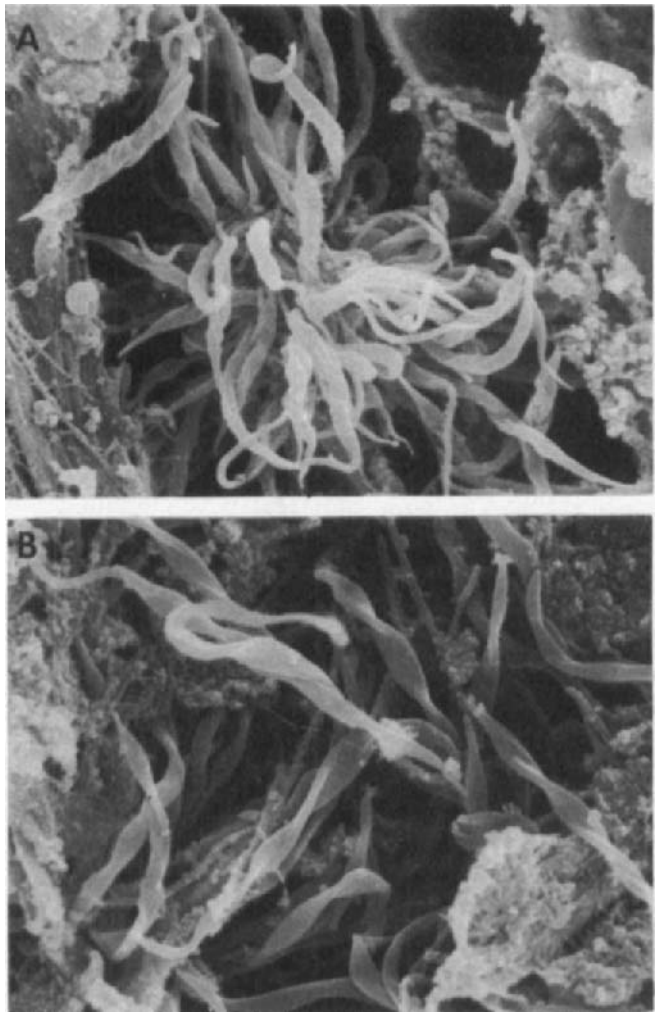


*Figure 7 Phytomonas francai* and *P. staheli.* Scanning electron micrographs of flagellates from (A) the lactiferous tubes of cassava and (B) the phloem vessels of the roots of coconut. (Courtesy of Dr Elliot Kitajima and Dr Wanderley De Souza, respectively.)

'after a lengthy search I found . . . in the divided tubes a large quantity of dark fusiform bodies. . . . With Giemsa the nuclei stained well, showing to me that we were dealing with a trypanosomatid, i.e., a *Phytomonas*, very similar to *Phytomonas davidi*' (translated from Stahel, 1931a). Stahel named the flagellate *Phytomonas leptovasorum* because of its habitat (Stahel, 1931a,b, 1933).

In the 1960s, Emdem (1962) and Vermeulen (1963, 1968) studied a new outbreak of coffee wilt in Surinam and confirmed all the findings of Stahel. Vermeulen continued the search for the vector and the exclusion of alternative aetiological agents. Although he found flagellates in the pentatomid hemipteran *Ochlerus*, which thrives on coffee plantations, he did not achieve disease transmission through this or any other insect. He could, however, transmit the disease from sick to healthy plants by root grafting. The role of nematodes and scale insects as vectors was considered, but without confirmation (Vermeulen, 1963).

The coffee plants affected by phloem necrosis were the species *Coffea liberica*, a cultivar of lesser economic importance. However, Vermeulen was able to transmit, by grafting, the flagellosis to various species of *Coffea* (*C. arabica*, *C. robusta*, *C. excelsa*) of great importance in other parts of the world (Vermeulen, 1968). Fortunately, in spite of the finding of phloem necrosis in plants from north-eastern Brazil sent to Stahel by Hardman and Pickel (Stahel, 1931b), the disease never became important in that country, which at the time was the world's largest coffee producer.

### 9.3. *Phytomonas staheli* and the Diseases of Palms

The disease known as *hartrot* (Dutch for bud or heart rot) was first reported at the beginning of the century in coconut (*Cocos nucifera*) plantations in the coastal areas of Surinam. According to Drost (in Kastelein, 1987), between 1918 and 1933 about 25 000 of the 60 000 coconut trees in the Coronie district died or had to be cut down and burned because of hartrot.

The disease known as *marchitez* or *muerte sorpressiva* is said to have appeared for the first time in 1963 in oil palm plantations (*Elaeis guineensis*) in Santander, Colombia (Lopez *et al.*, 1975). However, a disease of oil palms resembling *marchitez* had already been reported as early as 1921 by Drost in Surinam, near Paramaribo (in Slobbe *et al.*, 1978).

Both diseases, *hartrot* and *marchitez*, are acute lethal wilts. Their reported aetiological agent is *Phytomonas staheli* (see McGhee and McGhee, 1979). The diseases, although affecting two distinct genera of palms, have a roughly similar course. This led many authors (Slobbe *et al.*, 1978; Silveira and Pereira, 1983; Kastelein, 1987) to name both diseases *hartrot*, although in oil palms the rotting of the bud is not always present (Lopez *et al.*, 1975). The flagellosis preferentially affects adult trees and infects all of their parts. The first disease symptom is the progressive browning of the leaves, first the older ones and then the younger ones. Next, inflorescences begin to show rusty spots. The fruit colouration becomes dull and progressively turns brown as fruits rot and fall. Finally,

the spear and the roots rot and the plant dies, withered and wasted, within a couple of months from the onset of the symptons (Lopez *et al.*, 1975; Parthasarathy and Slobbe, 1978; Slobbe *et al.*, 1978; Waters, 1978; Thomas *et al.*, 1979; Renard, 1989).

The aetiology of *hartrot* and *marchitez* remained nebulous for more than 60 years after the first finding of diseased coconut trees. Viruses, phytoplasms, bacteria, and nematodes have all been considered as possible aetiologial agents. On further scrutiny, none met the original expectation. Nowadays, when one sees the abundance of flagellates swimming in the sap of diseased trees, one wonders how this could have passed unnoticed by skilled researchers. Even Stahel, the discoverer of the flagellates of coffee, reported inconclusively on *hartrot* (Stahel, 1938). It was only in 1976 that Parthasarathy *et al.* found trypanosomatids in the phloem of coconut palms. One year later, trypanosomatids were reported in the phloem of oil palms (Dollet *et al.*, 1977). Trypanosomatids also occur in other genera and species of Palmae (see Table 2) but, besides *Cocos* and *Elaeis*, aetiologically diagnosed *hartrot* has been reported only in the royal palm, *Roystonea regia*, in Bahia, Brazil (Attias *et al.*, 1989).

Flagellates of palms (Figure 7B) do not show any special diagnostic trait by either light or electron microscopy (Parthasarathy *et al.*, 1976; Dollet *et al.*, 1977; Dollet and Lopez, 1978; Parthasarathy and Slobbe, 1978; Waters, 1978; McGhee and McGhee, 1979; Thomas *et al.*, 1979; McCoy and Martinez-Lopez, 1982; Attias *et al.*, 1987a). Long twisted promastigotes coexist with amastigotes in the sieve tubes of diseased palms (Dollet and Lopez, 1978). According to Thomas *et al.* (1979), 'flagellates were observed only in mature sieve elements of the protophloem and metaphloem'. Their distribution was not uniform. Dollet and Lopez (1978) reported that 50–80% of the vascular bundles examined contained flagellates. These figures are different from the 17% rate reported by Thomas *et al.* (1979), but these authors referred to palms in different geographical areas. The latter authors also reported that 50% of the tubes within a vascular bundle were infected. Both reports described flagellates in the pores of the sieve plates. Dollet and Lopez (1978) measured the diameters of the pores (0.5–1.5 μm) and of the cell body of the flagellates (0.6–1.2 μm) and suggested that the ease with which the trypanosomatids could cross the sieve plate barrier explained their dissemination along the plant. One may add that the twisted, corkscrew-shaped body of sap flagellates must facilitate crossing the plate pores.

The flagellosis of Palmae is a cause of permanent economic concern to many countries in Latin America. Fortunately, the infection does not affect Palmae in other regions of the world, like South-East Asia, where most of the plantations of oil palm are concentrated.

In Latin America, the wilts of coconut and oil palms were first noticed in Surinam (see reviews by Parthasarathy and Slobbe, 1978; Slobbe *et al.*,

1978; Kastelein, 1987). It does not necessarily follow that they originated there. It may be merely that the agricultural services of the former Dutch colony were more alert than their counterparts elsewhere in the continent. Hoof (1962) redescribed the disease of oil palms in Oema, Surinam, at the same place where Slobbe *et al.* (1978) subsequently found flagellates in oil palms.

After Surinam, *marchitez* was reported in Colombia (Corrado, 1970; Lopez *et al.*, 1975; McCoy and Martinez-Lopez, 1982), Peru (Dollet *et al.*, 1977), Ecuador (Thomas *et al.*, 1979) and Brazil (Oliveira and Bezerra, 1982; Oliveira *et al.*, 1987). *Hartrot* was successively reported in Trinidad-Tobago (Barrow and Oliver, 1977; Waters, 1978), Colombia (McCoy and Martinez-Lopez, 1982), French Guyana (Louise *et al.*, 1986) and Brazil (Bezerra and Figueiredo, 1982).

It has not been proved experimentally that trypanosomatids are in fact the cause of *marchitez* and *hartrot*. However, all the epidemiological evidence favours this hypothesis.

Another subject of study is the association between disease and the presence of virus-like particles in palm flagellates (see Dollet *et al.*, 1996). Initially, Marché *et al.* (1993) reported that viral particles, as diagnosed by electron microscopy and the presence of a double-stranded RNA of ~4.7 kilobases (kb), were present in flagellates isolated from diseased coconut or oil palms but in none of the latex or insect trypanosomatids examined. However, the available data do not demonstrate conclusively that virus must be present for the flagellates to be pathogenic, especially because no viral RNA was detected in single isolates from the oil palm and coconut by Marché *et al.* (1995).

The transmission of the flagellates of palms (see Section 10) is also a controversial subject (Segeren and de Jong, 1985). Griffith (1980) achieved infection and disease in coconut palms after exposing 3-year-old plants to nymphs and adults of the hemipteran *Oncopeltus cingulifera* [sic], which had been collected on *Asclepias curassavica* and were naturally infected with trypanosomatids. From these experiments, Griffith (1980) explicitly concluded that the coconut wilt disease was caused by a flagellate 'originating in the milkweed plant on coconut estates'. He also postulated that *Micrococcus roseus* was a partner of the flagellates in the aetiology of the disease.

Trying to unveil the vector of *marchitez* in Ecuador, Chenon (1984) transferred *Lincus* sp. (actually *Lincus lethifer*; Hemiptera: Pentatomidae) collected on infected palms to palms in areas from which the disease was absent. Since the latter acquired *marchitez*, he concluded that *Lincus* sp. was the vector of *marchitez*. Later, Perthuis *et al.* (1985) repeated the transfer of *Lincus lethifer* to a caged oil palm which shortly thereafter displayed the symptoms of *marchitez*.

Louise *et al.* (1986) have also succeeded in transmitting *hartrot* to caged coconut palms through nymphs and adults of *Lincus* spp. (*L. cropius*, *L. apollo* and *L. dentiger*) collected on infected trees.

Most authors favour *Lincus* as vector of *Phytomonas staheli*, in spite of the negative results obtained by Dollet *et al.* (1997) with laboratory-reared infected insects (see Section 10). However, the pentatomid *Macropygium* sp., feeding on the roots of palms and harbouring flagellates in its salivary glands (Liceras and Hidalgo, 1987), has been largely overlooked as a possible disease vector.

## 9.4. New Diseases Caused by Phloem Flagellates

In the second *Phytomonas* workshop, held in Santa Marta, Colombia, Gargani *et al.* (1992) incidentally reported on the finding by P. Hunt of flagellates in the phloem of decaying plants of *Alpinia purpurata* in Grenada. *Alpinia* is an ornamental plant widely cultivated in the Caribbean and many other parts of the world. Neither in the report at the Santa Marta meeting nor elsewhere (Muller *et al.*, 1994, 1995; Marché *et al.*, 1995) were details given about the disease affecting alpinias. According to M. Dollet (personal communication), symptoms of the disease include the wilting of the flowers in addition to the progressive withering of the leaves. Plants die shortly thereafter.

The flagellate from alpinias was compared by kDNA and rDNA restriction analysis with latex, fruit and phloem flagellates and found to be indistinguishable from the phloem flagellates, although distinct from the others (Muller *et al.*, 1994, 1995; Marché *et al.*, 1995).

## 9.5. Trypanosomatids of Fruit: *Phytomonas serpens* and *Phytomonas mcgheei*

Only two species of trypanosomatids of fruit have been named so far, *Phytomonas serpens* isolated from tomatoes (Gibbs, 1957; Jankevicius *et al.*, 1989) and *Phytomonas mcgheei* isolated from maize (Jankevicius *et al.*, 1993; Teixeira *et al.*, 1997). Nevertheless, flagellates are widespread in fruit; 33 different species of fruit have been reported to be harbouring flagellates, one in Africa (Gibbs, 1957), three in Europe (Franchini, 1922a; Sanchez-Moreno *et al.*, 1995b) and the remainder in Brazil (see Table 3). This uneven distribution, rather than indicating a strange fondness of flagellates for Brazilian fruit, merely reflects the presence in Brazil of researchers looking for plant flagellates. The ease with which flagellates are found in fruit suggests that they are ubiquitous and that finding them anywhere in

the world is dependent only on looking for them. Fruit flagellates appear to be restricted to fruits and have never been reported to spread to other plant parts.

Nothing is known about the pathogenicity of flagellates in fruit. Fruits in general are programmed to rot. Thus, if the deleterious effect of flagellates on their juices favours rotting, this would hardly be noticed and could even be beneficial by helping fruits to fulfil their destiny. However, matters may be different with respect to seeds and, from a commercial standpoint, edible fruit.

Unfortunately, there is not a single published paper on the pathogenicity of trypanosomatids in fruit. However, many researchers, in different circumstances, have informally voiced their opinion on the subject, which makes up the bulk of the discussion that follows.

It is generally believed that, in tomatoes, the bites of infected insects result in circular, yellowish, easily noticeable spots on the surface of the fruits. However, bites of non-infected insects may also produce similar effects, although of a different magnitude. It is believed that these yellow spots reduce the commercial value of tomatoes, although this has not been measured, and it is possible that only persons interested in *Phytomonas* pay any attention to these tiny spots.

Another very simple question that has never been addressed is whether infected tomatoes, or any other fruit, rot faster than non-infected ones. The ethanol and carboxylic acids produced in the processing of carbohydrates by plant trypanosomatids (see Section 6.1) might render the juice of infected fruits sour or acidic. In certain fruit, however, acidity is not necessarily bad and ethanol may even be appreciated. Nevertheless, these by-products of flagellate infection and their effect on the quality of fruit have never been evaluated.

With respect to seeds, lack of information also prevails. Insect piercing generates minuscule cavities in the seed body, which are filled with flagellates and, eventually, yeast and bacteria. Logical reasoning predicts that such an infection would lead to diminished fertility. Not so, according to J.V. Jankevicius, who observed just the opposite in maize, although he did not quantify the observation (personal communication). In summary, the pathogenicity of trypanosomatids in fruits and seeds is a totally open question.

Phytophagous hemipterans are certainly involved in the transmission of trypanosomatids of fruit (see Section 10). It has been firmly established that the coreid *Phthias picta* is the vector of *Phytomonas serpens* (see Jankevicius *et al.*, 1989), although it is not necessarily the only one (Kastelein and Camargo, 1990). The vector of *Phytomonas mcgheei* is the coreid *Veneza zonata*, formerly known as *Leptoglossus zonatus* (see Jankevicius *et al.*, 1993).

## 10. TRANSMISSION

Camargo and Wallace (1994) reviewed the observational and experimental evidence related to the transmission of plant flagellates. No new evidence has been produced that would justify a reappraisal of the problem. Thus, only a summarized view of the subject is presented here.

Lafont did not hesitate in considering the many phytophagous Hemiptera that he noticed piercing the euphorbias of Mauritius as possible vectors of *Leptomonas davidi*. His prime suspect, the lygaeid *Nysius euphorbiae*, was soon demonstrated to be able to infect flagellate-free euphorbias (Lafont, 1911b). Since then, piercing phytophagous bugs have been generally regarded as the main vectors of plant flagellates. Flagellates are frequently found in their salivary glands and are thought to be inoculated with the saliva injected before or during their feeding on the host plant (Camargo and Wallace, 1994).

Indeed, the presence of trypanosomatids in Hemiptera is conspicuous. About 100 different species of phytophagous hemipterans were recorded by Camargo and Wallace (1994) as hosts of trypanosomatids. Just one new host has been added to the list in recent years, the pentatomid *Euchistus herus* (see Fiorini *et al.*, 1993a), plus one bug overlooked by Camargo and Wallace (1994) in their review, the pentatomid *Ochlerus* sp. (Vermeulen, 1963). Most of the flagellate-harbouring species listed were members of the family Coreidae (34), followed by the Pentatomidae (30), Lygaeidae (21), Pyrrhocoridae (9), Miridae (6) and Corimelaenidae (1). These data, however, were not the product of a systematic study on the prevalence of flagellates in insects, but the result of incidental observations by various researchers in diverse circumstances.

The only study deliberately done to assess the prevalence of flagellates in bugs was that by Sbravate *et al.* (1989), which revealed the following prevalence rates: Pyrrhocoridae, 44%; Coreidae, 40%; Lygaeidae, 19%; and Pentatomidae, 18%. The overall prevalence of flagellates in phytophagous Hemiptera was 35%. Trypanosomatids could be found in the digestive tube, haemocoel (seldom) and salivary glands of their insect hosts. Of the infected insects, 68.4% had flagellates exclusively in the digestive tract, whereas 31.6% harboured flagellates in their salivary glands, often together with digestive tract infections (29.3%). Most of the trypanosomatids were promastigotes (99.9%), some with large twisted bodies; a few were epimastigotes or choanomastigotes, usually mixed with promastigotes.

Flagellates in phytophagous bugs are not necessarily obligatory plant flagellates. They may be monoxenic insect parasites. It is impossible to tell from morphology alone which flagellates are plant parasites. It is possible, however, to say which are of the genus *Phytomonas* by testing with

monoclonal antibodies (Teixeira and Camargo, 1989; Teixeira *et al.*, 1995) or spliced leader gene probing (Nunes *et al.*, 1995; Teixeira *et al.*, 1996). By these methods, *Phytomonas* was detected in 12 of 38 phytophagous insects (31.5%) harbouring trypanosomatids, i.e. roughly one-third of the flagellates in the bugs were *Phytomonas* spp. These occurred in 7 of 15 different species (50%). The species harbouring *Phytomonas* were *Fabrictilis gonagra*, *Holymenia histrio*, *Hypselonotus fulvus*, *Veneza zonata*, *Veneza* sp., *Phthias picta* (all coreids) and *Nezara viridula* (a pentatomid). It is worth noting that these species may also harbour other genera of trypanosomatids (Sbravate *et al.*, 1989; Teixeira *et al.*, 1996).

There is presently no means of telling whether or not the *Crithidia*, *Leptomonas* or *Herpetomonas* found in phytophagous insects are plant parasites. Therefore, nothing can be said about the vector role of these insects. The only foolproof way to ascertain that an insect is a vector of plant flagellates is to infect a host plant via its bite (or defecation?). In a few cases, this has been done experimentally.

Early authors quickly identified phytophagous insects as vectors of the flagellates of euphorbias (Bouet and Roubaud, 1911; França, 1919 1922a,b; Strong, 1924). Holmes (1925c) demonstrated that the lygaeid *Oncopeltus fasciatus* transmitted the flagellosis of asclepiads. Bugs of the same genus have, on different occasions, also been considered as vectors of flagellates of various species of Asclepiadaceae (Vickerman, 1962; Hanson and McGhee, 1963; McGhee and Hanson, 1964). The vector of the flagellates of Cecropiaceae was shown to be *Edexa loxdali*, a pentatomid (Kastelein, 1985).

In a series of well-documented cage experiments, Jankevicius *et al.* (1989) transmitted *Phytomonas serpens* from laboratory-bred tomatoes to the coreid *Phthias picta* and vice versa. In addition to *P. picta*, the pentatomid *Arvelius albopunctatus* was found to be capable of transmitting flagellates between fruit of various species of Solanaceae (Kastelein and Camargo, 1990). The ubiquitous presence of this insect in shrubs of Solanaceae makes it the major candidate for disseminating flagellates within this large family of commercially important angiosperms. Jankevicius *et al.* (1993) demonstrated that the flagellates present in the salivary glands and gut of *Veneza zonata* (formerly *Leptoglossus zonatus*) could be transmitted to the seeds of maize (*Zea mays*) and vice versa.

The transmission of phloem flagellates was more difficult to establish because of understandable operational difficulties involved in the caging of palms and the long period of observation required, since the first symptoms of the disease take months to develop. Nevertheless, it was possible to demonstrate that species of the pentatomid bug *Lincus* were responsible for the transmission of the disease of palms (Chenon, 1984; Dolling, 1984; Asgarali and Ramkalup, 1985; Perthuis *et al.*, 1985; Louise *et al.*, 1986;

Rezende *et al.*, 1986). However, adult *Lincus* sp. experimentally infected with cultures of the coconut flagellate did not transmit the infection to young palms, and although there were flagellates in their gut they were never present in the salivary glands (Dollet *et al.*, 1997).

The transmission of the flagellosis of coffee and cassava remains a mystery.

## 11. RESERVOIRS

The discovery of flagellates in palms immediately raised questions about their transmission and about the possible existence of reservoirs for these parasites. From the start, lactiferous plants were candidates for the latter role and, accordingly, those proliferating around palm plantations have been thoroughly examined. Although many lactiferous weeds were found to be harbouring flagellates in palm plantations (Kastelein *et al.*, 1984a,b), it could not be determined whether the flagellates of weeds and palms were identical. This was because, until the 1980s, only morphological criteria were available and morphology was useless for this purpose.

Isoenzyme studies (Guerrini *et al.*, 1992; Muller *et al.*, 1994) and ribosomal RNA sequence comparisons (Marché *et al.*, 1995) made it clear that isolates of flagellates from palms were different from those from lactiferous plants and fruits. However, the same methods have shown differences of a comparable order of magnitude between isolates within each group. Therefore, data of this kind do not permit one to say that flagellates isolated from palms could not have a latex or fruit origin. In contrast, the data showing that palm flagellates have an *Eco*RI site in the SSU of the ribosomal gene, which latex and fruit flagellates do not have (see Sections 6.7 and 12.3), are strong evidence against the latex or fruit origin of palm flagellates.

However, the definitive answer to the question about reservoirs of palm flagellates could be obtained by infecting palms with flagellates of other origins. In attempts at experimental infection, Griffith (1980) obtained evidence that insects which had acquired flagellates from lactiferous plants were able to transmit the flagellates (and *hartrot*) to coconut trees (see Section 9). If these findings are confirmed, then latex plants would indeed be reservoirs of palm flagellosis.

There is another aspect of the problem that may be more pertinent to fruit flagellates but could apply to palm flagellates as well. This concerns the possibility that certain insects, in addition to their vector role, might also act as reservoirs. In other words, it is possible that plant flagellates circulate for long periods of time exclusively among insects, passing from

insect to insect of the same or different species. Thus, species of *Phytomonas* could be perpetuated in insects, their transmission to plants being an eventual step in their life cycles. Obviously, this is only a hypothesis, but it is not entirely groundless. It is known that insects can harbour flagellates throughout their lives, as best exemplified by the existence of living flagellates in the salivary glands of overwintering phytophagous insects (França, 1920). In addition, in contrast to *Leishmania* and *Trypanosoma*, 'lower' trypanosomatids can be transmitted from insect to insect. The facility with which trypanosomatids sometimes spread to an uninfected colony of phytophagous insects from a single infected insect has been a recurrent problem in our laboratory, although we could not determine at the time whether the infecting flagellates were of the genus *Phytomonas* (see Moraes *et al.*, 1994). Finally, it is known that flagellates of a given species of Solanaceae can infect and colonize various insect species (Jankevicius *et al.*, 1989; Kastelein and Camargo, 1990). Thus, the vector–reservoir role of insects is a working hypothesis that deserves further attention.

## 12. DIAGNOSIS

With regard to diagnosis, four aspects must be considered: (i) the diagnosis of the disease; (ii) the detection of flagellates in the plants; (iii) the identification of the genus of the flagellates; and (iv) the characterization of the infecting strain.

The diagnosis of the disease is made by the external symptons (see Section 9), although these are not pathognomonic. Therefore, the diagnosis must be confirmed by demonstrating the presence of flagellates in the diseased plant. This can be done microscopically or by indirect means.

### 12.1. Detection of Flagellates in Plants

In heavy infections, finding flagellates swimming in plant juices by light microscopy is easy for a trained person. However, untrained observers may fail to detect them. It should be remembered that, although *hartrot* was already known at the beginning of the century and palms have been thoroughly examined ever since, flagellates escaped light microscopical detection until 1977, when electron microscopy revealed their presence in coconut and oil palms and later in cassava.

In light infections or at the onset of the symptoms in palms, the detection of flagellates may be very difficult and time-consuming. In fruit, natural infections are never exuberant; usually it is very difficult to find

flagellates by light microscopy. In these circumstances it may be necessary to resort to other methods.

The absence of antibodies in plants precludes the use of immunological methods for diagnosis. However, in principle, anti-*Phytomonas* antibodies could be used to detect flagellate antigens in plant juices, but this has never been tested. In contrast, the polymerase chain reaction (PCR) has proved to be an efficient method for aetiological diagnosis in this case as well as in other diseases of plants and animals. Teixeira *et al.* (1994) developed an assay for the detection of flagellates in fruit by PCR amplification of an 800-bp fragment of the SSU of the ribosomal gene. A probe derived from the SSU of *Trypanosoma cruzi* by Dietrich *et al.* (1990) was used to hybridize with the amplified product. This coupled PCR/hybridization procedure was diagnostic for trypanosomatids in general and was used to detect trypanosomatid infections in tomatoes, yielding positive results a couple of days before microscopy and detecting as little as 10 ng of flagellate DNA. So far, PCR is the only procedure available, other than microscopy, for detecting flagellate infection in plants.

## 12.2. Genus Identification

Once flagellates have been detected in plants, it may be necessary to identify their genus. If cultures are available, *Phytomonas* can be diagnosed by the methods described in Section 7, particularly using monoclonal antibodies, ribosomal gene digestion and SL3′ probing. However, in addition to being complicated and time-consuming, primary cultures are very seldom obtained. Thus it would be desirable to have a method that does not require primary cultures.

The PCR amplification/hybridization of the ribosomal SSU described by Teixeira *et al.* (1994) can be rendered diagnostic of the genus *Phytomonas* if the amplified product is examined for the presence of the 360 *Pvu*II site. This can be done either by *Pvu*II digestion or by probing with the oligonucleotides SSU3 (Camargo *et al.*, 1992) and SSU4 (Teixeira *et al.*, 1997), which recognize the sequences flanking the *Pvu*II site. The 360 *Pvu*II site is present in all trypanosomatid genera except *Phytomonas*. Therefore, lack of hybridization with SSU3 and SSU4 would be diagnostic of *Phytomonas*, were it not for the existence of some poorly characterized trypanosomatids that also lack the *Pvu*II site (Teixeira *et al.*, 1994, 1997).

Serrano *et al.* (1996) reported the development of a PCR assay for amplification of the transcribed region of the spliced leader gene followed by hybridization with the SL3′ oligonucleotide (see Section 6.7.2). The method is supposed to be diagnostic of *Phytomonas*, but the results were very preliminary and the use of larger samples is necessary.

### 12.3. Strain Identification

The last step in the diagnostic process is the identification of the isolate (strain, species?) in question. This can be done by comparing isolates by isoenzyme analysis, and by rDNA or kDNA restriction analysis (Ahomadegbe *et al.*, 1990, 1992; Camargo *et al.*, 1992; Guerrini *et al.*, 1992; Muller *et al.*, 1994, 1995). Although proven to be reliable for the identification of isolates, these methods have not been used in practice. This is because they depend on the existence of archives containing the profiles of each isolate, as is the case for the identification of humans by fingerprints, and these archives are not yet available.

However, there is one finding that, if properly explored, could be of great practical importance. This is the presence exclusively in phloemicolous *Phytomonas* of a unique *Eco*RI site at position 752 of the SSU of the ribosomal gene. Coupling restriction cleavage to PCR amplification, Marché *et al.* (1995) detected the site exclusively in flagellates from the phloem of palms and of *Alpinia purpurata*, an ornamental member of the Zingiberaceae. None of the 14 latex or fruit flagellates examined contained the site, which is in agreement with the data of Camargo *et al.* (1992). Since the sequences flanking the *Eco*RI site are known, it should not be difficult to design a specific oligonucleotide probe for phloem flagellates. PCR amplification/*Eco*RI-probing, because of its simplicity, could become the method of choice not only for disclosing palm flagellates in all sorts of insects and plants that may function as their reservoirs, but also for identifying healthy carriers among palms.

Finally, it is necessary to emphasize that any method for the diagnosis of *Phytomonas* in plants can also be used for the detection of *Phytomonas* in insects, as has already been done (Sbravate *et al.*, 1989; Camargo *et al.*, 1992; Serrano *et al.*, 1996; Teixeira *et al.*, 1996).

## 13. TREATMENT AND PROPHYLAXIS

There are many drugs active against trypanosomes of humans and other animals. Some should also be active against palm flagellates. However, the problem in the chemotherapy of palms does not rest on drug availability but on the feasibility of their use in the field. There is no report of large-scale attempts to treat infected palms. Limited tests revealed that pentamidines and phenanthridine were active against the flagellates, but the work was never extended to field studies (Dollet *et al.*, 1979).

With regard to prophylaxis, field experiments date from the beginning of the century. At that time, diseased trees were simply cut down and burned,

a questionable practice if applied to humans, but nevertheless effective for plant diseases. Perhaps too effective, because it may have killed more plants than the disease alone would have done (Slobbe, 1977; Parthasarathy and Slobbe, 1978; Slobbe *et al.*, 1978; Kastelein, 1987). In more recent times, but before the aetiology of the disease was known, general measures for the improvement of plantations have not been effective in curbing the diseases (Corrado, 1970; Lopez *et al.*, 1975). Some success came from a wrong hypothesis. For some time it was believed that larvae of the lepidopteran *Sagalassa valida* were causing the disease by eating the roots of the palms. Therefore, insecticides were applied at the bases of the trees, leading to a reduction of the disease in several parts of Latin America (Lopez *et al.*, 1975).

After flagellates were indicted as aetiological agents and phytophagous bugs as their vectors, the prophylactic use of insecticides found its rationale and met with reasonable success (Dollet *et al.*, 1979; Louise *et al.*, 1986; Oliveira *et al.*, 1987). Additional measures, such as cleaning up riversides, slashing weeds within the plots, and cutting the leaves of palms in contact with the soil 'led to spectacular improvements in the phytosanitary situation' (Renard, 1989). These measures, plus permanent surveillance, have brought *marchitez* and *hartrot* to a halt in many places where they were rampant before. The diseases have also been eradicated from some large commercial plantations, but their global eradication remains a remote goal.

## APPENDIX

### 1. Addresses of *Phytomonas* Depositaries

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## 2. Labelling Isolates

At the First *Phytomonas* Workshop (Dollet and Wallace, 1987) it was agreed that new isolates of *Phytomonas* should be coded as follows:

P or V (respectively for plant or vector)
Family and genus of the host (two letters)
Country where isolated (two or three letters)
Year of isolation (four digits)
Author's initials (two letters).

Although agreed upon, this identification system (which is a variation of the World Health Organization identification system for *Leishmania*) has not been widely adopted. The subject was discussed again at the last *Phytomonas* workshop, but no consensus was reached.

## 3. *Phytomonas Newsletter*

There is an informal publication on plant flagellates, the *Phytomonas Newsletter*, founded in 1987 under the editorship of F.G. Wallace and which is presently in its 15th issue. The newsletter disseminates information about *Phytomonas* and activities related to them. Anyone interested in receiving the publication (free of charge) may write to its current editor, Dr Michel Dollet, at the address given above under *MD* in part 1 of this Appendix.

## 4. A Note on Systematics

Table 4 lists all the plant flagellates that have been given new specific names. As discussed in Section 4, all these flagellates should have been placed in the genus *Phytomonas* by the original authors or by later reviewers (Wenyon, 1926; McGhee and McGhee, 1979; Camargo *et al.*, 1990; Wallace

*et al.*, 1992). However, at least four species were not so placed. These include *Crithidia oncopelti*, which clearly does not belong to *Phytomonas* according to the new generic definition (see Section 7). The remaining species, since cultures are not available to verify their generic affiliation by current criteria, should be placed in *Phytomonas* according to the former host origin criterion. They are listed below.

1. *Phytomonas funtumiae.* Original name: *Herpetomonas funtumiae* Franchini, 1922. Host: Apocynaceae, *Funtumia elastica.* Tissue: latex. Continent: Europe. Reference: Franchini (1922c).
2. *Phytomonas euphorbiae.* Original name: *Trypanosoma euphorbiae* Franchini, 1922. Host: Euphorbiaceae, *Euphorbia coerulescens.* Tissue: latex. Continent: Europe. Reference: Franchini (1922b).
3. *Phytomonas nieschulzi* n. comb. Original name: *Herpetomonas euphorbiae* Nieschulz, 1924. Host: Euphorbiaceae, *Euphorbia cereiformis.* Tissue: latex. Continent: Europe. Reference: Nieschulz (1924). Note that this species cannot be renamed *Phytomonas euphorbiae* because the species from *Euphorbia coerulescens* described by Franchini has precedence. Therefore we are proposing *P. nieschulzi* in homage to the researcher who described it.
4. After these amendments, there remain 16 valid species of *Phytomonas*: *P. davidi*, *P. elmassiani*, *P. bordasi*, *P. funtumiae*, *P. euphorbiae*, *P. nieschulzi*, *P. ficuum*, *P. francai*, *P. bancrofti*, *P. ganorae*, *P. leptovasorum*, *P. tirucalli*, *P. tortuosa*, *P. serpens*, *P. staheli* and *P. mcgheei* (see Table 4 for authors and dates).

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# Paragonimiasis and the Genus *Paragonimus*

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## ABSTRACT

*The review concentrates on literature that has appeared since the 1960s. Since then, numerous species of* Paragonimus *have been described, mainly from Asia but also from Africa and the Americas. Some of these cause disease in humans. Recent information on life cycles and routes of transmission is summarized. All described species and their hosts are listed, with synonyms where known. For well-known species such as* Paragonimus westermani, *subspecific taxa and strains are reviewed and genetic studies discussed. Paragonimiasis in humans and experimental animals is discussed with emphasis on clinical manifestations and pathology, diagnosis, immune interactions with the host, treatment and public health issues.*

## 1. INTRODUCTION

Over 40 species have been described in the genus *Paragonimus* Braun, 1899 (syn. *Pagumogonimus* Chen, 1963) and the related genus *Euparagonimus* Chen, 1962. Species have been reported from Asia (from Pakistan east to south-eastern Russia and south to Sri Lanka, Indonesia and Papua New Guinea), the Americas (from the Great Lakes region of Canada south to Brazil and Peru) and Africa (West, Central, Southern and, questionably, North).

Adult worms are generally found in the lungs of their mammal hosts. Human infections, due to several species, occur in Asia, Africa and the Americas. It has been estimated that 20 million people are infected worldwide (Toscano *et al.*, 1995). The best-known species is *Paragonimus westermani* (Kerbert, 1878), which infects millions of people in Asia. Human infection occurs through eating raw crustaceans containing metacercariae or ingesting uncooked meat of paratenic hosts such as pigs.

A number of excellent reviews of *Paragonimus* and paragonimiasis have covered the early literature. Material presented in them is treated as briefly as possible. Yokogawa *et al.* (1960, with updates by Yokogawa in 1965 and 1969), in particular, have given an excellent historical account. Yokogawa

(1982a) and Miyazaki (1982a) have discussed paragonimiasis as a zoonosis. More recently there have been reviews, largely taxonomic, in Russian (Kurochkin, 1987) and Chinese (Chen, 1985). Reviews by Miyazaki (1974a, 1991), in English, are valuable but not readily available. Over 1400 relevant publications have appeared since Yokogawa's (1965) review.

## 2. MORPHOLOGY AND TAXONOMY

Taxonomy of *Paragonimus* species has traditionally depended on details of morphology and life cycle. These are summarized here.

### 2.1. Adult

Adult specimens of *Paragonimus* species are large in comparison with many other trematodes, the largest resembling a coffee bean in size and appearance. Figure 1 shows the general structure of a specimen compressed to show the internal organs.

Interspecific differences in size and patterns of lobation of the ovary and testes are well documented (Miyazaki, 1974a) and are used as taxonomic characters. For example, the ovary possesses six club-shaped lobes in *P. westermani* but many delicate branches in *P. mexicanus*. Testes are unusually large in *P. macrorchis*, but relatively small in *P. harinasutai* and *P. amazonicus*.

In most species, cuticular spines resemble individual thorns spaced over the body surface. In some species (*Euparagonimus cenocopiosus*, *P. ohirai*, *P. bangkokensis*, *P. compactus*, *P. menglaensis* and *P. siamensis*), spines occur in groups (Figure 2) resembling teeth of a comb. Although diagrams such as Figure 2 seem clear, there is potential for confusion because the form of spines can vary across the body surface. For this reason, Chen (1965) proposed a formula for describing spination, but it is not often used. Further, some single spines tend to become split distally and hence come to resemble grouped spines. Changes in spination also occur during maturation in the definitive host. For example, spines on metacercariae of *P. ohirai* (see Higo and Ishii, 1987) and *E. cenocopiosus* (see Peng, 1984) are singly spaced, but develop the grouped pattern seen in adults as they mature. Spines on metacercariae of *P. heterotremus* are single (Sugiyama *et al.*, 1990a) whereas those on adults tend to have serrated ends (Pariyanonda *et al.*, 1991).

Body proportions differ among *Paragonimus* species. Specimens of *P. skrjabini* and *P. amazonicus*, for example, are slender whereas those of *P. westermani* and *P. siamensis* are almost as wide as long.

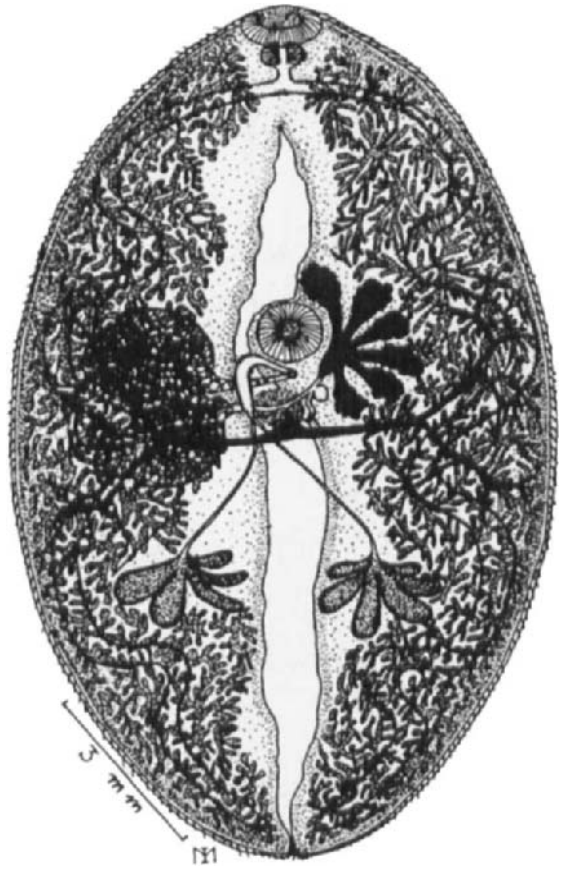
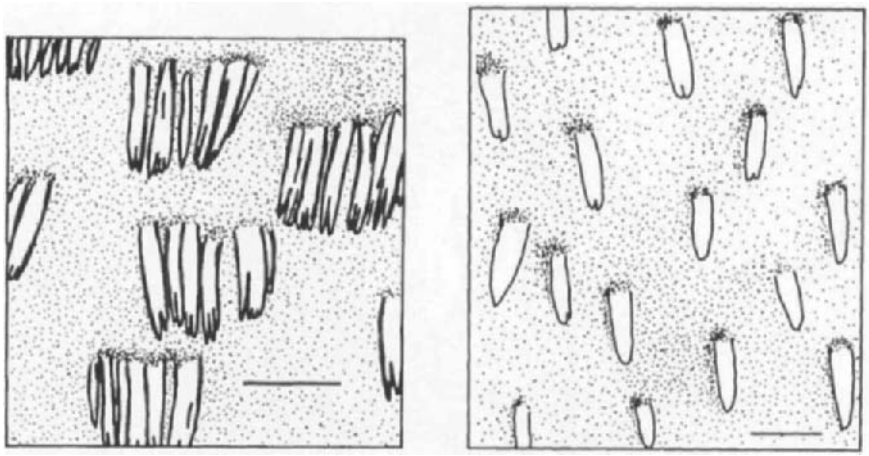


*Figure 1* Whole-mount of adult specimen of triploid *Paragonimus westermani* compressed to show internal organs. (Slightly modified from Miyazaki, 1991, with permission of International Medical Foundation of Japan, Tokyo.)

Developmental changes in other surface features such as papillae have been reported (e.g. Zheng and Zhang, 1991). Other studies of surface details of adult worms are by Fan *et al.* (1979), He and Ma (1980), He, Y.X. *et al.* (1982a, b), Li, Y.S. (1987) and Li, G.Y. *et al.* (1987a, b).

## 2.2. Egg

Eggs differ in size, in the position of the greatest width relative to the equator of the egg, in the extent of thickenings at either pole, and in surface sculpturing among species. Size can vary considerably even within a



(a) (b)

*Figure 2* Appearence of spines from surface of adult specimen of (a) *Paragonimus ohirai* (scale bar, 40 μm) and (b) *P. westermani* (scale bar, 20 μm). (After Miyazaki, 1991.)

species. Eggs of diploid *P. westermani* (see Section 4.1.1) are much smaller than those of triploids and tend to have their greatest width near the equator, instead of closer to the opercular pole as in triploids (Miyazaki, 1978). Thickenings of the abopercular pole are common in some species including *P. westermani*. Such a thickening assisted Sugiyama *et al.* (1989a) in distinguishing *P. harinasutai* from other Thai species. Egg shells of most species are smooth. In all American species except for *P. kellicotti*, however, the shell exhibits characteristic dimpling or pitting of the surface (e.g. Tongu *et al.*, 1995). Bayssade-Dufour *et al.* (1994) were able to distinguish four different types of *Paragonimus* eggs from West Africa using a multivariate analysis of egg dimensions and inferred that four different species were present. Similarly, Yuan and He (1986) could discriminate between eggs from triploid and diploid *P. westermani* in China.

## 2.3. Cercaria

Cercariae (Figure 3) of all species, where known, resemble one another closely. Slight differences have been reported in the numbers of gland cells, even between samples of the same species from the same locality (compare Ito *et al.*, 1985 with Rangel-Ruiz and Lamothe-Argumedo, 1986). This might reflect the difficulty of observation rather than real differences.


*Figure 3* Cercaria of triploid *Paragonimus westermani*. (Slightly modified from Miyazaki, 1991, with permission of International Medical Foundation of Japan, Tokyo.)

Similarly, numbers of flame cells are reported to differ among species. However, there is probably an increase in number as a cercaria ages, creating potential for confusion (Ito *et al.*, 1969, 1985); 60 is the usual number in a fully developed cercaria. The large posteroventral 'pseudo-sucker', said by Ito *et al.* (1985) to be unique to cercariae of *P. mexicanus*, might be a depression in the body surface present also in other species (Rangel-Ruiz and Lamothe-Argumedo, 1986). Surface features that might aid in separation of species have been studied in only a few species using scanning electron microscopy. Different arrangements of spines and papillae were noted for *P. westermani* by Higo *et al.* (1980; Japanese material) and Peng (1987; Chinese material). Higo and Ishii (1983) noted some differences among Japanese species in size and shape of the long spines

on the posteroventral part of the body. Rangel-Ruiz (1989) found no major differences in surface spination between the cercaria of *P. mexicanus* and the Japanese species studied by Higo and Ishii (1983), although there were some differences in numbers of papillae.

## 2.4. Metacercaria

Metacercarial characters have been widely used in distinguishing among species (Miyazaki, 1974a). Some species lack cysts and are found free in their crab hosts (e.g. *P. mexicanus*). Most species have either one or two cyst walls, visible using light microscopy and differing in thickness among species (Figure 4). Lou *et al.* (1992), using transmission electron microscopy, have characterized cyst walls of Japanese species at the ultrastructural level. In one species, *P. ohirai*, the inner cyst wall may be present or absent, a polymorphism apparently due to alleles at a single locus (see Section 4.1.2). The possible existence of such a polymorphism should be borne in mind when using metacercarial characters to distinguish between species. Cyst size is of limited use for discriminating among species. Cysts of *P. miyazakii* are regarded as larger on average than those of *P. westermani* (Miyazaki, 1991). However, a wide range of cyst sizes has been reported for *P. westermani* (e.g. Miyazaki, 1981a) and cysts of diploid forms are usually significantly smaller than those of triploids or tetraploids (e.g. Terasaki *et al.*, 1995). The size of the inner cyst may be more useful than overall cyst size.

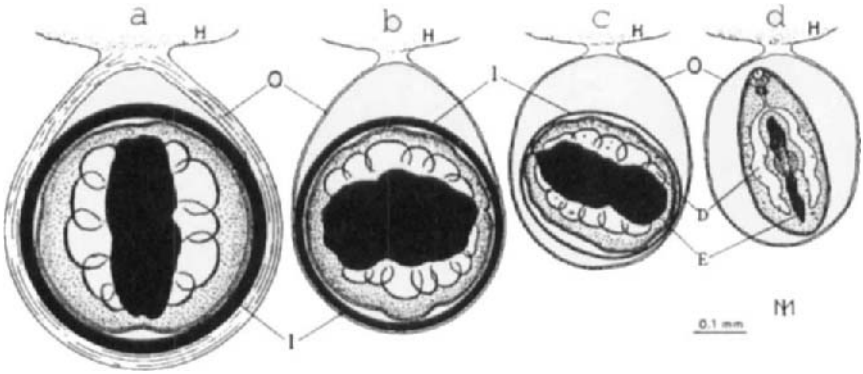



*Figure 4* Appearences of metacercarial cysts of several species of *Paragonimus*: (a) *P. miyazakii*; (b) *P. westermani*; (c) *P. ohirai*; (d) *P. ohirai* (morph with no inner cyst wall that was originally described as *P. iloktsuenensis*). D, intestine; E, excretory bladder; H, host tissue; I, inner cyst; O, outer cyst. (Slightly modified from Miyazaki, 1991, with permission of International Medical Foundation of Japan, Tokyo.)

Body proportions of the excysted metacercaria (Figure 5) vary among species. These include the relative diameters of the suckers, the anterior extent of the excretory bladder, and the presence and length of the stylet associated with the oral sucker. Coloured granules in the body and coloured gut contents have been reported for some species (e.g. Miyazaki *et al.*, 1980). Reported numbers of flame cells differ among species. This feature was used to separate the genera *Paragonimus* (60 flame cells) and *Pagumogonimus* (72 flame cells) (see Chen, 1964). Given that *P. caliensis* and *P. kellicotti* each has 96 flame cells in the metacercaria (Little, 1968) and 40 have been reported for *P. heterotremus* (see Chen, 1985), this does not seem a good basis for separating genera. Metacercariae of most species possess 60 flame cells. It is not known whether numbers of flame cells increase with age, as seems to happen in cercariae.

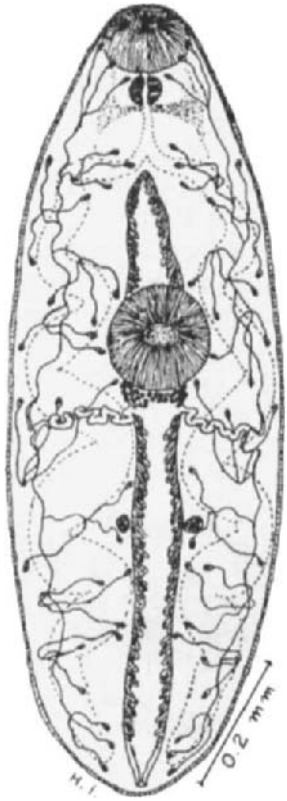



*Figure 5* Excysted metacercaria of triploid *Paragonimus westermani*. (Slightly modified from Miyazaki, 1991, with permission of International Medical Foundation of Japan, Tokyo.)

Surface structures of the excysted metacercaria have been examined using scanning electron microsopy. The extent of surface spination and the number and arrangement of papillae, especially around the suckers, are useful aids to identification (Higo and Ishii, 1987). Using, in part, patterns of papillae around the ventral sucker of the metacercaria, Tongu *et al.* (1990, 1995) suggested that a *Paragonimus* species from Venezuela might be new to science. Similarly, Hernandez and Monge (1989) noted two different patterns of papillae among metacercariae from Costa Rica, leading them to suggest that two species might be present. Other studies on surface ultrastructure of metacercariae are by Aji *et al.* (1984), Higo and Ishii (1984, 1987), Tongu *et al.* (1985, 1987, 1995), Li *et al.* (1986), Sugiyama (1987), Sugiyama *et al.* (1990a), Jiang and Xia (1993) and Paulin *et al.* (1993).

## 3. LIFE CYCLES OF *PARAGONIMUS* SPECIES

### 3.1. Introduction

The life cycles of *Paragonimus* species were among the first aspects to receive attention from biologists. The excellent and detailed early work in this area has been reviewed by Yokogawa *et al.* (1960).

The life cycle is (briefly) as follows. Operculated eggs are voided from the host either in sputum or faeces. Eggs develop (see Weina, 1986, concerning variation in development time depending on route of voiding) and hatch in fresh (or brackish) water, releasing a ciliated miracidium that swims to a suitable snail host. The miracidium penetrates the tissues of the snail, localizing in the haemocoel where it becomes a mother sporocyst. This produces, asexually, first and second generations of rediae. Cercariae emerging from second-generation rediae possess a stylet anteriorly and a short, stumpy tail. In some species of *Paragonimus*, cercariae emerge readily from the snail host; in others they do not. Development time within the snail is 9–13 weeks (e.g. Shimazu, 1981; Gyoten, 1986). Entry into the crustacean is by direct penetration by active cercariae and/or by ingestion of infected snails by the crustacean (see Section 3.3). Transformation of a cercaria into an infective metacercaria can take several weeks (reviewed in Gyoten, 1986). The fate of a metacercaria ingested by a mammal depends on the species of mammal. In those suitable as final hosts, metacercariae excyst in the intestine, migrate to the pleural cavity where they meet mates, then pair in cysts in the lungs and commence egg production. Maturation times for several species are shown in Table 1. In at least some unsuitable hosts, the juvenile worms persist in the tissues but grow only slightly or not at all (see

*Table 1* Prepatent periods for some species of *Paragonimus*.

| *Paragonimus* species | Host species | Days to start of egg production | Reference |
|---|---|---|---|
| *P. westermani* (2*n*) (Japan) | Cat | 66[a] | Kanazawa *et al.* (1987) |
| *P. westermani* (Philippines) | Rat | 60[b] | Miyazaki and Habe (1979) |
| | Rat | 70[a,b] | Yokogawa *et al.* (1979) |
| *P. westermani* (Taiwan) | Dog | 60[a] | Fan (1966) |
| | Monkey | 111[a] | Fan (1966) |
| *P. ohirai* | Rat | 28–35[b] | Okura (1963) |
| | Rat | 41–45[a], 31[b] | Chiu (1962b) |
| *P. yunnanensis* | Cat, dog | 88–89[a] | Ho *et al.* (1973) |
| *P. siamensis* | *Bandicota indica* | 47[a] | Ahmad *et al.* (1977), Yaemput *et al.* (1994) |
| | Cat | 63–91[a] | Sugiyama *et al.* (1989b), Yaemput *et al.* (1994) |
| *P. bangkokensis* | Cat | 55[a] | Sugiyama *et al.* (1989b) |
| *P. skrjabini* (as *P. szechuanensis*) | Cat | 55–64[a] | Chung *et al.* (1974) |
| *P. heterotremus* | Cat | 55–58[a] | Sugiyama *et al.* (1989b) |
| *P. miyazakii* | Nutria | 66–80[a] | Hatsushika *et al.* (1979) |
| *P. kellicotti* | Cat | 36[a] | Stromberg and Dubey (1978) |
| *P. caliensis* | *Didelphis marsupialis* | 74[a] | Little (1968) |

[a] Eggs first detected in faeces of experimentally infected hosts.

[b] Mature worms found by dissection of experimentally infected hosts at intervals.

Section 3.5). If the paratenic host is eaten by a suitable final host, the juvenile worms can mature in the new host.

Topics relating to life cycles that have received attention since the work reviewed by Yokogawa *et al.* (1960) are discussed in the following sections.

## 3.2. Molluscan Host Specificity and Interactions with Other Trematodes

Mirroring the situation in some schistosomes, there appears to be co-adaptation between local races of snails and their *Paragonimus* parasites. Habe (1993) found that populations of *Angustassiminea parasitologica* from areas endemic for *P. ohirai* were more susceptible to the parasite than populations from non-endemic areas. Similarly, Hamajima *et al.* (1989) observed differences in miracidial penetration by *P. westermani* of *Semisulcospira libertina* from different localities.

Despite apparent co-evolution of snails and parasites, it is striking that many species of *Paragonimus* have a much larger range of snail hosts than do, for example, *Schistosoma* species. Populations of *P. ohirai* utilize at least nine species within the families Assimineidae and Pomatiopsidae (Davis *et al.*, 1994) and experimental infections of the American pomatiopsid *Pomatiopsis lapidaria* are possible (LoVerde and Yasuraoka, 1972). *Paragonimus skrjabini* has been reported from some 22 species in the family Pomatiopsidae (Davis *et al.*, 1994). *Paragonimus westermani* occurs in snails of the families Pleuroceridae and Thiaridae (Davis *et al.*, 1994). It may be that these broad host specificities reflect underlying taxonomic divisions that are as yet unrecognized.

At least one genus of snails, the genus *Oncomelania* (Pomatiopsidae), appears to be a permissive host for many species of *Paragonimus*. Japanese *Oncomelania hupensis nosophora* is susceptible to experimental infection by a number of *Paragonimus* species for which it is not normally a host. These include two American species, *P. kellicotti* (normal host *Pomatiopsis lapidaria* also in Pomatiopsidae) (Basch, 1959) and *P. mexicanus* (normal host *Aroapyrgus* spp. in family Hydrobiidae) (Hata *et al.*, 1987a). Only a small proportion of snails yielded mature infections in the latter case. Asian species for which *O. hupensis nosophora* can act as an experimental host include *P. ohirai* (normal hosts in Assimineidae and Pomatiopsidae) (Kawashima and Miyazaki, 1963a, b), *P. miyazakii* and *P. heterotremus* (normal hosts also in Pomatiopsidae) (Kawashima and Miyazaki, 1964; Asavisanu *et al.*, 1985a). Other successful experimental infections of *Oncomelania* species and subspecies by *Paragonimus* species to which they are not normally exposed have been reported by LoVerde and Yasuraoka (1972), Kawashima *et al.* (1984) and Asavisanu *et al.* (1985a).

Interactions of various kinds are known to occur among trematode species inhabiting the same snail. Hata *et al.* (1988) demonstrated an antagonistic relationship between *P. ohirai* and *Schistosoma japonicum* in the snail *Oncomelania nosophora* in which *P. ohirai* was the dominant species. Prior infection of *Semisulcospira libertina* by another species of trematode may be required for successful establishment of *P. westermani* in this snail (experimental studies by e.g. Hamajima *et al.*, 1981, 1989; Shimazu and Oshima, 1983). Field surveys by Tomimura *et al.* (1989) found that, in natural Japanese populations of *S. libertina*, diploid *P. westermani* co-occurred with other trematodes significantly more often than predicted by chance alone.

### 3.3. Route of Infection of Crab

Experimental infections of crabs with cercariae of *P. westermani* have generally had limited success. Two routes of infection have been proposed: (i) oral, by ingestion of infected snails; and (ii) by penetration of the body surface by free cercariae (Shibahara, 1991). Both routes led to infection of *Geothelphusa dehaani* in experiments reported by Shibahara (1991). Although recovery of metacercariae was highest from crabs that had eaten infected molluscs or rediae dissected from these, the overall infection success remained low in all cases. Similarly, poor recovery rates of *P. miyazakii* metacercariae from crabs exposed to free cercariae have been reported by Gyoten (1986, 1995). This is despite the fact that cercariae of *P. miyazakii* readily emerge from the snail host (Gyoten, 1986). Distribution of metacercariae within a crab may depend on the species of trematode (e.g. Kino, 1994) or on the species of crab (Habe *et al.*, 1993 and references therein for *P. westermani*). Experimental infection of crabs with *P. siamensis* seems to have been straightforward (Yaemput *et al.*, 1994)

### 3.4. Other Second Intermediate Hosts

Frogs (*Rana boulengeri*) have been reported to contain metacercariae of *P. skrjabini* in China (Xie *et al.*, 1985). These matured in experimentally infected cats and rats. Although the term 'paratenic host' appeared in the title of the paper, this does not seen appropriate. The metacercariae were apparently encysted in the frogs.

### 3.5. Paratenic Hosts

Several species of *Paragonimus* are known to utilize paratenic hosts (see host details in Section 5.2 for the following species: *P. heterotremus*, *P. kellicotti*, *P. mexicanus*, *P. miyazakii*, *P. skrjabini* and *P. westermani*). Metacercariae eaten by these hosts remain in the tissues, usually the muscles, as juveniles only slightly developed beyond the metacercarial stage. Although most known paratenic hosts are mammals, Miyazaki and Habe (1976) recovered juveniles from hens fed metacercariae of *P. westermani* and Zhang *et al.* (unpublished results) have demonstrated the possibility of ducks and geese acting in this role for *P. westermani* in China.

The role of paratenic hosts in human paragonimiasis was demonstrated by Miyazaki and Habe (1976) and Miyazaki *et al.* (1978a) who found that wild boars are a potential source of *P. westermani* infection in Japan among

people who had never eaten freshwater crabs. Wild boars are also experimental paratenic hosts for *P. miyazakii* in Japan (Shibahara *et al.*, 1992a).

Rodents are common experimental paratenic hosts for *Paragonimus* species (see Section 3.5). A small proportion of worms might mature in the rodent (e.g. Kanazawa *et al.*, 1987; Su *et al.*, 1988; Yan *et al.*, 1991, 1993); however, the adults generally do not persist for long. Oral transfer of juveniles between potential paratenic hosts is also possible (e.g. Peng and Dong, 1987) and a proportion of worms will sometimes mature in the recipient. Yan *et al.* (1991) noted this for *P. skrjabini* as did Fan *et al.* (1994) for *P. westermani* (inoculation by intraperitoneal route in this case) and Bespozvannykh (1994) for *P. westermani ichunensis.* The last case is particularly interesting. In the Amur River region of south-east Russia, *P. westermani ichunensis* is probably the cause of both larval *Paragonimus* infection (Sukhanova, 1984) and pulmonary infection in humans. Bespozvannykh (1994) suggested that the former condition is due to human consumption of crustaceans containing metacercariae (i.e. humans act as potential paratenic hosts) and the latter due to consumption of meat from paratenic hosts.

It is unclear whether a period of development is required in a paratenic host before the juvenile is infective to a definitive host. Fan and Khaw (1965) found that juvenile *P. westermani* that had been in rats for as little as one day could successfully transfer to cats eating the rats. By contrast, Bespozvannykh (1994) determined experimentally that a period of about 42 days must elapse before juveniles of *P. westermani ichunensis* in mice were infective to cats.

Predation on paratenic hosts is probably the means by which tigers and other large carnivores become infected (Lim and Betterton, 1977; Habe *et al.*, 1996). Kobayashi (1942 cited in Fan and Hsu, 1965) regarded consumption of infected paratenic hosts as the only means by which a tiger that had spent all its life in Seoul Zoo (Korea) could have become infected.

A proportion of the metacercariae entering normal carnivorous definitive hosts might remain as juveniles but be capable of maturation when experimentally transferred to another host individual. Habe *et al.* (1996) worked with Malaysian material of *P. westermani.* Some juvenile worms from cats and dogs (both, particularly the latter, are poor definitive hosts when inoculated with metacercariae of the Malaysian strain), experimentally infected several months previously, matured when orally inoculated into uninfected cats and dogs. The maturation rate was higher in cats. Fan and Hsu (1965) have cited a number of older papers reporting maturation of juvenile worms transferred from one carnivorous host to another. Juveniles developing towards adulthood normally in a definitive host apparently cannot withstand oral transfer to other hosts after about the first 7–10 days of development (Kong *et al.*, 1994).

### 3.6. Migration in Definitive Host

Metacercariae excyst and penetrate through the small intestine wall (rarely the stomach, except in *P. siamensis*; see Waikagul *et al.*, 1986) into the peritoneal cavity. Small haemorrhagic spots can be detected where the worms penetrate the gut wall and lesions can be seen subsequently in other tissues (e.g. Sogandares-Bernal and Seed, 1973; Waikagul *et al.*, 1986). The time taken to reach the abdominal cavity is from 30 min to several hours. Thereafter, the activities of young worms depend to some extent on the species of worm and of host. In normal definitive hosts, worms of some species [Japanese *P. westermani* (see Yokogawa *et al.*, 1962; Kanazawa *et al.*, 1987), *P. heterotremus* (see Waikagul *et al.*, 1986), *P. siamensis* (see Waikagul *et al.*, 1986) and *P. kellicotti* (see Sogandares-Bernal and Seed, 1973, but Stromberg and Dubey, 1978 seem to contradict this)] migrate into the abdominal wall and remain there for a week or more before re-entering the abdominal cavity. Then they migrate to and penetrate the diaphragm to reach the pleural spaces where they find a mate. Finally they penetrate the lungs where a pair will form a cyst. Philippine *P. westermani* behave in this way in rats, in which they can mature (Yokogawa *et al.*, 1979). Other forms of *P. westermani* fail to mature in rats and behave differently during migration in this host. Juveniles enter the abdominal wall and body musculature but remain in the muscles without developing further (Kanazawa *et al.*, 1987) (see Section 3.5).

Juveniles of *P. ohirai* (Okura, 1963) and *P. skrjabini* (Hu *et al.*, 1982) forgo the sojourn in the abdominal wall, remaining instead in the abdominal cavity prior to migrating towards the liver and diaphragm. In *P. miyazakii*, about one-third of the juveniles enter the abdominal wall for a few days, the remainder staying in the abdominal cavity. The extent to which migrating juveniles enter and remain in the liver seems to differ according to the host–parasite combination (Zhong *et al.*, 1981; Hu *et al.*, 1982; Kanazawa *et al.*, 1987). A liver migration phase might be necessary in *P. miyazakii*. Metacercariae of this species surgically placed in the pleural cavities of rats undertook such a migration, whereas those of *P. ohirai* did not (Hashiguchi and Takei, 1969, 1971).

The structure and formation of cysts containing adult worms has been reviewed most recently by Sogandares-Bernal and Seed (1973). Since then, there has been little work in this area.

### 3.7. Pairing and Reproductive Biology

Early research (reviewed by Yokogawa, 1965) demonstrated that it is usual for adults of *Paragonimus* species to occur in pairs in cysts in the lungs.

Eggs laid within the cyst eventually discharge to the outside via the respiratory passages. An exception to the rule of paired worms in cysts seemed to be *P. westermani* in which unpaired worms were often found in lung cysts in human cases and in experimental infections of carnivores, leading to the assumption that the worms self-fertilized (e.g. Fan and Chiang, 1970). This anomaly was explained by the discovery of diploid and triploid forms of *P. westermani* in East Asia (Miyazaki, 1978). Diploid, sexually reproducing forms require partners for exchange of sperm and for cyst formation. In contrast, each triploid worm, reproducing parthenogenetically, is capable of cyst formation and egg production by itself (although more than one worm may occupy a cyst in heavy infections) (Miyazaki *et al.*, 1981a). Spermatogenesis in triploid *P. westermani* is aberrant and normal spermatozoa are not produced (Fujino and Ishii, 1982). Triploid worms are larger than diploids and are more pathogenic in humans. Recently, tetraploid forms of *P. westermani*, which might produce viable gametes, have been found in China (see Section 4.1.1).

Self-fertilization does not occur in *Paragonimus* species (Miyazaki *et al.*, 1981a, b; Habe and Agatsuma, 1993). Individuals of *P. ohirai* (see Hashiguchi *et al.*, 1969; Yoshimura, 1972) and diploid *P. westermani* (see Miyazaki *et al.*, 1981b) in experimental single-worm infections are capable of egg production, although this occurs in the thoracic cavity rather than in a cyst in the lungs and the eggs are unfertilized and largely unviable. Unpaired worms of *P. kellicotti* (following experimental infection of cats with a single metacercaria each) were found as immature subadults in the visceral pleura (Sogandares-Bernal, 1966). Unpaired worms of some species in the pleural cavity will continue to move around, causing damage. The tendency of unpaired worms to wander in the body might partly explain the high frequency of ectopic parasitism observed in human patients and the consequent pathogenic effects.

Arrival of potential mates in the pleural cavity need not be synchronous. Single metacercariae of *P. kellicotti* administered to cats developed only to the subadult stage in the pleura by 8 weeks (normal prepatent period 6 weeks) (Sogandares-Bernal, 1966). When a second metacercaria was administered to each cat between 8 and 12 weeks after the first, the worms could find each other, pair and form a typical lung cyst.

In mixed experimental infections, the different forms (diploid and triploid) of *P. westermani* are capable of cohabiting within a single cyst and the triploid can receive sperm from the diploid (Miyazaki *et al.*, 1981a; Terasaki *et al.*, 1996). Distinct species are also capable of sharing lung cysts. Hatsushika *et al.* (1975), in mixed infections of three species, found that individuals of *P. ohirai* and *P. miyazakii* frequently shared a cyst whereas those of *P. westermani* and *P. miyazakii* were never found together. Worms in mixed-species cysts were mature adults.

## 4. STRAINS, SPECIES AND GENETICS

### 4.1. Intraspecific Differences

#### 4.1.1. *Geographic and Strain Variation of* P. westermani

(a) *Hosts and morphology.* Among diploid populations of *P. westermani*, distinct geographical differences are known with respect to molluscan hosts. The snail hosts utilized in Malaysia and the Philippines belong to the genus *Brotia* (Thiaridae) and in east and north-east China (and adjacent Russia), Japan, Korea and Taiwan to the genus *Semisulcospira* (Pleuroceridae). Claims in the literature that other thiarids act as hosts remain unproven (Davis GM, personal communication). Nothing is known about molluscan hosts in any other endemic country.

Little analysis has been done of the distribution and specificity of crustacean hosts of *P. westermani*. Different crustacean hosts tend to be used in Japan and Taiwan by diploid (hosts *Geothelphusa* spp. in upland streams) and triploid (*Eriocheir japonicus* in lowland rivers and brackish waters) forms of *P. westermani*. In north-east China, both forms utilize freshwater crayfish of the genus *Cambaroides* (e.g. Terasaki *et al.*, 1995); in Fujian Province, both use crabs of the genus *Sinopotamon* (e.g. Li, Y.S. *et al.*, 1987).

Dogs and cats are good hosts for diploid (and triploid) *P. westermani* in most places. However, dogs inoculated with metacercariae appear to be poor hosts in Malaysia, with no worms maturing and most remaining as juveniles in the musculature (Habe *et al.*, 1996). In cats, about half the Malaysian metacercariae administered remained as juveniles (Habe *et al.*, 1996). Rodents usually act only as paratenic hosts. However, there is geographic variation in the proportion of diploid metacercariae capable of maturing in rats. In Japan, small proportions of diploid worms from Hyogo, Chiba and Mie could mature in rats, whereas none from Akita or Oita was capable of this (summarized in Kanazawa *et al.*, 1987). A small proportion of (presumably triploid) *P. westermani* from Korea can mature in rats (Fan *et al.*, 1993). In the Philippines, the rat is a normal definitive host (Miyazaki and Habe, 1979), whereas in Malaysia the rat is a paratenic host only (Habe *et al.*, 1996); see also Section 3.5 concerning the *P. westermani* strain in eastern Russia.

Some morphological variation has been noted across the geographic range of *P. westermani*. Between sympatric triploid and diploid forms in East Asia, differences are primarily in the sizes of eggs, adult worms (Miyazaki, 1978) and of metacercariae (Terasaki *et al.*, 1995). Metacercariae can also differ considerably in size among diploid populations (e.g. Miyazaki, 1981a) but exhibit little variation otherwise (Sugiyama,

1987). Morphological variations of adults among diploid populations have been used by Miyazaki and Habe (1979) and Miyazaki (1983) in proposing a number of subspecies. In some Philippine worms (*P. w. filipinus*), and a few diploids from Taiwan, one lobe of one or both testes is detached from the remainder (Miyazaki, 1981a). Miyazaki and Chiu (1980) have suggested a close relationship between Philippine and Taiwanese populations based on this observation, a relationship not supported by genetic evidence (Blair *et al.*, 1997). Japanese adult diploids (*P. w. japonicus*) tend to show more variation in numbers of ovarian lobes and have the testes slightly more anteriorly placed than in other populations (Miyazaki, 1983).

(b) *Polyploidy.* The discovery (reviewed by Miyazaki, 1978) in Japan of a triploid form ($3n = 33$) of *P. westermani* prompted further investigations into genetic aspects of the species. The triploid exhibited aberrant spermatogenesis, producing few or no normal sperm (Fujino and Ishii, 1982; Zhao and Huang, 1989) and was apparently parthenogenetic with eggs not undergoing a reduction division and remaining triploid (Sakaguchi and Tada, 1980). It was quickly discovered that triploid populations also occurred in Korea (Terasaki, 1980a), Taiwan (Miyazaki and Chiu, 1980) and China (He, L.Y. *et al.*, 1982; Li, D.Y. *et al.*, 1985; Li, Y.S. *et al.*, 1987). Miyazaki (1978) proposed that triploids should be referred to as a separate species, for which the name *P. pulmonalis* (Baelz, 1880) was available. This proposal has not met with general agreement (see Section 5.2). Diploid populations of *P. westermani* occur sympatrically with triploids, except in much of Korea. Individual *P. westermani* exhibiting different ploidies ($2n$, $3n$ or $4n$) in different gonadal cells have been reported from Liaoning and Zhejiang, China (Yuan and Li, 1987; Duan *et al.*, 1994).

Two explanations have been put forward for the origins of triploid *P. westermani.* Agatsuma and Habe (1985) proposed that they were allotriploids, i.e. produced by crossing between a Japanese diploid and a worm, of the same or a different species, from elsewhere. This suggestion was based on both allozyme and cytogenetic studies. Triploid individuals from Japan, China and Korea all possess the same alleles at each locus (Agatsuma *et al.*, 1989), suggesting that they are clonal and have a single recent origin (but note that Blair *et al.*, in press, have detected variation among triploids in a mitochondrial gene). Alleles present in triploids suggested affinities with both Japanese and north-eastern Chinese populations (Agatsuma *et al.*, 1989), indicating a possible crossing between worms from the two regions. C-band staining of chromosomes reinforced this view (Hirai *et al.*, 1985; Hirai and Agatsuma, 1991). Two of the three homologues in each triploid were identical with those found in Japanese diploids. However, the third homologue of several chromosomes was distinctly different from chromosomes found in Japanese diploids or in other Japanese species of *Paragonimus.* Nor were appropriate homologues

found in diploid populations of *P. westermani* from Malaysia, the Philippines or Taiwan (Hirai, 1987). In China, Zhao *et al.* (1989) also noted that the third homologue of several chromosomes in triploids differed from those in diploids.

Further evidence for allopolyploidy came from a study of mitochondrial DNA from Japanese diploids and triploids, which exhibited different patterns of bands in a gel when digested with restriction endonucleases (Agatsuma *et al.*, 1994). Similarly, Wang *et al.* (1991) and Zhang *et al* (1994) found differences in repetitive DNA between diploids and triploids from north-east China. In the latter study, differences were also noted among diploids from Zhejiang Province.

In Japan, Terasaki (1980b), studying Giemsa-stained chromosomes, proposed that triploids arose by autopolyploidy from diploids. Tan (1987), using the more sensitive C-banding technique on worms from north-east China, also proposed that the triploid form was an autotriploid. The differences noted among homologous chromosomes in triploids might be explained by chromosomal polymorphisms occurring within an ancestral diploid population. Such polymorphisms have now been reported from China by Tan and Li (1990) and Hirai and Agatsuma (1991) and from Japan by Terasaki *et al.* (1996), and are known for other species of *Paragonimus* (e.g. Hirai *et al.*, 1989). A reciprocal translocation between two chromosomes leading to a polymorphism has also been described from *P. westermani* from the Philippines (Hirai *et al.*, 1992). Allozyme studies might also support the view that triploids in north-east China arose through autopolyploidy. Chen and Li (1991) found that diploid and triploid worms from that region could not be distinguished using allozyme data.

Two tetraploid individuals ($4n = 44$) of *P. westermani* have been discovered in north-east China, sympatric with diploid and triploid specimens (Terasaki *et al.*, 1989). The tetraploids possessed different alleles at several loci (Agatsuma *et al.*, 1992), but among the alleles they possessed at all loci were those occurring in sympatric diploids and triploids. This suggested that tetraploidy has arisen more than once, and possibly as a result of fusion of a (haploid) sperm from a diploid worm with the (unreduced) triploid egg from a sympatric triploid worm. Terasaki *et al.* (1996) obtained experimental evidence for this when they observed sperm from diploids penetrating the unreduced eggs of triploids inhabiting the same lung cyst. Terasaki *et al.* (1995) observed production of spermatids in the tetraploid specimens and considered that these worms were autotetraploids, capable of producing viable diploid gametes. Given this, the lack of differences in karyotypes among the various ploidy forms and the probable involvement of triploids in production of the tetraploids, they proposed that the triploids were also autotriploids. They also speculated that triploidy might

arise as a result of the fusion of a diploid gamete from a tetraploid individual with a haploid gamete from a diploid individual.

(c) *Other genetic studies.* Allozyme studies have been reviewed by Blair (1993) and Agatsuma *et al.* (1993). Briefly, the most genetically variable populations are those in China, and the least variable those in Japan. Trees constructed from measures of genetic difference among the populations indicated geographically correlated genetic structure within *P. westermani.* Populations from China, Japan, Korea and Taiwan differed relatively little from one another compared with distances between these and populations from Malaysia and the Philippines. Populations from the last two countries were, however, quite distant from one another.

Cytological surveys of diploid and triploid populations revealed that C-band staining of chromosomes could detect differences among them. Hirai (1987) constructed a tentative phylogeny of four diploid populations (Malaysia, Philippines, Japan and Taiwan) based on C-band patterns.

#### 4.1.2. *Geographic and Strain Variation of Other* Paragonimus *Species*

(a) *Hosts and morphology.* There are few reports of intraspecific morphological differences among other species of *Paragonimus.* Peng (1984) noted that surface spination on *P. cenocopiosus* from Guangdong differed from that reported by He, Y.X. *et al.* (1982a) in specimens from other parts of China. Higo and Ishii (1987) noted slight differences in the distribution of surface papillae on metacercariae of two races of *P. ohirai* (*P. ohirai* and *P. iloktsuenensis*).

(b) *Genetic differences.* A striking polymorphism in structure of the metacercarial cyst has been noted among races of *P. ohirai* (reviewed in Habe *et al.*, 1992; Blair, 1993; Habe and Agatsuma, 1993). Differences in number of cyst walls appear to be controlled by a single gene and are probably inherited in a simple Mendelian fashion.

### 4.2. Interspecific Differences

The few comparative studies using allozymes have been reviewed in Blair (1993).

A number of studies have compared karyotypes and C-band staining of chromosomes among the species. The haploid chromosome number is 11 in all species studied (e.g. Sakaguchi and Tada, 1976, 1980; Terasaki, 1977, 1978, 1983; LoVerde, 1979; He, L.Y. *et al.*, 1982; Li and Zheng, 1983; Lei *et al.*, 1985). Conventional Giemsa staining of chromosomes has been inadequate for detecting differences among species (He, L.Y. *et al.*, 1982; Hirai *et al.*, 1992 and see comments in Terasaki, 1983 about the findings of

LoVerde 1979). C-band staining, however, reveals additional features and Hirai *et al.* (1985) have proposed a phylogeny of Japanese species of *Paragonimus* based on these features.

## 5. THE SPECIES OF *PARAGONIMUS*

### 5.1. Introduction

The genus *Paragonimus* has been variously placed in the families Troglotrematidae Odhner, 1914 and Paragonimidae Dollfus, 1939. Yokogawa *et al.* (1960) have given a detailed account of the history of the genus. Here we recognize two related genera, *Paragonimus* Braun, 1899 and *Euparagonimus* Chen, 1962. Other, more distantly related genera are discussed briefly in Section 5.5. Synonyms of *Paragonimus* are *Polysarcus* Looss, 1899 and *Pagumogonimus* Chen, 1963. Chen (1963; see also Chen, 1964) proposed three subgenera (*Paragonimus* Braun, 1899, *Rodentigonimus* and *Megagonimus*), but these names have seldom been used.

It would be impossible to list every host record for each species of *Paragonimus*. Instead, we have restricted ourselves to examples of recent or significant reports for each country. The taxonomy of snail and crustacean families utilized by *Paragonimus* species is in a state of considerable flux. As far as possible, the current name for each host has been used. Where different, names used in the literature appear in parentheses as synonyms. Names of mammal hosts follow Wilson and Reeder (1993); names of mollusc hosts follow Davis (1980) and Davis *et al.* (1994). Names of crustacean hosts are from many sources, including correspondence with appropriate taxonomists. Names of domestic or laboratory animal species, and of humans, are given as 'dog', 'cat', 'rat', etc. where these are the names used in the literature.

### 5.2. Alphabetical Listing of Species

5.2.1. Paragonimus africanus *Voelker and Vogel, 1965*

(a) *Type locality.* Lower Bakossi, West Cameroon.
(b) *Other localities.* Cameroon, Nigeria, Equatorial Guinea, Ivory Coast (tentative identification).
(c) *Molluscan hosts.* The mollusc hosts of *Paragonimus* in Africa remain unknown despite considerable enquiry. Assuming that aquatic snails must be responsible for the transmission of *P. africanus*, and finding no other

feasible candidate, Vogel and Crewe (1965) settled on snails of the genus *Potadoma* (Pleuroceridae) as the host. Neither they nor later researchers (reviewed in Sachs and Cumberlidge, 1989) found infected specimens. At a locality endemic for *P. uterobilateralis* in Liberia, Sachs and Cumberlidge (1989) searched for snails in a stream and on the nearby forest floor. The only snails found, *Homorus striatellus* (Rang, 1931), land snails of the family Subulinidae, shed cercariae resembling those of *Paragonimus* species. The amphibious habit of the crab host could lead to their infection on the forest floor. However, Sachs and Cumberlidge (1991a) later decided that the cercariae could not be those of a *Paragonimus* species. Moyou-Somo and Simo (1995) found cercariae they referred to as *P. africanus* in land snails of the family Achatinidae in Cameroon.

(d) *Crustacean hosts*

1. Family Potamonautidae
   *Sudanonautes africanus* (A. Milne-Edwards, 1869): Cameroon (Voelker and Sachs, 1977a).
   *Sudanonautes granulatus* (Balss, 1929): Cameroon (Voelker and Sachs, 1977a; misidentified as *S. pelii* according to Cumberlidge, 1994).
   Note: *Sudanonautes aubryi* (H. Milne Edwards, 1853) (syn. *S. pelii*) and *Sudanonautes floweri* (de Man, 1901) have been listed as hosts from Cameroon, but this might be due to misidentification (see Cumberlidge, 1994).

(e) *Mammalian hosts*

1. Family Loridae
   *Perodicticus potto* (Müller, 1766): Cameroon (Voelker and Sachs, 1977b).
2. Family Cercopithecidae
   *Cercocebus* sp.: Cameroon (experimental, Voelker and Vogel, 1965).
   *Chlorocebus aethiops* (Linnaeus, 1758) (syn. *Cercopithecus aethiops*): Cameroon (experimental, Voelker and Vogel, 1965).
   *Macaca mulatta* (Zimmermann, 1780): metacercariae from Cameroon (experimental, Voelker and Sachs, 1977b).
   *Mandrillus leucophaeus* (F. Cuvier, 1807): Cameroon (Voelker and Sachs, 1977b).
3. Family Hominidae
   'Human': Cameroon (Oelerich and Volkmer, 1976; Kum and Nchinda, 1982); Equatorial Guinea (Simarro *et al.*, 1991; parasite tentatively identified); Nigeria (Voelker *et al.*, 1975); Ivory Coast (Nozais *et al.*, 1980; parasite tentatively identified).
4. Family Canidae
   'Dog': Cameroon (experimental, Voelker and Vogel, 1965).
5. Family Herpestidae
   *Crossarchus obscurus* F.G. Cuvier, 1825: Cameroon (Voelker and Vogel, 1965).

6. Family Viverridae
   *Civettictis civetta* (Schreber, 1776) (syn. *Viverra civetta*): Cameroon (Voelker and Vogel, 1965).
7. Family Muridae
   *Sigmodon hispidus* Say and Ord, 1825: metacercariae from Cameroon (experimental, Voelker and Sachs, 1977a).
8. Family Caviidae
   'Guinea-pig': metacercariae from Cameroon (experimental, Voelker and Vogel, 1965).

5.2.2. Paragonimus amazonicus *Miyazaki, Grados and Uyema, 1973*

(a) *Type locality.* Peru.
(b) *Crustacean host*
1. Family Pseudothelphusidae
   *Hypolobocera chilensis* (H. Milne Edwards and Lucas, 1844) (syn. *Pseudothelphusa chilensis*): Peru (Miyazaki *et al.*, 1973; circumstantial evidence only that this is a host).
(c) *Mammalian hosts*
1. Family Didelphidae
   *Chironectes minimus* (Zimmermann, 1780): Peru (Miyazaki *et al.*, 1973, 1974),
   *Philander opossum* (Linnaeus, 1758): Peru (Miyazaki *et al.*, 1973, 1974).

5.2.3. Paragonimus asymmetricus *Chen, 1977*

See *Paragonimus westermani.*

5.2.4. Paragonimus bangkokensis *Miyazaki and Vajrasthira, 1967a*

(a) *Type locality.* Thailand, Nakorn-Nayok Province.
(b) *Other localities.* China, Hainan Island.
(c) *Crustacean hosts*
1. Family Potamidae
   *Apotamonautes hainanensis* (Parisi, 1916) (syn. *Potamonautes hainanensis*): China, Hainan (He, L.Y *et al.*, 1982).
   *Larnaudia beusekomae* (Bott, 1970): Thailand (Kawashima *et al.*, 1989a).
   *Potamiscus smithiana* (Kemp, 1923) (syn. *Potamon smithianus*): Thailand (Miyazaki and Vajrasthira, 1967a).
(d) *Mammalian hosts*
1. Family Felidae
   'Cat': Thailand (experimental, Miyazaki and Vajrasthira, 1967a); China, Hainan (He, L.Y. *et al.*, 1982).

2. Family Viverridae
   *Herpestes javanicus* (E. Geoffroy Saint-Hilaire, 1818): Thailand (Miyazaki and Vajrasthira, 1967a).
3. Family Muridae
   *Bandicota indica* (Bechstein, 1800): Thailand (experimental, Miyazaki and Vajrasthira, 1967a).

5.2.5. Paragonimus caliensis *Little, 1968*

(a) *Type locality.* Colombia.
(b) *Other localities.* Peru, Panama, Mexico, Costa Rica.
(c) *Molluscan host*
1. Family Hydrobiidae
   *Aroapyrgus colombiensis* Malek and Little, 1971: Colombia (Malek and Little, 1971).
(d) *Crustacean hosts*
1. Family Pseudothelphusidae
   *Hypolobocera chilensis* (H. Milne Edwards and Lucas, 1844) (syn. *Pseudothelphusa chilensis*): Peru (Miyazaki and Grados, 1972).
   *Potamocarcinus magnus* (Rathbun, 1895): Costa Rica (Rojas *et al.*, 1976), *Ptychophallus exilipes* (Rathbun, 1898): Panama (Miyazaki and Hendricks, 1975).
   *Ptychophallus tristani* (Rathbun, 1898): Costa Rica (Rojas *et al.*, 1976).
   *Strengeria* sp.: Colombia (Little, 1968).
(e) *Mammalian hosts*
1. Family Didelphidae
   *Didelphis marsupialis* Linnaeus, 1758: Colombia (natural and experimental, Little, 1968); Panama (Little, 1968; parasite tentatively identified); Mexico (Miyazaki and Ishii, 1968a; tentative identification as '*Paragonimus* species B').
   *Philander opossum* (Linnaeus, 1758): Colombia (Little, 1968).
2. Family Canidae
   'Dog': Peru (experimental, Miyazaki and Grados, 1972).

5.2.6. Paragonimus cheni *Hu, 1963*

Synonym: *Pagumogonimus cheni* (Hu, 1963) Chen, 1964.
(a) *Type locality.* China, Sichuan Province.
(b) *Other locality.* China, Yunnan Province (Hsia and Chen, 1964).
(c) *Crustacean host*
1. Family Potamidae
   *Sinopotamon denticulatum* (H. Milne-Edwards, 1983): China (Hu, 1963, Sichuan).

(d) *Mammalian hosts*
1. Family Muridae
   'Rat': China (experimental, Hu, 1963; worms did not mature).
2. Family Felidae
   'Cat': China (experimental, Hu, 1963).

5.2.7. Paragonimus compactus *(Cobbold, 1859)*

(a) *Type locality.* India.
(b) *Other locality.* Sri Lanka.
(c) *Mammalian hosts*
1. Family Felidae
   *Prionailurus viverrinus* (Bennett, 1833) (syn. *Felis vivverina*): Sri Lanka (Dissanaike and Paramananthan, 1962).
2. Family Herpestidae
   *Herpestes edwardsii* (E. Goeffroy Saint-Hilaire, 1818) (syn. *Viverra mongos, Mungos mungo*): India (Vevers, 1923; Ravikumar *et al.*, 1979).
3. Family Viverridae
   *Viverricula indica* (Desmarest, 1804): Sri Lanka (Dissanaike and Paramananthan, 1962).

5.2.8. Paragonimus divergens *Liu, Luo and Gu, 1980*

This species might be a junior synonym of *P. paishuihoensis* Tsao & Chung 1965. The appearance of the excretory bladder of the metacercaria is diagnostic.
(a) *Type locality.* China, Sichuan Province.
(b) *Crustacean host*
1. Family Potamidae
   *Sinopotamon denticulatum* (H. Milne Edwards, 1853): China (Liu *et al.*, 1980).
(c) *Mammalian hosts*
1. Family Canidae
   'Dog': China (Chen, 1985).
2. Family Muridae
   'Rat': China (Chen, 1985).

5.2.9. Paragonimus ecuadoriensis *Voelker and Arzube, 1979*

See *Paragonimus mexicanus.*

5.2.10. Paragonimus edwardsi *Gulati, 1926*

See *Paragonimus westermani.*

5.2.11. Paragonimus filipinus *Miyazaki, 1978*

See *Paragonimus westermani.*

5.2.12. Paragonimus fukienensis *Tang and Tang, 1962*

Specimens originally reported by Tang (1940) were regarded as belonging to *Paragonimus iloktsuenensis* by Chen (1940a).

(a) *Type locality.* China, Fujian.

(b) *Molluscan hosts*

1. Family Pomatiopsidae
   *Oncomelania hupensis tangi* (Bartsch, 1936) (syn. *Katayama tangi*: China (Tang and Tang, 1962, Fujian).

(c) *Crustacean hosts*

1. Family Parathelphusidae
   *Somanniathelphusa sinensis* (H. Milne-Edwards, 1853) (syn. *Parathelphusa sinensis*) (see Ng and Dudgeon, 1992 concerning this species): China (Tang and Tang, 1962, Fujian; Li, 1989; Fujian).

(d) *Mammalian hosts*

1. Family Felidae
   *Panthera tigris* (Linnaeus, 1758): China (Chen, 1985).
2. Family Muridae
   *Niviventer fulvescens* (Gray, 1847) (syn. *Rattus fulvescens*): China (Tang and Tang, 1962, Fujian).
   *Rattus losea* (Swinhoe, 1871); China (Tang and Tang, 1962, Fujian),
   *Rattus norvegicus* (Berkenhout, 1769): China (Tang and Tang, 1962, Fujian).
3. Family Leporidae
   'Rabbit': China (Chen, 1985).

5.2.13. Paragonimus harinasutai *Miyazaki and Vajrasthira, 1968*

(a) *Type locality.* Thailand.

(b) *Other locality.* China, Zhejiang.

(c) *Crustacean hosts*

1. Family Parathelphusidae
   *Siamthelphusa paviei* (de Man, 1898): Thailand (Hinz, 1992).
2. Family Potamidae
   *Larnaudia beusekomae* (Bott, 1970): Thailand (Sugiyama *et al.*, 1989b).

*Larnaudia larnaudii* (A. Milne-Edwards, 1869): Thailand (Sugiyama *et al.*, 1989b),
*Potamiscus smithianus* (Kemp, 1923) (syn. *Potamon smithianus*): Thailand (Miyazaki and Vajrasthira, 1968).
*Sinopotamon chekiangense* Tai and Sung, 1975: China (Song *et al.*, 1990, Zhejiang).

(d) *Mammalian hosts*

1. Family Felidae
'Cat' Thailand (experimental, Miyazaki and Vajrasthira, 1968); China (Song *et al.*, 1990, Zhejiang).

5.2.14. Paragonimus heterorchis *(Zhou, Pang and Hsiang, 1982)*

Synonym: *Pagumogonimus heterorchis* Zhou, Pang and Hsiang, 1982.

(a) *Type locality.* China, Hubei.

(b) *Crustacean host*

1. Family Potamidae
*Sinopotamon* spp.: China (Zhou *et al.*, 1986, Hubei).

(c) *Mammalian host*

1. Family Canidae
'Dog' China (experimental, Zhou *et al.*, 1986, Hubei).
*Vulpes vulpes* (Linnaeus, 1758): China (Zhou *et al.*, 1982, Hubei).

5.2.15. Paragonimus heterotremus *Chen and Hsia, 1964*

Synonym: *Paragonimus tuanshanensis* Chung, Ho, Cheng and Tsao, 1964 according to Chen, 1965.

(a) *Type locality.* China, Guangxi Province.

(b) *Other localities.* China (Yunnan and Guizhou provinces), Thailand, Laos, Vietnam.

(c) *Molluscan hosts*

1. Family Assimineidae
*Assiminea* sp.: Liu, D. *et al.*, 1982).
2. Family Pomatiopsidae
*Neotricula aperta* (Temcharoen, 1971) (syn. *Tricula aperta*): Thailand (experimental, Yaemput *et al.*, 1988).
*Oncomelania hupensis chiui* (Habe and Miyazaki, 1962) (syn. *Oncomelania chiui*): Thailand (experimental – snails from Taiwan – Asavisanu *et al.*, 1985a).
*Oncomelania hupensis formosana* (Pilsbry and Hirase, 1905) (syn. *Oncomelania formosana*): Thailand (experimental – snails from Taiwan – Asavisanu *et al.*, 1985a).
*Oncomelania hupensis hupensis* Gredle, 1881; Thailand (experimental – snails from China – Asavisanu *et al.*, 1985a).

*Oncomelania hupensis nosophora* (Robson, 1915) (syn. *Oncomelania nosophora*): Thailand (experimental – snails from Japan – Asavisanu *et al.*, 1985a).

*Oncomelania hupensis quadrasi* (Möllendorff, 1895) (syn. *Oncomelania quadrasi*): Thailand (experimental – snails from the Philippines – Asavisanu *et al.*, 1985a).

*Tricula* sp.: China, Guangxi (natural and experimental, Hu *et al.*, 1994).

(d) *Crustacean hosts*

1. Family Parathelphusidae

*Esanthelphusa dugasti* (Rathbun, 1902) (syn. *Somanniathelphusa dugasti*) (see Ng and Dudgeon, 1992 concerning this species): Thailand (Hinz, 1992).

*Siamthelphusa paviei* (de Man, 1898): Thailand (Hinz, 1992).

2. Family Potamidae

*Larnaudia beusekomae* (Bott, 1970) (syn. *Tiwaripotamon beusekomae*): Thailand (Komalamisra *et al.*, 1988).

*Larnaudia larnaudii* (A. Milne-Edwards, 1869): Thailand (Sugiyama *et al.*, 1990b).

*Malayopotamon* sp.: China (Liu, D) *et al.*, 1982, Guangxi).

*Potamiscus smithianus* (Kemp, 1923) (syn. *Potamon smithianus*): Thailand (Miyazaki and Vajrasthira, 1966).

*Potamon flexum* Dai *et al.*, 1980: China (Hu and Zhou, 1995, Guangxi).

*Sinolapotamon patellifer* (Wu, 1934): China (Liu, D *et al.*; 1982, Guangxi).

(e) *Mammalian hosts*

1. Family Cercopithecidae

*Macaca fascicularis* (Raffles, 1821) (syn. *Macaca irus*): Thailand (experimental, Vajrasthira and Radomyos, 1966, cited in Asavisanu *et al.*, 1985b).

2. Family Hominidae

'Human': Laos (Miyazaki and Fontan, 1970); Thailand (Vanijanonta *et al.*, 1984); China (Mao, 1991).

3. Family Canidae

'Dog': Thailand (experimental, Miyazaki and Vajrasthira, 1966); Vietnam (Kino *et al.*, 1995).

4. Family Felidae

'Cat': Thailand (natural and experimental, Miyazaki and Vajrasthira, 1966; experimental, Pariyanonda *et al.*, 1991); China (experimental, Chung *et al.*, 1964, Yunnan, as *Paragonimus tuanshanensis*).

5. Family Sciuridae

*Menetes berdmorei* (Blyth, 1849): Thailand (Hinz, 1992).

6. Family Muridae

*Meriones unguiculatus* (Milne-Edwards, 1867) (Mongolian gerbil): Thailand (experimental, Asavisanu *et al.*, 1985b).

'Mouse': Thailand (experimental paratenic host, Sugiyama *et al.*, 1990b); China (experimental paratenic host, Yan *et al.*, 1993, worms could probably mature in mice, Guangxi).
'Rat': Thailand (experimental paratenic host, Sugiyama *et al.*, 1990b); China (experimental, Fan *et al.*, 1979, Guangxi).
*Rhizomys pruinosus* Blyth, 1851: China (Chen, 1985).

7. Family Leporidae
'Rabbit': Thailand (experimental, Vajrasthira, 1969, cited in Asavisanu *et al.*, 1985b).

### 5.2.16. Paragonimus hokuoensis *Ho, Chung et al. 1964 (published 1965)*

The name was proposed following the discovery of a small number of distinctive metacercariae from *Sinopotamon* species in Yunnan. Kurochkin (1987) treated this as a *species inquirenda* and it is not included in Chen's (1985) compilation. Chung *et al.* (1975a) include the metacercaria in a table distinguishing among species.
(a) *Type locality.* China, Yunnan Province.

### 5.2.17. Paragonimus hueitungensis *Chung, Hsu, Ho, Kao, Shao, Chiu, Pi, Liu, Ouyang, Shen, Yi and Yao, 1975b*

See *Paragonimus skrjabini.*

### 5.2.18. Paragonimus iloktsuenensis *Chen, 1940*

See *Paragonimus ohirai.*

### 5.2.19. Paragonimus inca *Miyazaki, Mazabel, Grados and Uyema, 1975*

Lamothe-Argumedo (1985) regarded this species as a synonym of *P. mexicanus* but his does not seem to be a general view. The thickening of the eggshell at the abopercular pole is diagnostic of *P. inca.* Miyazaki *et al.* (1975) considered that specimens reported by Little (1968) from *Felis weidii* and *Felis pardalis* in Colombia may also belong to this species.
(a) *Type locality.* Peru, Tingo Maria.
(b) *Other locality.* Colombia (tentative).
(c) *Crustacean hosts*

1. Family Pseudothelphusidae
*Hypolobocera chilensis* (H. Milne Edwards and Lucas, 1844) (syn. *Pseudothelphusa chilensis*): Peru (Miyazaki *et al.*, 1975, (presumed host).
*Hypolobocera gracilignatha* (Pretzmann, 1972): Peru (Miyazaki and Uyema, 1980).

2. Family Trichodactylidae
   *Valdivia latidens* (A. Milne-Edwards, 1869) (syn. *Rotundovaldivia latidens*): Peru (Miyazaki and Uyema, 1980).

(d) *Mammalian hosts*

1. Family Felidae
   'Cat': Peru (Miyazaki *et al.*, 1975; experimental, Miyazaki and Uyema, 1980).
   *Herpailurus yagouarondi* (Lacépède, 1809) (spelling variants of specific name exist): Peru (Miyazaki *et al.*, 1975).
   *Leopardus pardalis* (Linnaeus, 1758): Peru (Miyazaki *et al.*, 1975).
   *Puma concolor* (Linnaeus, 1771): Peru (Miyazaki *et al.*, 1975).
2. Family Mustelidae
   *Eira barbara* (Linnaeus, 1758): Peru (Miyazaki *et al.*, 1975).

### 5.2.20. Paragonimus jiangsuensis *Cao, Liu, Zhao and Qiu, 1983*

(a) *Type locality.* China, Jiangsu.

(b) *Crustacean host*

1. Family Potamidae
   *Sinopotamon yangtsekiense* Bott, 1967: China (Cao *et al.*, 1983, Jiangsu).

(c) *Mammalian host*

1. Family Felidae
   'Cat': China (experimental, Cao *et al.*, 1983, Jiangsu).

### 5.2.21. Paragonimus kellicotti *Ward, 1908*

Worms from North America have sometimes been referred to erroneously as *P. westermani* (e.g. Short and Hendrickson, 1960; Alden, 1995) or as *P. rudis* (e.g. Stewart and Jones, 1959). Similarly, worms from Central and South America were often assumed to be *P. kellicotti* before the existence of other species was recognized in that region (e.g. Iturbe, 1942). Vevers (1923) also used this name for specimens (later identified as *P. westermani* by Lee and Miyazaki, 1965) from a Malaysian tiger. The name *P. kellicotti* used to be applied to worms from Japan that were later recognized as a separate species, *P. miyazakii* (see Miyazaki, 1964). Some reports of worms from South Africa (e.g. McCallum, 1975, identification apparently based on eggs alone) have also used the name *P. kellicotti*. Harrus *et al.* (1997) reported *P. kellicotti* from a naturally infected dog from Israel.

(a) *Type locality.* USA (Midwest).

(b) *Other localities.* American states of the Mississippi Basin and the Atlantic coast, Ontario and Quebec in Canada.

(c) *Molluscan hosts*

1. Family Pomatiopsidae
   *Oncomelania hupensis nosophora* (Robson, 1915) (syn. *Oncomelania nosophora*): USA (experimental – snails derived from Japanese stock – Basch, 1959).
   *Pomatiopsis cincinnatiensis* Lea, 1840: USA (Gesinski *et al.*, 1964; experimental, Basch, 1959).
   *Pomatiopsis lapidaria* (Say, 1817): USA (experimental, Basch, 1959; Sogandares-Bernal, 1965a).

(d) *Crustacean hosts*

1. Family Astacidae
   *Cambarus bartoni* (Fabricius, 1798): USA (Harley, 1972).
   *Cambarus robustus* Girard, 1852: USA (Ameel, 1932).
   *Cambarus virilis* (Hagen, 1870): USA (Ameel, 1932).
   *Orconectes propinquus* (Girard, 1852) (syn. *Cambarus propinquus*): USA (Ameel, 1932; Ishii, 1966; Gesinski *et al.*, 1964).
   *Orconectes rusticus* (Girard, 1852) (syn. *Cambarus rusticus*, *Orconectes juvenilis*): USA (Ameel, 1932; Harley, 1972; Stromberg *et al.*, 1978).
   *Procambarus blandingi acutus* (taxonomic status unclear): USA (Sogandares-Bernal (1965b).
   *Procambarus clarkii* (Girard, 1852): USA (Sogandares-Bernal, 1965b; experimental crayfish from stock introduced into Japan – Yoshida and Nishimura, 1968).
2. Family Potamidae
   *Geothelphusa dehaani* (White, 1847) (syn. *Potamon dehaani*): Japan (experimental – using material of *P. kellicotti* imported from the USA – Yoshida and Nishimura, 1968).

(e) *Mammalian hosts*

1. Family Didelphidae
   *Didelphis virginiana* Kerr, 1792: USA (McKeever, 1958, host referred to as *D. marsupialis*).
2. Family Tupaiidae
   *Tupaia glis* (Diard, 1820): USA (experimental, Sogandares-Bernal and Seed, 1973, single immature worm found).
3. Family Cercopithecidae
   'Rhesus monkey': USA (experimental, Sogandares-Bernal and Seed, 1973, single immature worm found).
4. Family Hominidae
   'Human': USA (Mariano *et al.*, 1986).
5. Family Canidae
   *Canis latrans* Say, 1823: Canada (Ramsden and Presidente, 1975).
   'Dog': USA (Kern, 1991; experimental, Bowman *et al.*, 1991); Canada (Gillick, 1972).

*Urocyon cineroeoargenteus* (Schreber, 1775): USA (Davidson *et al.*, 1992a).
*Vulpes vulpes* (Linnaeus, 1758) (syn. *Vulpes fulva*): USA (Davidson *et al.*, 1992b); Canada (Ramsden and Presidente, 1975).

6. Family Felidae
'Cat': USA (Johnson *et al.*, 1981; experimental, Bowman *et al.*, 1991).
*Lynx rufus* (Schreber, 1777) (syn. *Felis rufus*): USA (Snyder *et al.*, 1991).
7. Family Mustelidae
*Mephitis mephitis* (Schreber, 1776): USA (Alexander *et al.*, 1972).
*Mustela vison* Schreber, 1777: USA (Zabiega, 1996); Canada (Ramsden and Presidente, 1975).
8. Family Procyonidae
*Procyon lotor* (Linnaeus, 1758): USA (Ishii, 1966; Harkema and Miller, 1964, as *Paragonimus rudis*; experimental, Sogandares-Bernal and Seed, 1973, worms did not mature).
9. Family Suidae
'Pig': USA (Stewart and Jones, 1959).
10. Family Bovidae
'Goat': USA (Hall, 1925).
11. Family Muridae
*Ondatra zibethicus* (Linnaeus, 1766): USA (Harley, 1972).
'Rat': USA (experimental, Ameel, 1932, paratenic host).
'Syrian hamster': USA (experimental, Weina and Burns, 1992).

### 5.2.22. Paragonimus macacae *Sandosham, 1953*

See *Paragonimus westermani.*

### 5.2.23. Paragonimus macrorchis *Chen, 1962*

(a) *Type locality.* China, Hainan Island.
(b) *Other localities.* China (Fujian Province), Sri Lanka, Thailand.
(c) *Crustacean hosts*

1. Family Parathelphusidae
*Ceylonthelphusa rugosa* (Kingsley, 1880) (syn. *Parathelphusa rugosa*): Sri Lanka (Kannangara, 1969).
*Perbrinckia enodis* (Kingsley, 1880) (syn. *Parathelphusa enodis*) (see Ng, 1995 concerning this genus): Sri Lanka (Kannangara, 1969).
2. Family Potamidae
*Isolapotamon sinense* Tai and Sung, 1975: China (Chen, 1985).
*Larnaudia beusekomae* (Bott, 1970): Thailand (Waikagul *et al.*, 1989a, parasite tentatively identified).

*Potamiscus smithianus* (Kemp, 1923) (syn. *Ranguna smithiana*): Thailand (Waikagul *et al.*, 1989a, parasite tentatively identified).
*Sinopotamon denticulatum* (Milne-Edwards, 1853): China (Chen, 1985).

(d) *Mammalian hosts*

1. Family Canidae
'Dog': Sri Lanka (experimental, Kannangara, 1969).
2. Family Felidae
'Cat': Sri Lanka (experimental, Kannangara, 1969).
3. Family Muridae
*Bandicota bengalensis* (Gray and Hardwicke, 1833): Thailand (Miyazaki and Vajrasthira, 1967b).
*Bandicota indica* (Bechstein, 1800): Thailand (Miyazaki and Vajrasthira, 1967b).
'Rat': Sri Lanka (experimental, Kannangara, 1969).
*Rattus norvegicus* (Berkenhout, 1769) (syn. *Mus norvegicus*): China (experimental, Chen, 1964).
*Rattus rattus* (Linnaeus, 1758): Thailand (Miyazaki and Vajrasthira, 1967b).

### 5.2.24. Paragonimus menglaensis *Chung, Ho, Cheng and Tsao, 1964*

See *Paragonimus proliferus.*

### 5.2.25. Paragonimus mexicanus *Miyazaki and Ishii, 1968b*

Synonyms: *Paragonimus ecuadoriensis* Voelker and Arzube, 1979; *Paragonimus peruvianus* Miyazaki, Ibañéz and Miranda, 1969. Lamothe-Argumedo (1985) regarded *P. inca*, *P. peruvianus* and *P. ecuadoriensis* as junior synonyms. Other recent authors (e.g. Miyazaki *et al.*, 1980; Vieira *et al.*, 1992) regard only the last two as synonyms of *P. mexicanus.*

(a) *Type locality.* Mexico, Colima.

(b) *Other localities.* Peru, Ecuador, Costa Rica, Panama, Guatemala.

(c) *Molluscan hosts*

1. Family Hydrobiidae
*Aroapyrgus allei* Morrison, 1946: Mexico (Ito *et al.*, 1985; experimental, Rangel-Ruiz and Lamothe-Argumedo 1986.
*Aroapyrgus colombiensis* Malek and Little, 1971: Ecuador (Amunárriz, 1991a); Peru (experimental, Malek *et al.*, 1985, as *Paragonimus peruvianus*).
*Aroapyrgus costaricensis* (Morch, 1861): Costa Rica (natural and experimental, Malek *et al.*, 1975; Brenes *et al.*, 1980).
2. Family Pomatiopsidae
*Oncomelania hupensis nosophora* (Robson, 1915) (syn. *Oncomelania*

*nosophora*): Mexico and Peru (experimental – using snails from Japan – Hata *et al.*, 1987a).

(d) *Crustacean hosts*

1. Family Pseudothelphusidae

*Hypolobocera aequatorialis* (Ortmann, 1897): Ecuador (Vieira *et al.*, 1992).

*Hypolobocera chilensis* (H. Milne Edwards and Lucas, 1844) (syn. *Pseudothelphusa chilensis*): Peru (Hata *et al.*, 1987a).

*Hypolobocera gracilignatha* (Pretzmann, 1972): Peru (Malek *et al.*, 1985, as *Paragonimus peruvianus*).

*Odontothelphusa maxillipes* (Rathbun, 1898) (syn. *Potamocarcinus maxillipes*): Mexico (Lamothe-Argumedo, 1984).

*Potamocarcinus magnus* (Rathbun, 1895): Costa Rica (Miyazaki, 1974b, as *Paragonimus peruvianus*).

*Pseudothelphusa americana belliana* Rathbun, 1898: Mexico (Brenes *et al.*, 1980).

*Pseudothelphusa dilatata* Rathbun, 1898: Mexico (Lamothe-Argumedo *et al.*, 1977, 1979); Costa Rica (Tongu *et al.*, 1987).

*Pseudothelphusa nayaritae* Álvarez y Villalobos, 1994: Mexico (Lamothe-Argumedo, 1995).

*Pseudothelphusa propinqua* Rathbun, 1905: Guatemala (Miyazaki *et al.*, 1980).

*Pseudothelphusa terrestris* Rathbun, 1893: Mexico (Lamothe-Argumedo, 1995).

*Ptychophallus cocleensis* Pretzmann, 1965 (syn. *P. montanus cocleensis*): Panama (Miyazaki and Hendricks, 1975, as *Paragonimus peruvianus*).

*Ptychophallus costaricensis*: Costa Rica (natural and experimental, Brenes *et al.*, 1980).

*Ptychophallus richmondi* (Rathbun, 1893) (syn. *Pseudothelphusa richmondi*, *Potamocarcinus richmondi*): Panama (Miyazaki, 1972; Miyazaki and Hendricks, 1975; both as *P. peruvianus*); Costa Rica (Monge and Brenes, 1986).

*Ptychophallus tristani* (Rathbun, 1898): Costa Rica (natural and experimental, Brenes *et al.*, 1980).

*Ptychophallus tumimanus* (Rathbun, 1898): Costa Rica (Monge and Brenes, 1986).

*Raddaus bocourti* (A. Milne-Edwards, 1866) (syn. *Pseudothelphusa cobanensis*): Guatemala (Miyazaki *et al.*, 1980).

*Raddaus tuberculatus* (Rathbun, 1897) (syn. *Potamocarcinus tuberculatus*): Mexico (Lamothe-Argumedo, 1984).

2. Family Trichodactylidae

*Zilchiopsis emarginatus* (H. Milne Edwards, 1853) (syn. *Z. ecuadoriensis*): Ecuador (Amunárriz, 1991a).

(e) *Mammalian hosts*

1. Family Didelphidae
   *Didelphis albiventris* Lund, 1840 (syn. *Didelphis paraguayensis*): Peru (Miyazaki *et al.*, 1978b).
   *Didelphis marsupialis* Linnaeus, 1758: Mexico (Miyazaki and Ishii, 1968b); Ecuador (Vaca *et al.*, 1989, cited in Vieira *et al.*, 1992); Panama (Miyazaki, 1972, as *Paragonimus peruvianus*); Costa Rica (Brenes *et al.*, 1980).
   *Didelphis virginiana* Kerr, 1792: Mexico (Rangel Ruiz, 1989).
   *Philander opossum* (Linnaeus, 1758) (syn. *Metachirops opossum pallidus*): Mexico (Lamothe-Argumedo, 1982); Costa Rica (Brenes *et al.*, 1980).
2. Family Cebidae
   *Ateles geoffroyi* Kuhl, 1820: Costa Rica (experimental, Brenes *et al.*, 1980).
   *Cebus capucinus* (Linnaeus, 1758): Costa Rica (experimental, Brenes *et al.*, 1980).
3. Family Hominidae
   'Human': Mexico (Lamothe-Argumedo, 1985); Costa Rica (Saborio *et al.*, 1995); Peru (Yokogawa *et al.*, 1983, as *Paragonimus peruvianus*); Ecuador (Calvopiña *et al.*, 1993; Guevara *et al.*, 1995).
4. Family Canidae
   'Dog': Mexico (experimental, Hata *et al.*, 1987a); Costa Rica (experimental, Brenes *et al.*, 1980); Peru (experimental, Hata *et al.*, 1987a).
   *Urocyon cinereoargentateus* (Schreber, 1775): Costa Rica (Brenes *et al.*, 1980).
5. Family Felidae
   'Cat': Mexico (Lamothe-Argumedo *et al.*, 1978; Barquín *et al.*, 1982; Rangel Ruiz, 1989); Costa Rica (natural and experimental, Brenes *et al.*, 1980); Guatemala (experimental, Tongu *et al.*, 1995); Peru (Malek *et al.*, 1985, as *Paragonimus peruvianus*); Ecuador (experimental, Guevara *et al.*, 1995).
   *Herpailurus yaguarondi* (Lacépède, 1809) (syn. *Felis yagouaroundi*) (spelling variants of specific name exist): Mexico (Lamothe-Argumedo and Cruz, 1993).
   *Leopardus pardalis* (Linnaeus, 1758) (syn. *Felis pardalis*): Ecuador (Amunárriz, 1991a); Costa Rica (Brenes *et al.*, 1980).
6. Family Mustelidae
   *Mephitis macroura* Lichtenstein, 1832: Guatemala (Caballero, 1946, as *Paragonimus rudis*, specimens re-examined by Brenes *et al.*, 1980).
7. Family Procyonidae
   *Nasua nasua* (Linnaeus, 1766): Ecuador (Amunárriz, 1991b).
   *Procyon lotor* (Linnaeus, 1758): Costa Rica (Brenes *et al.*, 1980).

8. Family Suidae
   *Tayassu pecari* (Link, 1795): Ecuador (Amunárriz, 1991a).

5.2.26. Paragonimus microrchis *Hsia, Chou and Chang, 1978*

(a) *Type locality.* China, Yunnan Province.
(b) *Mammalian hosts*
1. Family Canidae
   'Dog': China (experimental, Hsia *et al.*, 1978).
2. Family Felidae
   'Cat': China (experimental, Hsia *et al.*, 1978).
3. Family Muridae
   'Rat': China (experimental, Hsia *et al.*, 1978).

5.2.27. Paragonimus minqinensis *Li and Chen, 1983*

(a) *Type locality.* China, Fujian Province.
(b) *Crustacean host*
1. Family Potamidae
   *Sinopotamon fujianense* Dai and Chen, 1979: China (Li and Chen, 1983, Fujian).
(c) *Mammalian hosts*
1. Family Canidae
   'Dog': China (experimental, Li and Chen, 1983, Fujian).
2. Family Felidae
   'Cat': China (experimental, Li and Chen, 1983, Fujian).

5.2.28. Paragonimus miyazakii *Kamo, Nishida, Hatsushika and Tomimura, 1961*

In the older Japanese literature, *P. miyazakii* was misidentified as *P. kellicotti*. Miyazaki (1965) has given a review of the species.
(a) *Type locality.* Japan.
(b) *Molluscan hosts*
1. Family Pomatiopsidae: (see comments in Davis *et al.*, 1994 concerning identities of snails)
   '*Bythinella*' *kubotai* Kuroda and Habe, 1957: Japan (Nishida *et al.*, 1994).
   '*Bythinella*' *nipponica akiyoshiensis* Kuroda and Habe, 1957: Japan (experimental, Kawanaka *et al.*, 1979).
   '*Bythinella*' *nipponica* (Mori, 1937): Japan (experimental, Gyoten, 1995).

*Oncomelania hupensis nosophora* (Robson, 1915) (syn. *Oncomelania nosophora*): Japan (experimental, Kawashima and Miyazaki, 1964).

(c) *Crustacean hosts*

1. Family Potamidae
   *Geothelphusa dehaani* (White, 1847) (syn. *Potamon dehaani*): Japan (Lou *et al.*, 1992; experimental, Gyoten, 1995).

(d) *Mammalian hosts*

1. Family Hominidae
   'Human': Japan (Ono *et al.*, 1992; Okamoto *et al.*, 1993).
2. Family Canidae
   'Dog': Japan (Sugano *et al.*, 1989, diagnosis based on eggs only).
   *Nyctereutes procyonoides* (Gray, 1834): Japan (Hirai *et al.*, 1978).
3. Family Felidae
   'Cat': Japan (Uga *et al.*, 1983; experimental, Shibahara *et al.*, 1992b).
4. Family Mustelidae
   *Martes melampus* (Wagner, 1841): Japan (Ashizawa *et al.*, 1976).
   *Meles meles* (Linnaeus, 1758) (syn. *Meles meles anakuma*): Japan (Habe *et al.*, 1977).
   *Mustela sibirica* Pallas, 1773: Japan (Ashizawa *et al.*, 1980b).
5. Family Suiidae
   *Sus scrofa* Linnaeus, 1758 (wild boar): Japan (Hamajima and Miyazaki, 1968; experimental, Shibahara *et al.*, 1992a, potential paratenic host).
6. Family Muridae
   'Hamster': Japan (experimental, Yoshida, 1970, only immature worms recovered).
   'Mouse': Japan (experimental, Hata and Kojima, 1989a, only immature worms recovered).
   'Rat': Japan (experimental, Hata *et al.*, 1991).
7. Family Caviidae
   'Guinea-pig': Japan (experimental, Hata *et al.*, 1991, paratenic host suitable as a model for human infection).
8. Family Myocastoridae
   *Myocastor coypus* (Molina, 1782): Japan (experimental – host introduced from S. America – Hatsushika *et al.*, 1979).
9. Family Leporidae
   'Rabbit': Japan (experimental, Hatsushika, 1967).

### 5.2.29. Paragonimus mungoi *Mishra, Patal, Acharya and Chatterji, 1976*

A *nomen nudum*. The name was applied to worms from the lungs of a mongoose (*Herpestes edwardsii* (E. Geoffroy Saint-Hilaire, 1818) syn. *Herpestes mungo*) from Orissa, India.

5.2.30. Paragonimus napensis *Amunárriz, 1991*

This species was described from the metacercarial stage only. Amunárriz (1991b) found small, encysted metacercariae in the crab host.
(a) *Type locality.* Ecuador, Napo River.
(b) *Crustacean host*
1. Family Trichodactylidae
   *Zilchiopsis emarginata* (H. Milne Edwards, 1853) (syn. *Z. ecuadoriensis*): Ecuador (Amunárriz, 1991b).

5.2.31. Paragonimus ohirai *Miyazaki, 1939*

Synonyms: *Paragonimus iloktsuenensis* Chen, 1940; *Paragonimus sadoensis* Miyazaki, Kawashima, Hamajima and Otsuru, 1968.
(a) *Type locality.* Japan.
(b) *Other localities.* China (coastal regions from Canton (Guangdong) to Liaoning provinces), Korea (south coast), Taiwan.
(c) *Molluscan hosts*
1. Family Assimineidae
   *Angustassiminea nitida* Pease, 1865: Japan (natural and experimental, Kawashima and Hashiguchi, 1973).
   *Angustassiminea parasitologica* (Kuroda, 1958) (syn. *Assiminea parasitologica*): Japan (Habe, 1993); Korea (Seo *et al.*, 1977, as *Paragonimus iloktsuenensis*).
   *Angustassiminea yoshidayukioi* (Kuroda, 1960) (syn. *Assiminea yoshidayukioi*): Japan (Miyazaki and Yoshida, 1960).
   *Assiminea lutea* A. Adams, 1861: China (natural and experimental, Chen, 1940a, b, as *Paragonimus iloktsuenensis*, Guangdong).
   *Assiminea violacea* Heude, 1882: China (in Davis *et al.*, 1994, Shanghai).
2. Family Pomatiopsidae
   *Oncomelania hupensis chiui* (Habe and Miyazaki, 1962) (syn. *Tricula chiui* Habe and Miyazaki, 1962): Japan (experimental, Yoshimura *et al.*, 1970, as *Paragonimus sadoensis*); Taiwan (natural and experimental, Chiu, 1965, as *Paragonimus iloktsuenensis*).
   *Oncomelania hupensis nosophora* (Robson, 1915): Japan (experimental, Hata and Kojima, 1989b).
   *Oncomelania minima* (Bartsch, 1936) (syn. *Tricula minima*): Japan (experimental, Miyazaki *et al.*, 1968, as *Paragonimus sadoensis*).
   *Oncomelania quadrasi* (Möllendorff, 1895): Japan (experimental – using snails from the Philippines – Kawashima *et al.*, 1984).
   *Pomatiopsis lapidaria* (Say, 1817): Japan (experimental – using snails from USA – LoVerde and Yasuraoka, 1972).

(d) *Crustacean hosts*

1. Family Grapsidae
   *Chasmagnathus convexus* (de Haan, 1835): Japan (Miyazaki and Mannoji, 1950).
   *Helice tridens* De Haan, 1835 (taxonomy of this genus is confused): Japan (Miyazaki and Mannoji, 1950); Korea (Yokogawa *et al.*, 1971, see summary of paper, as *Paragonimus iloktsuenensis*).
   *Sesarma dehaani* H. Milne-Edwards, 1853: Japan (natural and experimental, Hashiguchi *et al.*, 1976); Korea (Yokogawa *et al.*, 1971, as *Paragonimus iloktsuenensis*); China (Chen, 1940a, as *Paragonimus iloktsuenensis*, Guangdong; Li and Wang, 1985, Liaoning).
   *Sesarma haematocheir* (de Haan, 1835): Japan (natural and experimental, Hashiguchi *et al.*, 1976).
   *Sesarma sinensis* H. Milne-Edwards, 1853: China (Miyazaki, 1943, mouth of Yangtze River).
   *Sesarmops intermedius* (Ortmann, 1874) (syn. *Sesarma intermedia*): Japan (Sano *et al.*, 1981); Korea (Seo and Kwak, 1972, as *Paragonimus iloktsuenensis*).
2. Family Potamidae
   *Geothelphusa dehaani* (White, 1847): Japan (Hirai, 1988).
   *Geothelphusa miyazakii* (Miyake and Chiu, 1965) (syn. *Potamon miyazakii*): Taiwan (Miyake and Chiu, 1965, as *Paragonimus iloktsuenensis*).

(e) *Mammalian hosts*

1. Family Hominidae
   'Human': Japan (Yamaguchi *et al.*, 1988; Miyazaki, 1991, pp. 112, 136, 141).
2. Family Canidae
   'Dog': Japan (experimental, Miyazaki, 1940); Korea (experimental, Lee *et al.*, 1989a, as *Paragonimus iloktsuenensis*).
   *Nyctereutes procyonoides* (Gray, 1834): Japan (Shibahara and Nishida, 1985).
3. Family Felidae
   'Cat': Japan (natural and experimental, Hamajima, 1970, as *Paragonimus sadoensis*); China (experimental, He, Y.X. *et al.*, 1982a, as *Paragonimus iloktsuenensis*); Korea (experimental, Lee *et al.*, 1989a, as *Paragonimus iloktsuenensis*).
4. Family Mustelidae
   *Meles meles* (Linnaeus, 1758): Japan (Yokogawa *et al.*, 1957).
   'Mink' (=*Mustela vison*): Japan (experimental, Mannoji, 1953).
   *Mustela sibirica* Pallas, 1773: Japan (Hamajima and Miyazaki, 1968).
   *Martes* sp.: Japan (Yagisawa, 1978).
5. Family Suidae

'Pig': Japan (Kamegai *et al.*, 1964); China (experimental, Chen, 1940a, b, Guangdong) (as *Paragonimus iloktsuenensis*, regarded as poor host).
*Sus scrofa* Linnaeus, 1758: Japan (Ashizawa *et al.*, 1979).

6. Family Muridae
*Apodemus speciosus* (Temminck, 1844): Japan (experimental, Hamajima, 1970, as *Paragonimus sadoensis*).
'Hamster': Japan (experimental, Miyazaki *et al.*, 1954).
*Microtus montebelli* (Milne-Edwards, 1872): Japan (experimental, Hamajima, 1970, as *Paragonimus sadoensis*).
'Mouse': Japan (experimental, Yokogawa *et al.*, 1959); China (experimental, Chen, 1940a, b, Guangdong, as *Paragonimus iloktsuenensis*, regarded as poor host).
*Mus musculus* Linnaeus, 1758 (syn. *Mus molossinus*): Japan (experimental, Hamajima, 1970, as *Paragonimus sadoensis*).
*Niviventer coxingi* (Swinhoe, 1864) (syn. *Rattus coxingi*): Taiwan (Miyazaki and Chiu, 1962, as *Paragonimus iloktsuenensis*).
'Rat': Korea (experimental, Lee *et al.*, 1989a, as *Paragonimus iloktsuenensis*).
*Rattus norvegicus* (Berkenhout, 1769): Japan (experimental, Hamajima, 1970, as *Paragonimus sadoensis*); China (natural and experimental, Chen, 1940a, b, Guangdong, as *Paragonimus iloktsuenensis*); Taiwan (Miyazaki and Chiu, 1962, as *Paragonimus iloktsuenensis*).
*Rattus rattus* (Linnaeus, 1758): Japan (experimental, Hamajima, 1970, as *Paragonimus sadoensis*); China (Chen, 1940a, b, Guangdong, as *Paragonimus iloktsuenensis*).

7. Family Caviidae
'Guinea-pig': Japan (experimental, Miyazaki, 1940).

8. Family Leporidae
'Rabbit': Japan (experimental, Miyazaki, 1940).

### 5.2.32. Paragonimus paishuihoensis *Tsao and Chung, 1965*

Possible synonym: *Paragonimus divergens* Liu *et al.*, 1980. A very small number of metacercariae of striking appearance were found on a single occasion. Subsequent efforts to collect more failed. Kurochkin (1987) regarded this species as a *species inquirenda*.

(a) *Type locality.* China, Sichuan Province.
(b) *Other locality.* China, Yunnan Province.
(c) *Crustacean hosts*

1. Family Potamidae
*Potamon hispidum* (Wood-Mason, 1871): China (Chen, 1985, Yunnan).
*Sinopotamon denticulatum* (Milne-Edwards, 1853) (syn. *Potamon denticulatus*): China (Tsao and Chung, 1965, Sichuan).

(d) *Mammalian host*
1. Family Felidae
'Cat': China (experimental, Tsao and Chung, 1965, worms did not mature, Sichuan).

5.2.33. Paragonimus pantheri *Mishra, Patal, Acharya and Chatterji, 1976*

A *nomen nudum*. The name was applied to worms from the lungs of a tiger (*Panthera tigris* Linnaeus, 1758) from Orissa, India.

5.2.34. Paragonimus peruvianus *Miyazaki, Ibañéz and Miranda, 1969*

See *Paragonimus mexicanus*.

5.2.35. Paragonimus philippinensis *Ito, Yokogawa, Araki and Kobayashi, 1978a*

See *Paragonimus westermani*.

5.2.36. Paragonimus proliferus *Hsia and Chen, 1964*

Synonym: *Paragonimus menglaensis* Chung, Ho, Cheng and Tsao, 1964 according to Chen, 1965 and Miyazaki, 1974.
(a) *Type locality.* China, Yunnan Province.
(b) *Molluscan hosts.* See notes 7 and 9 in Davis *et al.*, 1994 concerning the identification of these species.
1. Family Pomatiopsidae
*Tricula gregoriana* Annandale, 1924: China (experimental, Yang and Xia, 1992; Chung *et al.*, 1964, as 'probable' host for *Paragonimus menglaensis*).
*Tricula cristella* (Gredler, 1887): China (experimental, Yang and Xia, 1992).
(c) *Crustacean hosts*
1. Family Potamidae
*Potamon chinghungense* Dai *et al.*, 1975: China (Zhou and Xia, 1989, Yunnan).
*Potamon hispidum* (Wood-Mason, 1871): China (Zhou and Xia, 1989, Yunnan).
(d) *Mammalian hosts*
1. Family Felidae
'Cat': China (experimental, Chung *et al.*, 1964, as *Paragonimus menglaensis*, Yunnan).
2. Family Muridae
'Rat': China (experimental, Hsia and Chen, 1964, Yunnan).

5.2.37. Paragonimus pulmonalis *(Baelz, 1880)*

See *Paragonimus westermani.*

5.2.38. Paragonimus ringeri *(Cobbold, 1880)*

See *Paragonimus westermani.*

5.2.39. Paragonimus rudis *(Diesing, 1850) Stiles and Hassall, 1900*

*Distoma rude* was described by Diesing (1850) from lungs of an otter, *Pteronura braziliensis* (Gmelin, 1788), from Mato Grosso State, Brazil. The original description and a later redescription by Braun (1901) were too brief to permit clear identification of the species. Despite this, the name *P. rudis* has been applied to lung worms from the Americas by many authors (see Miyazaki and Ishii, 1968b). Some authors (e.g. Thatcher, 1967) have also regarded *P. rudis* as a senior synonym of *P. kellicotti.* Miyazaki (1974a) regarded *P. rudis* as a *species inquirenda*, an opinion shared by subsequent authors (although Voelker *et al.*, 1981 preferred to regard it as a *nomen nudum*). There have been two recent attempts to find *Paragonimus* specimens at the type locality. Voelker *et al.* (1981) examined many crabs and one otter without success. Tongu *et al.* (1993), also without success, examined crabs and several mammals other than otters.

5.2.40. Paragonimus sadoensis *Miyazaki, Kawashima, Hamajima and Otsuro, 1968*

See *Paragonimus ohirai.*

5.2.41. Paragonimus siamensis *Miyazaki and Wykoff, 1965*

A small proportion of worms recovered from experimentally infected cats in the Philippines were identified by Cabrera and Vajrasthira (1972, 1973) as *P. siamensis.* Miyazaki (1981a) examined large numbers of metacercariae and experimentally obtained adults from the same location in the Philippines. All belonged to *P. westermani.* He stated that *P. siamensis* probably does not exist in the Philippines.

(a) *Type locality.* Thailand.
(b) *Other localities.* Sri Lanka.
(c) *Molluscan hosts*
1. Family Viviparidae
   *Filopaludina* (*Siamopaludina*) *martensi martensi* (Frauenfeld): Thailand (Yaemput *et al.*, 1994).

(d) *Crustacean hosts*

1. Family Parathelphusidae
   *Ceylonthelphusa rugosa* (Kingsley, 1880) (syn. *Parathelphusa rugosa*): Sri Lanka (Kannangara and Karunaratne, 1969b).
   *Chulathelphusa brandti* (Bott, 1968) (syn. *Somanniathelphusa brandti*): Thailand (experimental, Yaemput *et al.*, 1994).
   *Esanthelphusa dugasti* (Rathbun, 1902) (syn. *Parathelphusa dugasti*) (see Ng and Dudgeon, 1992 concerning this species): Thailand (Waikagul *et al.*, 1986).
   *Oziothelphusa senex* (Fabricius, 1798) (syn. *Parathelphusa ceylonensis*): Sri Lanka (Kannangara and Karunaratne, 1969b).
   *Sayamia bangkokensis* (Naiyanetr, 1982) (syn. *Somanniathelphusa bangkokensis*): Thailand (experimental, Yaemput *et al.*, 1994).
   *Sayamia germaini* (Rathbun, 1902) (syn. *Parathelphusa germaini*, *Somanniathelphusa germaini*): Thailand (Miyazaki and Wykoff, 1965; Ahmad *et al.*, 1977).
   *Sayamia sexpunctata* (Lanchester, 1906) (syn. *Somanniathelphusa sexpunctata, S. juliae*): Thailand (experimental, Yaemput *et al.*, 1994).
   *Sundathelphusa grapsoides* (H. Milne Edwards, 1853) (syn. *Parathelphusa grapsoides*): Philippines (Cabrera and Vajrasthira, 1973, but see Miyazaki, 1981a).
2. Family Potamidae
   *Larnaudia beusekomae* (Bott, 1970): Thailand (Hinz, 1992).

(e) *Mammalian hosts*

1. Family Felidae
   'Cat': Thailand (Miyazaki and Wykoff, 1965; experimental, Yaemput *et al.*, 1994); Philippines (natural and experimental, Cabrera and Vajrasthira, 1973, but see Miyazaki, 1981a); Sri Lanka (experimental, Kannangara and Karunaratne, 1969a).
2. Family Herpestidae
   *Herpestes edwardsii* (E. Geoffroy Saint-Hilaire, 1818) (syn. *Herpestes lanka*): Sri Lanka (Kannangara and Karunaratne, 1969a).
3. Family Muridae
   *Bandicota indica* (Bechstein, 1800): Thailand (experimental, Yaemput *et al.*, 1994).
   *Bandicota savilei* Thomas, 1916: Thailand (Hinz, 1992).
   *Berylmys berdmorei* (Blyth, 1851) (syn. *Rattus berdmorei*): Thailand (Hinz, 1992).
   *Maxomys rajah* (Thomas, 1894) (syn. *Rattus rajah*): Thailand (Hinz, 1992).
   'Rat': Sri Lanka (experimental, Kannangara and Karunaratne, 1969a); Thailand (experimental, Waikagul *et al.*, 1986).

5.2.42. Paragonimus skrjabini *Chen, 1959*

Synonyms: *Paragonimus szechuanensis* Chung and Tsao, 1962a, b; *Paragonimus hueitungensis* Chung, Hsu, Ho, Kao, Shao, Chiu, Pi, Liu, Ouyang, Shen, Yi and Yao, 1975; *Pagumogonimus veocularis* Chen and Li, 1979.

Chen (1959) reported *P. skrjabini* from the lungs of the viverrid *Paguma larvata* purchased in the markets in Canton (Guangzhou) and gave more morphological details in 1960. Chen (1962a) reported mature adults of this species from cats from Sichuan, and immature specimens from human cases in the same province. In the same year, Chung and Tsao (1962a, b) described *Paragonimus szechuanensis* from the lungs of naturally and experimentally infected cats from Sichuan Province. They distinguished their species from *P. skrjabini* on the basis of the cuticular spines, size and shape of eggs and body proportions of adults. Chen (1963) discussed these points in detail and dismissed them as being inadequate justification for proposing a separate species. Chen (1965) was able to infect the snail *Assiminea lutea* from Guangdong Province with *P. skrjabini* originating in both Guangdong and Sichuan. He saw no reason to separate *P. szechuanensis* from *P. skrjabini* and his view has tended to prevail in the literature (e.g. see Tung and Hwang, 1965; Zhan and Hsu, 1990); see Chung *et al.* (1974, 1975b, 1977) for opposing views.

*Paragonimus veocularis* was described from Qingchuan County, Sichuan and also noted from Guangdong Province (Chen, 1985). Li and Chen (1992) regarded *P. hueitungensis* as a synonym of *P. skrjabini*.

(a) *Type locality.* China, Guangdong Province.

(b) *Other localities.* China (Shanxi, Shaanxi, Zhejiang, Jiangxi, Fujian, Henan, Hubei, Hunan, Guangxi, Sichuan, Guizhou, Yunnan, Gansu), Thailand (Miyazaki, 1991, p. 123).

(c) *Molluscan hosts*

1. Family Assimineidae
   *Assiminea lutea*: China, Guangdong (experimental, Chen, 1965).
2. Family Pomatiopsidae
   There is great confusion concerning the names of pomatiopsid snails in China and the identities of hosts of *P. skrjabini* (discussed at length in Davis *et al.*, 1994). We have therefore only provided a summary of the information from that paper.
   *'Akiyoshia' chinensis*: China (Hunan).
   *Erhaia* spp.: China (Hunan, Hubei, Fujian).
   *Tricula* spp.: China (Hunan, Hubei, Guangxi, Fujian, Guangdong).
   *Tricula* sp. (*Tricula cristella* of Chung *et al.*, 1975b; see Davis *et al.*, 1994): China (Chung *et al.*, 1975b, as *Paragonimus hueitungensis*, Hunan).
   *Neotricula* spp.: China, Hunan.

(d) *Crustacean hosts*

1. Family Potamidae

*Aparapotamon grahami* (Rathbun, 1929): China (Anon., 1984, Hubei, Tang *et al.*, 1993, Hunan).

*Huananpotamon angulatum* (Dai and Lin, 1979) (syn. *Nanhaipotamon angulatum*): China (Li and Lin, 1994, Fujian).

*Isolapotamon papilionaceus* Tai *et al.*, 1975: China (Tang *et al.*, 1993, as *Paragonimus hueitungensis*, Hunan).

*Isolapotamon sinense* Tai and Sung, 1975: China (Tang *et al.*, 1993, as *Paragonimus hueitungensis*, Hunan).

*Malayopotamon fukienensis* Dai and Lin, 1979: China (Lin *et al.*, 1980, Fujian).

*Nanhaipotamon formosanum* (Parisi, 1919): China (Li and Lin, 1994, Fujian, see Ng and Dudgeon, 1992 concerning this species).

*Sinopotamon* sp.: China (Chen and Li, 1979, as *Pagumogonimus veocularis*, Sichuan; Lin *et al.*, 1980, Fujian; Anon., 1984, Hubei).

*Sinopotamon decrescentum* Dai, Chen, Zhang and Lin, 1986: China (Tang *et al.*, 1993, Hunan).

*Sinopotamon denticulatum* (H. Milne Edwards, 1853) (syn. *Potamon denticulatus*): China (Chen, 1963, Guangdong; Lin *et al.*, 1991, Hubei; Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan; Tang *et al.*, 1993, as *Paragonimus skrjabini* and *P. hueitungensis*, Hunan).

*Sinopotamon davidi* (Rathbun, 1904): China (Zheng and Zhang, 1991, Shaanxi).

*Sinopotamon fujianense* Dai and Chen, 1979: China (Li and Lin, 1994, Fujian).

*Sinopotamon honanense* Dai *et al.*, 1975: China (Lin *et al.*, 1991, Hubei).

*Sinopotamon introdigitum* Dai, Chen, Zhang and Lin 1986: China (Lin et al., 1991, Hubei).

*Sinopotamon kwanhsienense* Tai and Sung, 1975: China (Tai and Sung, 1975, as *Paragonimus szechuanensis*, Sichuan).

*Sinopotamon linguum* Dai, 1993: China (Tang *et al.*, 1993, Hunan).

*Sinopotamon lingxiengense* Dai, 1993; China (Tang *et al.* (1993, Hunan).

*Sinopotamon loudiense* Dai, 1995: China (Tang *et al.*, 1993, Hunan).

*Sinopotamon shaanxiense* Dai, 1993: China (Zheng and Zhang, 1991, Shaanxi).

*Sinopotamon shangxianense* Dai and Chen, 1981: China (Lin *et al.*, 1991, Hubei).

*Sinopotamon shaoyangense* Dai, 1993: China (Tang *et al.*, 1993, Hunan).

*Sinopotamon shensiense* (Rathbun, 1904): China (Anon., 1984, Hubei).

*Sinopotamon teritisum* Dai, Chen, Zhang and Lin, 1986: China (Lin *et al.*, 1991, Hubei).

*Sinopotamon jiangkuoense* Dai, 1995: China (Tang *et al.*, 1993, Hunan).

*Sinopotamon huitongense* Dai, 1995: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon xiangxiense* Dai, 1995: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon xingguingense* Dai, 1993: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon convexum* Dai, 1995: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon yaanense* (Chung and Tsao, 1962) (syn. *Potamon yaanensis*): China (Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan).
*Tenuilapotamon joshuiense* (Dai *et al.*, 1975) (syn. *Sinopotamon joshuiense*): China (He, L.Y. *et al.*, 1982, as *Paragonimus hueitungensis*, Hunan).
*Tenuilapotamon latilum* (Chen, 1980) (syn. *Sinopotamon latilum*): China (Lin *et al.*, 1991, Hubei).

(e) *Other second intermediate hosts*

*Rana boulengeri*: China (Xie *et al.*, 1985, Hunan, see Section 3.4).

(f) *Mammalian hosts*

1. Family Cercopithecidae
   *Macaca mulatta* (Zimmermann, 1780): China (experimental, Hu *et al.*, 1982, outcome not reported).
   'Monkey': China (experimental, Chung *et al.*, 1974, as *Paragonimus szechuanensis*).
2. Family Hominidae
   'Human': China (Hu *et al.*, 1982, Sichuan; Chung *et al.*, 1975b, as *Paragonimus hueitungensis*, Hunan).
3. Family Canidae
   'Dog': China (experimental, Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan; experimental, Peng *et al.*, 1989, Hubei; experimental, Chung *et al.*, 1975b, as *Paragonimus hueitungensis*, Hunan).
   *Nyctereutes procyonoides* (Gray, 1834): China (Anon., 1984, Hubei).
   *Vulpes vulpes* (Linnaeus, 1758): China (Anon., 1984, Hubei).
4. Family Felidae
   'Cat': China (natural and experimental, Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan; experimental, Chung *et al.*, 1975b, as *Paragonimus hueitungensis*, Hunan).
   *Prionailurus bengalensis* (Kerr, 1792) (syn. *Felis bengalensis chinensis*): China (Lin *et al.*, 1980, Fujian), Anon. (1984, Hubei).
5. Family Mustelidae
   *Mustela sibirica* Pallas, 1773: China (Anon., 1984, Hubei).
   'Weasel': China (experimental, Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan).
6. Family Viverridae
   *Paguma larvata* (Smith, 1827): China (Chen, 1960, Guangdong;

Anon., 1984, Hubei; experimental, Chung *et al.*, 1974, as *Paragonimus szechuanensis*).
*Viverricula indica* (Desmarest, 1804) (syn. *Viverricula* sp.): China (Wang *et al.*, 1985, Fujian).

7. Family Muridae
'Mouse': China (experimental paratenic host, Chung and Tsao, 1962a, as *Paragonimus szechuanensis*; Yan *et al.*, 1990).
'Rat': China (experimental, Chung and Tsao, 1962a, as *Paragonimus szechuanensis*, Sichuan; experimental, Chung *et al.*, 1975b, as *Paragonimus hueitungensis*, Hunan).
*Rattus norvegicus* (Berkenhout, 1769): China (Zhou *et al.*, 1993, Hubei).
*Rattus tanezumi* Temminck, 1844 (syn. *Rattus flavipectus*): China (Zhou *et al.*, 1993, Hubei).
8. Family Hystricidae
*Hystrix brachyura* Linnaeus, 1758 (syn. *Hystrix hodgsoni*): China (Chen *et al.*, 1988, Hubei).

### 5.2.43. Paragonimus szechuanensis *Chung and Tsao, 1962*

See *Paragonimus skrjabini.*

### 5.2.44. Paragonimus taipingini

This name was mentioned in Kurochkin (1987, p. 13) as a *species inquirenda.* No further information is available.

### 5.2.45. Paragonimus tuanshanensis *Chung, Ho, Cheng and Tsao, 1964*

See *Paragonimus heterotremus.*

### 5.2.46. Paragonimus uterobilateralis *Voelker and Vogel, 1965*

(a) *Type locality.* Cameroon.
(b) *Other localities.* Liberia, Nigeria, Gabon.
(c) *Molluscan hosts.* See comments under *Paragonimus africanus.*
(d) *Crustacean hosts*

1. Family Potamonautidae
*Liberonautes chaperi* (A. Milne-Edwards, 1886): Liberia (Sachs and Cumberlidge, 1991b.
*Liberonautes latidactylus* (de Man, 1903): Liberia (Sachs and Cumberlidge, 1991c.

*Liberonautes nanoides* Sachs and Cumberlidge, 1989: Liberia (Sachs and Cumberlidge, 1991d.

*Liberonautes paludicolis* Cumberlidge and Sachs, 1989: Liberia (Cumberlidge and Sachs, 1989).

*Sudanonautes africanus* (A. Milne-Edwards, 1869): Nigeria (Voelker and Sachs, 1977a, misidentified as *S. aubryi* according to Cumberlidge, 1994); Gabon (Voelker and Sachs, 1985).

*Sudanonautes aubryi* (H. Milne Edwards, 1853, syn. *S. pelii*): Nigeria (Voelker *et al.*, 1975, misidentified as *S. africanus* according to Cumberlidge, 1994).

*Sudanonautes floweri* (de Man, 1901): Nigeria (Voelker *et al.*, 1975, misidentified as *S. aubryi* according to Cumberlidge, 1994).

*Sudanonautes granulatus* (Balss, 1929): Cameroon (Voelker and Sachs, 1977a, misidentified as *S. pelii* according to Cumberlidge, 1994).

(e) *Mammalian hosts*

1. Family Soricidae
   *Crocidura flavescens* (I. Geoffroy, 1827): Liberia (experimental, Voelker, 1973).
2. Family Cercopithecidae
   *Macaca mulatta* (Zimmermann, 1780): Nigeria (experimental, Oelerich, 1977).
3. Family Hominidae
   'Human': Liberia and Guinea (Sachs and Voelker, 1982; Monson *et al.*, 1983); Ivory Coast (Nozais *et al.*, 1980, parasite tentatively identified); Nigeria (Voelker and Nwokolo, 1973; Onuigbo and Nwako, 1974; Udonsi, 1989); Gabon (Sachs *et al.*, 1983).
4. Family Canidae
   'Dog': Cameroon (Voelker and Vogel, 1965); Liberia (Sachs and Cumberlidge, 1990); Nigeria (experimental, Voelker and Sachs, 1977c).
5. Family Felidae
   'Cat': Nigeria (experimental, Voelker and Nwokolo, 1973); Gabon (experimental, Sachs *et al.*, 1983); Liberia (experimental, Voelker, 1973).
6. Family Herpestidae
   *Atilax paludinosus* (G. Cuvier, 1829): Cameroon (Voelker and Vogel, 1965.
   *Crossarchus obscurus* F.G. Cuvier, 1825: Liberia (experimental, Voelker, 1973).
7. Family Mustelidae
   *Lutra maculicollis* Lichtenstein, 1835: Liberia (Sachs and Cumberlidge, 1988, eggs in faeces of otter).
8. Family Viverridae
   *Civettictis civetta* (Schreber, 1776) (syn. *Viverra civetta*): Liberia (Sachs and Voelker, 1982; experimental, Voelker, 1973); Nigeria (Voelker *et al.*, 1975).

9. Family Muridae
   *Hybomys planifrons* (Miller, 1900): Liberia (experimental, Voelker, 1973).
   *Lophuromys sikapusi* (Temminck, 1853): Liberia (experimental, Voelker, 1973).
   *Malacomys edwardsi* Rochebrune, 1885: Liberia (experimental, Voelker, 1973).
   *Mastomys natalensis* (Smith, 1834): Liberia (experimental, Zillmann *et al.*, 1987).
   'Mouse': Liberia (experimental, Voelker, 1973).
   *Praomys tullbergi* (Thomas, 1894): Liberia (experimental, Voelker, 1973).
   'Rat': Nigeria (experimental, Voelker and Nwokolo, 1973); Liberia (experimental, Voelker, 1973).
   *Sigmodon hispidus* Say and Ord, 1825: Liberia (experimental, Weber and Sonntag, 1989).

5.2.47. Paragonimus veocularis *(Chen and Li, 1979)*

See *Paragonimus skrjabini.*

5.2.48. Paragonimus westermani *(Kerbert, 1878)*

Probable synonyms: *Paragonimus ringeri* (Cobbold, 1880) Ward and Hirsch, 1915; *Paragonimus edwardsi* Gulati, 1926; *Paragonimus macacae* Sandosham, 1953; *Paragonimus asymmetricus* Chen, 1977; *Paragonimus pulmonalis* (Baelz, 1880) Miyazaki, 1978; *Paragonimus filipinus* Miyazaki, 1978; *Paragonimus philippinensis* Ito, Yokogawa, Araki and Kobayashi, 1978.

Subspecific names: *Paragonimus westermani westermani* (Kerbert, 1878) Braun, 1899; *Paragonimus westermani* Szechuan (Sichuan) variety (see Chung and Tsao, 1962, Zhong 1981); *Paragonimus westermani ichunensis* Chung, Hsu and Kao, 1978; *Paragonimus westermani filipinus* Miyazaki, 1978; *Paragonimus westermani japonicus* Miyazaki, 1983.

*Paragonimus westermani* has probably the widest distribution of any member of the genus (Asia, from Pakistan to Japan and the Philippines, north to south-east Russia and south to Indonesia and possibly Papua New Guinea). Genetic and biological evidence suggests that the nominal *P. westermani* may consist of a complex of strains and species reminiscent of the situation in schistosomes. Records of this species from the Americas and Africa are due to misidentification often owing to the assumption that *P. westermani* was the sole species of lung fluke (e.g. Ward, 1894; Stiles and Hassall, 1900). Recent reports of *P. westermani* from Gabon (Kombila *et al.*, 1995; Vuong *et al.*, 1996) are of interest.

Miyazaki (1978) proposed that triploid *P. westermani* (see Section 4.1.1) should be placed in a separate species. Of the two names available for this, Miyazaki chose *Paragonimus pulmonalis* (Baelz, 1880). This name had been published in September 1880 for material from a human patient in Japan (see Yokogawa, 1982b for details). Contemporary morphological studies (summarized in Miyazaki, 1978) suggest strongly that Baelz's material did indeed represent triploid *P. westermani*. In August 1880, Cobbold had published the name *Distoma ringeri* for a lung fluke taken *post mortem* from a Portuguese resident of Taiwan and sent to him by Manson. The history of the case suggests that this form also was triploid *P. westermani*. Yokogawa (1982b) and Li (1995) have pointed out that the name *P. ringeri* (Cobbold, 1880) has priority. The type and sole specimen of *P. ringeri* is in poor condition and Miyazaki (1981b) was unable to see any diagnostic characters. He preferred to regard it as a *species inquirenda*, as did Kurochkin (1987). The worm reported from humans in Japan as *P. ringeri* by Ward and Hirsch (1915) apparently has cuticular spines arranged in groups and probably belongs to *P. ohirai* (see Miyazaki, 1991). Morphological, cytological, molecular and allozyme studies suggest that the triploid form has arisen from diploid populations within its range. Because of this, most workers prefer not to accept a separate specific name for it (e.g. see Yokogawa, 1982b; Tan and Li, 1990; Blair *et al.*, 1997).

Chen (1977) fed six metacercariae (from Huiyang, Guangdong) of distinctive shape to a dog and obtained a single adult worm for which he proposed the name *P. asymmetricus*. Liu, S. *et al.*, 1982) and Li and Liu (1984) demonstrated experimentally that similar metacercariae gave rise to adult *P. westermani* in cats. Zhang *et al.*, 1995) noted no difference in isoenzyme patterns between *P. asymmetricus* and *P. westermani*; see also Li and Chen (1992).

Sandosham (1953) described *Paragonimus macacae* from a crab-eating monkey from Malaysia. Miyazaki (1956) and Lee and Miyazaki (1965) re-examined these and other specimens from Malaysia, concluding that all should be referred to *P. westermani*.

*Paragonimus edwardsi* was described by Gulati (1926) from the lungs of a civet from India.

*Paragonimus filipinus* Miyazaki, 1978 and *P. philippinensis* Ito, Yokogawa, Araki and Kobayashi, 1978 were described from the Philippines. Miyazaki and Habe (1979) and Miyazaki (1981a) regarded these as belonging to a single subspecies, and preferred the name *P. westermani filipinus*.

*Paragonimus westermani ichunensis* Chung, Hsu and Kao, 1978 was erected for worms from north-east China and the adjacent part of Russia. In people ingesting uncooked crayfish, the juveniles of this subspecies do not mature but continue to migrate throughout the pleura. Recently, Bespozvannykh (1994) has shown that metacercariae from crayfish, when

ingested by any of a number of mammal species, tend to remain immature, utilizing the host as a paratenic host. When a paratenic host is eaten by another mammal, some or all of the larvae will mature in the new host. C-banding studies of chromosomes (Tan, 1987) and allozyme studies (Chen *et al.*, 1990) showed no differences between *P. westermani ichunensis* and diploid *P. westermani*. Li and Chen (1992) did not regard this subspecies as distinct from diploid *P. westermani*.

Miyazaki (1983) proposed that diploid *P. westermani* from Japan be referred to *P. w. japonicus* and other diploid populations of *P. westermani* (excepting those from the Philippines) to *P. w. westermani*. This subdivision does not fit well with genetic data (Blair *et al.*, 1997).

Chung and Tsao (1962a) found a form of *P. westermani* in Sichuan Province that differed in size of egg and metacercaria from populations of this species elsewhere in China. They named this the Szechuan (= Sichuan) variety. Chung *et al.* (1978) distinguished between the clinical effects of this form and those of *P. westermani* from elsewhere. Chen (1963) proposed that the dimensions given by Chung and Tsao (1962a) were not an adequate basis for recognizing a separate form.

Where the information is included in the cited papers, for records from Japan, China, Taiwan and Korea, the ploidy of the specimens is stated below. Worms from other localities are assumed to be diploid.

(a) *Type locality.* Probably India. Kerbert (1878, 1881) used the name *Königstiger* (= Bengal tiger) for the host. Records of the Amsterdam Zoo, in which the host tiger died, make no mention of its origin. Tigers in the collection at that time had come from India, Indonesia and China (Plantenga, *in litt.*).

(b) *Other localities.* Pakistan, Nepal, Thailand, Philippines, Indonesia, Papua New Guinea, Myanmar, Vietnam, Cambodia, Laos, Korea, China (Jilin, Heilongjiang, Liaoning, Hebei, Jiangsu, Anhui, Zhejiang, Jiangxi, Fujian, Henan, Hubei, Hunan, Guangdong, Hainan, Sichuan, Guizhou, Yunnan), Japan, Taiwan, Far East of Russia.

(c) *Molluscan hosts*

1. Family Pleuroceridae. Note comments concerning our poor taxonomic understanding of *Semisulcospira* in Davis *et al.*, 1994).

   *Juga buettneri*: Russia (Bespozvannykh, 1989, as *Paragonimus w. ichunensis*).

   *Juga tegulata* (von Martens, 1894): Russia (Bespozvannykh, 1989, as *Paragonimus w. ichunensis*).

   *Semisulcospira amurensis* (Gerstfeld, 1859): China (Chung *et al.*, 1978, Heilongjiang).

   *Semisulcospira calculus* (Reeve, 1859): northern China (Davis *et al.*, 1994).

   *Semisulcospira cancellata* (Benson, 1842): China (Wu *et al.*, 1991, Zhejiang).

*Semisulcospira extensa* (von Martens, 1894) (syn. *Juga extensa*): Korea (Davis *et al.*, 1994)); Russia (Bespozvannykh (1989, as *Paragonimus w. ichunensis*).

*Semisulcospira gottchei* (von Martens, 1886): Korea (Davis *et al.*, (1994).

*Semisulcospira libertina* (Gould, 1859) (syn. *Semisulcospira toucheana* Heude, 1880; *Melania libertina*; and probably *Semisulcospira bensoni*): Japan (Tomimura *et al.*, 1989, diploid; Shibahara, 1991, diploid; experimental, Shibahara, 1993, diploid; experimental, Hamajima *et al.*, 1981, triploid); China (Peng, 1987, Hubei, diploid; Zhong *et al.*, 1979, as *Paragonimus w. ichunensis*, Heilongjiang); Taiwan (Chiu, 1962a); Korea (Joo *et al.*, 1985).

*Semisulcospira mandarina* (Deshayes, 1874): China (Ho and Jiang, 1989).

*Semisulcospira multicincta* (von Martens, 1894): Korea (Davis *et al.*, 1994).

*Semisulcospira nodiperda* (von Martens, 1894): Korea (Davis *et al.*, 1994).

*Semisulcospira peregrinorum* (Heude, 1890): China (Wu *et al.*, 1991, Zhejiang. See Davis *et al.*, 1994 concerning the identity of this species.

2. Family Thiaridae

*Brotia asperata* (Lamarck, 1822) (syn. *Antemelania asperata*): Philippines (Ito *et al.*, 1978, as *Paragonimus philippinensis*).

*Brotia costula* (Rafinesque, 1833): Malaysia (Kim, 1978).

*Melanoides tuberculata* (Muller, 1774): China (Ho and Jiang, 1989; Wu *et al.*, 1991, Zhejiang). Status as host not proven according to Davis *et al.* (1994).

*Tarebia granifera* (Lamarck, 1822): China (Ho and Jiang, 1989). Status as host not proven according to Davis *et al.*, 1994).

(d) *Crustacean hosts*

1. Family Natantia

*Macrobrachium* sp.: Taiwan (Kunz, 1969).

*Macrobrachium nipponensis* (De Haan, 1849): China (Wu *et al.*, 1991, Zhejiang); Korea (Soh *et al.*, 1966).

2. Family Astacidae

*Cambaroides dauricus* (Pallas, 1772): China (Terasaki *et al.*, 1995, Liaoning, diploids, triploids and tetraploids; Chung *et al.*, 1978, as *Paragonimus w. ichunensis*, Heilongjiang); Russia (Shimazu and Oshima, 1983, as *Paragonimus w. ichunensis*).

*Cambaroides similis* (Koelbe): Korea (Agatsuma and Habe, 1985, triploid; Fan *et al.*, 1994).

*Cambaroides schrenki* (Kessler): China (Chung *et al.*, 1978, as *Paragonimus w. ichunensis*, Heilongjiang; Russia (experimental, Bespozvannykh, 1994).

*Procambarus clarki* (Girard, 1852) (syn. *Cambarus clarki*): Japan (Yokogawa, 1953). Crayfish introduced to Japan.

3. Family Grapsidae
   *Eriocheir japonicus* (de Haan, 1835) (this species regarded as the usual host for triploid *Paragonimus westermani* in Japan): Japan (Lou *et al*, 1992, triploid); Taiwan (Miyazaki and Chiu, 1980, triploid); Korea (Cho *et al.*, 1991).
   *Eriocheir sinensis* H. Milne Edwards, 1853: China (Li, 1989, diploid, Fujian).
   *Varuna litterata* (Fabricius, 1798): Philippines (Cabrera and Valeza, 1979).
4. Family Parathelphusidae
   *Ceylonthelphusa rugosa* (Kingsley, 1880) (syn. *Parathelphusa rugosa*): Sri Lanka (Kannangara and Karunaratne, 1969b).
   *Irmengardia pilosimana* (Roux, 1936): Malaysia (Habe *et al.*, 1993).
   *Oziothelphusa senex* (Fabricius, 1798) (syn. *Parathelphusa ceylonensis*): Sri Lanka (Kannangara and Karunaratne, 1969b).
   *Parathelphusa maculata* (de Man, 1879) (syn. *P. incerta*): Malaysia (Habe *et al.*, 1993).
   *Parathelphusa malaysiana* Ng and Takeda, 1992: Malaysia (Habe *et al.*, 1993).
   *Siamthelphusa improvisa* (Lanchester, 1901): Thailand (Kawashima *et al.*, 1989b, identification of parasite tentative).
   *Sundathelphusa philippina* (von Martens, 1868) (syn. *Parathelphusa mistio*, also misidentified as *Sundathelphusa picta*: see Cabrera, 1975): Philippines (Miyazaki and Habe, 1979, as *Paragonimus w. filipinus*; Zhong *et al.*, 1986; Hirai *et al.*, 1992).
5. Family Potamidae
   *Candidiopotamon rathbuni* (de Man, 1914): Taiwan (Su *et al.*, 1989, diploid).
   *Geothelphusa candidiensis* Bott, 1967: Taiwan (Su *et al.*, 1989, diploid). Host previously misidentified in Taiwan as *G. dehaani*).
   *Geothelphusa dehaani* (White, 1847) (syn. *Potamon dehaani*) (this species regarded as the usual host for diploid *P. westermani* in Japan): Japan (Miyazaki, 1983, as *Paragonimus w. japonicus*, diploid; natural and experimental, Shibahara, 1993, diploid; Miyazaki, 1982b, triploid, rare as host for triploid); Korea (Soh *et al.*, 1966).
   *Geothelphusa miyazakii* Miyake and Chiu, 1965: Taiwan (Su *et al.*, 1989, diploid).
   *Huananpotamon angulatum* (Dai and Lin, 1979) (syn. *Nanhaipotamon angulatum*): China (Li *et al.*, 1984, Fujian).
   *Huananpotamon obtusum* (Dai and Chen, 1979) (syn. *Nanhaipotamon obtusum*): China (Li, 1989, Fujian).

*Isolapotamon nasicum* Dai and Chen, 1979: China (Dai *et al.*, 1979, Fujian, not clear if the parasite is *P. westermani*).
*Isolapotamon sinense* Tai and Sung, 1975: China (Tai and Sung, 1975, Hunan).
*Johora johorense* (Roux, 1936) (syns. *Potamon johorense*, *Potamiscus johorensis*): Malaysia (Lee and Miyazaki, 1965).
*Johora tahanensis* (Bott, 1966): Malaysia (Habe *et al.*, 1993).
*Malayopotamon fukienense* Dai and Lin, 1979: China (Li, 1989, diploid, Fujian).
*Parapotamon hsingyiense* Tai and Sung, 1975 (syn. *P. spinescens hsingyiense*): China (Tai and Sung, 1975, identification of parasite tentative, Guizhou).
*Potamiscus smithianus* (Kemp, 1923) (syn. *Potamon smithianus*): Thailand (Miyazaki, 1982b).
*Potamon hispidum* (Wood-Mason, 1871): China (Ho and Jiang (1989).
*Potamon hokuoense* Dai *et al.*, 1975: China (Ho and Jiang (1989).
*Sinopotamon anhuiense* Dai and Fan in Dai *et al.*, 1979: China (Ho and Jiang, 1989).
*Sinopotamon bilobatum* Dai and Jiang, 1991: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon chekiangense* Tai and Sung, 1975: China (Lei *et al.*, 1987, diploid, Zhejiang).
*Sinopotamon davidi* (Rathbun, 1904): China (Ho and Jiang (1989).
*Sinopotamon decrescentum* Dai, Chen, Zhang and Lin, 1986: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon denticulatum* (H. Milne Edwards, 1853): China (He, L.Y. *et al.*, 1982, diploid, Fujian).
*Sinopotamon depressum* Dai and Fan, 1979: China (He, L.Y. *et al.*, 1982, diploid, Hunan), Lin *et al.*, 1991, Hubei).
*Sinopotamon fujianense* Dai and Chen, 1979: China (Li and Lin, 1994, diploid and triploid, Fujian).
*Sinopotamon honanense* Dai *et al.*, 1975: China (Ho and Jiang, 1989).
*Sinopotamon jichiense* Du *et* al.: China (Ho and Jiang, 1989).
*Sinopotamon kwanhsienense* Tai and Sung, 1975: China (Tai and Sung, 1975, Sichuan).
*Sinopotamon lansi* (Doflein, 1902): China (Ho and Jiang, 1989).
*Sinopotamon lingxiengense* Dai, 1993: China (Tang *et al.*, 1993, Hunan).
*Sinopotamon liuyangense* Dai, 1995: China (Tang *et al.* 1993, Hunan).
*Sinopotamon shensiense* (Rathbun, 1904): China (Ho and Jiang, 1989).
*Sinopotamon wuyiense* Li *et al.*, 1985: China (Li, 1989, Fujian).
*Sinopotamon yaanense* (Chung and Tsao, 1962): China (Ho and Jiang, 1989).

*Sinopotamon yangtsekiense* Bott, 1967: China (Zhang *et al.*, 1991, Anhui).

*Stoliczia tweediei* (Roux, 1934, syn. *Potamiscus tweediei, P. cognatus*): Malaysia (Miyazaki and Kwo, 1969). Records of this species from Baling, Malaysia probably refer to *Stoliczia karenae* Ng, 1993; see comments in Ng, 1993).

(e) *Mammalian hosts*

1. Family Cercopithecidae

*Macaca arctoides* (I. Geoffroy, 1831): China (experimental, Yuan *et al.*, 1990, diploid and triploid).

*Macaca cyclopis* (Swinhoe, 1863): Japan (experimental – imported animal – Sugiyama *et al.*, 1984, paratenic host).

*Macaca fascicularis* (Raffles, 1821) (syn. *Macaca irus*): Japan (experimental – imported animal – Sugiyama *et al.*, 1984, paratenic host); Malaysia (Lee and Miyazaki, 1965).

*Macaca fuscata* (Blyth, 1875): Japan (experimental, Sugiyama *et al.*, 1984, paratenic host).

*Macaca mulatta* (Zimmermann, 1780): China (experimental, Yuan *et al.*, 1990).

2. Family Hominidae

'Human': Japan (Miyazaki, 1981c, as *Paragonimus pulmonalis*); China (Yu *et al.*, 1994); Philippines (Cabrera, 1984); Papua New Guinea (Barnish and Ashford, 1989); Vietnam (Landmann *et al.*, 1961, as *Paragonimus ringeri* or *P. westermani*); India (Razaque *et al.*, 1991); Myanmar (Than *et al.*, 1994); Nepal (Sherchand, 1997); Taiwan (Liu and Cross, 1971); Korea (Shin and Joo, 1990); Gabon (Vuong *et al.*, 1996); Russia (Sukhanova, 1984, as *Paragonimus w. ichunensis*).

3. Family Canidae

'Dog' Japan (experimental, Fujino and Ishii, 1982, triploid); Taiwan (experimental, Miyazaki and Chiu, 1980, diploid and triploid); China (experimental, Li, Y.S. *et al.*, 1987, diploid and triploid, Fujian; experimental and natural, Chung *et al.*, 1978, as *Paragonimus w. ichunensis*; experimental, Terasaki *et al.*, 1995, diploid, triploid and tetraploid, Liaoning); Korea (experimental, Song and Kim, 1994); Malaysia (experimental, Habe *et al.*, 1996, paratenic host); Philippines (experimental, Hirai *et al.*, 1992); India (Pythal *et al.*, 1993a); Russia (experimental, Shimazu and Oshima, 1983).

'Fox': Russia (Posokhov and Shabanova, 1977).

*Nyctereutes procyonoides* (Gray, 1834): Japan (Shibahara *et al.*, 1985, diploid; experimental, Shibahara, 1985, diploid and triploid); Russia (Posokhov and Shabanova, 1977); China (Wu *et al.*, 1991, Zhejiang).

*Vulpes vulpes* (Linnaeus, 1758): Japan (Ashizawa *et al.*, 1980b; experimental, Shibahara and Nishida, 1986a, diploid).

'Wolf': Russia (Posokhov and Shabanova, 1977).

4. Family Felidae
   'Cat': Japan (Uga *et al.*, 1983; experimental, Miyazaki *et al.*, 1981b, diploid and triploid); China (natural and experimental, Chung *et al*, 1978, as *Paragonimus w. ichunensis*, Heilongjiang); Malaysia (experimental, Habe *et al.*, 1996); Philippines (experimental, Miyazaki and Habe, 1979, as *Paragonimus* w. *filipinus*; experimental, Ito *et al.*, 1979, as *Paragonimus philippinensis*); Taiwan (experimental, Miyazaki and Chiu, 1980, diploid and triploid); Korea (experimental, Miyazaki, 1978, triploid); India (Srivastava, 1938); Malaysia (Rohde, 1967); Indonesia (Yokogawa *et al.*, 1960, Java); Sri Lanka (experimental, Kannangara and Karunaratne, 1969b); Russia (experimental, Bespozvannykh (1994, as *Paragonimus w. ichunensis*).
   *Catopuma temminckii* (Vigors and Horsfield, 1827) (syn. *Felis temminckii*): Malaysia (Lim and Betterton, 1977).
   *Neofelis nebulosa* (Griffith, 1821): India (Hiregoudar amd Pethkar, 1970); Malaysia (Rohde, 1967).
   *Panthera pardus* (Linnaeus, 1758): Thailand (Kawashima *et al.*, 1989b); India (Pythal *et al.*, 1993b); Sri Lanka (Dissanaike and Paramananthan, 1962); Malaysia (Miyazaki, 1978).
   *Panthera tigris* (Linnaeus, 1758): China (Li, G.Y. *et al.*, 1987c, Guangdong); Korea (Miyazaki, 1978); Russia (Miyazaki, 1978); India (Gaur *et al.*, 1980; Arora and Das, 1988); Malaysia (Lee and Miyazaki, 1965; Rohde, 1967); Indonesia (Kwo and Miyazaki, 1968, Sumatra).
   *Prionailurus bengalensis* (Kerr, 1792) (syn. *Felis bengalensis*): China (Lin *et al.*, 1980, Fujian); Malaysia (Fishchthal and Kunz, 1965, north Borneo), Lim and Betterton, 1977); India (Vevers, 1923); Russia (Posokhov and Shabanova, 1977).
   *Prionailurus planiceps* (Vigors and Horsfield, 1827) (syn. *Felis planiceps*): Malaysia (Lim and Betterton, 1977).
   *Prionailurus rubiginosus* (I. Geoffroy Saint-Hilaire, 1831) (syn. *Felis rubiginosa*): Sri Lanka (Dissanaike and Paramananthan, 1962).
5. Family Herpestidae
   *Herpestes edwardsii* (E. Geoffroy Saint-Hilaire): India (Shyamasundari and Hanumantha Rao, 1978).
   *Herpestes urva* (Hodgson, 1836, syn. *Terpestis urva*): Taiwan (experimental, Yokogawa *et al.*, 1960).
6. Family Viverridae
   *Arctictis binturong* (Raffles, 1821): India (Dutt and Gupta, 1978, parasite tentatively identified).
   *Paguma larvata* (Smith, 1827): China (Wu *et al.*, 1991, Zhejiang).
7. Family Mustelidae
   *Melogale moschata* (Gray, 1831): China (Wu *et al.*, 1991, Zhejiang).

8. Family Suidae
   'Pig' (domestic animals): Japan (experimental, Shibahara and Nishida, 1986b, diploid, potential paratenic host); China (experimental, Zhu and Zhu, 1986, potential paratenic host, Zhejiang; Liu *et al.*, 1990, triploid, Liaoning); Russia (experimental, Bespozvannykh, 1994, as *Paragonimus w. ichunensis*, paratenic and final host).
   *Sus scrofa* Linnaeus, 1758 (wild animals): Japan (Miyazaki *et al.*, 1978a, paratenic host; Shibahara and Nishida, 1985, diploid; experimental, Shibahara and Nishida, 1986b, diploid, potential paratenic host); Pakistan (Cheema *et al.*, 1992).
9. Family Muridae
   'Hamster': Japan (experimental, Habe, 1978, potential paratenic host).
   'Mouse': Japan (experimental, Habe, 1978, paratenic host); Korea (experimental, Fan *et al.*, 1994, paratenic host); China (experimental, Dong *et al.*, 1984, paratenic host); Russia (experimental, Bespozvannykh, 1994, as *Paragonimus w. ichunensis*, paratenic host); Taiwan (experimental, Su *et al.*, 1988, diploid, potential paratenic host).
   'Rat': Japan (experimental, Miyazaki and Habe, 1976, paratenic host; experimental, Shibahara, 1984, diploid, paratenic and definitive host); China (experimental, Shibahara *et al.*, 1989, diploid and triploid, experimental paratenic hosts); Korea (experimental, Fan *et al.*, 1994, paratenic host); Russia (experimental, Bespozvannykh, 1994, as *Paragonimus w. ichunensis*, paratenic and definitive host); Taiwan (experimental, Su *et al.*, 1988, diploid, few worms matured); Malaysia (experimental, Habe *et al.*, 1996, paratenic host); Philippines (experimental, Miyazaki and Habe, 1979, as *Paragonimus w. filipinus*).
   *Rattus norvegicus* (Berkenhout, 1769): Taiwan (Chiu, 1962b); Philippines (Cabrera, 1977); China (Zhou *et al.*, 1993, Hubei).
   *Rattus tanezumi* Temminck, 1844 (syn. *Rattus rattus mindanensis*, *R. flavipectus*); Philippines (Jueco and Zabala, 1990); China (Zhou *et al.*, 1993, Hubei).
10. Family Caviidae
    'Guinea-pig': Japan (Habe, 1978, potential paratenic host).
11. Family Leporidae
    'Rabbit': Japan (experimental, Habe, 1978, potential paratenic host).

(f) *Bird hosts*
   'Hen': Japan (Habe, 1978, potential paratenic host).
   'Duck': China (Zhang *et al.*, unpublished, Liaoning).
   'Goose': China (Zhang *et al.*, unpublished, Liaoning).

5.2.49. Paragonimus xiangshanensis *He, Ma, Xiu and Gao, 1995*

(a) *Type locality* China, Zhejiang Province, Xiangshan County.
(b) *Crustacean hosts*
*Sinopotamon chekiangense* Tai and Sung, 1975: China (He *et al.*, 1995, Zhejiang).
(c) *Mammalian hosts*
1. Family Canidae
   'Dog': China (experimental, He *et al.*, 1995).
2. Family Felidae
   'Cat': China (experimental, He *et al.*, 1995).

5.2.50. Paragonimus yunnanensis *Ho, Chung et al. 1959*

Ho *et al.* (1973) noted that the species is similar to *P. harinasutai.*
(a) *Type locality.* China, Yunnan Province.
(b) *Crustacean hosts*
1. Family Potamidae
   *Potamon* sp.: China (Chung *et al.*, 1964, Yunnan).
   *Potamon hokuoense* Dai *et al.*, 1975: China (Chen, 1985).
   *Potamon chinghungense* Dai *et al.*, 1975: China (He, L.Y. *et al.*, 1982, Yunnan).
   *Potamon hispidum* (Wood-Mason, 1871): China (Chen, 1985).
(c) *Mammalian hosts*
1. Family Canidae
   'Dog': China (experimental, Ho *et al.*, 1973, poor host, Yunnan).
2. Family Felidae
   'Cat': China (experimental, Ho *et al.*, 1973, Yunnan).

## 5.3 *Paragonimus* sp. Not Otherwise Identified

Evidence of infection with species of *Paragonimus* in humans or animals is often reported. Some of these records are worth mentioning because they indicate the presence of lung flukes in hosts, or geographical locations, otherwise overlooked. Thus a single human case in the interior of Peru prompted surveys that led to the discovery of two new species (Miyazaki *et al.*, 1973, 1975). Similarly, the finding of *Paragonimus* specimens in Sri Lanka (Dissanaike and Paramananthan, 1961) prompted workers there to investigate further, leading to the eventual recognition of four species in the country. In Assam, India, Baruah *et al.* (1985) found, but did not identify to species, *Paragonimus* in pigs.

Despite recent descriptions of several species from the Americas, it is likely that more await discovery. Alarcón de Noya *et al.* (1985a, b) reported a *Paragonimus* species in humans and opossums (*Didelphis marsupialis*) in north-east Venezuela. This remains unidentified despite a number of studies (Tongu *et al.*, 1990; Noya *et al.*, 1992) and might represent a new species. As yet undetermined specimens of *Paragonimus* have been collected from marsupials in Brazil (Brenes *et al.*, 1980). In Central America, metacercariae resembling those of the genus *Euparagonimus* have been found in crabs (Miyazaki et al., 1980). Species of *Euparagonimus* are otherwise known only from China. Evidence for the occurrence of this genus in Africa has been provided by Bayssade-Dufour *et al.*, 1995) and Ollivier *et al.*, (1995).

Paragonimiasis has been reported from humans, dogs and cats in South Africa. Adult worms were found on two occasions (Curson, 1928 in a cat; Van Rensburg *et al.*, 1987 in a dog) but not properly identified. Finding of eggs was the means of diagnosis in two other reports (Proctor and Gregory, 1974; McCallum, 1975). A case reported by Fossati (1971) from Libya might have been acquired in West Africa. Other reports of unidentified lung flukes from Africa are reviewed in Yokogawa *et al.* (1960) and Nozais *et al.* (1980).

Worms recovered from humans are often not in a condition that permits identification. A number of such cases have been of ectopic parasitism (Brenes *et al.*, 1983 in Honduras; Thamprasert, 1993 in Thailand). An interesting recent report (Møller *et al.*, 1995) of cerebral paragonimiasis was in a woman who had lived only in Denmark and Poland, far distant from any known endemic area.

### 5.4. Related Species: Genus *Euparagonimus* Chen, 1962

Distinctive characters of the metacercaria (excretory bladder not extending anterior to the ventral sucker) led Chen (1962) to erect a new genus.

#### 5.4.1. Euparagonimus cenocopiosus *Chen, 1962*

(a) *Type locality.* China, Guangdong Province.
(b) *Other localities.* Southern and eastern provinces of China.
(c) *Crustacean hosts*

1. Family Parathelphusidae
   *Somanniathelphusa sinensis* (H. Milne Edwards, 1853) (see Ng and Dudgeon, 1992 concerning this species): China (Yian *et al.*, 1990, Fujian).
2. Family Potamidae
   *Malayopotamon fukienense* Dai and Lin, 1979: China (Li, 1989, Fujian).

*Nanhaipotamon formosanum* (Parisi, 1919): China (Yian *et al.*, 1990, Fujian, see Ng and Dudgeon, 1992 concerning this species).
*Potamon hispidum* (Wood-Mason, 1871): China (Yian *et al.*, 1990, Fujian).
*Sinopotamon chekiangense* (Tai and Sung 1975): China (Wu *et al.*, 1991, Zhejiang).
*Sinopotamon denticulatum* (H. Milne-Edwards, 1953): China (He, L.Y. *et al.*, 1982, Fujian; Peng, 1984, Guangdong).
*Sinopotamon fujianense* Dai and Chen, 1979: China (Li and Lin, 1994, Fujian).
*Sinopotamon wuyiense* Li *et al.*, 1985: China (Li, 1989, Fujian).
*Sinopotamon yangtsekiense* Bott, 1967: China (Wu *et al.*, 1991, Zhejiang).

(d) *Mammalian hosts*

1. Family Canidae
   'Dog': China (experimental, He, Y.X. *et al.*, 1982a, Fujian).
2. Family Felidae
   'Cat': China (experimental, He, L.Y. *et al.*, 1982, Fujian).
3. Family Mustelidae
   *Martes* sp.: China (Wu *et al.*, 1991, Zhejiang).
4. Family Muridae
   'Rat': China (experimental, Shen and Zhang, 1987).

5.4.2. Euparagonimus hongzesiensis *Hu* et al., *1990*

(a) *Type locality.* China, Jiangsu.
(b) *Mammalian hosts*

1. Family Canidae
   *Nyctereutes procyonoides* (Gray, 1834): China (Hu *et al.*, 1990, Jiangsu).

## 5.5. Similar Tissue-dwelling Species

The family Achillurbainiidae Dollfus, 1939 was proposed for a fluke from an abscess in the eyelid of a panther from Malaysia. Since then, members of the genus *Achillurbaina* Dollfus, 1939 (syn. *Poikilorchis* Fain and Vandepitte, 1957) have been found in humans in several parts of the world. It is unclear whether the Achillurbainiidae are related to the Paragonimidae. However, there is potential for these tissue-dwelling trematodes to be confused with *Paragonimus* species. In all human cases of achillurbainiasis, large cysts have occurred behind the ear. Adult worms or their eggs have been recovered from these. The eggs are much smaller than those of *Paragonimus* species and are flattened at the opercular end. Early reports of this condition

are summarized in Yokogawa *et al.* (1960). Subsequent reports and reviews are by Chen (1965), Beaver *et al.* (1977), Nozais *et al.*, 1980) and Miyazaki (1991).

## 6. *PARAGONIMUS* SPECIES AS PARASITES OF HUMANS AND DOMESTIC ANIMALS

### 6.1. Reviews Based on Geographical Regions

There are a number of recent reviews dealing with paragonimiasis in humans and/or domestic animals in specific countries or regions, including Sogandares-Bernal and Seed (1973, the Americas), Zhong *et al.*, 1981, China), Lamothe-Argumedo (1985, the Americas), Choi (1990, Korea), Xu (1991, China), Nwokolo (1991, West Africa), Shim *et al.*, 1991, Korea) Im *et al.* (1993, Korea) and Hinz (1996, Thailand).

### 6.2. Clinical Manifestations and Pathology

Paragonimiasis is a chronic condition, persisting in some cases for many years (Yokogawa *et al.*, 1960). Spontaneous cures can occur (Sadun and Buck, 1960). Pathological effects are due to the presence of adult worms and their eggs in the lungs and other sites, to the movement of worms through tissues, and to the metabolites produced by worms.

#### 6.2.1. *Lungs*

Species of *Paragonimus* normally mature in the lungs of their hosts. Pulmonary signs and symptoms are therefore common. Worms pair and lie together in cysts of host origin that can be 1–2 cm in diameter. Sogandares-Bernal and Seed (1973) and Choi (1990) have discussed cyst formation and associated pathology for *P. kellicotti* and *P. westermani* respectively. Eventually, the cysts communicate with bronchioles and eggs and matter discharge into them and hence to the outside in faeces or sputum. Most pulmonary infections with *Paragonimus* species are probably inapparent or produce mild symptoms only (Zhong *et al.*, 1981; Shim *et al.*, 1991). The most common symptoms are chronic cough and vague respiratory discomfort. In symptomatic cases, bouts of coughing yield sputum that contains blood and is often brown in colour due to the presence of worm eggs. Microscopic examination may reveal eggs, necrotic tissue, blood, pus cells and occasionally Charcot–Leyden crystals. Eggs are not

invariably found. In very rare cases, expectoration of adult worms has been noted (Vanijanonta *et al.*, 1981). Haemoptysis may be severe and associated with paroxysms of coughing. Pneumothorax and secondary bacterial infections may occur. The pulmonary form of paragonimiasis is best known in infections with triploid *P. westermani*, where solitary individuals can form large cysts in the lungs. The diploid form of *P. westermani* is generally regarded as less pathogenic; individuals are smaller than the triploids, and can only form lung cysts if partners are found. The situation is similar for other species (e.g. *P. heterotremus*, *P. mexicanus*), all of which are diploid, maturing in the lungs of humans. Recent reviews of pulmonary paragonimiasis are by Nana and Bovornkitti (1991), Shim *et al.* (1991) and Im *et al.* (1993).

### 6.2.2. *Other Sites*

Extrapulmonary paragonimiasis can be caused by migration of young flukes through the tissues prior to reaching the lungs. Experimental studies in dogs (e.g. Zhong *et al.*, 1981) have shown that there can be marked pathological changes caused by this. Symptoms associated with early, acute infections in humans (abdominal pain, fever, diarrhoea) were reported by Zhong *et al.* (1981).

Extrapulmonary manifestations can also occur while worms are seeking sexual partners, usually in the pleural cavities, before establishment in the lungs. This is quite common in human infections with diploid *P. westermani* (Miyazaki, 1991). The active worms cause damage to the pleura. Pleural effusion is common and sometimes contains eggs.

For some species (e.g. *P. miyazakii*, *P. skrjabini*), humans might be unsuitable hosts in which to mature. Worms remain juvenile and eggs are rarely or never found (Chung *et al.*, 1978; Okamoto *et al.*, 1993). Haemoptysis is rare or does not occur but pleural effusion is common. Various symptoms of larva migrans occur of which the most common, in the case of *P. skrjabini*, is the appearance of migratory subcutaneous nodules that usually contain juvenile flukes (Chung and Tsao 1962b; Zhong *et al.*, 1981). Brain involvement is relatively frequent.

Some adult worms may spontaneously migrate from the lungs and lodge in other sites. This can occur with *P. westermani*, for which ectopic sites include (Yokogawa *et al.*, 1960; Choi, 1990; Harinasuta and Bunnag, 1990) primarily the brain (especially the occipital and temporal lobes) but also the spinal cord, thoracic muscle, subcutaneous tissues (e.g. Ogata *et al.*, 1990), liver (e.g. Nabeshima *et al.*, 1991), spleen, greater omentum, peritoneal cavity (e.g. Shimao *et al.*, 1994), pericardial cavity and heart muscles, mediastinum, uterus, fallopian tubes, ovaries, scrotum and urinary tract. Eggs may be produced by worms in such sites. Infection in the brain

is particularly life-threatening (Kusner and King, 1993). Lesions here vary in size up to several centimetres in diameter and may eventually become calcified. Cerebral paragonimiasis due to species other than *P. westermani* has been reported from the Americas (Brenes *et al.*, 1982).

In all forms of extrapulmonary paragonimiasis, toxic or allergenic metabolites released by the worms may be pathogenic (Zhong *et al.*, 1981), but this aspect has received little attention.

## 6.3. Diagnosis

### 6.3.1. *General*

A definitive diagnosis requires the finding of parasite eggs. These may be found in sputum, faeces, pleural effusion and biopsies of nodules and cysts as well as at post-mortem examination. Eggs in faeces should be differentiated from those of other trematodes and pseudophyllidean cestodes. Eggs from nodules might be confused with eggs of *Achillurbainia* species (Miyazaki, 1991). Unfortunately for the diagnostician, eggs are frequently not found (e.g. Shim *et al.*, 1991) and other symptoms may be insufficiently specific for diagnosis of paragonimiasis (Sadun and Buck, 1960).

Chest X-rays may reveal few or no abnormalities, even in patients expectorating eggs in the sputum (Nwokolo, 1980; Toscano *et al.*, 1995). Changes that are seen include patchy infiltration, cavitation, fibrosis and pleural thickening (Harinasuta and Bunnag, 1990). In general, chest X-rays rather resemble those seen in cases of tuberculosis and misdiagnosis is possible unless paragonimiasis is suspected (Toscano *et al.*, 1995). Computerized tomography may provide better visualization of lesions (Shim *et al.*, 1991; Im *et al.*, 1993). Extrapulmonary paragonimiasis can also be misdiagnosed. The cerebral form must be differentiated from brain tumour and other parasitic cysts, especially cysticercosis, and the cutaneous form from cutaneous larva migrans such as gnathostomiasis.

As a consequence of these diagnostic difficulties, immunodiagnosis is very useful for pulmonary paragonimiasis, and usually the only option for prepatent stages and extrapulmonary forms.

### 6.3.2. *Immunodiagnosis*

Yokogawa *et al.*, 1960) and Yokogawa (1965) have reviewed the older literature on immunodiagnosis in detail. Choi (1990) has reviewed various tests from a Korean perspective. Intradermal (ID) tests, complement fixation (CF) tests (see Section 6.3.2(c)), enzyme-linked immunosorbent assay (ELISA), gel precipitation methods (diffusion methods and immun-

oelectrophoresis) and agglutination tests are among those that have been used.

(a) *ID test.* The ID test is very simple, rapid and cheap. A small quantity (0.01–0.1 ml) of a dilute antigen extract (usually a relatively crude worm extract) is injected into the skin (reviewed in Yokogawa *et al.*, 1960; Yokogawa, 1965; Kim, 1969). To be scored as positive, within 15 min a wheal significantly larger than that surrounding a control injection must appear on the skin. Yokogawa *et al.* (1960, p. 179) were of the opinion that the ID test is more efficient than either serological methods or sputum and stool examinations for the diagnosis of paragonimiasis. It is also useful in distinguishing between paragonimiasis and other chest diseases, especially tuberculosis. Although it is often stated to be free of adverse effects, Chen (1985) has reported cases of allergic reactions after one or more skin tests. The reaction increased in severity with successive tests and was most common in people with a long history of paragonimiasis. Such reactions are likely to be very rare.

Major disadvantages of the ID test are that it can remain positive for many years after cure or death of the parasites, and that cross-reactions are common if other trematodes, such as schistosomes or *Clonorchis sinensis*, are present in the host. Apparent false positives are quite common, but false negatives are rare. Because of this, it is often suggested that people with a positive ID test should then be screened using other methods, such as ELISA or CF.

Despite the disadvantages, huge numbers of people have been tested through the years using this technique. Zhong *et al.*, (1981) reported that over 2 million people in China were tested between 1952 and 1981; overall, 20% were positive. Bi and Zhang (1996) reported the results of ID tests in Liaoning Province, China, after a control programme. In 1963, of 42 365 people tested, 10 246 (24.2%) returned a positive result and almost half of those proved to be infected. Of 81 487 people tested in 1990–1991, 7362 (9%) returned a positive result but only 322 (0.4%) were confirmed as infected. Liu *et al.* (1995) have reported results of tests on 46 698 people in Sichuan Province; of these, 19.7% were positive, with the highest rate in those under 15 years of age. Singh *et al.* (1993), in India, have reported another recent example of the use of the ID test for mass screening.

(b) *ELISA.* ELISA tests are now very commonly used. The first ELISA tests for paragonimiasis were reported from Thailand by Quicho *et al.* (1981) using experimentally infected cats and from China by Zhong *et al.* (1981). Since then, there have been numerous reports of ELISA tests for paragonimiasis (e.g. Knobloch and Lederer, 1983, Africa and Ecuador; Imai, 1987, Japan; Zhang *et al.*, 1989, China; Waikagul, 1989 and Pariyanonda *et al.*, 1990, both Thailand; Lim *et al.*, 1990, Korea, using experimentally infected rats; Guevara *et al.*, 1995, Ecuador). Yokogawa *et*

*al.* (1983), in Peru, compared ELISA with CF tests, double diffusion and ID tests. The ELISA proved more sensitive than other tests and its results were well correlated with those of other methods. Cho *et al.* (1983) reported similar findings from Korea. The suitability of ELISA for mass screening in Japan has been discussed by Choi *et al.* (1992).

In most of the studies listed in the previous paragraph, crude parasite extracts were used and cross-reactivity with sera from patients with fascioliasis, clonorchiasis or schistosomiasis was observed. ELISA methods have been used to assess the diagnostic sensitivity and specificity of antigens purified in various ways. Thus Wongkham *et al.* (1994) used preparative isoelectric focusing to purify an antigen of 31.5 kDa, known from previous immunoblotting experiments (Maleewong *et al.*, 1991) to be a diagnostic antigen for *P. heterotremus*. ELISA tests using this antigen were very specific for human paragonimiasis. A substance partially purified by Indrawati *et al.* (1991) and evaluated using ELISA might represent the same antigen (Wongkham *et al.*, 1994). Indrawati *et al.* (1991) demonstrated that this antigen was not a protein. Carbohydrate epitopes have been demonstrated in metacercarial antigens (e.g. Zhang *et al.*, 1991).

Reduction or elimination of cross-reactions can be achieved by absorbing test sera with heterologous antigens prior to ELISA (Waikagul, 1989; Ono *et al.*, 1992). ELISA inhibition tests, using a *Paragonimus*-specific monoclonal antibody to determine the availability of antigenic epitopes following exposure of these to test sera, provide an alternative way to reduce cross-reactions (Yong *et al.*, 1993).

Cysteine proteases (see Section 6.4.2) are emerging as a class of strongly antigenic substances of potential use in immunodiagnosis. These are excretory/secretory (E/S) antigens, produced in the gut wall and eventually voided from the worm through its mouth. It is possible that some of the strongly antigenic bands from worms and their E/S products detected by immunoblotting experiments (e.g. Sugiyama *et al.*, 1987; Itoh and Sato, 1988; Sugiyama and Ketudat, 1989) and against which specific monoclonal antibodies have been raised (e.g. Sugiyama *et al.*, 1988) are in fact cysteine proteases (Ichikawa and Ikeda, 1995). Use of crude E/S products of *Paragonimus* species as antigens in ELISA gave better results than did somatic antigens (Ikeda *et al.*, 1992). Partially purified cysteine proteases have now been tested in ELISAs and found to be free of cross-reactions with distantly related parasites (Ikeda *et al.*, 1996). However, epitopes appear identical among *Paragonimus* species and species-specific diagnosis was not possible. However, this situation can be exploited in that antigens from common species, such as *P. westermani* or *P. ohirai*, can be used to diagnose paragonimiasis due to any species (Kojima *et al.*, 1983; Sugiyama and Ketudat, 1989; Ikeda *et al.*, 1996). Recent reports on the purification and characterization of cysteine proteases from *Paragonimus* species include

those by Yamakami and Hamajima (1989, 1990), Song and Dresden (1990), Song and Kim (1994), Yamamoto *et al.* (1994), Yamakami *et al.* (1995) and Chung *et al.* (1995).

An alternative to coating ELISA plates with parasite antigens is to coat the plates with anti-parasite antibodies. These will capture parasite antigens (from sera) that are detected by addition of further anti-parasite monoclonal antibodies (sandwich ELISA). Zhang *et al.* (1996 and see references therein) have developed such a test, which is very specific and sensitive (and see Section 6.3.2(c)).

Itoh and Sato (1990) proposed a dot ELISA in which antigens were dotted onto nitrocellulose membranes and dried. The remaining steps were essentially as in plate-format ELISA except that the final colour intensity was scored by eye. The dot method showed stronger cross-reactions than the conventional plate format, but homologous sera always gave the strongest reactions. Further dilution of sera reduced cross-reactivity. This method requires fewer reagents and less equipment than plate-format ELISA, and prepared nitrocellulose membranes have a long half-life. Dot ELISAs are commonly used in Japan (e.g. Okamoto *et al.*, 1993; Tomita *et al.*, 1996). A variant dot ELISA was described by Zhang *et al.* (1991, 1993). Sera were dotted onto nitrocellulose membranes, probed with monoclonal antibodies to adult or juvenile antigens of *P. westermani* and the quantity of parasite antigens in the sera estimated in an manner similar to that described above for the sandwich ELISA.

(c) *Monitoring post-treatment recovery.* Researchers and clinicians have used immunodiagnostic tests as an indication of cure after treatment. The ID test is of little value in this regard because it is known to remain positive for >10 years after cure (reviewed by Yokogawa, 1965). Shim *et al.* (1991) noted that, in endemic areas of Korea, fewer than 20% of positive reactors to the skin test have an active infection. According to Yokogawa *et al.* (1960), Yokogawa (1965) and Tsuji (1984) the CF test is the most closely correlated with the course of active infection (although it is also a technically demanding and laborious test, as pointed out by Shim *et al.* (1991). However, Oelerich and Volkmer (1976) found that the CF test was not suitable because of the long persistence of antibody (>1 year) after cure.

Serum IgG levels may be slightly elevated following cure, but then decline slowly over a period of many months (Knobloch, 1984; Knobloch *et al.*, 1984; Cho *et al.*, 1989). Consequently, a long period must elapse for confirmation of cure by IgG assays. Ikeda *et al.* (1992) proposed that IgE ELISA might be more useful in determining recovery after treatment because of the short half-life of circulating IgE. However, Knobloch (1984) reported that after cure with praziquantel, serum IgE changes were not very informative.

Assaying parasite antigens may be more informative. Zhang *et al.* (1993, 1996) used a sandwich ELISA (see Section 6.3.2(b)). They captured antigens from human sera using anti-*Paragonimus* IgG from hyperimmune rabbits, then quantified the antigens using *Paragonimus*-specific monoclonal antibodies. In addition to its use as a diagnostic test, it was used to monitor changes in antigenaemia after treatment with praziquantel. There is a marked but transient increase shortly after treatment followed by a slow decline in antigen levels in the blood, perhaps because the cyst wall retards clearance. However, this decline is apparent within 2 months after cure.

## 6.4. Immunology

It is unclear to what extent acquired immunity can develop and be effective against *Paragonimus* species (discussed briefly by Sogandares-Bernal and Seed, 1973 and by Choi, 1990).

### 6.4.1. *Modes of Immune Killing*

The vertebrate host responds to infection by *Paragonimus* species with an increase in circulating antibody. Levels of IgE, in particular, can be greatly elevated (Knobloch *et al.*, 1984; Ikeda *et al.*, 1991), especially in pleural exudates, suggesting local synthesis of the antibody (Yokogawa *et al.*, 1976; Ikeda *et al.* (1992). In rats infected with *P. ohirai*, Ikeda and Fujita (1980) and Ikeda *et al.* (1982) were able to demonstrate experimentally that specific IgE could be produced locally by the mediastinal lymph node. They also demonstrated that older worms might actually suppress IgE formation in the rat. Much of the IgE produced as a response to paragonimiasis is non-specific (Yokogawa *et al.*, 1976; Shin and Min, 1997) and this appears to interfere with immune killing of the parasites (Shin and Min, 1997). Guevara *et al.* (1995) noted that levels of IgM and IgG and its subclasses were significantly elevated in humans infected with *P. mexicanus*. $IgG_4$ levels were particularly high. It was proposed that this subclass may play a role in obstructing the action of IgE-mediated immunopathology.

The role of cells in immune responses to *Paragonimus* has not been much investigated. It is assumed that clearance of *Paragonimus* is due to antibody-dependent cell-mediated cytotoxicity (Guevara *et al.*, 1995). However there is as yet little direct evidence for this. Eosinophilia is usual and can be extreme (e.g. Yokogawa *et al.*, 1976; Ono *et al.*, 1992; Kan *et al.*, 1995). Eosinophils and neutrophils are attracted to extracts of adults of *P. westermani* or their E/S products (Duan and Wu, 1989). Min *et al.* (1990) studied the effects of rat peritoneal macrophages on excysted metacercariae of

*P. westermani*. They demonstrated that, although metacercariae can induce the non-specific activation of peritoneal macrophages, cytotoxicity is greatly enhanced in the presence of antibodies and complement.

### 6.4.2. *Immune Evasion*

In some trematodes (e.g. *Fasciola hepatica*), the tegument and its covering glycocalyx are heavily involved in immune evasion processes. Sloughing off and replacement of glycocalyx complexed with antibodies is probably one means of immune evasion. Some evidence suggests a similar role for the tegument in immune evasion in *Paragonimus* species. Formation and turnover of glycocalyx seems most active during migration through the tissues of the host, but is greatly diminished in the adult worm (Fujino *et al.*, 1989). In immature *P. westermani* in the muscles of a paratenic host, antibody–glycocalyx complexes seem to form a physical barrier to inflammatory cells (Fukuda *et al.*, 1993). In *P. ohirai*, there are changes in the molecular weight (Ikeda, 1992), antigenicity (Ohara *et al.*, 1985; Oikawa and Ikeda, 1989) and sugar composition (Ikeda *et al.*, 1990) of the glycoproteins constituting the glycocalyx during maturation of the worms.

The E/S products of adult *P. westermani* contain peroxidase, catalase and superoxide dismutase, which may help provide protection against oxidative killing by host cells (Chung *et al.*, 1993). These enzymes are present in higher levels in juvenile worms or metacercariae than in adults (Chung *et al.*, 1992).

Various proteases have also been detected in E/S products and their roles may include mediation of immune evasion. *Paragonimus westermani* possesses at least two cysteine proteases, one with a neutral pH optimum, the other with an acid optimum (Yamakami *et al.*, 1995 and references therein). These enzymes might act together in digesting host tissues for nutrition and to facilitate migration of juveniles. They are capable of degrading collagen and haemoglobin as well as immunoglobulins and other molecules associated with the mammalian immune system (Song and Kim, 1994; Yamakami *et al.*, 1995 and references therein). The neutral thiol protease in *P. westermani* metacercariae suppresses a number of immune responses and might induce tolerance to specific antigens (Hamajima *et al.*, 1994). For example, it suppressed rejection of skin grafts, leading Hamajima *et al.* (1994) to suggest a therapeutic use in human medicine.

Ohara *et al.* (1985), using *P. ohirai* in rats, showed that specific antibody production dropped after the worms had matured. This might be because mature flukes, when surrounded by the cyst wall in lungs, may be less apparent to the host immune response. Zhang *et al.* (1993) have suggested that lowering of metabolic activity by the encysted worm might be a better explanation.

### 6.5. Treatment

The early literature (reviewed in Yokogawa *et al.*, 1960) revealed problems of high toxicity and low efficacy with many drugs. The situation changed with the introduction of bithionol in 1962, which rapidly replaced earlier drugs (Yokogawa, 1965). In about 30% of patients, bithionol causes diarrhoea and nausea and relapses can occur (Coleman and Barry, 1982). Niclofan was introduced in about 1980 and found to be very effective, but to have a narrow safety margin (Shim *et al.*, 1991). Praziquantel, released about the same time, is an effective, well-tolerated anthelmintic and only a short course of treatment is required. Praziquantel is given at a dose of 25 mg $kg^{-1}$ three times daily after meals for 3 days (Udonsi, 1989; Harinasuta and Bunnag, 1990). Side-effects are mild and transient (Fu *et al.*, 1988). Cure rates are generally very high, with no eggs being found several weeks after treatment. The lesions in chest X-rays are cleared after a few months. Praziquantel is also effective against extrapulmonary forms. It has been used in veterinary practice for treatment of *P. kellicotti* infections in dogs and cats (e.g. Bowman *et al.*, 1991). One human case of *P. kellicotti* was also successfully treated with praziquantel (Mariano *et al.*, 1986), and treatment of *P. mexicanus* (e.g. Nicanor Ibáñez and César Jara, 1992; Saborio *et al.*, 1995) and *P. miyazakii* (e.g. Ono *et al.*, 1992) infections have also been reported. Shim *et al.* (1991) commented that complications due to pleural effusion and fibrosis are the main causes of the failure of praziquantel to effect a cure.

Triclabendazole has been successfully used in experimental paragonimiasis (Weber *et al.*, 1988) and human infections in Africa (Ripert *et al.*, 1992). In the latter case, a single dose was effective. Triclabendazole cured three Ecuadorian patients infected with *P. mexicanus* who had failed to respond to bithionol and praziquantel (Calvopiña *et al.*, 1993). Use of other drugs has been reported (e.g. by Nwokolo and Volkmer, 1977 and reviewed by Yokogawa 1984). Zhong *et al.* (1981) and Xu (1991) have summarized drug therapy in China.

Surgical treatment of paragonimiasis was sometimes used before effective drugs were developed (Yokogawa *et al.*, 1960). Surgical intervention is now rare except in the case of subcutaneous nodules, which can be relatively easily excised (e.g. Ogata *et al.*, 1990), and for surgical decortication of pleural lesions (e.g. Shim *et al.*, 1991; Tomita *et al.*, 1996).

### 6.6. Control Measures and Public Health

Most human infection is acquired by eating inadequately cooked or treated crabs or crayfish. In many endemic areas, local customs enhance transmis-

sion. Culinary use of crab juice (Micozzi and Ongchangco, 1980; Cabrera, 1984, Philippines) or ingestion of raw or undercooked crabs (e.g. Udonsi, 1987, Nigeria; Choi, 1990, Korea; Gyoten, 1994, Japan) are obvious sources of infection. Ingestion of raw crabs because of their supposed medicinal properties is common (Vogel and Crewe 1965, Cameroon; Choi, 1990, Korea; Vieira *et al.*, 1992, Ecuador). Marinating or salting crabs does not kill all metacercariae and ingestion of such crabs is a principal route of infection in China and Taiwan (Fan and Khaw, 1964a, b) and the Philippines (Micozzi and Ongchangco, 1980). Kawashima and Miyahara (1974) have suggested that infection with *P. ohirai* is possible among fishermen who crush infected crabs in their mouths before using them as bait.

Some infection is undoubtedly also due to eating raw meat of paratenic hosts such as pigs (Miyazaki and Habe, 1976). Other modes of infection have been suggested. Release of metacercariae from dead crustaceans and their survival in water used for drinking has been proposed (Yokogawa *et al.*, 1960). It has been shown that metacercariae can survive for days or weeks after removal from their host, and can even withstand freezing (Loh *et al.*, 1969; Fan *et al.*, 1993). Infection via cuts or surgical wounds has also been postulated or demonstrated experimentally (Wu *et al.*, 1988; Waikagul *et al.*, 1989b). Pythal *et al.* (1993a) reported possible infection of a puppy in India with *P. westermani* via ingestion of its mother's regurgitus. There have been a few human cases where no apparent route of infection could be demonstrated (e.g. Woolley, 1941; Mizuki *et al.*, 1992).

Paragonimiasis is primarily a zoonosis. The extent to which eggs from infected humans contribute to the continuation of the cycle is unclear. At least in some parts of Japan, however, humans are the principal mammalian host (Yokogawa, 1965). Reductions in prevalence and intensity of infections among cats, dogs and crustaceans in parts of China following public education and mass chemotherapy (World Health Organization, 1995) suggest that humans are also principal hosts there. The usual hosts of *P. westermani* in Asia are a range of carnivores, especially felids and canids. Hosts of *P. miyazakii* in Japan are principally wild boars and mustelids (Sano *et al.*, 1978). In the Americas, marsupials are important hosts of *P. mexicanus* (Table I of Sogandares-Bernal and Seed, 1973) and in Africa, viverrids and herpestids (Voelker *et al.*, 1975) host local species. With recent increases in human populations and displacement of wildlife, it seems likely that domestic animals have become major reservoir hosts for *Paragonimus* species in some areas. In Liberia (Sachs and Cumberlidge, 1990) and Vietnam (Kino *et al.*, 1995), domestic dogs might play this role. Domestic animals presumably function as reservoir hosts for *P. skrjabini*, which cannot mature in humans (and see Chung and Tsao, 1962a).

Shortages of food, caused by wars and economic difficulties, may cause people to supplement their diet with 'wild' food. This situation was blamed

for the great increase in paragonimiasis in Nigeria after the civil war there (Nwokolo, 1972) and again more recently during a period of economic austerity (Udonsi, 1987).

Few large-scale control programmes have been instituted against paragonimiasis. Zhong *et al.* (1981), Xu (1991) and Bi and Zhang (1996) have described control measures in China. Currently, paragonimiasis is not a notifiable disease in China and it is accorded low priority by provincial institutes for parasitic disease control. In Korea, laws were passed as early as the 1920s prohibiting collection and transportation of freshwater crustaceans (Seo, 1984). During the Japanese occupation and the Second World War, similar regulations were enforced in both Korea and Taiwan, but subsequently lapsed (Sadun and Buck, 1960). Infected crabs are still sold in markets in Korea (Cho *et al.*, 1991).

Reductions in incidence of paragonimiasis have been noted in many places. This has been a side-effect of social and economic changes in endemic countries, with increased industrialization, land clearance and environmental pollution (Choi, 1990). The effects of this have been particularly striking in Korea (Cho *et al.*, 1997). Reduction of reservoir host numbers through hunting and the effects of deforestation have probably reduced the incidence of paragonimiasis in parts of Ecuador (Vieira *et al.*, 1992) and Cameroon (Ollivier *et al.*, 1995). Extensive clearing of rain forest in Mato Grosso, Brazil, the type locality for *P. rudis*, was a possible reason for the failure to rediscover the species there (Voelker *et al.*, 1981).

Concerns have been expressed that introduction of intermediate hosts of *Paragonimus* species might spread the parasites to new regions. The crab host of triploid *P. westermani*, *Eriocheir* sp., has now spread in Europe (Freund, 1938). Fears have been expressed that the snails *Melanoides tuberculatus* and *Tarebia granifera*, introduced to the Caribbean area as biological control agents of schistosome snail hosts, might act locally as hosts for *Paragonimus* species (McCullough and Malek, 1984; Noblet and Damian, 1991). However, neither of these snail species is confirmed as a host for any *Paragonimus* species (Davis *et al.*, 1994 and personal communication) despite statements to the contrary in the literature.

## 7. BIOCHEMISTRY AND PHYSIOLOGY

Interest in the functioning of *Paragonimus* species has centred on their interactions with the immune system of the host (see Section 6.4), aspects of their metabolism, and ultrastructure relating to nutrition and development. Although less work has been done than on schistosomes and fasciolids, *Paragonimus* species are suitable subjects for investigation

because of their relatively large size and the ease with which they can be grown to maturity in laboratory animals. *Paragonimus ohirai*, which matures in rats in only 4 weeks, is a common model organism (see Section 8). *P. miyazakii* can also be raised in rats, but takes somewhat longer. *Paragonimus westermani*, although a human pathogen, requires longer periods in cats or dogs to mature.

Nucleic acid metabolism has been poorly studied. Pyrimidines can be synthesized *de novo* by *P. ohirai* (Kobayashi *et al.*, 1984). There appear to be no studies dealing with protein or lipid metabolism. Carbohydrate catabolism the principal source of energy in adult trematodes (Smyth and Halton, 1983), is better understood. Trematodes may experience very different environmental oxygen tensions at different life-cycle stages. In some species, a switch from aerobic metabolism in the larval stages to anaerobic in the adult has been documented (Smyth and Halton, 1983). The oxygen tensions to which migrating juvenile and encysted adults of *Paragonimus* species might be exposed are unknown. Unlike adults of *Fasciola hepatica*, adult *P. westermani* have mitochondria capable of aerobic and anaerobic carbohydrate catabolism (Takamiya *et al.*, 1994). Fujino *et al.* (1995) noted that parenchymal cells of *P. ohirai* (juveniles and adults) could be divided into two categories according to the morphology and cytochrome *c* oxidase activity of their mitochondria. Mitochondria in the tegument showed intense cytochrome *c* oxidase activity and were clearly involved in aerobic respiration.

A combination of biochemical and ultrastructural studies has assisted understanding of the means of nutrition and food uptake by *Paragonimus* species. The gastrodermis of a newly excysted metacercaria of *P. ohirai* is a layer of flattened epithelial cells with short, simple, luminal cytoplasmic projections (Fujino and Ishii, 1990). Within 15 days of entry into the definitive host, the gut epithelium has effectively reached its adult form. During this process, epithelial cells increase in size and cytoplasmic projections develop into extensive lamellae protruding into the gut lumen (Fujino *et al.*, 1987; Fujino and Ishii, 1990). Protein synthesis is intense in juvenile worms (Fujino and Ishii, 1990). Enzymes such as acid phosphatase (Fujino and Ishii, 1990) and glycosidases (Fujino *et al.*, 1983) can be detected in the gastrodermis within a few days. Other enzymes detected are proteases in the lumen, which might be associated with migration through the host in juveniles and with immune interactions with the host (see Section 6.4). Adult gastrodermal cells cycle between secretory and absorptive phases and phagocytosis occurs while a cell is in the absorptive phase (Fujino and Ishii, 1988; Fujino, 1994). The lamellar surfaces carry arrays of rods of glycosaminoglycans, which may adsorb food particles (Fujino *et al.*, 1987). These are absent from metacercariae but develop as the worm matures (Fujino and Ishii, 1990). These rods appear to replace the more

conventional glycocalyx found in the guts of other trematodes. Excretion of lipid droplets into the gut lumen has been observed (Fujino and Ishii, 1988). Regurgitated gut contents void such excretory products from the worm and also gut-associated antigens into the host (see Section 6.4). No studies have discussed nutrient take-up across the tegument in *Paragonimus* species.

Ultrastructural studies of the excretory system and bladder of *P. ohirai* (Orido, 1990 and references therein) have demonstrated that waste material accumulated during the metacercarial phase is passed into the bladder and excreted only after entry into the definitive host. The epithelium of the excretory bladder is metabolically very active for the first 2 weeks in the final host, but is thin and inactive in the adult.

Other recent ultrastructural studies on *P. ohirai* have dealt with the development and structure of parts of the female reproductive system (Orido *et al.*, 1994 and references therein) and with aspects of gametogenesis and spermatozoon structure (Orido, 1988a,b; Orido and Hata, 1988).

*In vitro* cultivation has been useful for many physiological studies on trematodes (Smyth and Halton, 1983). Specimens of *Paragonimus* have often been held *in vitro* for short periods of time. However, few workers have attempted to cultivate worms from metacercaria to adult. Kannangara (1974) and Hata *et al.* (1987b) have reported efforts to do this. In the latter paper, growth of *P. ohirai* to maturity was reported but the eggs produced were abnormal.

## 8. *PARAGONIMUS* SPECIES AS MODEL ORGANISMS

*Paragonimus* species and their experimental hosts have been used in various ways as model organisms. Non-human species have been used as models for those causing human disease. They have also been used as models for host–trematode interactions in general.

A common theme has been the search for suitable host–parasite systems to investigate experimentally the pathological effects of worms in an easily managed host (examples include: Nishimura, 1966, *P. ohirai* in rats, cats and dogs; Hoover and Dubey, 1978 and Weina and England, 1990, *P. kellicotti* in cats; Weina and Burns, 1992, *P. kellicotti* in hamsters; Rácz *et al.*, 1977, Volkmer, 1977 and Oelerich, 1977, African species in monkeys; Im *et al.*, 1993, *P. westermani* in cats). These studies are often associated with assessment of the effects of drugs on worms (e.g. Weber, 1987 and Weber *et al.*, 1988, *P. uterobilateralis* in cotton rats; Yokogawa, 1984, Lee *et al.*, 1989b and Fujino, 1994, *P. ohirai* in rats; Hashiguchi and Okamura, 1988, 1989, *P. ohirai* and *P. miyazakii* in rats; Zheng and Zhang, 1988, *P. skrjabini* in dogs; Jiang and Xia, 1992, *P. heterotremus* in rats).

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Note: Methods for rendering Chinese names in Roman script have varied through the years. For each Chinese paper, we have used the romanization method current at the time of its publication.

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# Immunology and Biochemistry of *Hymenolepis diminuta*

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## ABSTRACT

*This review is an account of modern research into the immunology and biochemistry of the rat tapeworm,* Hymenolepis diminuta. *The first half of the review is devoted to the immunological responses of the host to the parasite. It describes the specific responses that occur when the host is exposed to a primary infection, and the changes that occur when further infections are superimposed on the primary one. The aquisition of immunity to the tapeworm and its persistence in the absence of the infection are also discussed, as well as the non-specific responses of the host to the parasite. The second half of the review is concerned with biochemistry, summarizing the early biochemical work that has been carried out on the tapeworm and describing the metabolic pathways now thought to be characteristic of the parasite. What little information that exists on intermediary metabolism in eggs and larvae is summarized here. Much of this section is concerned with the role of mitochondria in* H. diminuta, *especially the control of the critical branchpoint (PK/PEPCK), which partitions carbon into either the cytosol or the mitochondrion. The role of 5-hydroxytryptamine in controlling both worm behaviour and metabolism is discussed, followed by a brief look at some other effectors that may prove in the future to have great significance in regulating the parasite. Finally, there is a detailed consideration of strain variation within* H. diminuta *and of the impact on the tapeworm of components of the immune system, formerly described as the 'crowding effect'. The review concludes with a brief discussion of evolutionary aspects of the rat–tapeworm relationship and a comprehensive bibliography.*

## 1. INTRODUCTION

The rat tapeworm, *Hymenolepis diminuta* is a denizen of almost every parasitology laboratory in the world. Indeed, it has been promoted in the *Journal of Biological Education* as a suitable organism for teaching in schools (Arme, 1983). *Hymenolepis diminuta* has a relatively simple life cycle. The adult helminth lives in the small intestine of the rat; embryonated eggs are passed in the faeces and ingested by an insect intermediary host. A variety of beetles can so act but the most commonly encountered ones are the mealworm, *Tenebrio molitor*, or the flour beetle, *Tribolium confusum*. After the insect has ingested the egg, an oncosphere (also called a hexacanth because it has six hooks) is liberated from the egg. This penetrates the midgut and enters the haemocoel where, at 26°C, it develops after 10 days into a cysticercoid. At this point it is infective: if the insect is eaten by a rat, the cysticercoid is activated and becomes mature, with mature male and female reproductive organs, in the small intestine in 2 or 3 weeks.

In the laboratory, its ease of maintenance makes it a most accessible helminth and it is possible, without too much effort, to raise it in quantities exceeding tens of grams. On the face of it this is a simple tale; it should be

possible to breed the parasite to establish a uniform cultivar and studies of the biology, immunology and biochemistry should be straightforward and uncontentious.

The literature tells a very different story. Over the last 50 years our understanding of immunity to *H. diminuta* has progressed from a stage at which it was even doubted that the tapeworm was immunogenic to a point where we are beginning to understand how much we do not know about this complex and subtle host–parasite relationship. Our understanding of the biochemistry of this parasite is equally perplexing. We have left the days far behind when it was felt that to describe the metabolism of one helminth was to describe the metabolism of all of them and we are acutely aware that there are large species and generic differences between helminths. However, the *Hymenolepis*–rat association has much more than that to teach us. Even something as straightforward as incubating the adult worm in a standardized nutrient medium and analysing the end-products of metabolism so formed, involving a technology that was certainly available 50 years ago, produced markedly different results in different laboratories. Could so many research workers have got it wrong?

The answer is, of course, no; they were reliably reporting what they observed. It was the parasite, behaving in different ways in different places, that was causing the confusion. There is an urban myth in parasitological circles (it may be true, but we have not seen the evidence) about 9 o'clock worms and 5 o'clock worms. Two laboratories were arguing about the characteristics of a particular cultivated parasite. In the end, the differences between them turned out to be due to a simple difference in the method of cultivation. In one laboratory, it was the practice to collect eggs from rat faeces in the morning; in the other, evening collection was the rule. Diurnal variation in the quality of the rat faeces exposed the eggs to different selection processes. The consequence was that the two laboratories were effectively working with different cultivars.

A rather more sophisticated version of this is frequently encountered in the study of the biochemistry of *H. diminuta*. It may vary during development, and during growth and maturation. It may vary depending on the definitive host and on the intermediate host. It may vary depending on which cultivar is used and on which laboratory maintained and supplied the parasite. And it may vary depending on the physiological and immunological status of the host.

Adult tapeworms living in the intestinal lumen were considered, even quite recently, to be commensals and not real parasites (Sprent, 1962, 1963; Insler and Roberts, 1976). Even when they were granted parasite status, tapeworms were not considered capable of stimulating immune reactions in the host (Heyneman, 1962). It is now accepted, however, that intestinal worms, including tapeworms, secrete or excrete substances that may be

antigenic to the host, or that the parasite surface itself may stimulate host immune reactions of one kind or another. Although low infections with tapeworms are not expelled from the so-called normal host, they are usually affected by host immune mechanisms in one way or another. Most of these effects have been ascribed to 'crowding effects', i.e. intraspecific interactions between the tapeworms. This is discussed in detail in Section 4.

Tapeworms, however, are probably affected more by the host's immune reactions than by other cohabiting tapeworms. Far from being a 'simple' tapeworm, *H. diminuta* is a very complex organism indeed. Much of recent research into the biology of this tapeworm has been devoted to sorting out these complexities.

## 2. THE NATURE OF IMMUNITY TO *HYMENOLEPIS DIMINUTA* IN THE RAT

Parasites and hosts evolve together; parasites become adapted to the host's defensive response (the immune response) as hosts become adapted to the deleterious effects of the parasite (pathology). During this coevolution, successful parasites develop defences against the immune reponse of the host. These include avoidance responses, immunosuppression of the host or becoming immunologically invisible to the host. Often tapeworms avoid stimulating the host's intestinal inflammatory reactions, which would otherwise destrobilate and expel the worm. However, if so many parasites become established that a host is at risk of being killed by the parasite, then the host may have the capacity to reduce the number of parasites by one or more immunological reactions.

### 2.1. Primary Infections

In mice infected with *H. diminuta* it has been shown that host immune reactions are mounted against even a single tapeworm. A single worm grows until about day 9–12 when it destrobilates and is expelled (Hopkins *et al.*, 1972a,b). Thymus-deficient nude mice, on the other hand, are unable to reject a single worm (Isaak *et al.*, 1975; Bland, 1976). Although a rat is unable to reject a single tapeworm, it is quite likely that the worm is affected by host immune reactions, just as the growth of a single worm in a normal mouse is affected by the host immune reactions. Preliminary results (Andreassen, unpublished data) have shown that *H. diminuta* also grows better in nude rats than in normal rats of the Hooded Rowett strain.

With an increasing number of worms, the size of the adult tapeworms, measured in length and/or weight, decreases (Chandler, 1939; Hager, 1941; Read, 1951; Roberts, 1961; Hasselberger and Andreassen 1975; Pike and Chappell, 1981; Keymer *et al.*, 1983; Quinnel, 1988). Egg production per worm decreases (Hager, 1941; Hesselberg and Andreassen, 1975; Quinnel, 1988) and their mean position in the small intestine (about 15–20% from pylorus in infections with one to five worms) changes to a more posterior position (35–40% from pylorus in infections with 10–20 worms) and they occupy more of the length of the small intestine than in infections with one to five worms (Hesselberg and Andreassen 1975). The cause of these reactions between adult tapeworms is probably a combination of intraspecific competition for space and food, secretion by the worms of so-called 'crowding substances' and host immune reactions (see Section 4). It is interesting to note that Goodchild and Harrison (1961) found that, during the first 3 days of development in a rat, worms show no significant differences in size whether the rat had 10 or 100 worms, indicating that at least during this time no crowding effects are taking place.

Although immunity to intestinal cestodes was demonstrated by Seddon in sheep infected with *Moniezia expansa* as early a 1931, immunologically dependent expulsion was not detected in rats infected with *H. diminuta*, and Hager (1941) felt justified in writing that 'spontaneous loss of the tapeworm *H. diminuta* does not occur readily from the rat'. However, in 1968, Roberts and Mong demonstrated that rats infected with 50 cysticercoids and monitored for 150 days often showed a partial spontaneous loss of the infection. However, they did not associate this phenomenon with host immune reactions against the tapeworms.

Read (1951) calculated a 'relative surface area' (using data from 1964 worms from infections with 1–100 worms, all 38–44 days old and from male rats weighing about 200 g, employing the formula weight$^{0.67}$/weight) and showed it to be nearly constant (increasing only a little) in parasite burdens of up to 8–10 worms, while with more than 10 worms the relative surface area increased in proportion to the logarithm of the number of worms. Read speculated as to what the limiting factor might be. He concluded that there was no reason to believe that it was due to insufficient carbohydrates, proteins or fat in the host diet, and stated: 'the limiting factor is probably not a food substance'. Read (1951) suggested that oxygen could be the limiting factor, although the metabolism of tapeworms is predominantly anaerobic: an increasing number of worms would result in less contact of the individual worm with the mucosal surface of the gut, where the oxygen tension is highest.

However, it is interesting to note that, in rats with up to 10 *H. diminuta*, between 90 and 100% of the worms established and were present 4, 8 or 14 weeks or even 8 months after infection (Harris and Turton, 1973; Hesselberg

and Andreassen 1975; Pike and Chappell, 1981; Quinnel, 1988). However, an average of 10 normal worms was found 8 weeks after infection of rats with 12, 20, 40, 50, 100 or 200 cysticercoids (Hesselberg and Andreassen, 1975). The same reduction to about 10 worms per rat was also found by Harris and Turton (1973) with an initial infection of 25 cysticercoids, Chappell and Pike (1976) with 30, and Pike and Chappell (1981) with 50 cysticercoids. Furthermore, Pike and Chappell (1981) found that infections of 50 worms established in newly weaned rats, but around day 28 and thereafter worms were lost from the rats and less than 10 worms were present 5 months after infection. In mature rats, the same expulsion occurred approximately 3 weeks earlier. Quinnel (1988), using 80 cysticercoids in 1- and 5-month-old rats, showed that 4 weeks after infection a mean of seven to eight worms (about 9% recovery) were found in the older rats compared with about 83% in the 1-month-old rats. This indicated that increased host age, and presumably increased immune responsiveness, was the cause of the decrease in worm recovery.

While Chappell and Pike (1976) concluded that their results would fit either a competitive or an immunological model, Keymer *et al.* (1983) stated that their results indicated that the crowding effect cannot be explained simply in terms of inter-worm competition for carbohydrates. Quinnell (1988) suggested that immunological mechanisms may have an important role in the crowding effect.

At high infection doses (40–100 worms), a number of destrobilated worms were found, showing that destrobilation takes place at these infection doses and probably also at infection doses of 12 and 20 worms. The so-called 'limiting factor' for worm infections above 10 worms could therefore be the host immune response, which in the rats used by Hesselberg and Andreassen (1975) was strong and caused destrobilation, while in the rats used by Read (1951) was weaker and only caused inhibition of growth. In the latter case, it was perhaps due to the use of another strain of rat, or younger rats, or rats having immunosuppressive intestinal protozoan infections.

Ishih *et al.* (1992) showed clear strain-dependent variation in six inbred and one outbred strain of rats in their susceptibility to *H. diminuta*. It is therefore likely that the reason why infections with 50 worms in Sprague–Dawley rats are characterized both by progressive loss and decrease in individual worm weight compared with little worm loss and no worm growth changes in Hooded Lister rats (Chappell and Pike, 1977) is because of strain differences in immune responses to *H. diminuta*. However, different strains of *H. diminuta* have also been used and they too have different physiological characteristics. This may in turn influence the host immune response to the tapeworm; see, for example, Ovington and Bryant (1981), Mettrick and Rahman (1984), Bryant and Flockhart (1986), Kino and Kennedy (1987) and Bennet *et al.* (1990).

That host reactions against intestinal tapeworms are under the control of host genes has been nicely demonstrated in white-footed deer mice (*Peromyscus maniculatus*) infected with *H. citelli*. Experimentally, all deer mice become infected with *H. citelli*, but those mice with a single or double autosomal dominant gene (*R*) destrobilate and expel all their worms. Primary infections survive in mice with a double recessive gene combination (*rr*) and these hosts are incapable of resisting reinfection, and secondary infections can be superimposed upon initial ones (Wassom *et al.*, 1973, 1974, 1986). As expected, host genes have also been shown to have an impact on other helminths (Wakelin and Apanius, 1997) but more information about tapeworm–host relationships is necessary, and little is known about the rat–*H. diminuta* relationship.

## 2.2. Superimposed Infections

Superimposed infections in rats with *H. diminuta* were, except for an attempt by Palais (1934) who failed to produce superinfections, first used by Chandler (1939). Chandler infected rats with 1, 2, 4, 10, 20 or 40 cysticercoids and 4 weeks later superimposed them with a further 10 cysticercoids. At autopsy 10 days after the superimposed infection, a decreasing number and size of superimposed worms were found with an increasing number of primary worms present. When an average of 36 primary worms were present no superimposed worms were found. As Chandler (1939) could not demonstrate secondary (acquired) immunity to *H. diminuta* in rats, he concluded that the effect of primary infection on a superimposed infection ('premunition') in tapeworm infections is due to crowding rather than to immunity in the ordinary sense. Although Roberts and Mong (1968) could not confirm Chandler's results directly, they also found a negative correlation between the number of primary worms and secondary worm establishment and calculated that 58 and 113 primary worms, from two separate experiments respectively, were required to give complete protection against superimposed worms. Furthermore, they also found that weights of individual secondary worms were inversely proportional to the numbers of primary worms, although they suggested avoidance of the term 'premunition' with reference to lumen-dwelling cestodes until clear experimental demonstration had been reported.

Resistance to superimposed infections caused by host immune reactions without the host being able to kill an existing infection was termed 'concomitant immunity' by Smithers and Terry (1969) to describe the immune status of rhesus monkeys resistant to a superimposed infection with *Schistosoma mansoni* while maintaining an existing infection.

Since Roberts and Mong (1968) published the results of their experiments on superimposed infections, only Hopkins and Andreassen (unpublished results) have carried out such infections with *H. diminuta* in rats. Their results indicated that concomitant immunity may exist in cestode–host relationships. Eight-week-old outbred Wistar rats were infected with five cysticercoids, superimposed with 50 cysticercoids 4 weeks later and autopsied 7 days thereafter. At autopsy, the total dry weight of the five primary worms per rat varied from about 500 to 1000 mg in the first experiment and from about 150 to 900 mg in the second experiment (carried out 3 years later). When correlating the biomass of superimposed worms with the biomass of primary worms in individual rats, Hopkins and Andreassen found that the greater the total dry weight of the primary worms, the greater the total dry weight of the superimposed worms. In both experiments a positive, linear, logarithmic correlation existed. It seems clear that these results cannot be explained by intraspecific competition because larger primary worms would then result in smaller secondary worms because of competition between the two types of worms. However, the results can be explained in immunological terms, in that the rats were presumably showing differences in their immune responses. High-responder rats not only give rise to small primary worms but, naturally, also to small superimposed worms, while low responders give rise to both heavier primary and superimposed worms. The probable biochemical basis of this phenomenon is discussed at length in Section 4.

Another experiment, with 6–8-month-old Wistar rats infected in the same way but using 100 cysticercoids in the superimposed infection, revealed that the superimposed infection was destrobilated and reduced in number. This indicated a higher immune response in 6–8-month-old rats compared with 8-week-old rats and/or a stronger response to the higher doses in the superimposed infection (Andreassen, unpublished results).

### 2.3. Non-specific Host Responses

It has long been known that in rats concurrently infected with 10 acanthocephalan cystacanths of *Moniliformis moniliformis* (previously *M. dubius*) and five cysticercoids of *H. diminuta*, the tapeworms are not only restricted in growth but also have a more posterior position than in a single infection with five *H. diminuta* (Holmes, 1961). Furthermore, it has been shown that an infection with 100 *M. moniliformis*, which causes an increased leakage of plasma into the intestinal lumen of rats and is expelled 4–8 weeks after infection, causes destrobilation of a concurrent infection with five *H. diminuta* (Andreassen *et al.*, 1978a). Intestinal nematodes such as *Trichinella spiralis*, which cause more severe inflammatory reactions in the

small intestine than acanthocephalans, have been shown to destrobilate adult *H. diminuta* and eventually bring about expulsion of the tapeworm together with the nematodes, If cysticercoids were given at the time of expulsion of the nematode, they did not establish (Christie *et al.*, 1979; Silver *et al.*, 1980).

If rats infected with adult *H. diminuta* are starved, the tapeworms will destrobilate. Some worms will be expelled unless the rats start normal feeding again or are fed intravenously. In this case the destrobilated tapeworms will grow again (Castro *et al.*, 1976; Cornford, 1977). Under natural conditions where the host hibernates, as in ground squirrels of the genus *Citellus*, it has been shown that the tapeworm *H. citelli* will destrobilate; some will be expelled but others will survive the hibernation (Ford, 1972). Thereafter, if anorexia, which is known to occur in some hosts infected with protozoans or helminths (including a bird tapeworm, according to Symons, 1985), also occurs in mammals infected with tapeworms, tapeworms would be destrobilated and some would be expelled. At present, we do not know what causes destrobilation *in vivo*, but an obvious possibility is substances present in the plasma leaking into the intestinal lumen in the above-mentioned cases.

One such system is the complement system, which has been shown to cause lysis of bacteria, protozoans and helminths (see, for example, Jokiranta *et al.*, 1995). Experiments *in vitro* have shown that newly excysted cysticercoids of *H. diminuta* were completely lysed within 1–2 min by complement in 50% fresh normal rat serum (Bøgh *et al.*, 1986), but concentrations of rat serum as little at 6–8% were able to lyse newly excysted cysticercoids (Christensen *et al*, 1986). All 4-day-old worms of *H. diminuta* were shown to be either lysed completely or to be lysed from the strobila up to the neck region when placed in 50% fresh normal rat serum. In the case of 10-day-old worms, only 1 of 11 worms had its scolex lysed and the other 10 worms survived as destrobilated worms with an intact scolex and neck region (Bøgh *et al.*, 1986). The region between the lysed strobila and the nearly intact neck region was shown to be morphologically distinct, comprised of two constrictions between the neck region and the lysed strobila. These constrictions were also found on worms destrobilated *in vivo* in the small intestine of both rats and mice (Malmberg *et al.*, 1985; Christensen *et al.*, 1986; Robinson *et al.*, 1987).

Since concentrations of complement as low as 6–8% are able to completely lyse newly excysted worms *in vitro*, even higher concentrations may reasonably be assumed to occur when rats are starved or when plasma leakage, associated with inflammatory reactions, has taken place in the small intestine as a result of a helminth infection. The complement system may thus be able to destrobilate and kill *H. diminuta in vivo*. However, other mechanisms may also be able to cause damage to the tapeworm

tegument and cause destrobilation and/or expulsion, such as leakage of C-reactive plasma proteins (Taylor and Hoole, 1997), mucus from goblet cells, or major basic protein and cationic protein from eosinophils. One or more of these or other mechanisms may be responsible for the expulsion of *H. diminuta* from DBA/2 mice, deficient in the complement component C5, which is needed for the *in vitro* lysis of a tapeworm. Furthermore, tapeworms have probably developed one or more mechanisms to resist complement-mediated lysis, such as anticomplement factors, prevention of complement activation and perhaps, restricted membrane fluidity, as suggested by Taylor *et al.* (1997).

## 2.4. Acquired Immunity and Immunological Memory

Although immunity to adult tapeworm infections of *Moniezia expansa* was demonstrated in sheep by Seddon as early as 1931, acquired immunity to *H. diminuta* in rats could not be demonstrated by Chandler (1939), and it was not until the 1960s that immunity was demonstrated in mice against *H. nana* and *H. microstoma* (Heyneman, 1962; Weinmann, 1966; Tan and Jones, 1967). The real breakthrough came with the thorough studies by Hopkins *et al.* (1972a,b) of mice infected with *H. diminuta*.

Acquired immunity to *H. diminuta* in rats was first demonstrated by Andreassen *et al.* (1974) who showed that SPF Wistar rats primarily infected with 100 cysticercoids, treated with an anthelmintic after 6 weeks and reinfected with another 100 cysticercoids at week 7 after infection exhibited an earlier and increased expulsion of the secondary infection.

Immunological memory following a heavy (50 worm) infection was demonstrated by Andreassen and Hopkins (1980), who showed that the growth of worms in a secondary infection, given 8, 23 or 41 days after the primary infection had been removed by anthelmintic treatment, was 0.5, 3 and 25% respectively of that in the control worms. Thus, there is a strong initial resistance to a challenge infection, but this resistance weakens 50-fold between days 8 and 41 after infection. Even primary infections of five worms, which would have survived indefinitely if not expelled by anthelmintics, depressed the growth of secondary infections given 8 days later by over 80%, but did not cause destrobilation or expulsion of secondary infections. Alghali (1986) also showed that secondary infections with six *H. diminuta* in rats (after removal of a 22-day-old 50-worm infection) were significantly inhibited in growth after 8 and 14 days. In strongly sensitized rats, worm growth was inhibited severely (or the worms destrobilated), partial worm expulsion occurred and the remaining worms migrated to a more anterior position in the intestine (Hindsbo *et al.*, 1975). The biolo-

gical importance of the responses appears to be that it prevents the rat from accumulating a heavy load of worms.

While a primary infection of *H. diminuta* in mice results in an at least 8 months of strong secondary response (Hopkins, 1982), the immunological memory in rats infected with *H. diminuta* has been shown to be rather short-lived (Hopkins and Andreassen, 1991). They showed that the biomass of 8-day-old worms in secondary infections, administered to rats 3–10 days after a primary infection was chemotherapeutically expelled, was 70–90% less and the worms more posteriorly distributed than in naive control rats. The strong depressive effect on growth waned rapidly over 2–5 weeks; even in rats not challenged until 17 months after infection, worm growth was depressed by 30%. The extent to which growth was depressed in a secondary infection was independent of the number of worms in the challenge infection but increased with the number of worms in the immunizing infection up to four to eight worms, while further increase up to 64 worms had little effect.

## 2.5. Host Immune Responses

### 2.5.1. *Natural Immunodepression*

In mammals it has been shown that during pregnancy, and especially during lactation the female host is immunodepressed (Lloyd, 1983) and that the host's immune reactions against intestinal helminths are therefore weakened, resulting in failure to expel intestinal nematodes (see, for example, Selby and Wakelin, 1975). Unpublished results by Andreassen and Heilskov showed that an infection with 100 *H. diminuta* in normal non-lactating Wistar rats was partly destrobilated and expelled during the second and third week after infection, leaving 34 worms of which 10 were destrobilated at the third week of infection. In lactating rats, 94 worms survived as normal worms, reaching a total dry weight of 1959 ± 539 mg dry weight of worms per rat, while normal non-lactating rats had 263 ± 113 mg dry weight of worms per rat. Furthermore, 1 week after removal of the pups from the infected lactating mothers, i.e. 28 days after infection, destrobilation had taken place and an average of 69 scolices were found, of which 23 were destrobilated. The total dry weight of worms was reduced by about 80 mg, to 390 ± 125 mg. It has also been shown in mice that *H. diminuta* not only grew faster but was also expelled 4–5 days later in lactating mice compared with normal mice (Hopkins, 1980).

Young animals do not have a fully developed immune system and therefore are unable to react immunologically as well as do adult animals. As a consequence, young animals have been shown not to expel intestinal helminths as well as adult animals, e.g. *H. diminuta* in 4-week-old mice (Befus and Featherston, 1974). It has also been shown that newly weaned

Sprague-Dawley rats do not expel an infection with 50 *H. diminuta* as quickly as do mature rats (Pike and Chappell, 1981) and 1-month-old Wistar rats do not expel an 80-worm infection 4 weeks after infection; in 5-month-old rats, the infection is reduced to only seven to eight worms (Quinnel, 1988).

While some concurrent infections cause destrobilation and expulsion of tapeworms (see above), others cause immunosuppression of the host. This results in a chronic tapeworm infection in hosts that normally expel the tapeworm infection spontaneously. In mice, the nematode *Heligmosomoides polygyrus* (previously *Nematospiroides dubius*) causes impairment of the efferent arm of the response to tapeworms which as a result grow larger (*H. microstoma*, Courtney and Forrester, 1973) or survives longer (*H. diminuta*, Hopkins, 1980; *H. citelli*, Alghali *et al.*, 1985). A number of protozoan infections also cause immunosuppression in their hosts and therefore may inhibit the destrobilation and expulsion of tapeworms. Delayed expulsion of *H. diminuta* has in fact been demonstrated in mice concurrently infected with *Trypanosoma cruzi* (Machnicka and Choromanski, 1980) and *T. brucei* (Fagbemi and Christensen, 1984). Immunosuppression of rats infected with other parasites may also result in larger growth and/or longer survival of *H. diminuta*, although experimental evidence is lacking.

### 2.5.2. *Artificial Immunodepression*

Immunologically dependent expulsion of intestinal helminths has, in a number of experiments, been shown to be inhibited by different treatments with immunodepressive agents. For *H. diminuta*, inhibition has been shown with treatments of corticosteroids (Andreassen *et al.*, 1974 in rats; Hopkins and Stallard, 1976 in mice), methotrexate (Hopkins *et al.*, 1972b in mice), cyclophosphamide (Choromanski, 1980, 1983 in mice) and antithymocyte serum (Hopkins *et al.*, 1972b in mice; Hindsbo *et al.*, 1982 in rats).

In rats infected with 100 *H. diminuta* the normal destrobilation and expulsion has been shown to be completely inhibited by corticosteroid treatments, resulting in the recovery of an average of 99 worms 6 weeks after infection (Hindsbo *et al.*, 1975). Not only had the worms survived but they had grown in the rat's small intestine to a total length of about 20 m. In rats treated with antithymocyte serum and infected with 100 *H. diminuta* destrobilation and expulsion of the tapeworm was delayed (Hindsbo *et al.*, 1982).

### 2.5.3. *Immunodeficiency*

Thymus-deficient *nu/nu* (nude) mice and rats are naturally deficient in T-cells and not able to mount the same immune responses, or responses to the

same degree, as their normal counterparts, the +/*nu* or +/+ animals. One would expect, therefore, that immune-dependent mechanisms against intestinal tapeworms would be delayed or inhibited in nude hosts, and this is exactly what has been found. First it was demonstrated that nude mice infected with one or three *H. diminuta* did not destrobilate or expel their worms for at least 33 days after infection, whereas their thymic littermates did (Isaak *et al.*, 1975; Bland, 1976). Furthermore, nude mice were shown to be able to keep one or two *H. diminuta* for as long as they lived or at least 136 days (Andreassen *et al.*, 1982). However, if nude mice are infected with five or more worms, the worms are destrobilated and expelled, although the reaction is delayed compared with that in normal mice (Andreassen *et al.*, 1978, 1982). Experiments with *H. diminuta* in nude rats have also been carried out, and it has been shown that although destrobilation and expulsion does occur in 100-worm infections in nude Hooded Rowett rats, they are delayed compared with heterozygous normal rats (Andreassen, 1980). However, as pointed out in the review by Ito and Smyth (1987), further work is needed in this area.

From the above-mentioned results it seems justified to conclude that destrobilation and expulsion of *H. diminuta*, whether from mice or rats, is under the influence of T-cells, which in one way or another accelerate destrobilation and expulsion. Proof of this comes from experiments where injection of T-cells into naive nude mice was shown to cause elimination of the tapeworm (Isaak *et al.*, 1975). However, since *H. diminuta* can be destrobilated and expelled from high infections in both nude mice and rats, T-cell-independent mechanisms may be operating when the antigenic stimulus is sufficiently high, or the necessary low number of T-cells may be stimulated by a high concentration of antigens.

## 2.6. Specific Immune Responses

### 2.6.1. *Humoral Responses*

Antibody responses against adult tapeworms have been studied mainly in mice infected with *H. diminuta* or *H. microstoma*. All the main immunoglobulin classes (IgA, IgE, IgG and IgM) have been demonstrated in serum (Moss, 1971; Murray *et al.*, 1984; Dhont *et al.*, 1988; Ito *et al.*, 1989). Specific antibodies against tapeworms have been found in the intestinal lumen of mice infected with *H. microstoma* by Murray *et al.*, (1984), who suggested that these mucosal antibody responses were responsible for the protective responses. In mice infected with *H. diminuta*, Dhont *et al.*, (1988) have found both IgA and IgE. Furthermore, Van der Vorst *et al.*

(1988) found IgE and IgA bound to intestinal eosinophils in mice infected with *H. diminuta*.

The first to demonstrate serum antibodies in rats infected with *H. diminuta* were Coleman *et al.* (1968), who showed that these antibodies cross-reacted with *H. nana*. Of the antibody classes found in rats infected with *H. diminuta*, IgE was the first to be demonstrated (Harris and Turton, 1973). They found IgE antibodies against a chronic infection with five *H. diminuta* in Sprague–Dawley rats, with titres similar to or higher than antibodies produced against an infection with 25 *H. diminuta*. However, a 25-worm infection is gradually reduced to a mean of 10–11 worms 14 weeks after infection, indicating that some rats were unable to expel all or nearly all tapeworms, while rats infected with five cysticercoids harboured four or five worms from weeks 1 to 14 after infection, irrespective of the IgE titres.

With regard to IgE, it is interesting to note that in some strains of rats where doses of *H. diminuta* are destrobilated and expelled (e.g. the Wistar strain) no specific IgE could be demonstrated, while in Hooded Lister rats, where *H. diminuta* is expelled more slowly (Andreassen, 1991), the presence of IgE could be demonstrated. Chappell and Pike (1977) found that the response of Hooded Lister rats against *H. diminuta* is much lower that that of Sprague-Dawley rats. In chronic *H. microstoma* infections in mice, a high titre of IgE was produced (Moss, 1971). This raises the interesting question that IgE may be beneficial for some tapeworms. This was suggested for other helminths by Pritchard (1993). On the other hand, Watanabe *et al.* (1994) found an increase in worm burden in IgE-deficient mice infected with *H. nana*, although the infection was direct via eggs.

Although antibodies have been shown not to be essential for expulsion of *Raillietina cesticillus* in bursa-deficient chickens (Elowni and Hopkins, 1981), this does not exclude the possibility that antibodies may play a role in rejection of other tapeworms or even that some antibodies play a role in retarding expulsion. Further studies designed to detect other antibodies in rats infected with *H. diminuta* are urgently needed, in both chronic and acute infections, to throw light on the role(s) of antibodies in the expulsion of tapeworms from the normal host.

It has been demonstrated that specific antibodies are produced against secretions from special glands, such as the rostellar glands in the scolex of *Echinococcus granulosus* (Jenkins and Rickard, 1986). Precipitation *in vitro* of mouse serum antibodies (IgG and IgM) against secretions from the rostellar glands of *H. microstoma* have been demonstrated (Andreassen and Birklund, 1993), who also showed similar precipitates on the tegument of *H. diminuta*, *H. microstoma* and *H. nana* and inhibition of the growth *in vitro* of *H. microstoma* in immune mouse serum. Electron microscope studies later showed that sloughed-off microtriches were present in the

precipitates, indicating that antigen–antibody reactions may have a deleterious effect directly on the tegument (Hoole *et al.*, 1994).

### 2.6.2. *Cell-mediated Immune Reponses*

Cell-mediated immune responses have been much less studied in adult tapeworm infections than in intestinal nematode infections. However, significant increases in the number of intestinal mast cells and eosinophils have been shown in Wistar rats infected with 100 *H. diminuta* (Hindsbo *et al.*, 1982). In Sprague–Dawley rats infected with 40 *H. diminuta*, Featherston and Copeman (1990) found an increasing mastocytosis from day 30 to 47 after infection and a decreasing mean number of worms from 32 at day 20 to 20 at day 30 to 11 at day 47. Furthermore, the number of mast cells was reduced after anthelmintic treatment but remained high in rats with a secondary infection; establishment of the secondary worms was reduced. That mast cells are important and perhaps involved in the protective host response to *H. diminuta* was also shown in the DA strain of rats, where the number of worms was significantly reduced 6 weeks after infection at the time when the mast cell number was significantly increased (Ishih, 1992). In F344/N rats, however, no mastocytosis and no worm loss occurred during a 6-week infection. Using two different populations of Sprague–Dawley rats infected with 40 *H. diminuta*, Featherston *et al.* (1992) found no correlation between serum mast cell protease II and worm loss and concluded that mucosal mast cells have no direct role in expulsion of a primary *H. diminuta* infection from rats. Whether this reflects a lack of worm antigen-specific IgE or of worm degranulators, or both, needs further investigation. The above data on this mucosal mastocytosis do show that *H. diminuta* in rats is fully capable of activating the mucosal T-lymphocyte–mucosal mast cell precursor axis, and as suggested by Hopkins and Andreassen (1991) the mastocytosis may be associated with, or be part of, the intestinal inflammatory responses that contribute to the subsequent maintenance of a dynamic equilibrium between the rat and tapeworm once a threshold worm burden has been established. Furthermore, the mastocytosis stimulated by a primary infection may also play a role in the reduction of a secondary or superimposed infection.

In rats infected with 10 *H. diminuta*, no significant increase in histamine levels in the intestinal lumen could be demonstrated compared with uninfected rats (Yonge and Webb, 1989).

In mice infected with the bile duct tapeworm *H. microstoma*, a significant increase in the number of intraepithelial mast cells in the duodenum was observed during a primary five-worm infection: a significant increase during a superimposed infection correlated with inhibition of survival of the superimposed worms (Novak and Nombrado, 1988). Although mast cells

may play a role in superimposed infections, destrobilation and expulsion of *H. diminuta* has been shown to take place in nude mice where intestinal mast cells could not be found (Andreassen *et al.*, 1982).

In intestinal nematode infections, such as *Nippostrongylus brasiliensis* in rats, it has been shown that mast cells can bring about expulsion; however, when mast cells are not present, mucosal goblet cells may increase in number and biochemical changes in mucus may cause expulsion of the worms (Wakelin, 1996). In *H. diminuta* infections in mice, it has been shown that numbers of goblet cells increase significantly until day 8 in a primary infection. After 8 days they decrease to the normal level, together with expulsion of the worms. They again increase significantly during a secondary infection (McKay *et al.*, 1990). The same authors also looked at goblet cells in a chronic infection with 10 *H. diminuta* in rats but only found a significant increase in goblet cell numbers in a posterior section of the small intestine on the tenth day after infection. This was not observed in an anterior section and had disappeared after 15 months of infection. Further studies are needed during an acute infection with a high dose of worms in rats, and not only should the number of goblet cells be analysed but also the mucus itself.

Eosinophilia has been demostrated in the guts of rats infected with 100 *H. diminuta* (Hindsbo *et al.*, 1982) and in the blood of experimental self-infections in humans. (Turton *et al.*, 1975) but its role, if any, in destrobilation and expulsion of tapeworms is unknown.

Stimulation of cell-mediated immunity in rats infected with *H. diminuta* has also been shown by Machnicka and Choromanski (1983), who observed an inhibition of macrophage migration from days 7 to 22 in infections with one worm and to day 16 in infections with three to six worms. Whether these results mean that rats are immunosuppressed by a low *H. diminuta* infection and that a high *H. diminuta* infection stimulates immunity needs further investigation.

It has been shown *in vitro* that peritoneal exudate cells from rats are able to adhere to and kill *H. diminuta* at such low serum concentrations that there is not enough complement to lyse the worms (Andreassen *et al.*, 1990). Further *in vitro* studies with peritoneal exudate cells from mice infected with *H. microstoma* have shown that they are able to kill newly excysted worms in serum without C5 and more quickly when immune serum is present (Andreassen, unpublished results).

Passive protection of naive mice by transferring mesenteric lymph node cells or peritoneal exudate cells from tapeworm-infected hosts have only been successful using *H. nana* egg-infected mice (see, for example, Palmas *et al.*, 1984, 1988). Asano *et al.* (1986) have shown that mesenteric lymph node cells from mice infected with eggs of *H. nana* can transfer immunity to nude mice when taken 4 days after an egg infection but not when taken

21 days after, and that $CD4^+$ T-helper cells are necessary for effective rejection of cysticercoids. This may indicate that adult tapeworms suppress the activity of these cells or stimulate the proliferation of supressor T-cells, but as yet we do not know. The only known example of successful transfer of protective cells from hosts infected with adult tapeworms to naive recipients comes from *Peromyscus maniculatus* infected with *H. citelli* (Wassom *et al.*, 1974).

## 3. METABOLISM

### 3.1. The Early History

Studies of tapeworm biochemistry have a respectable antiquity, going back more than 60 years. Von Brand (1933) demonstrated that incubations of *Moniezia expansa* produced succinate, while Friedheim and Baer (1933), working on *Diphyllobothrium latum*, concluded that cestodes had a respiration markedly different from that observed in other organisms. Van Grembergen (1944) detected cytochromes and measured a cytochrome oxidase-like activity in *Moniezia benedeni*. Most work on helminths at that time, however, concentrated more on the composition of parasites (the reader is referred to Von Brand, 1973 for a whole series of painstakingly compiled tables) than on dynamic aspects of metabolism. The latter were too difficult in the face of the insensitivity of the available techniques, the lability of parasite material and the difficulty of harvesting it in sufficiently large quantities.

The end of the Second World War marked a watershed in biochemistry. The availability of new techniques, especially those involving the use of radioactive tracers, saw a proliferation of investigative methods in enzymology and metabolism. Modern studies of respiration in *H. diminuta* began with Clark Read's series of papers quaintly titled 'Contributions to cestode enzymology' (Read, 1952). In the first paper of this series, Read described succinic dehydrogenase activity in *H. diminuta*, the presence of which was later confirmed by Goldberg and Nolf (1954). Read was also able to demonstrate a cytochrome *c* oxidase activity that was susceptible to inhibition by malonate. The link between the cytochrome system and succinic dehydrogenase was lost in aged homogenates.

Later, Read (1956) showed that lactate was a major excretory product of *H. diminuta*, and Fairbairn *et al.* (1961) established that succinate was also important as an excretory product, confirming the observations of Von Brand (1933). Fairbairn *et al.* were also the first clearly to enunciate the need to define, as accurately as possible, the conditions for producing

uniform infections in rats. In their system, they found that carbohydrates but not lipids were utilized during starvation. Cholesterol was present and so, too, were all the normally recognized phospholipids except for sphingomyelin. Lipids tended to be aggregated in the more mature segments of the worm. During anaerobic incubation, ammonia, urea and α-amino nitrogen were excreted. Fermentation of exogenous glucose was greater in starved worms; even in undepleted worms carbohydrate continued to be synthesized. In Fairbairn's system, succinate was the major excretory product followed by acetic acid and lactate. Much later, Webster (1972) was able to demonstrate the presence of these products in the protonephridial canal of *H. diminuta.*

In 1965, Prescott and Campbell showed that radioactive $CO_2$ was incorporated into organic, keto and amino acids but less was incorporated into polysaccharide under anaerobic conditions, suggesting that the increased activity of anaerobic respiration overcame glycogenesis. Malate was formed during this process, from which they inferred the participation of phosphoenolpyruvate carboxylase (now usually referred to as phosphoenolpyruvate carboxykinase) and malic enzyme.

Scheibel *et al.* (1968) first observed the incorporation of inorganic phosphate into ATP under anaerobic conditions and also noted its inhibition by uncouplers of oxidative phosphorylation. They found that $CO_2$ was also necessary. During the process, fumarate was reduced by NADH in aged mitochondria and, like Read (1952), who also remarked that this activity disappeared from aged preparations of the worm, found no cytochrome oxidase activity. In the same year, a peroxidase-like activity was demonstrated and found to be associated with the mitochondria of the surface syncytium but not, significantly, with muscle mitochondria, demonstrating for the first time specialization within mitochondrial populations from different cestode tissues (Threadgold *et al.*, 1968). It thus became clear that variations in metabolism were possible within the tissues of a single individual

Meanwhile, Rybicka (1967) had turned her attention to the earlier stages of development. She found high activities of succinic dehydrogenase, NADH oxidase and cytochrome oxidase in gonads and embryos. Towards the end of development these activities decreased and cytochrome oxidase was not found during embryogenesis. As she remarked. 'the study raises the problem of terminal oxidation processes in cestode embryos', and in so doing raised the spectre of variations in metabolism during development.

So, at the beginning of the 1970s these studies, together with inferences drawn (with shaky justification in hindsight) from those of other intestinal helminths, had painted a picture of a parasite that was probably anaerobic and which, if exposed to oxygen was a producer of peroxide. This suggested that the oxidase-like activity observed might be artefactual, a product of

the direct interaction between a haemoprotein and oxygen. Under anaerobic respiration, oxidative phosphorylation occurred with a concomitant and necessary uptake of $CO_2$. $CO_2$ uptake was mediated by phosphoenolpyruvate carboxykinase, leading to the formation of oxaloacetate and malate. During the further metabolism of malate, succinate, acetate and lactate were formed as end-products of metabolism.

### 3.2. Eggs, Larvae and Early Development

The saccharide fraction from 10-day-old worms included much glucose, followed by galactose, glucosamine, fucose, mannose and, possibly, rhamnose. In the egg, glucose and galactose were equally abundant, together with the same sugars that were found in 10-day-old worms, plus xylose. Eggs may also contain heparin and/or heparin sulphate; 10-day-old worms have the polyglycans but possibly neither chondroitin sulphate nor hyaluronic acid (Robertson *et al.*, 1984).

According to Pappas amd Durka (1993a,b, 1995), each egg of *H. diminuta* contains 9.6 ng carbohydrate and 3.6 ng glucose. Glucose, galactose (probably from the eggshell), glycerol, *N*-acetylglucosamine and 2-deoxyribose were obtained from hydrolysed eggs. There were also free pools of mannose and glycerol but no glucose, which suggests that eggs may have a different metabolism from adults. Intact eggs, shelled eggs (treated with NaOCl), activated larvae (with both shell and inner envelope removed) and oncospheres (activated larvae treated with papain to remove the embryophore) absorb and metabolize radioactive glucose. Small amounts are incorporated into endogenous polysaccharide. However, glucose is incorporated into material of high molecular weight, which is then excreted into the incubation medium. There is an increase in the excretion of this material with time, suggesting that metabolism changes with time.

Mercer *et al.* (1987a,b) found that eggs and adult worms contained free and conjugated ecdysteroids that were not detected in cysticercoids. They suggest that, as in insects, these compounds might have a role in embryogenesis. Cysticercoids of *H. diminuta* were unable to use exogenous trehalose (Pappas and Morrison, 1995).

### 3.3. Enzymology

In common with studies on other invertebrates, much of the knowledge of metabolic pathways derives from inference based upon formation of end-products, analysis of intermediates formed during metabolism and extrapolation from other organisms. Generally, researchers have contented

themselves with detailed studies only of those enzymes considered to exert control over metabolism. As a result, knowledge is patchy and often confined to those enzymes that are stable and easily measured. However, Siddiqui and Podesta (1985) determined the distribution of a number of enzymes as a method of assessing the integrity of preparations of subcellular fractions. Enzymes assayed were 5′-nucleotidase, acid and alkaline phosphatase, ATPase, thiamine pyrophosphatase, uricase, α-glucosidase, *N*-acetyl-β-D-glucosaminidase, succinate dehydrogenase; and lactate dehydrogenase.

Glucose concentrations in the parasite are similar to those of the intestine. The highest concentration of glucose is found in the scolex neck region, with a diminishing concentration towards the rear, mature proglottides. Absorption of glucose by *H. diminuta* is concentration dependent. *H. diminuta* regulates its body water content in the range 210–335 mosmol $l^{-1}$. Outside this range the parasite rapidly gains or loses water. Glucose uptake and metabolism increase with osmolarity within the regulatory range (Uglem, 1991). The rate of glucose utilization is very high and glucose is rapidly metabolized, especially at the anterior end of the parasite (Cornford, 1990). Uptake of glucose was determined in the latter study by observing the uptake of 3-*O*-methyl glucose, which is not metabolized. However, analysis of 3-*O*-methyl glucose uptake (Uglem and Pappas, 1991) indicated that the mechanism is simple diffusion only and its use in glucose uptake studies may not be justified.

Glycogen synthase is the enzyme that regulates the deposition and utilization of glycogen in *H. diminuta* and other animals. It exists in two forms, an active form and an inactive one depending on the physiological condition of the host, and the transition from inactive to active regulates the rate of deposition of glycogen. Dendinger and Roberts (1977) and Mied and Bueding (1979a,b) found that the ratio of active to inactive enzyme is low in worms isolated from a fasted host (and therefore, one presumes, probably falsely, from a fasted parasite), suggesting that glycogen is being mobilized for respiration. The values increase 1 hour after the host is fed and the increase appeared to be due to the presence of glucose in the gut. *In vitro*, glucose also caused activation of glycogen synthase; if the worm contained high concentrations of glycogen that also inhibited activation. Ratios of active to inactive glycogen synthase and glycogen phosphorylase were correlated with the host feeding regime before recovery. Glycogen synthase was in the inactive form in worms from both fed and fasted hosts, but 1 hour after refeeding 80% was in the active form. The reverse was true for the phosphorylase. *In vitro*, after glucose deprivation for 18 hours, addition of glucose increased the proportion of active synthase and decreased that of phosphorylase. The effects indicate that synthesis and mobilization are reciprocal (Wages and Roberts, 1990). Activation was also

found to be a function of crowding. Activity was greatest in crowded worms, which suggests that there is intensive competition for glucose. The distribution of the enzyme activity varied along the length of the parasite, being highest in the pregravid proglottides at the midpoint of the strobila. Activity increased during development, and was not found to be due to an increase in the ratio of active to inactive enzyme but rather to increased synthesis. Glycogen also modified activity, the effect depending on the molecular weight: the higher the molecular weight, the greater the inhibition.

Hexokinase catalyses the first step in the metabolism of glucose. In *H. diminuta* it exists in both a soluble and a membrane-bound form. This is similar to the distribution found in *Ascaris* preparations, where it is surmised that it acts to sequester glucose 6-phosphate differentially for glycogenesis and glycolysis (Supowit and Harris, 1976). Fructose is unimportant as an energy source (Komuniecki and Roberts, 1977a).

*Hymenolepis diminuta* has been shown to possess galactokinase, galactose 1-phosphate uridyl transferase and UDP-galactose epimerase activities at low specific activities. Galactose 1-phosphate is a non-competitive inhibitor of galactose phosphorylation, which explains the inadequacy of galactose as a carbohydrate source (Komuniecki and Roberts, 1977b) and Read's (1967) observation that although galactose is absorbed at a high rate, it does not serve as a substrate for glycolysis.

Hexokinase and glycogen synthase are important enzymes that initiate glycolysis. The next major control point that has been studied in some detail occurs at the level of phosphoenolpyruvate. Two enzymes compete for this substrate: pyruvate kinase and phosphoenolpyruvate carboxykinase. Both are present in *H. diminuta*. According to Bueding and Saz (1968) fermentation in the direction of succinate or lactate depends upon their competing activities under conditions of allosteric regulation (called the PK/PEPCK branchpoint). Pyruvate kinase is multienzymic, and at different stages of development *H. diminuta* may contain as many as five isozymes (Carter and Fairbairn, 1975). Competition between pyruvate kinase and phosphoenolpyruvate carboxykinase is probably controlled at the fructose 1, 6-diphosphate level. Pyruvate kinase activity at phosphoenolpyruvate levels consistent with $K_m$ and $V_{max}$ is unaffected by ATP, alanine, fructose 1, 6-diphosphate or hydrogen ion concentrations; 50% inhibition is caused by 6.3 mM lactate and 30 mM bicarbonate (Moon *et al.*, 1977a).

One product of the activity of pyruvate kinase is pyruvate, which is converted to lactate by lactate dehydrogenase. Lactate dehydrogenase from *H. diminuta* was found by Burke *et al.* (1972) to have a molecular weight of 141 000. However, these workers detected one form only kinetically similar to heart lactate dehydrogenase, that had a $K_m$ for pyruvate of

0.17 mM; the $K_m$ for lactate was 6.3 mM. Logan *et al.* (1977) reported the presence of two isozymes of dehydrogenase in *H. diminuta*. Activity of the A isozyme occurs in all tissues, but activity of the B isozyme is restricted to ovarian tissues. This supports the view of Walkey and Fairbairn (1973) and is further supported by Moon *et al.* (1977a). The latter found that partially purified lactate dehydrogenase comprises two isozymes and has a pI of 6.9–7.0. While its activity could be modulated by pH, no other effectors were detected.

Phosphoenolpyruvate carboxykinase from *H. diminuta* is similar to that from rat liver but possesses a stronger affinity for metal ions, phosphoenolpyruvate and is more susceptible to inhibition by 3-mercaptopicolinate and quinolinate (Reynolds, 1980). There are at least two possible regulators of the enzyme: GTP and ITP (Wilkes *et al.* 1981). Under conditions of high bicarbonate concentration phosphoenolpyruvate carboxykinase is maximally activated and lactate is inhibitory. These results led Moon *et al.* (1977b) to suggest that the $HCO_3^-/CO_2$ system is a major factor in determining end-products of respiratory metabolism. This may be so from a long-term, evolutionary point of view but does not appear to be the case in short-term adaptation.

The activity of phosphoenolpyruvate carboxykinase leads, as mentioned above, to the formation of oxaloacetate. The conversation of this to malate requires malic dehydrogenase. In *H. diminuta* malic dehydrogenase occurs as four isozymes, with pI of 7.45, and is very sensitive to pH change (Moon *et al.*, 1977a). At all pH values, it is favoured over lactate dehydrogenase if oxaloacetate is present. Unlike pyruvate kinase and phosphoenolpyruvate carboxykinase, which are modulated by a number of allosteric effectors such as nucleotide phosphate and cation availability, malic dehydrogenase and lactate dehydrogenase are controlled primarily by substrate availability.

There is one other malate-metabolizing enzyme of considerable importance in *H. diminuta*. Malic enzyme, more properly referred to as malate dehydrogenase (decarboxylating) is located primarily in the mitochondrion and is responsible for decarboxylating malate. It has a molecular weight of about 120 000, utilizes NADP and decarboxylates oxaloacetate at pH 7.5 (Li *et al.*, 1972).

Only a very few of the enzymes that metabolize amino acids in *H. diminuta* have been identified. A lyophilized extract from *H. diminuta* generated $CO_2$ from aspartate and 2-oxoglutarate, yielding alanine and glutamate (Nations *et al.*, 1973). This activity is similar to that of aspartate 4-decarboxylase, but dissimilar in that activity did not occur at allosteric concentrations of 2-oxoglutarate and did not occur at all if it was absent. It is probable, therefore, that the $CO_2$ originates from the following sequence of reactions:

*Aminotransferase*
aspartate + 2-oxoglutarate → oxaloacetate + glutamate

*Decarboxylation*
oxaloacetate → pyruvate + $CO_2$

*Aminotransferase*
glutamate + pyruvate → alanine + 2-oxoglutarate

Glutamate dehydrogenase is present in the cytosol of the adult worm. When the pH of the reaction was decreased to 6.5 from 7.4 the apparent $K_m$ for 2-oxoglutarate was reduced to one-tenth of the original value, while that for glutamate remained unchanged. At the lower pH glutamate formation is thus favoured and pH may exert a regulatory role. NADH oxidation is favoured at low substrate concentrations (Mustafa *et al.*, 1978).

Data suggest the *H. diminuta* is capable of acetate-dependent synthesis of isoprenoids and that this occurs via the intermediate step of the conversion of hydroxymethylglutaryl CoA to mevalonic acid. The appropriate reductase, hydroxymethylglutaryl CoA reductase, was found in mitochondrial and microsomal fractions. It was specific for NADPH and the evidence suggests that the mitochondrial activity is due to contamination (Fioravanti *et al.*, 1989).

## 3.4. Mitochondria and the PK/PEPCK Branchpoint

### 3.4.1. *Introduction*

In the intermediary metabolism of *H. diminuta*, the flow of carbon from glycogen or glucose via the Embden–Meyerhof pathway to the level of phosphoenolpyruvate is unremarkable in that it follows the sequence of reactions seen in most other organisms. Indeed, it is so unremarkable that not all the enzymes of this part of the pathway have been formally identified; rather, their presence is inferred from the substrates and products of their reactions. There are differences, but they are subtle and manifested as changes in the way the reactions respond to allosteric regulation.

In the part of the pathway that begins at the level of phosphoenolpyruvate, however, there are marked differences from the pathways encountered in the higher mammals, yet it is not dissimilar from some that are encountered in other metazoans (e.g. annelids and molluscs) or in prokaryotes. Where helminths differ from other metazoans is that this part of the pathway is permanent and constitutive, whereas in annelids and molluscs such metabolism is generally a temporary adaptation to a certain suite of environmental conditions (for a collection of reviews on the subject of anoxic life, see Bryant, 1991).

In the previous section it was remarked that the competing activities of pyruvate kinase and phosphoenolpyruvate carboxykinase (the PK/PEPCK branchpoint) determined the flow of carbon to either lactate or malate. The former pathway is cytosolic; the latter occurs in the mitochondrion. The responses of *H. diminuta* to perturbations of metabolism were studied by Precious and Barrett (1993). Calculation of flux coefficients confirmed that hexokinase, phosphofructokinase, phosphoenolpyruvate carboxykinase and pyruvate kinase were capable of modulation; however, the flux coefficient for glycogen synthase showed that regulation was achieved by the distribution of metabolic flux through alternative pathways. It is not necessary for both phosphoenolpyruvate carboxykinase and pyruvate kinase to be modulated to control the flow of carbon through the PK/PEPCK branchpoint, as changes in the activities of enzymes more remote from the branchpoint will also have an effect (Barrett and Precious, 1995).

### 3.4.2. *ATP Synthesis*

There are several published accounts of mitochondrial preparations from *H. diminuta*. That of Harlow and Byram (1971) is accompanied by detailed electron micrographs showing that generally the mitochondria conform to the expected pattern, with an outer and inner membrane separated by an intermembrane space, and an internal space, the matrix, traversed by cristae. A detailed analysis of the morphology indicates that the mitochondria are smaller than rat liver mitochondria and possess longitudinal rather than transverse cristae.

As mentioned earlier, Scheibel *et al.* (1968) were the first to observe the incorporation of $^{32}P$ into ATP by intact adults of *H. diminuta* and $^{32}P$ exchange into ATP in mitochondrial preparations. The presumption was that a $CO_2$-dependent phosphorylation of ADP occurred but the authors did not demonstrate a net increase in the amount of ATP in their system. According to Rahaman and Meissner (1973), mitochondria from *H. diminuta* showed coupled respiration, i.e. respiration with associated phosphorylation of ADP to yield ATP when α-glycerophosphate, succinate, pyruvate plus malate, glutamate plus malate or 2-oxoglutarate plus malate were substrates. The ADP/O ratios varied from 0.6 to 1.7 and the addition of cytochrome *c* increased respiration rate.

### 3.4.3. *Malic Enzyme and the Transhydrogenase*

In parasitic helminths, the conventional wisdom is that phosphoenolpyruvate is carboxylated to yield oxaloacetate, which is then reduced to malate. Malate enters the mitochondrion, where it suffers two simultaneous fates,

the so-called dismutation reaction. In a true dismutation, such as the one catalysed by superoxide dismutase, a single molecule is simultaneously oxidized and reduced. In the malate dismutation, two molecules of malate are involved, while one enzyme catalyses the oxidation and two enzymes catalyse the reduction. The reduction to succinate, is carried out by fumarase and fumarate reductase; the oxidation to pyruvate by malic enzyme:

*Oxidative decarboxylation*
malate → pyruvate + $CO_2$, etc.

*Reduction*
malate → fumarate → succinate, etc.

Fumarate reduction is carried out by an electron transport system and requires NADH. In *H. diminuta*, however, mitochondrial malic enzyme generates NADPH and in fact the enzyme is NADP dependent. NADPH cannot be used directly in fumarate reduction; there is an additional enzyme, a non-energy-linked mitochondrial NADP:NAD transhydrogenase, first observed by Saz *et al.* (1972). It is associated with the inner mitochondrial membrane and an anaerobic malate-dependent electron transport system that catalyses ATP synthesis and is inhibited by uncouplers of oxidative phosphorylation (Walker and Fioravanti, 1995).

Fioravanti (1982a,b) and coworkers have carried out an extensive programme of research on mitochondria from *H. diminuta*, in particular focusing on the reactions that NAD and NADP undergo. Mitochondria possess a membrane-associated rotenone-sensitive NADH oxidase and an NADH-dependent fumarate reductase. NADH is the preferred substrate in each case. NADH:NAD transhydrogenase and lipoamide dehydrogenase activities were detected in cysticercoids, 14-day-old worms and in mitochondria from all parts of the adult cestode (Walker and Fioravanti, 1995; Walker *et al.*, 1997). On the other hand, malic enzyme proved to be NADP specific, and generated reducing equivalents that brought about the synthesis of ATP by the electron transport system. The malic enzyme from *H. microstoma* was similar. Malic enzyme activity, yielding oxaloacetate in disrupted mitochondria, occurred in the presence of NADP, indicating the operation of an NADPH:NADP transhydrogenase (McKelvey and Fioravanti, 1984).

Both NADH oxidase and NADH reductase are relatively insensitive to antimycin A, KCN and sodium azide. Malonate inhibited mitochondrial NADH oxidase, suggesting malate involvement. Succinate or NADPH oxidation resulted in peroxide formation (Fioravanti, 1981, 1982b). Lumsden *et al.* (1969) had previously identified peroxidase activity by histochemical means in *H. diminuta* mitochondria and concluded that it was due not to peroxidase *per se* but to an ancillary, or even artefactual, function of the cytochrome system.

In the light of these data, the role of a transhydrogenase becomes crucial, for how else can the reducing equivalents represented by reduced NADP be transferred to NAD for use in the electron transport-mediated synthesis of ATP?

Saz *et al.* (1972) had already reported such a transhydrogenase from *H. diminuta*. Fioravanti (1981) confirmed that the enzyme was indeed mitochondrial, transferring hydride from NADPH to NAD, thus coupling it with electron transport. *Hymenolepis microstoma* also possesses an NADPH:NAD transhydrogenase (Fioravanti, 1982b). The enzyme is dependent on phospholipid for maximum activity; phosphatidylcholine, when tested, proved stimulatory (Fioravanti and Kim, 1983), from which it can be concluded that the enzyme is membrane bound.

This is indeed the case. Both fumarate reductase and the NADPH:NAD transhydrogenase were found on the inner mitochondrial membrane, whereas malic enzyme and fumarase were in the soluble, matrix compartment. Thus NADPH and fumarate are accumulated within the matrix and the formation of NADH from NADPH occurs on the matrix side of the inner membrane. Electron transport-dependent NADH oxidation and fumarate reduction also occur on the matrix side of the inner membrane (McKelvey and Fioravanti, 1985).

Submitochondrial particles, designated 'phosphorylating', from adult *H. diminuta* contain a pyridine nucleotide transhydrogenase that catalyses an energy-linked NADH–$NADP^+$ transhydrogenation. The reaction is inhibited by rotenone but not by oligomycin. In the presence of rotenone and $Mg^{2+}$, ATP brought about an ATP-specific three-fold increase in activity. Succinate was without effect. Thus, energy for the reaction may be derived either from electron transport-dependent NADH oxidation or ATP utilization via the phosphorylating mechanism (Fioravanti *et al.*, 1992).

### 3.4.4. *Other Components of Electron Transport*

Mitochondria from *H. diminuta* are capable of oxidizing reduced cytochrome *c* and succinate in the presence of oxygen. One of the end-products is peroxide, which suggests that these reactions may not be physiological. They are consistent with the type of electron transport system observed in *Ascaris* and *Moniezia expansa* (Cheah, 1972). In these helminths, NADH is oxidized to NAD and at the same time fumarate is reduced. This process is also observed in *H. diminuta* and indicates that a similar sequence of reactions is taking place. Figure 1 illustrates these reactions. A flavoprotein-dependent NADH oxidase accepts electrons from NADH and transfers them via a quinone carrier to cytochromes and thence to fumarate, which becomes converted to succinate. A number of other reactions

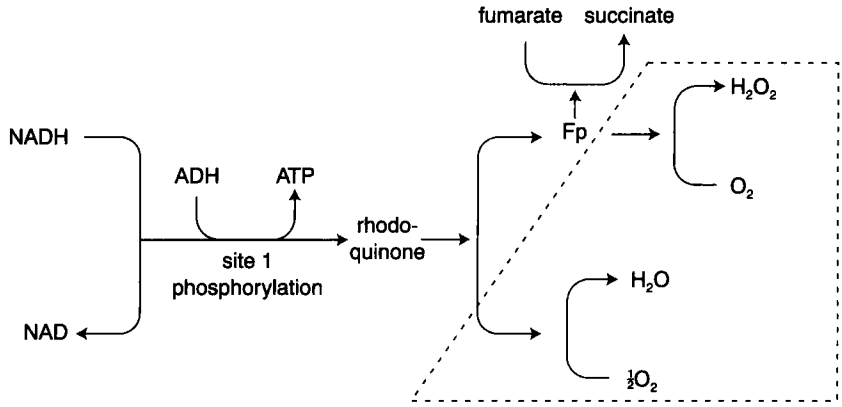


*Figure 1* A model for electron transport in *Hymenolepis diminuta* mitochondria. Only those reactions drawn outside the box are considered to be physiological. Fp, flavoprotein component of fumarate reductase. (Redrawn from Fioravanti and Reisig, 1990.)

are possible, but their physiological significance is questioned. This model is supported by several studies made by Fioravanti's group (Fioravanti, 1982b; Fioravanti and Kim, 1988; Fioravanti and Reisig, 1990; Fioravanti *et al.*, 1992).

Pentane extracts from lyophilized mitochondria and subsequent spectral analysis identified rhodoquinone as the quinone carrier. This is in contrast to conventional systems, where ubiquinone is often encountered, but similar to that of *Ascaris*, Both NADH oxidase and fumarate reductase from *H. diminuta* require rhodoquinone for maximum activity, in conformity with the model described in the previous paragraph (Fioravanti and Kim, 1988).

Membranes from mitochondria isolated from *H. diminuta* catalyse inhibitor-sensitive ferricytochrome *c* reduction. Cytochrome *c* reductase activity occurs with either NADPH or succinate. The membranes also contain NADH oxidase and succinoxidase, and produce hydrogen peroxide. A ferricytochrome *c* oxidase is also present, as is cytochrome *c* peroxidase. These two enzymes may combine to limit peroxide accumulation within the cell. Peroxide production suggests that there are two routes for oxygen reduction, only one of which leads to hydrogen peroxide. Neither route is sensitive to cyanide. Inhibition by malonate suggests that the peroxide-forming pathway is associated with the malonate-sensitive fumarate reductase (Kim and Fioravanti, 1985; Fioravanti and Reisig, 1990).

### 3.4.5. *Nuclear Magnetic Resonance Studies*

Nuclear magnetic resonance (NMR) is a technique used routinely by chemists for verifying the product of reactions and syntheses but it is

only in the last decade that it has been used by parasitologists. It is a potentially powerful technique as it can be used to determine metabolic end-products excreted into incubation media and metabolic intermediates and structural components within live parasites. If the parasite is small enough it can be recovered and used in other experiments.

The first NMR study of cestodes was carried out by Wasylishen and Novak (1983). Among the cestodes they used was *H. diminuta*. They obtained natural abundance $^{13}$C-NMR spectra from live worms that showed glycogen, triglycerides and choline derivatives; *H. diminuta* was shown to have a lower concentration of polyunsaturated fatty acids than either proliferating *Mesocestoides corti* tetrathyridia or cysts of *Echinococcus multilocularis*. Signals were routinely gathered for 8 hours, which casts a shadow on the continued integrity of the intact, gravid specimens of *H. diminuta* throughout the experiment.

In an NMR analysis of excretory products resulting from the metabolism of D-[$^{13}C_6$] glucose, Blackburn *et al.* (1986) found label in lactate, acetate, succinate and alanine, confirming decades of observations by more antiquated means. Novak *et al.* (1991) subsequently confirmed the excretion of lactate, acetate and succinate, and showed that histamine increased glycolysis, as evidenced by increases in lactate and acetate production; however, 5-hydroxytryptamine (5-HT) had a concentration-dependent effect, low concentrations enhancing and high concentrations inhibiting glycolysis (see Section 3.5). Another study of glucose metabolism using $^{13}$C-NMR showed carbon from [U-$^{13}$C]glucose in succinate, lactate and acetate and also in glycogen. Parasites from fed hosts utilized glucose less rapidly than those from starved hosts. When $^{13}$C was present as $NaH^{13}CO_3$, it appeared in both the succinate carboxyl carbons, at C-1 and C-4, and in lactate at C-1. These observations led the authors to conclude that mitochondrial malate or pyruvate contribute to the cytosolic pyruvate pool (Behm *et al.*, 1987).

*In vivo* $^{31}$P spectra showed a variety of expected phosphate compounds; evidence suggests that the worms were viable during the experiment. While 2,4-dinitrophenol caused a lowering of nucleotide triphosphate, mebendazole did not. Thompson *et al.* (1987) concluded that mebendazole did not exert its primary effect on oxidative phosphorylation.

*In vivo* NMR spectra were collected for *Tenebrio molitor* fed with D-[1-$^{13}$C]glucose, both uninfected and infected with *H. diminuta*. Label was incorporated into glycogen, succinate, acetate and lactate. In extracts from cysticercoids, trehalose of parasite origin was most heavily labelled followed by alanine, succinate, lactate, glycogen and acetate (Schoen *et al.*, 1996).

### 3.5. The Role of 5-HT in Regulating Intestinal Migration

5-HT-like and acetylcholine-like reactivities are readily detected in *H. diminuta* and their distribution had been described in some detail (Webb, 1985; Fairweather *et al.*, 1988; Samii and Webb, 1990). A number of neuropeptides have also been demonstrated (McKay *et al.*, 1991; Fairweather *et al.*, 1988; Sukhdeo and Sukhdeo, 1994) and for a detailed account the reader is referred to the excellent short review by Fairweather and Skuce (1995). The present section is concerned more with the larger phenomenon of the circadian migration of *H. diminuta* along the rat intestine.

The circadian migration of *H. diminuta* correlates well with the daily variations of 5-HT in worm tissue and in the intestine, and with the amount of food present in the small intestine. When rats were fasted, there was no regular change in the concentration of intestinal 5-HT and the worms failed to migrate. However, if food was prevented from entering the small intestine from the stomach, the worms still migrated forward. These observations suggest that it is 5-HT that is responsible for migration rather than the food itself (Cho and Mettrick, 1982a). The 5-HT antagonist methysergide also abolished the response, confirming this conclusion (Mettrick and Cho, 1982). Insulin administration did not increase 5-HT concentration in host fluids, but caused a backwards migration of *H. diminuta*. Ligation of the pylorus prevented this, which once again suggests that gastric secretion may be involved (Cho, 1984). Histamine, on the other hand, promotes posterior migration.

Worms migrate forward following intragastric feeding (Sukhdeo and Mettrick, 1984). Reversing intestinal flow did not affect worm migration following feeding and neither did glucose when it was injected into the duodenum. However, when glucose was injected into the small intestine it did initiate forward migration. When gastric secretions into the stomach were rerouted to a more posterier point in the intestine, avoiding the anterior portion containing the worms, no migration was observed. If worms were present below the new point of entry of the gastric secretions, they did migrate.

Sukhdeo and Mettrick (1984) concluded that both pancreatic and bile secretions appear to contain a stimulator of anterior migration. The order of potency in producing migration was (i) pancreatic secretions, (ii) gastric secretions, (iii) biliary secretions and (iv) glucose. It is apparent that worm migration is effected in reponse to many factors, which include 5-HT as well as physicochemical gradients and the host's physiological condition (Mettrick and Podesta, 1982).

5-HT administered daily for 15 days to rats infected 1 day previously did not affect either the number of worms establishing or their growth,

although some evidence suggests that egg production may have been affected. Histamine, however, resulted in fewer worms establishing, and this resulted in a greater worm weight among those that did establish. Egg output, as measured by eggs per gram faeces, was reduced; so too was the number of cysticercoids developing in *Tribolium confusum* that were allowed to feed on gravid proglottides. Cho and Mettrick (1982b) concluded that histamine treatment may have emulated the non-specific immunological or inflammatory response often seen in worm infections.

Working with isolated worms *in vitro*, Thompson and Mettrick (1984) showed that 5-HT increased the motility of the whole strobila of *H. diminuta*, whereas acetylcholine inhibited it. Like 5-HT, somatostatin had a stimulatory effect on the mobility of the anterior and mid-regions, but the effect was small and only apparent at high concentrations. Histamine was inhibitory (Sukhdeo *et al.*, 1984). These preliminary studies on the neuromuscular physiology suggest that $Ca^{2+}$ concentration is important in regulating contractions.

5-HT in *H. diminuta* derives from two sources. Exogenous 5-HT is absorbed from the environment within the host and is of host origin. 5-HT can also be synthesized by *H. diminuta* tissue, using tryptophan as a precursor. In worms depleted of tryptophan, the amount of detectable 5-HT is reduced, confirming that synthesis takes place (Ribeiro and Webb, 1984). Studies of the release of exogenously supplied 5-HT showed that it was neither $Ca^{2+}$ dependent nor antagonized by $Mg^{2+}$, whereas the release of endogenous 5-HT was $Ca^{2+}$ dependent. Gordon and Webb (1989) concluded that endogenous 5-HT was released from nervous tissue in a neurotransmitter-like manner.

A kinetic study of the uptake of 5-HT showed that it has two components. One is dependent on sodium ions and displays a high affinity for 5-HT, whereas the other is insensitive to sodium ions and has a rather lower affinity. However, it is unclear whether this uptake is of physiological significance because of the ability of the parasite to synthesize its own 5-HT from tryptophan (Ribeiro and Webb, 1984; Webb, 1985). Tryptophan is rapidly transported into the worm by a high-affinity carrier (Cornford, 1992).

Specific binding sites for 5-HT are present in membrane preparations from which the tegument has been removed. Their role remains unclear, though the authors suggest that, in comparision with mammalian systems, the activity of 5-HT on motor activity is achieved by the modulation of neurotransmitter release (Ribeiro and Webb, 1986).

Metabolic effects of 5-HT on the metabolism of *H. diminuta* have been studied by Mettrick *et al.* (1981), who found that, *in vitro*, it induced only a small increase in glucose uptake, whereas in worms deprived of glucose glycogen mobilization was enhanced, One effect of 5-HT, therefore, seems

to be to increase glycolytic rate in the parasites. A similar effect is observed in *H. microstoma* (Rahman *et al.*, 1982). When these effects were examined more closely, it was found that 5-HT at a concentration of 0.5 mM enhanced glucose uptake in the anterior strobila and scolex of adult *H. diminuta* whereas there was no effect in the posterior region. There is also no effect of 5-HT on glucose uptake in the normal rat intestine; however, in the parasitized intestine it was inhibited (Gruner and Mettrick, 1984).

Novak *et al.* (1991) confirmed that 5-HT had a concentration-dependent effect on *H. diminuta*, low concentrations enhancing and high concentrations inhibiting glycolysis. They also found that histamine increased the rate of glycolysis, as evidenced by increases in lactate and acetate production. 5-HT may exert its effect on glycolysis in *H. diminuta* by elevating cyclic AMP and/or AMP levels, as these nucleotides can increase phosphofructokinase activity (Rahman and Mettrick, 1982; Sangster and Mettrick, 1987). It also causes increased incorporation of $^{32}P$ from ATP in crude membrane preparations; 5-HT agonists enhance this effect while antagonists inhibit it (Ribeiro and Webb, 1991). 5-HT also increased the incorporation of inorganic phosphate into phosphatidylinositol phosphatidylethanolamine, phosphatidic acid and phospholipid fraction in intact 10-day worms. Messenger molecules derived from inositides may thus be involved in 5-HT stimulation (Ip and Khan, 1989, 1990). Glucose inhibits $^{32}P$ incorporation into phosphatidylcholine, while serotonin and histamine increased it into all lipid classes (Khan *et al.*, 1987).

### 3.6. Emerging Areas of Importance in Regulation

In mainstream biochemistry, there is now much emphasis on regulation of cellular activity and the role of various messengers in that regulation. The information about such effectors in *H. diminuta* and the regulation of its metabolism, neurophysiology and behaviour is both sparse and patchy. Uptake of choline by tissue slices occurs by two mechanisms (Webb, 1994). One is sodium dependent and involves a carrier with a high affinity for choline (although this was not observed by Cornford, 1992). This 'low-activity' choline uptake is coupled chemiosmotically to the sodium electrochemical gradient and appears to be similar to that described in the high-affinity uptake in synaptosomes of locust. The other choline uptake process is independent of sodium concentration. Choline is only slowly converted into acetylcholine in *H. diminuta*, unlike the rapid conversion observed in mammalian tissue. Choline is also used in the synthesis of phosphatidylcholine, the predominant phospholipid in mammalian tissues.

Glutamate, as well as acetylcholine, stimulates the production of inositol 1, 4, 5-trisphosphate production in *H. diminuta* (Samii and Webb, 1996). Activation probably proceeds via a G protein that enhances phospholipase C activity. Nitric oxide is the latest in a series of neural messengers to assume great importance. The enzyme reponsible for its synthesis, nitric oxide synthase, has been detected in the nervous system, of adult *H. diminuta*.

$Ca^{2+}$ influx into anterior portions of *H. diminuta* with scolex was biphasic, with an initial fast uptake followed by a continued slower influx. Glucose starvation inhibited the fast uptake, which in turn was inhibited by praziquantel. Praziquantel increased $Ca^{2+}$ efflux (Tayal *et al.*, 1986). $Ca^{2+}$ influx into mitochondria from *H. diminuta* takes place via an electrophoretic uniport and causes, as does $Mg^{2+}$, a concentration-dependent hydrolysis of ATP. $Na^{+}$ induces an efflux of $Ca^{2}$. In this it is similar to mammalian mitochondria (Wani and Sirvastava, 1995; Wani *et al.*, 1995). Calmodulin has been implicated in numerous cellular events (Branford-White *et al.*, 1984). Branford-White and Hipkiss (1985) describe a $Ca^{2+}$-dependent activator protein from *H. diminuta* that has many of the properties of calmodulin. Calmodulin has subsequentely been identified in those structures classically associated with calmodulin in higher eukaryotes, where it appears to play a similar role in spite of the syncytial organization of the tapeworm (Eastlake *et al.*, 1994).

*Hymenolepis diminuta* possesses a cystathionine-β-synthase with wide substrate specificity, but which differs from that of mammals in having high serine sulphydrase and serine lyase activities. There was low γ-cystathionase, with a wide range of substrates. Cysteine aminotransferase was present but no 3-mercaptopyruvate sulphotransferase. Cysteine dioxygenase and cysteine sulphinate transaminase activities were also present. *Hymenolepis diminuta* was able to reduce cystine to cysteine via a glutathione–cysteine transhydrogenase system (Gomez-Bautista and Barrett, 1988). This process is illustrated in Figure 2.

*Hymenolepis diminuta* carries out a range of hydrolytic and reductive reactions whose role appears to be detoxification of xenobiotic compounds. Oxidative detoxification reactions were not detected, but neither have they been detected in other helminths (Munir and Barrett, 1985); *H. diminuta* also lacks sulphotransferase activity (Raines and Barrett, 1988).

A thymine–uracil transport system is present in the brush border of *H. diminuta* (Uglem *et al.*, 1983). It also contains a nuclear adenosyldiphosphoribosyl transferase activity, dependent on DNA containing strand breaks. It modifies previously synthesized proteins by the covalent attachment of the ADP-ribose moiety of NAD. This is not surprising as this activity is present in all nucleated cells (Alsharif *et al.*, 1990).

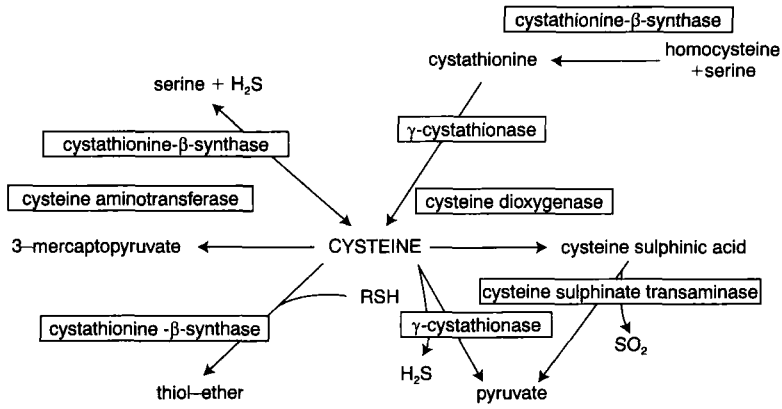


*Figure 2* Metabolism of cysteine in *Hymenolepis diminuta*. RSH is an additional thiol. (After Gomez-Bautista and Barrett, 1988.)

## 4. STRAIN VARIATION AND THE CROWDING EFFECT

During development in the definitive host, *H. diminuta*, in common with other cestodes, experiences a crowding effect, which is progressively more severe as higher numbers of worms establish in an individual host. In the crowded population, this is manifested as smaller and less mature worms. Indeed there is an inverse ratio between number of worms establishing and size of worm. This is independent of host intake of dietary carbohydrate (Roberts, 1966; Zavras and Roberts, 1984). The apparent adverse interactions between worms in a crowded infection led Roberts (1961) to propose the existence of a crowding substance or substances. Subsequently, Hesselberg and Andreassen (1975) referred to a deleterious factor that might have its origins in the host's immune system. It is now clear that crowding effects are, to a large extent, a product of the interaction of the tapeworm with the immune system of the host. These become manifest as both 'classical' crowding effect and changes in intermediary metabolism.

During *in vitro* incubation, *H. diminuta* secretes substances into the medium that inhibit DNA syntheses in the germinative region of normal uncrowded worms (Bolla and Roberts, 1971). Worm-conditioned saline inhibited thymidine incorporation into DNA in the anterior region of worms by more than 50%. If such a saline was prepared from worms that had been subject to even greater crowding, the inhibition was also greater; the crowding substances were also stable to heat treatment (Roberts and Insler, 1982). Zavras and Roberts (1985) found that D-glucosaminic acid was partially inhibitory, while a defined mixture of 12 mM succinate, 5 mM acetate, 25 mM D-glucosaminic acid and 2.8 nM cGMP

was as effective as worm-conditioned medium. An *in vivo* experiment confirmed this result showing that worms that had been exposed to the artificial mixture had fewer proglottides than the control group (Cook and Roberts, 1991).

It is not clear from the literature on the crowding effect whether it has adaptive significance. Roberts' (1961) detailed analysis of hypotheses that were current at the time sheds little light on this. As mentioned above, it is clearly not due to competition for carbohydrate as was suggested in the 1950s. The fact that the crowding effect manifests itself approximately 6–8 days after infection suggests that a host immune factor may be involved. That the effect may be non-specific is suggested by the work of Holmes (1961). He noticed that a reciprocal crowding effect was exerted by *Moniliformis dubius* on *H. diminuta*, worms of both genera being smaller in concurrent infections. Roberts (1961) had earlier remarked, with some prescience, that 'there may be fundamental differences in the metabolism of worms from different population densities'. Bennet *et al.* (1993) have noted that there are indeed metabolic differences between control worms and worms from hosts which have been given a non-specific immunological challenge.

Variation is the stuff of evolution, but how many ways can an organism vary? First, there is genetic variation. Stable variations, such as that observed in a balanced polymorphism, may arise as a result of physiological or spatial isolation of two populations and may be regarded as stations on the way to the emergence of new species. These variants are stable over generations and may be identified by changes in the genome, changes in morphology or changes in physiology and biochemistry.

There may also be variation brought about by adaptation to local changes in the environment. Goodwin (1994) devotes a whole book to this thesis and shows in a variety of organisms that morphology derives from environmental cues such as ion gradients or temperature. In other words, the genome defines the outer limits of what is possible, but the environment dictates what is permissible. Genes can only act within the context of the developing organism, which in turn provides a regulatory milieu for gene action at the prompting of the external environment. This makes for flexibility in development and may offer an escape hatch in the event of the appearance of some adverse environment characteristic, such as an immune response. The result may be startling differences in morphology and biochemistry.

Then there is variation within an organism. This is obvious to a human being, accustomed to the fact that kidney morphology and metabolism differ in significant respects from those of liver, for example. However, it has not been wholly obvious, or perhaps has been ignored as too difficult to cope with, to biochemical parasitologists who have wrestled with metabolic pathways in intransigent material.

Finally, there is variation that occurs within an organism during development. Morphologists are, of course, used to this but it is an area that has been rather neglected by biochemists. However, such variation has been long noted in *Ascaris* (Oya *et al.*, 1963) and more recently in *Fasciola* (Tielens *et al.*, 1984).

Research on *H. diminuta* over the last two decades had demonstrated that all these levels of variation are readily observed in the developing tapeworm. Using isoelectric focusing of proteins from three species of hymenolepids Dixon and Arai (1985, 1987, 1989) found marked species differences between them, although they also found that the species of host was without effect. Other workers, using different experimental systems, have found marked influences imposed by the host on the parasite. For example, four strains of *H. diminuta* were compared and found to differ in a number of important characteristics: size of eggs, size of oncospheres and numbers and distribution of testes (Papps and Leiby, 1986). This variability is confirmed in a cluster of papers by Stradowski (1995a,b,c, 1996a,b, 1997, in Polish, read only in abstract). In *H. diminuta* inbred over a maximum of 54 generations, Stradowski noted variations in genital ducts and pores, in testis number and proglottid form, and changes due to crowding.

There are marked differences between a Japanese wild strain of *H. diminuta* and one derived from the Rice Institute in Texas (Kino and Kennedy, 1987). The differences are manifest in infectivity, growth rate and fecundity. The Japanese parasites (from *Rattus rattus* but grown in *R. norvegicus*) showed much greater variation and heterogeneity and, on this basis, it was concluded that the Texas strain (normally maintained in *R. norvegicus*) was better adapted to its host than the Japanese strain at that time. However, this obviously does not preclude a closer adaptation in the future, after which, perhaps, the Texas and Japanese strains would be indistinguishable. It could come about by a simple mechanism. The host, as environment, will 'edit out' those individuals that cannot survive its particular characteristics. Those that do survive will exhibit compromises of morphology and physiology consistent with survival. If such compromises are the property of only a very small number of individuals within a wide spectrum of genetic possibilities, it is possible that a new strain will emerge. If, however, the ability to make such compromises is the property of the greater proportion of individuals, them the emergence of a new strain is unlikely.

Variation within an individual tapeworm has also been noted. Coles and Simpkin (1977) showed that a metabolic gradient existed along the length of adult *H. diminuta*. The first 2 cm, including the scolex, produced relatively more lactate and acetate, and less succinate, than the remainder of the worm.

Adult *H. diminuta* also show metabolic variation with time. Parasites recovered from immature, 6-day infections excreted equal amounts of succinic and lactic acids and less acetic acid. At 10 and 14 days, lactic acid excretion was small, whereas succinate accounted for about half the total (Watts and Fairbairn, 1974). Further, the rate of uptake of glucose by adult worms was found to decrease with age and as the number of worms per infection increased. The data suggest that either the uptake system changes with age or there are two components of glucose absorption, one of which acts at high concentrations of glucose, the other at low concentrations, the distribution of which varies with age (Henderson, 1977). Subsequently, Cornford (1991) showed that the $K_m$ for glucose uptake increased with age. In 8-day worms the value is 14 nM $min^{-1}$ $g^{-1}$ increasing to 18, 21 and 39 nM $min^{-1}$ $g^{-1}$ on days 10, 17 and 32 respectively (the last two readings are for the first part of the worm, which generally has a greater rate of glucose uptake). Evidence suggest that regional differences occur in the distribution of glucose transporter proteins in the worm. This is supported by observations made by Rosen *et al.* (1994) on excysted scoleces. When excysted scoleces of *H. diminuta* were incubated for periods of up to 8 hours in saline with tritiated glucose, two abrupt $Ca^{2+}$-dependent increases in uptake velocity occurred, a small one between 0.25 and 1.25 hours and a much larger one between 5 and 6 hours. Similar changes did not occur for either leucine or uracil, suggesting that there are specific $Ca^{2+}$-dependent glucose transporters that increase in number with time.

These intraindividual variations highlight a major problem that arises when different worms from different cultivars are compared. Ovington and Bryant (1981) addressed this problem after comparing the published results of experiments that described organic acid excretion in *H. diminuta*. Using the argument that reliable techniques for the measurement of acetic, lactic and succinic acids existed 50 years ago and after making due allowance for differences in incubation, they concluded that what was being reported was an accurate account of what was observed by these early workers. This left variation between cultivars or between worms as a possible cause of the disparity in observations. Ovington and Bryant (1981) therefore examined acid excretion in an Australian (ANU) strain of *H. diminuta*. They found that it excreted mainly lactic and acetic acids, succinic acid representing less than 10% of the total output. However, one important source of variation was identified. At high $pCO_2$, the amount of succinate produced increased while that of lactate decreased. Acetate production was not affected.

Metabolic variation between the ANU strain of *H. diminuta* and Toronto strain was reported by Kohlhagen *et al.* (1985). The ANU strain produced relatively more lactate and less succinate under both aerobic and anaerobic conditions compared with a Toronto strain. During the shift from aerobic to anaerobic conditions, both strains produced more succi-

nate. When enzyme activities were measured, it was found that the ANU strain had higher activities of hexokinase, pyruvate kinase, lactate dehydrogenase, α-glycerophosphate dehydrogenase, and cytosolic and mitochondrial malic enzyme. The Toronto strain had higher activities of fumarase, succinate dehydrogenase and fumarate reductase. The activity of the latter was more than an order of magnitude greater and its $K_m$ was one-third that of the ANU strain, and it was less sensitive to inhibition by NAD or ATP. There were no differences in the activities of phosphoenolpyruvate carboxykinase and malate dehydrogenase. It was considered that these differences reflected differences between true genetic strains (Kohlhagen *et al.*, 1985).

After much study of the possible source of metabolic variation in *H. diminuta*, assaying hundreds of individual worms from over 100 rats all using standardized protocols, there emerged a picture of the influence of individual hosts on the metabolism of the cestode. Mature worms show great variation in the production of organic acid end-products and two distinct metabolic types have been identified (Bennet *et al.*, 1990). In Type A worms about 76% of the carbon from carbohydrate is metabolized by mitochondrial fermentation to succinate and acetate, while 24% appears in lactate. In Type B worms, about 86% of the carbon appears in lactate. Type B worms have not undergone transition from cytosolic to mitochondrial metabolism during maturation but have retained a pathway more characteristic of early stages of development (Bennet *et al.*, 1993). It could be argued that the two worm types are due to genetic heterogeneity of the parasite. However, the crucial observation that all worms recovered from each rat have the same type of metabolism makes this extremely unlikely. Further, Type B worms (that metabolize substrate less efficiently) are usually smaller and are recovered from rats in which the proportion of infective larvae developing to maturity is low. It is clear that some rats provide a less favourable environment for *H. diminuta* than other rats.

The host environment thus affects growth, survival to maturity and metabolism of the tapeworm, The host attributes that induce changes in *H. diminuta* may be immunologically based and not parasite specific. Fewer *H. diminuta* establish in rats previously infected with the nematode *Nippostrongylus brasiliensis*; in those worms that do establish, growth is slower and the metabolic transition from cytosolic to mitochondrial pathways is suppressed (Bennet *et al.*, 1993).

In other laboratories the effect of concurrent rather than previous infection with *N. brasiliensis* on *H. diminuta* have been examined. Hopkins and Andreassen (1991) found that fewer and smaller *H. diminuta* develop in rats concurrently infected with *N. brasiliensis*. They noted that similar effects on *H. diminuta* are observed in heavy *H. diminuta* infections and therefore these authors suggested that worm loss and growth inhibition in

heavy or secondary infections, initially thought to be a consequence of intraspecific competition for resources (crowding effect), is due to a host inflammatory response.

There are thus two sources of variation in *H. diminuta*: (i) the 'founder effect' of the establishment of genetic strains, which derives from the limited genetic constitution of the original sample parasites that were used in a particular cultivar, followed by a long period of selection and adaptation to the particular conditions obtaining in the laboratory of the cultivators; and (ii) the variation observed when individuals of a particular cultivar adapted to rats in different physiological and immunological states. There is probably also a third type of variation.

Two strains of *H. diminuta* were cultivated in different beetle hosts, *Tenebrio molitor* and *Tribolium confusum*, After recovery from the intermediate host and *in vitro* cultivation for 1 hour, differences were observed that were considered to be due to the strain of the parasite. This was the expected result. There was, however, a marked variation that could be ascribed to the genus of intermediate host in which the adult tapeworm had passed its larval life. Metabolic activity was greater in worms grown in *Tribolium confusum*, while intermediate pool sizes were larger with worms from *Tenebrio molitor* (Mettrick and Rahman, 1984).

Yan and Norman (1995) compares susceptibility to infection by *H. diminuta* in two species of *Tribolium*, *T. confusum* and *T. castaneum*. They found intraspecific variation in susceptibility and also significant intraspecific variation with strain and sex of beetle. Although the authors were more interested in beetle susceptibility as a genetically variable trait, it is also apparent that the beetles are theoretically capable of exerting selection pressure that might bring about strain variation within the worms.

The mechanism by which this variation comes about is still not understood. Two obvious explanations spring to mind. The first depends on the fact that a tapeworm produces a large number of eggs whose genetic constitution covers a wide range of possibilities. The intermediate host may act as a genomic sieve, by providing conditions that allow only worms of a particular genetic constitution to develop. Obviously, *Tenebrio* and *Tribolium* provide different sets of conditions, and this selective process is reflected in differences in the biochemistry of the adult. Alternatively, the intermediate hosts exert no selective pressure but allow a true, random sample to go through larval development. However, because development in *Tenebrio* takes place under different conditions from development in *Tribolium*, differences occur in the milieu in which the tapeworm genes are acting. These produce changes in larval integrity that become manifest in the adult. Information about the phenomenon of variation imposed by intermediate hosts is scanty and no conclusions can as yet be drawn.

## 5. CONCLUSION

Medzhitov and Janeway (1997) have recently drawn the attention of immunologists to a concept much beloved by ecologists. These are the twin concepts of *r*- and *K*-selection (MacArthur and Wilson, 1967). An *r*-selected organism is one in which lifespan is short, development is rapid and major investment is placed in the production of large numbers of offspring, and immunity is innate. A *K*-selected organism has a relatively long lifespan and a relative slow development, produces few offspring and immunity is both innate and adaptive. Like all generalizations, this is a simplification and organisms in real life fall somewhere in a continuum between extreme *r* and extreme *K*, and may or may not possess all the characteristics indicated above.

However, the concept does produce some useful insights. Parasites in general are heavily *r*-selected while their hosts, axiomatically, are less *r*- and more *K*-selected. Mammals are the great exponents of *K*-selection; even mammals like the rat, which have a high fecundity, are far more *K*-selected than the majority of invertebrates. *Hymenolepis diminuta* is clearly an *r*-selected organism, manifesting rapid and opportunist development and great fecundity. As far as anyone knows, the immunity displayed by the parasite is innate only, whereas the rat possesses not only innate immunity but also a well-developed adaptive immunity.

The adaptive immunity of the rat is the major obstacle that the worm must overcome in order to complete its life cycle. Host and parasite coevolve running the Red Queen's race (Van Valen, 1973) to stay in the same evolutionary position with respect to one another. During this time of coevolution the rat has to deal with myriad would-be invaders and has placed heavy emphasis on an adaptive immune system. The tapeworm has but one important problem and that is the adaptive immune system of the rat (this discussion is, of course, simplified and ignores adaptation to a secondary host, even though the arguments may be similar, and the special problems of transmission between hosts).

The results of this process, as far as they are known, are summarized in this review. On the one hand, the rat has developed an immune system that is a generalist but is capable of acting with specificity against viruses and prokaryote invaders but which is less effective against eukaryotes like *H. diminuta*. Cell-mediated immunity is thus prominent in the rat's immunological armour. On the other hand, the tapeworm has adopted a number of survival strategies that enable it to survive the onslaught of the rat. These strategies may not permit the parasite to reach its maximum reproductive potential, but they do provide an optimum for a given individual

host–parasite association and provide an opportunity, albeit reduced, for completing the life cycle.

Evolution is a compromise. The strategies adopted by *H. diminuta* are crafted from what was already present. The parasite carries with it much of the early evolutionary baggage for its grade of organization (Bryant, 1994) and this has resulted in a metabolism that it both similar to, yet different in important respects from, that of its host. The result is an immensely complicated relationship, with important consequences for both partners. In the 40 years that have elapsed since *H. diminuta* was considered to be a non-immunogenic commensal, parasitologists have only just begun to unravel the intricacies of this host–parasite relationship.

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# Control Strategies for Human Intestinal Nematode Infections

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## ABSTRACT

*In recent years significant progress has been made in understanding the ecology, epidemiology and related morbidity and development of new tools for the control of soil-transmitted helminths. Such knowledge has recognized the impact of helminth infections on the health of infected groups and has created a rational basis for their control. Schoolchildren harbour some of the most intense helminthic infections, which produce adverse effects on health, growth and scholastic performance. However, although great effort has been put into targeting school-age children, women of child-bearing age and pre-school children are two other groups at high risk of morbidity due to intestinal nematode infections. Highly effective and safety-tested, single-dose anthelminthic drugs are now available, permitting periodical deworming of schoolchildren and other high-risk groups at affordable prices. Four anthelminthics against all intestinal nematodes are included in the WHO Essential Drug List (albendazole, levamisole, mebendazole and pyrantel). Recently ivermectin has also been registered for use against* Strongyloides stercoralis *in humans. Several well-monitored country experiences have shown that chemotherapy-based control of morbidity due to soil-transmitted helminths is possible and highly cost-effective.*

## 1. INTRODUCTION

Infection by soil-transmitted helminths has been increasingly recognized as an important public health problem, particularly in developing countries. Progress has been made in the understanding of ecology, epidemiology and related morbidity and in developing new tools for control. Such knowledge has recognized the impact of helminth infections on health and has created a rational basis for the solution of the public health problem related to these infections.

In the 1993 *World Development Report* intestinal helminths rank first as the main cause of disease burden in children aged 5–14 years and also rank highly as the disease that can be efficiently controlled by cost-effective intervention (World Bank, 1993). Schoolchildren harbour some of the most intense helminthic infections, which produce adverse effects on health, growth and school performance. Treatment of this group achieves

the maximum return in terms of reduction of morbidity. Schoolchildren are also one of the most accessible groups for treatment, and health care can be efficiently integrated with education programmes. Highly effective and safety-tested single-dose anthelminthic drugs are now available, permitting periodical deworming of schoolchildren at affordable prices (Savioli *et al.*, 1992).

Although much effort has been put into targeting school-age children, women of child-bearing age and pre-school children are other groups at high risk of morbidity due to intestinal nematode infections. New strategies for control have been developed in the last decade and promising control programmes and operational research have been implemented. The aim of this article is to review the knowledge and experience of the control measures for intestinal helminth infections and highlight advances that have been made in the past few years.

## 2. PUBLIC HEALTH RELEVANCE OF INTESTINAL HELMINTHS: RECENT ESTIMATES

Intestinal nematode infections are among the most widespread diseases worldwide. Current estimates suggest that at least one-quarter of the world population is chronically infected with intestinal helminths and that most of these infected people live in developing countries.

The World Health Organization (WHO) (1996a) reports that *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms (*Ancylostoma duodenale* and *Necator americanus*) infect 1.4, 1.0 and 1.3 billion people respectively. Other estimates, based on projections of mathematical models, are of the same magnitude (Bundy *et al.*, 1996). *Strongyloides stercoralis* infects not less than 200 million people (WHO, 1992) and the morbidity related to chronic infection is not well known at the community level. Demographic perspectives and increasing urbanization in developing countries do not predict a decreasing trend. About one-third of the population in the cities of developing countries live in slums and shanty towns. By the year 2025 it is estimated that about 57% of the population in developing countries will live in urban areas. The prevalence and intensity of *A. lumbricoides* and *T. trichiura* infections are significantly increasing among populations who are migrating to these urban and suburban settings due to the favourable conditions of transmission (Crompton and Savioli, 1993).

The public health impact of intestinal helminth infections has been consistently underestimated. This is partly due to the fact that tools for assessing the disease related to helminthic infections have been lacking and this huge number of infections appeared to be related to little evident

disease. Therefore the public health community viewed nematode infections as a common but usually unimportant problem that merited low priority for attention. The mortality rate related to intestinal helminths is considered to be low; even so, the absolute number of deaths is fairly high because of the high prevalence of infection in developing countries: about 60 000 deaths per year occur for *A. lumbricoides* infection, most of them due to intestinal obstruction in young children; *T. trichiura* is estimated to be responsible for 10 000 deaths per year, due to massive dysentery syndrome; hookworm infections cause the death of 65 000 people per year as a consequence of severe iron-deficiency anaemia (WHO, 1996a). Clinical disease associated with nematode infections is well documented. A review of studies conducted throughout the tropics reported that ascariasis was the cause of paediatric intestinal obstruction in 5–35% of all cases (Pawlowski and Davis, 1989). Trichuriasis is associated with chronic dysentery and rectal prolapse in 4% of children studied in the West Indies (Bundy and Cooper, 1989), while 36 million people suffer from hookworm-related iron-deficiency anaemia (Bundy *et al.*, 1996).

However, these figures do not consider other more insidious consequences of chronic helminth infections on the health and development of children and women of child-bearing age. It has been increasingly recognized that ascariasis plays a major role in the aetiology and persistence of childhood malnutrition (Crompton, 1992) and has also been associated with micronutrient, particularly vitamin A, deficiency (Curtale *et al.*, 1995). *Trichuris trichiura* has been recognized as a contributor to undernutrition, growth stunting and iron-deficiency anaemia in intense infections (Cooper *et al.*, 1990). Hookworm infections, apart from the well-accepted role in contributing to iron-deficiency anaemia, are associated also with stunting of linear growth (Crompton and Stephenson, 1990). In addition, intestinal nematodes are thought to be responsible for reduced physical fitness and physical activity (Shetty and Shetty, 1992; Stephenson *et al.*, 1993). Cognitive performance is another effect of moderate infection underestimated in past decades, as interpreted as a covariant of socioeconomic status. Educational achievement has been negatively associated with hookworm infections since the beginning of the century (Waite and Neilson, 1919); more recently, intensity of infection of other nematode species has been inversely related to mental development and cognitive function of infected children (Nokes *et al.*, 1992). As a consequence, school attendance and performance may be affected in infected children, although further studies are needed to evaluate the direct effect of chronic helminth infection on educational achievements (Connolly and Kvalsvig, 1993; Nokes and Bundy, 1994).

A characteristic of the biology of helminth infection is that the occurrence of disease is related to the intensity of infection: infections with a few

worms tend to be asymptomatic but as more worms are acquired signs and symptoms of disease are more likely to occur (Bundy *et al.*, 1990). Intensity is the average number of worms within infected individuals in the population. However, the occurrence of disease is not just related to the number of worms in a host, but is likely to be related also to the duration of infection and to the background health status of the host.

In order to calculate updated estimates of possible associated morbidity, epidemiological studies have developed methods for estimating the relationship between prevalence and mean intensity and potential morbidity, also incorporating age classes and geographical heterogeneity (Chan *et al.*, 1994a). These estimates are based on empirical data and chosen to be relatively conservative. According to these calculations the morbidity associated with *A. lumbricoides* infection amounts to 122–214 million cases, 8–15% of the total number infected. The morbidity associated with *T. trichiura* infection is 87–133 million cases, corresponding to 8–13% of total *T. trichiura* infections. The overall prevalence of 1297 million hookworm infections corresponds to 61–96 million cases of associated morbidity, about 5–7% of total hookworm infections. The higher thresholds for *A. lumbricoides* and *T. trichiura* are based on empirical observations of worm burdens associated with developmental deficits or impairment in growth and fitness. The lower thresholds are more conservative values intended to reflect more serious clinical consequences. Hookworm thresholds are based on upper and lower bound estimates of the relationship between infection intensity and anaemia (Lwambo *et al.*, 1992). About one-third of *A. lumbricoides* and *T. trichiura* infections and of related morbidity occurs in China, while the highest prevalence of morbidity related to hookworm infection is in the Indian subcontinent (Chan *et al.*, 1994a; Peng *et al.*, 1995).

Another important high-risk group is women of child-bearing age. Iron-deficiency anaemia in pregnant women could be precipitated by hookworm infection in areas where these infections are endemic. Current available data do not allow a precise and direct observation of the disease burden. However, some analysis has provided estimates of the number of women that are both pregnant and infected with hookworm at a worm burden likely to be associated with disease (Bundy *et al.*, 1995). The number of pregnant women worldwide in 1990 was estimated to be 124 million (World Bank, 1993), of whom 44 million were estimated to be infected with hookworms. In sub-Saharan Africa, data suggest that 24 million women were pregnant in 1990, of whom 7.5 million were infected. The infected women might represent the number at risk of morbidity if disease is associated with low worm burdens. If risk of morbidity is determined by higher worm burdens (between 100 and 200 hookworms), the number at risk of morbidity is 3–5 million (Bundy *et al.*, 1995). A WHO informal consultation was

called in 1994 to address the control of hookworm infection and anaemia in girls and women (WHO, 1996b). Research studies are underway in tropical Africa and in Asia to relate the intensity of hookworm infections to health status of pregnant women and to pregnancy outcome, and to examine the relative benefit of anthelminthic treatment and iron-folate and vitamin A supplementation (Dreyfuss *et al.*, 1996).

As confirmed by the relationship of intensity of infections with age, the morbidity due to infection with *A. lumbricoides* and *T. trichiura* peaks in school-age children, remaining stable in older age groups. The morbidity due to hookworm infections is usually higher in adults, as expected by the increase in prevalence and morbidity of infection with age. Intensity typically reaches maximum levels at 15–20 years but does not necessarily level off during adulthood, even continuing to increase in older adults (Bundy, 1990).

Present estimates suggest that the potential morbidity attributable to soil-transmitted helminthiases is greater than previously supposed by WHO (1992) (Table 1). The very significant difference between these two sets of estimates may reflect differences in the definition of morbidity. The WHO estimates may be concerned only with the most severe clinical consequences, such as prostrating anaemia, intestinal obstruction and rectal prolapse.

## 3. INFECTION CONTROL OR DISEASE CONTROL?

As can be seen from prevalence and morbidity data, a distinction should be clearly made between prevalence of infection and prevalence of disease. It is important to realize that the majority of individuals with worms are healthy and will almost certainly remain so. Thus, in helminthiasis there is a great difference between infection and disease, words that are often used interchangeably and sometimes misunderstood.

The explanation of this situation is relatively simple, both biologically and epidemiologically. The helminths of interest here, in contrast to all other infectious agents (viruses, bacteria, fungi and protozoa), do not replicate in the definitive human host, with a very few exceptions such as strongyloidiasis, intestinal capillariasis and hymenolepiasis nana. Thus, for each egg that is ingested or each larva that penetrates the skin, only one worm will develop. The first important consequence is that the occurrence of disease is related to the intensity of infection based on the number of worms that successfully enter the body. Secondly, intestinal helminths are neither uniformly nor randomly distributed amongst individuals in a

*Table 1* Morbidity associated with intestinal helminth infections: world estimates of prevalence 1990. (Adapted from Bundy *et al.*, 1996)

| | High intensity of infection | | Cognitive deficit | | Cognitive impairment | | Intestinal obstruction | | Massive dysentry syndrome | | Anaemia | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. ($\times 10^3$) | Rate (per 100 000) | No. ($\times 10^3$) | Rate (per 100 000) | No. ($\times 10^3$) | Rate (per 100 000) | No. ($\times 10^3$) | Rate (per 100 000) | No. ($\times 10^3$) | Rate (per 100 000) | No. ($\times 10^3$) | Rate (per 100 000) |
| Ascariasis | 61 847 | 1174 | 14 585 | 277 | | | 42 | 0.8 | | | | |
| Trichuriasis | 45 421 | 862 | | | 39 682 | 753 | | | 3596 | 68 | | |
| Ancylostomiasis and necatoriasis | 152 492 | 2895 | | | 9568 | 182 | | | | | 36 014 | 684 |

population but are highly aggregated, so that most individuals have few worms while a few people harbour disproportionately large worm burdens (Anderson and May, 1985; Bundy *et al.*, 1992). Field studies have found that typically about 70% of the worms occur in 15–30% of the people (Thein Hlaing, 1985; Elkins *et al.*, 1988). This minority of heavily infected individuals includes people more likely to develop disease and who are also major sources of infection in the community. The characteristic aggregated or overdispersed distribution of helminth infections has led some to hypothesize that certain 'wormy people' are predisposed to heavy infections because of some as yet undefined genetic, ecological, behavioural or social factor. Numerous studies conducted to test this hypothesis have found that the infection intensity prior to anthelminthic treatment is significantly correlated with the infection intensity from reinfection after treatment (Schad and Anderson, 1985; Haswell-Elkins and Anderson, 1987; Bradley and Chandiwana, 1990; Upatham *et al.*, 1992). The causes of this predisposition to helminth infection have yet to be fully elucidated, and could be due to either higher exposure to infection or increased susceptibility because of immune response or genetic factors. This epidemiological characteristic of the disease has led some to suggest that an efficient, economic and effective helminth control programme would target for chemotherapy treatment those individuals predisposed to heavy infection (Schad and Anderson, 1985). However, it has been shown that such targeting is effective only if aggregation in parasite burden is due to long-term genetic factors rather than heterogeneity of exposure to infective stages (Anderson and Medley, 1985).

On the other hand, Bundy *et al.* (1985) and Hall *et al.* (1992), in studies from Jamaica and Bangladesh respectively, did not find evidence for predisposition to *T. trichiura* and *A. lumbricoides* infection and raised questions about the predisposition dogma, claiming that mass treatment, targeted to a population group at high risk of morbidity, is more cost-effective than selective treatment of heavily infected individuals. Selecting heavily infected individuals for treatment may be difficult and costly in practice because of (i) the lack of simple methods for accurately identifying 'wormy people'; (ii) the fact that where prevalence is very high most of the children (70%) present as heavily infected in repeated surveys (Hall *et al.*, 1992); and (iii) the poor compliance of this approach (Asaolu *et al.*, 1991). Furthermore, such a strategy would not address the fact that light or moderate hookworm infection is sufficient to cause anaemia in people with low iron stores and low iron intake.

In clinical practice, individuals either with disease or likely to develop disease can be detected and treated; however, at the community level, how to identify population groups at highest risk and to quantify disease at the community level is more complex and costly in the use of scarce resources.

A simple and inexpensive method to quantify disease at the community level from the estimated prevalence of infection has been proposed by Guyatt and Bundy (1991).

Different strategies are adopted and different timing has to be considered according to the goal to be met. Most control programmes now aim to reduce morbidity as an urgent but short-term goal. As a long-term goal, transmission of infection can be reduced by a combined approach using periodic chemotherapy, promotion and implementation of sanitation, and health education. Current helminth control programmes are focused on reducing infection load and transmission potential in order to reduce morbidity and the avoidance of mortality associated with the disease rather than eradication of helminth infection (Gilles, 1985; WHO, 1987). With tools presently at hand, attempts to eradicate worm infections by multiple methods will be extremely costly and will almost certainly fail in most areas of the world. However, attempts to control disease, i.e. to reduce the worm burden, can be successful, at reasonable cost (Warren, 1982; Savioli *et al.*, 1992). This quantitative concept was first postulated more than 50 years ago by Andrews in an article entitled 'Hookworm disease control methods in Georgia'. He succinctly described a hookworm control programme as aimed 'at the destruction of the greatest possible number of hookworms with the least expenditure of time, travel and material' (Andrews, 1942).

Chemotherapy with single-dose, safety-tested, effective anthelminthic drugs is now available and is the mainstay of control programmes that aim to control morbidity due to intestinal nematodes as a short-term goal (Savioli *et al.*, 1992). Reductions in the number of cases and in the worm load will inevitably lead, in the long run, to reductions in the rate of transmission (Anderson and May, 1982). There is evidence that chemotherapy targeted to the population group that harbour the heaviest worm burden results in benefit also in the adult non-treated population, presumably by clearing the sources of infection, reducing faecal contamination of the environment and, eventually, diminishing transmission. This have been advocated by epidemiological modelling (Chan *et al.*, 1994b) and validated by field studies (Bundy *et al.*, 1990). However, mathematical models suggest that about 30% of the community should be treated every 2 months for many years in areas of moderate to high transmission to suppress prevalence of helminthic infections at very low levels (Anderson, 1989); this has been confirmed by field experience from control programmes in different countries (Seo and Chai, 1980; Thein Hlaing, 1985). In practice, improvements in hygiene and sanitation will have to occur if the long-term suppression of transmission is to be achieved and sustained.

## 4. STRATEGIES FOR CONTROL: AN UPDATE

### 4.1. Chemotherapy

Periodic anthelminthic chemotherapy, ideally in the context of efforts to promote sanitation, is the key intervention in current intestinal nematode control strategies. Recent information about recommended drugs for helminth infections is summarized in Table 2. Four anthelminthic drugs described in the WHO (1997a) Model List of Essential Drugs, albendazole, levamisole, mebendazole and pyrantel, are considered for the treatment of intestinal nematodes. Ivermectin is listed as an antifilarial drug, but has recently been registered in France, Australia and the USA for the treatment of strongyloidiasis and is now considered the drug of choice for this infection. Piperazine is still used in the treatment of ascariasis in some countries and thiabendazole is widely available for the treatment of strongyloidiasis.

#### 4.1.1. *Albendazole*

(a) *Pharmaceutical form.* Chewable tablets, 200 and 400 mg, oral suspension 100 mg per 5 ml.

(b) *Category.* Benzimidazole derivative.

(c) *Mode of action.* All benzimidazoles have a similar mechanism of action. The drugs selectively bind to nematode tubulin, inhibiting the tubulin polymerase, preventing the formation of microtubules and so stopping cell division. The loss of cytoplasmic microtubules disturbs the uptake of glucose and causes depletion of glycogen, hampering the formation of ATP required for survival and reproduction of the worms and impairing nutrient uptake by the parasite (Lacey, 1990). The effect on microtubules has been observed in developing helminth eggs, where it prevents hatching (Borgers and Nollin, 1975; Lacey *et al.*, 1987). Albendazole, mebendazole and thiabendazole also have larvicidal and ovicidal effects (Van Den Bossche *et al.*, 1982; Maisonneuve *et al.*, 1985; Lacey *et al.*, 1987). Thiabendazole and the other benzimidazole-2-carbamates have been also shown to inhibit mitochondrial fumarate reductase, which is specific for helminths (Sheth, 1975).

(d) *Pharmacokinetics.* The drug is poorly absorbed, with most of its anthelminthic action directly in the gastrointestinal tract. That which is absorbed is rapidly and extensively metabolized by the liver, and the active sulphoxide metabolite is found in plasma at peak concentrations of 0.04–0.55 g/ml$^{-1}$ after 1–4 hours when given at a higher dosage than 400 mg. When the drug is given with a fatty meal, a two to four-fold increase in plasma concentrations is observed. The plasma concentration of albendazole

*Table 2* Recommended drugs for the treatment of soil-transmitted nematode infections

| Substance | Therapeutic activity | | Dosage | Use in pregnancy and in children |
|---|---|---|---|---|
| Albendazole (tablet 200 and 400 mg, suspension 100 mg per 5 ml) | Ascariasis | +++ | 400 mg single dose | Not recommended in the first trimester of pregnancy and in children under 2 years |
| | Trichuriasis | ++ | 400 mg single dose | |
| | Hookworm infections | +++ | 400 mg single dose | |
| | Strongyloidiasis | ++ | 400 mg daily for three days | |
| Ivermectin (tablet 6 mg) | Ascariasis | +++ | 200 μg $kg^{-1}$ single dose | Not recommended in the first trimester of pregnancy |
| | Trichuriasis | + | 200 μg $kg^{-1}$ single dose | |
| | Hookworm infections | – | | |
| | Strongyloidiasis | +++ | 200 μg $kg^{-1}$ single dose | |
| Levamisole (tablet 40 mg, syrup 40 mg per 5 ml) | Ascariasis | +++ | 2.5 mg $kg^{-1}$ single dose | No evidence of teratogenicity |
| | Trichuriasis | + | 2.5 mg $kg^{-1}$ single dose | |
| | Hookworm infections | ++ | 2.5 mg $kg^{-1}$ single dose; for heavy necatoriasis repeat after 7 days | |
| | Strongyloidiasis | – | | |
| Mebendazole (tablet 100 and 500 mg, suspension 100 mg per 5 ml | Ascariasis | +++ | 500 mg single dose | Not recommended in the first trimester of pregnancy and in children under 2 years |
| | Trichuriasis | ++ | 100 mg twice daily for 3 days; 500 mg single dose (less effective) | |
| | Hookworm infections | +++ | 100 mg twice daily for 3 days; 500 mg single dose (less effective) | |
| | Strongyloidiasis | + | 100 mg twice daily for 28 days | |
| Piperazine (tablet 500 mg, elixir 500 mg per 5 ml) | Ascariasis | +++ | 75 mg $kg^{-1}$ single dose | Not recommended in the first trimester of pregnancy; the dosage in children under 2 years is 50 mg $kg^{-1}$ |
| | Trichuriasis | – | | |
| | Hookworm infections | – | | |
| | Strongyloidiasis | – | | |
| Pyrantel (tablet 250 mg, suspension 50 mg per 5 ml) | Ascariasis | +++ | 10 mg $kg^{-1}$ single dose | Not recommended in the first trimester of pregnancy |
| | Trichuriasis | – | | |
| | Hookworm infections | ++ | 10 mg $kg^{-1}$ single dose; for heavy necatoriasis repeat for 3 days | |
| | Strongyloidiasis | – | | |
| Thiabendazole (tablet 500 mg, suspension 100 mg per 5 ml) | Ascariasis | ++ | 25 mg $kg^{-1}$ daily for 2 days | Not recommended in the first trimester of pregnancy |
| | Trichuriasis | – | | |
| | Hookworm infections | – | | |
| | Strongyloidiasis | +++ | 25 mg $kg^{-1}$ daily for 3 days | |

is 15–49 times higher than that of mebendazole. Up to 70% of albendazole binds to plasma protein. The sulphoxide metabolite has a plasma elimination half-life of 9 hours. The absorbed fraction is largely excreted through the kidneys and to some extent in the bile as the sulphoxide (Marriner *et al.*, 1986; Gottschall *et al.*, 1990). Albendazole sulphoxide crosses the blood–brain barrier and attains a concentration in the cerebrospinal fluid one-third that in plasma (Jung *et al.*, 1990). The bioavailability of the active sulphoxide metabolite is enhanced by cimetidine, an inhibitor of cytochrome P450 that may suppress the metabolism of the drug (Wen *et al.*, 1994).

(e) *Dose.* A single dose of 400 mg is sufficient to eliminate ascariasis, enterobiasis and hookworm infections. Strongyloidiasis and heavy *T. trichiura* infection may require a 3-day course of treatment.

(f) *Efficacy.* As a single dose it is very effective against *A. lumbricoides*, with a cure rate (CR) and egg reduction rate (ERR) between 92 and 100%. The drug is effective against hookworms (CR 57–95% and ERR 79–99%) and has been shown to be more active than other anthelminthics against *N. americanus.* It is less effective against *T. trichiura*, with a CR between 10 and 77% and an ERR between 61 and 99% (Pene *et al.*, 1981; Ramaligam *et al.*, 1983; Rossignol and Maisonneuve, 1983; Coulaud and Rossignol, 1984; Ismail *et al.*, 1991; Long Qi *et al.*, 1992; Albonico *et al.*, 1994a). Heavy infections with *T. trichiura* respond well to a 3-day course of albendazole (Hall and Nahar, 1994). For the treatment of *S. stercoralis* infection, a course of 400 mg for 3 days was reported with a CR between 38 and 95% (Archibald *et al.*, 1993; Datry *et al.*, 1994; Pitisuttithum *et al.*, 1995; Marti *et al.*, 1996), but more controlled studies are needed to confirm its advantage over thiabendazole and to compare its efficacy with ivermectin, which is now the drug of choice (Table 3).

(g) *Side-effects.* After a single-dose treatment of albendazole 400 mg, minor and transient side-effects are seen. A very few cases report migration of *A. lumbricoides* through the mouth, occasional gastrointestinal symptoms (epigastric pain 0.3%, diarrhoea 0.3%, nausea 0.2%, vomiting 0.1%), central nervous system (CNS) symptoms (headache 0.2%, dizziness 0.1%) and rare allergic phenomena (oedema 0.7/1000, rashes 0.2/1000, urticaria 0.1/1000). All are transient and disappear after 48 hours (Rossignol and Maisonneuve, 1983; Coulaud and Rossignol, 1984; Albonico *et al.*, 1994a). Liver function abnormalities and leucopenia have been reported occasionally, but only at higher dosages and after prolonged treatment (Morris and Smith, 1987; Davis *et al.*, 1989). Although in the prescribing information the drug is not recommended in children below 2 years, there is no evidence that albendazole, when administered to children 9–23 months of age, has any side-effect or causes laboratory abnormalities (Mbendi *et al.*, 1988).

*Table 3* Efficacy and safety trials of albendazole, ivermectin, mebendazole and thiabendazole for the treatment of strongyloidiasis

| Study | Sample size | Regimen | Follow-up | Cure rate (%) | Side-effects |
|---|---|---|---|---|---|
| Beus (1989) | 110 | Thiabendazole 25 mg $kg^{-1}$ b.d. for 2 days | 3 months | 96 | Not reported |
| | 41 | Mebendazole 10 mg $kg^{-1}$ daily for 5 days | | 44 | |
| Shikiya *et al.* (1990) | 26 | Thiabendazole 500 mg t.d. for 5 days + mebendazole 100 mg daily for 9 days | 1 month | 100 | Nausea (19%), headache (15%), increased transaminase (52%) |
| | 33 | Mebendazole 100 mg b.d. for 28 days | | 83 | Constipation, headache (9%); increased transaminase (71%) |
| Gann *et al.* (1994) | 19 | Thiabendazole 25 mg $kg^{-1}$ b.d. for 3 days | 3 months | 89 | Disorientation, fatigue or nausea (95%) |
| | 16 | Ivermectin 200 μg $kg^{-1}$ single dose | | 100 | Itching, lightheadedness or fatigue (12%) |
| | 18 | Ivermectin 200 μg $kg^{-1}$ single dose for 2 days | | 100 | Itching, lightheadedness or fatigue (12%) |
| Shikiya *et al.* (1994) | 125 | Ivermectin 6 mg single dose repeated in 2 weeks | 2 weeks | 86 | Liver dysfunction (14%) |
| Archibald *et al.* (1993) | 47 | Albendazole 400 mg b.d. for 3 days | 6 months | 75 | Nausea and diarrhoea (0.5%) |
| Datry *et al.* (1994) | 24 | Albendazole 400 mg daily for 3 days | 3 months | 38 | Abdominal pain, nausea, vertigo (8%); mild increased transaminase (4%) |
| | 29 | Ivermectin 200 μg $kg^{-1}$ single dose | | 83 | Fatigue, nausea, tremor, increased transaminase, leucopenia (3%) |
| Pitisutthitum *et al.* (1995) | 12 | Thiabendazole 1 g b.d. for 5 days | 3 weeks | 100 | Anorexia, weakness, headache, insomnia (20%); nausea, rash, myalgia (10% |
| | 19 | Albendazole 400 mg b.d. for 5 days | | 95 | Increased transaminase (20%); weakness, headache (10%); rash, insomnia, nausea (2%) |
| Marti *et al.* (1996) | 149 | Albendazole 400 mg daily for 3 days | 3 weeks | 45 | Headache, loose stools (10%); dizziness (6%); cough, fever (5%); nausea, itching (3%) |
| | 152 | Ivermectin 200 μg $kg^{-1}$ single dose | | 83 | Headache, loose stools (10%); cough (7%); fever (6%); chest tightness, abdominal distension (4%); dizziness, nausea (3%); itching (2%) |

(h) *Pregnancy.* Albendazole has been shown to be teratogenic in rats and rabbits at high dosage. There are no reports of malformations following inadvertent exposure of 15 patients during the first trimester of pregnancy to high multiple doses; however, albendazole should not be administered in the first trimester (WHO, 1996b).

4.1.2. *Ivermectin*

(a) *Pharmaceutical form.* Chewable tablet, 6 mg.

(b) *Category.* Macrocyclic lactone.

(c) *Mode of action.* The drug causes paralysis in many nematodes and arthropods through the influx of chloride ions across cell membranes and from the disruption of nerve signal transmission mediated by γ-aminobutyric acid (GABA) (Mellin *et al.*, 1983; Abalis *et al.*, 1986).

(d) *Pharmacokinetics.* The drug is absorbed into the blood following oral administration and peak levels are reached 4 hours after oral administration. It is excreted almost entirely in the faeces (Lo *et al.*, 1985; Wilkinson *et al.*, 1985).

(e) *Dose.* Single administration of 200 $\mu g\ kg^{-1}$.

(f) *Efficacy.* Few trials have investigated the use of ivermectin for the treatment of intestinal helminth infections. Despite its multitude of uses in veterinary medicine and its activity against various life-cycle stages of many nematodes, in human medicine its use has been almost exclusively as a microfilaricide for the treatment of onchocerciasis. It is currently licensed in France for the treatment of *S. stercoralis.* The drug has been reported to be 100% effective against *A. lumbricoides* at dosages of 50–200 $\mu g\ kg^{-1}$ (Whitworth *et al.*, 1991; Njoo *et al.*, 1993), 82–95% effective against *S. stercoralis* in single or two administrations of more than 150 $\mu g\ kg^{-1}$ (Naquira *et al.*, 1989; Datry *et al.*, 1994; Shikiya *et al.*, 1994; Marti *et al.*, 1996), 11–100% effective against *T. trichiura* according to the different dosage used (from 200 $\mu g\ kg^{-1}$ single dose to 400 $\mu g\ kg^{-1}$ in two divided doses with administration over 2 days) (Freedman *et al.*, 1989; Naquira *et al.*, 1989; Njoo *et al.*, 1993; Marti *et al.*, 1996) and 0–20% effective on hookworms (Testa *et al.*, 1990; Richards *et al.*, 1995; Marti *et al.*, 1996). The comparative efficacy of drugs against *S. stercoralis* infection is shown in Table 3.

(g) *Side-effects.* Mild adverse reactions (e.g. borborygmus, diarrhoea, constipation, headache) have been reported in a small minority of patients (Whitworth *et al.*, 1991; Archibald *et al.*, 1993; Datry *et al.*, 1994; Marti *et al.*, 1996).

(h) *Pregnancy.* Ivermectin should not be administered during pregnancy (WHO, 1995a).

4.1.3. *Levamisole*

(a) *Pharmaceutical form.* Chewable tablet, 40 mg.

(b) *Category.* Laevorotatory isomer of tetramisole.

(c) *Mode of action.* Binds to the acetylcholine receptors of the autonomic ganglia of nematodes causing spastic contraction followed by tonic paralysis. The worms are then passively eliminated (van Wauwe and Janssen, 1991; Martin, 1993).

(d) *Pharmacokinetics.* Levamisole is highly soluble in water and is rapidly absorbed almost completely by the gastrointestinal tract. Peak plasma levels are reached within 2 hours of administration. The drug is metabolized in the liver and eliminated in the urine largely as metabolites within 2 days (Adams, 1978; Kouassi *et al.*, 1986).

(e) *Dose.* Single administration of 2.5 mg $kg^{-1}$.

(f) *Efficacy.* There are more than 60 clinical trials showing the high efficacy of levamisole against *A. lumbricoides* and hookworm infections. CRs of 86–100% and ERRs of 96–98% with a single dose have been reported for *A. lumbricoides* infection (Lionel *et al.*, 1969; Thienpoint *et al.*, 1969; Lucas and Oduntan, 1972; Moens *et al.*, 1978). For hookworm infection, CRs are 66-100% and ERRs of 86–99% have been reported (Lucas and Oduntan, 1972; Huys *et al.*, 1976; Farid *et al.*, 1977). In the case of severe hookworm infections (*N. americanus*) a second standard dose may be given 7 days after the first treatment (Al-Saffar *et al.*, 1971). The drug has a lower efficacy against *T. trichiura* (CR 16–18%, ERR 73%) (Ismail *et al.*, 1991).

(g) *Side-effects.* Occasional vomiting (5%), dizziness (3%), headache (3%) and weakness (2%) occur which are mild and transient (Lionel *et al.*, 1969; Farid *et al.*, 1977). No laboratory abnormalities are detected at single dose of 2.5 mg $kg^{-1}$. The drug has shown no teratogenic effect at doses up to 40 mg $kg^{-1}$ in rats and in farm animals.

(h) *Pregnancy.* Levamisole has shown no evidence of teratogenicity and embryotoxicity in animals and it may represent the safer drug for use in pregnant women, although no drug should be administered during the first trimester (WHO, 1996b).

4.1.4. *Mebendazole*

(a) *Pharmaceutical form.* Chewable tablets of 100 and 500 mg, oral suspension 100 mg per 5 ml.

(b) *Category.* Benzimidazole derivative.

(c) *Mode of action.* Same as for the other benzimidazoles.

(d) *Pharmacokinetics.* Almost insoluble in water, in humans it is poorly absorbed by the gastrointestinal tract with an oral bioavailability of less

than 20% (Dawson *et al.*, 1985). Its absorption can be increased several times if taken with a fatty meal. Peak plasma levels are reached within 4 hours (Munst *et al.*, 1980). About 95% of the drug is bound to plasma proteins. The portion absorbed is rapidly metabolized by the liver and excreted in the bile. Plasma concentrations of mebendazole are detectable only with high dosages (1.5 g) (Dawson *et al.*, 1985). The drug is largely excreted unchanged via the bile through the faeces (Gottschall *et al.*, 1990).

(e) *Dose.* The ideal dosage for hookworm and *T. trichiura* infections is 100 mg twice daily for three consecutive days. A second course may be given after 3–4 weeks if eggs persist in faeces. A 500-mg single-dose administration is effective for ascariasis and is used to control hookworm infections and trichuriasis in community-based controlled programmes (WHO, 1995a). For enterobiasis a single dose of 100 mg repeated after 2–3 weeks is sufficient to clear the infection.

(f) *Efficacy.* In open clinical studies in patients with ascariasis, CRs of 95–100% were reported after treatment with mebendazole 100 mg twice daily for 3 days (Hutchison *et al.*, 1975; Chavarria *et al.*, 1973; Wagner and Rexinger, 1978). Similar CRs have been achieved in patients infected with *N. americanus* and *Ancylostoma duodenale* (Chavarria *et al.*, 1973; Hutchison *et al.*, 1975) using the same regimen. In *T. trichiura* infection, CRs can vary from 45 to 100% (Blechman, 1975; Scragg and Proctor, 1977). The efficacy of the 500-mg single dose is similar to the 3-day regimen for the treatment of *A. lumbricoides.* It is less effective in curing *T. trichiura* and hookworm infections, although it significantly reduces the worm burden (ERR 48-100%) (Abadi, 1985; Cawenbergh, 1985; Evans *et al.*, 1987; Ismail *et al.*, 1991; Charoenlarp *et al.*, 1993; Albonico *et al.*, 1994a, b). Efficacy studies with mebendazole 500 mg as single dose are summarized in Table 2. Mebendazole has been reported of some efficacy in *S. stercoralis* infection if used at a dose of 200 mg daily for 4 weeks (Mravak *et al.*, 1983; Beus, 1989).

(g) *Side-effects.* A few cases are reported of erratic migration of *A. lumbricoides,* mild gastrointestinal disturbance, transient abdominal pain and diarrhoea (Chavarria *et al.*, 1973; Pereira *et al.*, 1979; Muttalib *et al.*, 1981; Albonico *et al.*, 1994a). Only high doses (50 mg $kg^{-1}$ for 3–4 weeks) may give rise to severe abdominal pain, raised transaminase levels, allergic conditions, CNS symptoms (vertigo, headache), neutropenia and bone marrow depression (Miskovitz and Javitt, 1980; Kammerer and Scantz, 1984; Fernandez Banares *et al.*, 1986). Although mebendazole is not recommended in children below 2 years, studies have shown that the drug can be administered to younger children without any observable side-effects (Albonico *et al.*, 1994b).

(h) *Pregnancy.* Mebendazole has been shown experimentally to have teratogenic potential in rats at doses of 40 mg $kg^{-1}$ (Van den Bossche

*et al.*, 1982). A survey in pregnant women who inadvertently took mebendazole in the first trimester has not shown spontaneous abortion or malformations greater than those of the general population (Food and Drug Administration, 1992). However, WHO recommends that drugs should not be administered during the first trimester (WHO, 1995a, 1996b).

#### 4.1.5. *Piperazine*

(a) *Pharmaceutical form.* Tablets of 500 mg hydrate, elixir 500 mg per 5 ml.

(b) *Category.* Heterocyclic organic base.

(c) *Mode of action.* Piperazine blocks the response of *A. lumbricoides* muscles to acetylcholine, inducing flaccid paralysis that results in expulsion of the worms by intestinal peristalsis (del Castillo *et al.*, 1964).

(d) *Pharmacokinetics.* It is rapidly absorbed through the oral route and about 20% is excreted unchanged in the urine (Fletcher *et al.*, 1982).

(e) *Dose.* A single dose of 75 mg $kg^{-1}$ up to 3.5 g for adults and 2.5 g for children between 2 and 12 years; a single dose of 50 mg $kg^{-1}$ in children below 2 years (WHO, 1995a).

(f) *Efficacy.* In the treatment of infection due to *A. lumbricoides*, CRs of 74% after 1 day of therapy and 94% after 2 days of therapy have been reported (Brown *et al.*, 1956).

(g) *Side-effects.* Very occasionally patients exhibit gastrointestinal disturbance (nausea, vomiting, diarrhoea), allergic reactions (urticaria, exanthema, bronchospasm) and neurological effects (vertigo, ataxia, hallucination, visual disturbance and increase in petit mal attacks). For this reason it is contraindicated in patients with a history of epilepsy (Abdi *et al.*, 1995).

(h) *Pregnancy.* Piperazine has been used in pregnancy without ill effects. However, it should preferably not be administered during the first trimester (WHO, 1995a).

#### 4.1.6. *Pyrantel*

(a) *Pharmaceutical form.* Chewable tablet 250 mg (as embonate).

(b) *Category.* Pyrimidine derivative.

(c) *Mode of action.* Binds to the acetylcholine receptors of nematodes depolarizing neuromuscular blocking agents. They induce nicotinic activation, causing spastic paralysis followed by passive elimination of the worms (Aubry *et al.*, 1970).

(d) *Pharmacokinetics.* Insoluble in water, it is poorly absorbed in the gastrointestinal tract. Partially and rapidly metabolized in the liver, a small percentage is excreted with the urine and a large amount is eliminated unchanged in faeces (Webster, 1991).

(e) *Dose.* Single administration of 10 mg $kg^{-1}$.

(f) *Efficacy.* In more than 40 trials, a single dose of 10 mg $kg^{-1}$ has been shown to be effective against *A. lumbricoides* (CR 81–100%, ERR 82–100%) (Farahmandian *et al.*, 1977; Tankhiwale *et al.*, 1989; Seo, 1990). A single dose of 5 mg $kg^{-1}$ has also proved to be effective against *A. lumbricoides*, giving a CR of about 97% (Kobayashi *et al.*, 1970). The drug is also effective against hookworms (CR 37–88%, ERR 64–90%) (Haswell-Elkins *et al.*, 1988; Nahmias *et al.*, 1989). It has been shown to be more effective against *Ancylostoma duodenale* (ERR 85%) than against *N. americanus*, where repeated doses are needed especially to cure heavy infections (ERR 71%) (WHO, 1995a).

(g) *Side-effects.* In a study on 1506 individuals, side-effects were mild and transient including occasional diarrhoea (4.3%), abdominal pain (4%), nausea (3.5%), vomiting (2%) and headache (3%) (Pitts and Migliardi, 1974). Transient raised serum transaminase was detected in 2% of patients.

(h) *Pregnancy.* Tests in rats, sheep and rabbits have shown no teratogenic effects, but as with other drugs pyrantel should not be administered during the first trimester of pregnancy.

### 4.1.7. *Thiabendazole*

(a) *Pharmaceutical form.* Chewable tablets of 500 mg, oral suspension 500 mg per 5 ml.

(b) *Category.* Benzimidazole derivative.

(c) *Mode of action.* Same as for the benzimidazoles.

(d) *Pharmacokinetics.* The drug can be administrated orally, topically and rectally. The drug is quickly absorbed and peak plasma levels of 13–18 mg $ml^{-1}$ are obtained 1–2 hours after drug administration. Drug plasma levels decline rapidly between 24 and 48 hours. About 87% is excreted with the urine in the form of glucuronides or sulphates of 5-hydroxy-thiabendazole (Tocco *et al.*, 1966; Gottschall *et al.*, 1990).

(e) *Dose.* The standard dose for *S. stercoralis* infection is 25 mg $kg^{-1}$ daily in three divided doses for three consecutive days, taken preferably after meals. The total daily dose should not exceed 3 g. A course of 2 days is required when treating cutaneous larva migrans; the condition has also been treated successfully by topical application of thiabendazole. Treatment with thiabendazole for disseminated strongyloidiasis should be continued for at least 5 days (Webster, 1991).

(f) *Efficacy.* The drug was reported to be effective in a clinical study on prisoners of war in the Far East infected with *S. stercoralis* (Gill and Bell, 1979). In randomized trials thiabendazole (50 mg $kg^{-1}$ given twice daily for 3 days) was compared with ivermectin and albendazole, giving CRs between 89 and 100% (Gann *et al.*, 1994; Pitisuttithum *et al.*, 1995) (see

Table 3). Satisfactory results have been reported after treatment of cutaneous larva migrans with topical application of 14% thiabendazole suspension applied five to six times for 2 weeks (Whiting, 1976) as well as oral treatment with thiabendazole 25 mg $kg^{-1}$ for 2 days. The results of thiabendazole treatment of *A. lumbricoides*, *Ancylostoma duodenale* and *N. americanus* have been variable. Questionable results have also been reported in patients with trichuriasis (Stuart and Welch, 1973; Farahmandian *et al.*, 1977; Kale, 1977).

(g) *Side-effects.* These are of fairly frequent occurrence and include nausea, vomiting, headache and dizziness. In two studies, 40–89% of the patients experienced side-effects, including nausea (67%), neuropsychiatric symptoms (23%), malaise (16%), anorexia (16%), vomiting and abdominal pain (7%). Side-effects occurred 1–4 hours after ingestion of the drug and lasted for up 8–12 hours (Farahmandian *et al.*, 1977; Grove, 1982). Occasionally cholestatic jaundice, skin reactions, crystalluria, diarrhoea, fatigue, pruritus, tinnitus, numbness and drying of mucous membrane may occur. Single cases of Stevens–Johnson syndrome and toxic epidermal necrosis have been reported (Robinson and Samorodin, 1976). Some patients may excrete a metabolite that imparts an odour to urine similar to that which occurs after eating asparagus (Robinson *et al.*, 1978). Concomitant administration of thiabendazole may raise serum levels of theophylline and other xanthine derivatives above the toxic threshold (Lew *et al.*, 1989).

(h) *Pregnancy.* Teratogenicity has not generally been reported in laboratory animals (Lankas and Wise, 1994; Wise *et al.*, 1994) except in one study in mice after giving high doses in early pregnancy (Ogata *et al.*, 1984). No ill effects have been reported in a baby born to a mother treated with the drug during pregnancy (Robinson *et al.*, 1978). However, it is recommended to avoid the drug during the first trimester, unless there is a strong indication for use (WHO, 1990a).

### 4.1.8. *Choice of Anthelminthic Drugs*

The choice of anthelminthic drug for use in a control programme depends on (i) its safety record, (ii) its therapeutic effect (cure rate or efficacy), (iii) its spectrum of activity, (iv) local health policy and (v) financial considerations. Confidence and experience in use of the drug should also be taken into account. With these elements in mind, the choice of anthelminthic drug in public health should be tailored to the local epidemiology of soil-transmitted helminth infections. Information should be collected on prevalence and intensity of helminth infections, the population groups at highest risk of morbidity and the health impact of helminth infections in the community that can benefit from treatment. This body of information

should be the rationale for local health planners to choose the anthelminthic drug to be used in public health and should be the backbone of a successful chemotherapy-based control programme.

#### 4.1.9. *Quality*

Drugs must be manufactured according to good practice and be of sufficient quality to perform as required. It is known that many counterfeit products exist and these may be used inadvertently in control programmes. With generic products there may be variations in pharmaceutical formulation, bioavailability and therapeutic equivalence from the originator product. It is necessary to demonstrate that a product is not counterfeit or substandard and actually contains the stated compound. A simple thin-layer chromatographic method has been produced for the identification of impurities and active ingredients in essential drugs, including albendazole and mebendazole (Pachaly *et al.*, 1994). It is also important to know how the product behaves with regard to features such as weight of active compounds and excipients used, disintegration and dissolution tests, using standard pharmacopoeial methods to compare the selected drug with the originator product. In the absence of reasonable similarity, the only approach would be a demonstration of efficacy *in vivo* (Albonico *et al.*, 1994a). In this context a careful balance between quality of product and cost must be made, and contracts to purchase anthelminthic drugs should be dependent on demonstration of adequate quality assured by independent analysis.

#### 4.1.10. *Strategies for the Use of Anthelminthic Drugs in the Treatment of Intestinal Helminth Infections in Control Programmes*

In the case of control programmes designed to deal with helminth infections, the general body of epidemiological knowledge suggests that drugs may be used in the community in one of three ways: universal, targeted or selective treatment (WHO, 1996c).

1. Universal: population-level application in which the community is treated irrespective of their ages, sex, worm burdens or other social characteristics of the individuals in the population.
2. Targeted: group-level application where the group may be defined by age, sex, religion or other social characteristics.
3. Selective: individual-level application of anthelminthics where selection is based on the intensity of current or past infection.

In practice, selective treatment is difficult to achieve because it requires extensive technical effort to identify heavily infected individuals and it is

not likely to be well accepted in the community. Targeted treatment, especially when aimed at schoolchildren who are relatively easy to reach and may comprise 30% of the community, has proved to be an extremely acceptable strategy (Thein Hlaing, 1989). Universal treatment is equally effective and well received, but requires more drug and delivery may be difficult (Asaolu *et al.*, 1991).

#### 4.1.11. *Measuring the Effects of Anthelminthic Drugs*

Three types of effect can be measured: (i) CRs can be estimated; (ii) reductions in prevalence and intensity rates (efficacy) can be measured by the collection and microscopic examination of stool samples; and (iii) feedback from the community in the control programme can be obtained.

The efficacy of anthelminthic drugs at different dosages for the treatment of intestinal nematode infections has been evaluated in numerous trials. Efficacy is usually measured with qualitative and quantitative diagnostic tests that assess the eggs or larvae in faeces after an optimal time interval variable for each parasite. CR and ERR are indicators commonly used to measure the reduction in prevalence and in intensity of infection. However, a wide variability of efficacy is found, even in trials in which the same drug is given at the same dosages (Table 4). The lack of use of a standardized parasitological technique, the different length of follow-up after treatment, the presence of single or multiple infections before treatment, the different pretreatment prevalence and intensity rates, the age group enrolled, the sample size and the fact that many trials were not placebo controlled account for the difference in efficacy of the same drug in epidemiological studies. In addition, diverse statistical measurements of intensity (arithmetic or geometric means) have been used, making the results of efficacy trials more difficult to compare and interpret. The need for more standardized guidelines in order to perform survey studies that can be compared has been sought in a recent informal consultation held in WHO (WHO, 1996c).

#### 4.1.12. *Determination of the Appropriate Interval for Chemotherapy*

A key determinant for the optimal use of anthelminthic drugs is to decide when and how frequently to treat the population of concern. The results of the epidemiological survey to determine the reinfection rate are important for determining the treatment schedules (Hall *et al.*, 1992). Usually, the treatment of intestinal helminth infections should be more frequent during the early phase of the programme, with up to three treatments a year in areas of high endemicity (Albonico *et al.*, 1995). Annual treatments or treatments at longer intervals may be acceptable thereafter, depending on

*Table 4* Review of some comparative trials among single-dose anthelminthics

| Study | Drug | No. of patients | *A. lumbricoides* | | *T. trichiura* | | Hookworms | | Technique and post-examination treatment |
|---|---|---|---|---|---|---|---|---|---|
| | | | CR[a] (%) | ERR[b] (%) | CR (%) | ERR (%) | CR (%) | ERR (%) | |
| Moens *et al.* (1978) (multicentre) | Levamisole | 768 | 91 | 98 | | | | | Kato–Katz, 30 days |
| | Pyrantel | 157 | 87 | 96 | | | | | |
| Kan (1986) (Malaysia) | Albendazole | 33 | | | 33[c] | 88 | | | Kato–Katz, 30–45 days |
| | Pyrantel | 51 | | | 68 | 90 | | | |
| Mbendi *et al.* (1988) (Zaire) | Albendazole | 450 | 100 | | 100 | | 100 | | Formol ether, 7 days |
| | Pyrantel | 125 | 100 | | 0 | | 88 | | |
| Sinniah *et al.* (1990) (Malaysia) | Albendazole | 50 | 91 | 99 | 42 | 71 | 100 | 100 | Flotation + Beavers, 21 days |
| | Pyrantel | 50 | 90 | 97 | 56 | 60 | 43 | 64 | |
| Ismail *et al.* (1991) (Sri Lanka) | Albendazole | 160 | 96 | 100 | 32 | 87 | 100[d] | 100 | Kato–Katz, 14–28 days |
| | Levamisole | 150 | 86 | 96 | 18 | 73 | 87[d] | 100 | |
| | Mebendazole | 181 | 97 | 99 | 36 | 80 | 90[d] | 100 | |
| | Pyrantel | 162 | 94 | 99 | 23 | 54 | 90[d] | 100 | |
| Jongsuksuntingul *et al.* (1991) (Thailand) | Albendazole | 53 | 100 | 100 | 67 | 87 | 84 | 96 | Kato–Katz, 14 days |
| | Mebendazole | 56 | 100 | 100 | 70 | 89 | 30 | 70 | |
| Long Qi *et al.* (1992) (China) | Albendazole | 100 | 99 | 100 | 42 | 69 | 95 | 99 | Kato–Katz, 28 days |
| | Pyrantel | 50 | 81 | 82 | 7 | 17 | 83 | 90 | |
| Bartoloni *et al.* (1993) (Bolivia) | Albendazole | 54 | 100 | 100 | 33[d] | 46 | 82 | 93 | Kato–Katz, 21–28 days |
| | Mebendazole | 54 | 100 | 100 | 60[d] | 15 | 17 | 62 | |
| Albonico *et al.* (1994a, b) (Zanzibar) | Albendazole | 1174 | 99 | 100 | 10 | 73 | 57 | 98 | Kato–Katz, 21 days |
| | Mebendazole | 1120 | 98 | 99 | 14 | 82 | 22 | 82 | |

[a] CR, cure rate.

[b] ERR, egg reduction rate.

[c] Double-intensity pretreatment.

[d] 10 cases or less.

the targets set to be maintained in the programme. From extensive studies on the transmission of *A. lumbricoides* carried out by the Asian Parasite Control Organization (APCO) research programme (Kobayashi, 1980), it is apparent that determination of intervals of treatment depends on (i) knowledge of rates of reinfection following chemotherapy and (ii) detection of the presence of seasonal influences on transmission of infective stages. Seasonal effects can be quite subtle, being related to such activities as the use of nightsoil as a fertilizer at times when particular vegetables are available and in demand (Figure 1). Ideally, community treatment should be applied soon after an identified transmission season is over.

The conclusion from the body of results obtained by the APCO experience indicates that the appropriate intervals for the application of anthelminthic chemotherapy to control morbidity induced by soil-transmitted nematodes will be twice annually for prevalence rates <50% and thrice annually for prevalence rates >50%. However, it is stressed that the decision about intervals between treatments must be taken after consideration of local circumstances.

### 4.1.13. *Delivery of Anthelminthic Drugs through Schools: the Partnership for Child Development*

Experience from several control programmes and from the efforts of the Partnership for Child Development (WHO, 1994a) has shown that treatment targeted at school-age children can reduce the intensity of infection in the remainder of the community that do not receive treatment, indicating that treating school-age children reduces transmission (Bundy *et al.*, 1990;

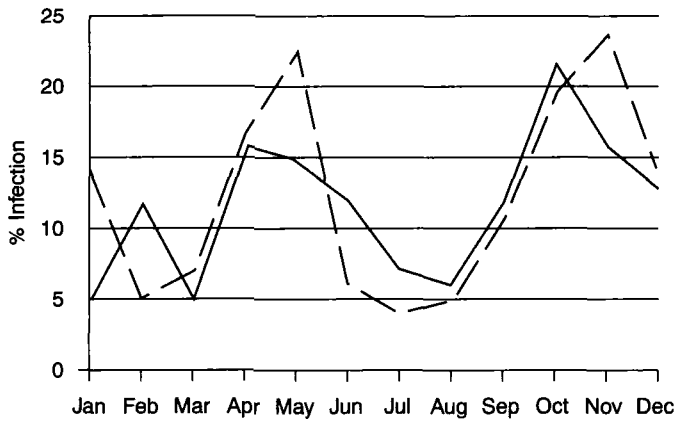


*Figure 1* Seasonal variation of *A. lumbricoides* infection. Solid line, Kobayashi (1954); broken line, Yajima (1955). (Adapted from Kobayashi, 1980.)

Asaolu *et al.*, 1991). The benefits of periodic anthelminthic treatment of school-age children are now clear.

The fact that children assemble in schools for numerous days of the year means that many can be treated regularly and as required by the control programme timetable. Secondly, the school system provides an existing infrastructure for delivering treatments. Thirdly, there are usually more schools than clinics and more teachers than health personnel, so the potential exists for treatments to be delivered to schools and be administered by teachers. Fourthly, school-based health programmes have the potential to integrate anthelminthic treatment against soil-transmitted nematode infections with actions against other diseases such as schistosomiasis and with treatment of micronutrient and vitamin deficiencies. Schools can also provide health education to support control programmes, attempting to change behaviour to reduce or even prevent transmission and enhancing support for health care in the community served by the school. Delivering anthelminthics through schools is relatively inexpensive and cost-effective and, in circumstances where enrolment in schools is good, such programmes are likely to achieve effective coverage.

A limitation of school-based programmes is that they do not reach those children who do not attend school because they have never enrolled or they have dropped out. Such children will be disadvantaged not only because they do not benefit from school education, but also because they miss school health programmes. Schools have the potential to act as a focus for health services for children out of school; efforts are needed to promote this service. The Partnership for Child Development and WHO are presently assessing alternative means to reach school-age children who are not enrolled in schools.

### 4.2. Health Education and Community Involvement

The contribution of health education to the success and sustainability of intestinal helminth control programmes is now accepted as being of major importance. Sustainable helminth control will not be achieved without health education and improvements in sanitation. Health education is mentioned as an important tool to make people aware and responsible for their own health and to create the demand for, and to involve the community in, latrine building, use and maintenance, but precise strategies are not well elaborated. There has been a tendency to view helminth control, as a health intervention in support of health education rather than a health intervention supported by health education (Trainer, 1989). Health education must be more than the information component of service delivery

programmes, and education in schools can be a major component of a school health programme.

However, health education programmes in sub-Saharan Africa are hampered by some common constraints: health education is given low priority in the education sector; planning of education is done centrally and is not based on needs assessment of communities; teachers are poorly trained and motivated and teaching materials are meagre. Yet there are examples in which helminth control can be a successful entry point for health education. Chemotherapy treatment is well accepted by the community and results are immediately visible. These advantages foster trust between the health officer or teacher who provides the medicine and the community. Also, the realization that treatment alone will not eliminate worms from the community will stimulate people to take action to eliminate and prevent helminth infection in their environment.

This is the approach that was successfully adopted by JOICFP (Japanese Organization for International Cooperation in Family Planning), which used mass screening and treatment of intestinal nematodes to stimulate people's interest in family planning and in environmental and family hygiene (Yokogawa, 1985). Another successful example is the Paunch-Khal Project in Nepal, where helminth control was made available to the community via anthelminthic treatment. Although latrine construction was not part of the original project, helminth control created a demand for the construction of sanitary facilities. By the end of 1983, 4 years from the start of the project, all families were using some kind of pit-latrine and 75% of the cost of construction was provided by the people (Upadhya, 1983). A well-known health education programme that has been widely implemented in developing countries is the Child to Child Initiative. The programme leaves implementation to local initiative: it involves primary schoolteachers and provides them with training material in order to disseminate information to schoolchildren and motivate them to take care of their younger siblings. Principal topics are nutrition and a healthy and safe environment. It is now known in more than 50 developing countries, and experiences from Brazil, Nigeria, Uganda, Zambia and India have shown its advantages and improvements (Webb, 1988).

Although stimulating changes in behaviour related to environmental and family hygiene is a long-term challenge, it is time to implement health care activities and promote health education within sanitation programmes. The necessarily regular visits to deliver treatment can serve as foci for readdressing health issues within the community. These activities are likely to be particularly closely linked in the case of school-based programmes. The current emphasis on delivering intestinal helminth control measures through schools offers an excellent opportunity for introducing this topic into the national curriculum. Starting with helminth control as an entry

point, other crucial health issues can be developed with children at school. However, this approach will require that teachers receive training and support for this aspect of their work (Albonico *et al.*, 1996).

### 4.3. Sanitation

Transmission of intestinal helminths occurs primarily through physical contact with an environment contaminated by human faeces. In most chemotherapeutic short-term interventions the gains made are often quickly hampered by reinfections, which in some cases may lead to higher worm burdens than those before treatment (Elkins *et al.*, 1988; Albonico *et al.*, 1995). Therefore, safe disposal of human faeces is essential for the long-term control and eradication of helminth infections. Defective latrines and septic tanks, overflowing soakaway pits and the discharge of untreated sewage into rivers, streams and ponds can jeopardize the progress made by a control programme and ruin prospects for sustainability. The provision of culturally acceptable sanitary facilities for disposal of excreta and their proper use are necessary components to be included in any programme aimed at controlling intestinal parasites. When developing school-based treatment programmes, consideration should be given to the importance of providing adequate sanitation in schools to promote hygienic behaviour in children. Most importantly, the safe sanitary disposal of human stools has health benefits far beyond those that will accrue from the control of helminth infections (Cairncross, 1990). Control programmes based on sanitation aim to reduce or interrupt transmission, prevent re-infection and gradually reduce worm loads (Wong and Bundy, 1990).

Despite the well-recognized role of effective sanitation in preventing transmission of intestinal helminths, the impact of improved water supply and sanitation intervention has not been well studied (Huttly, 1990). Data from St Lucia showed that prevalences of infection with *A. lumbricoides* and *T. trichiura* in children were significantly lower in areas with improved sanitary conditions (Henry, 1981), as was reinfection rate 6 months after chemotherapy (Henry, 1988). In a study in Tanzania, however, a sanitation programme associated with chemotherapy for hookworm infection achieved a reduction in intensity of infection for only up to 6 months after the intervention (Tanner *et al.*, 1987). Experience from Sri Lankan tea plantations showed that lower intensities of hookworm infection were correlated with the availability of latrine facilities, but these facilities did not have an impact in more crowded areas and congested living conditions (Sorensen *et al.*, 1994). Evaluation of a programme of latrine construction in Mozambique showed no association between *Ascaris* infection or presence of *Ascaris* eggs in the soil and the type of latrine (Muller *et al.*,

1989). Data from Thai villages showed that latrine availability was not a significant protective measure for hookworm infections in that situation (Chongsuvivatwong *et al.*, 1996).

The effect of improved sanitation on helminth infection is slow to develop and, in practice, may take decades to achieve measurable impact (Huttly, 1990). Sanitation alone is not the solution in the short term: Esrey *et al.* (1991) reported only a 4% reduction in prevalence of hookworm infection due to the use of latrines. This is not an argument against improvements in sanitation, but rather a reason for maintaining anthelminthic interventions until sanitation has had its effect. The use and availability of latrines combined with periodic chemotherapy greatly enhanced the reduction in intensity of infection and the time-span of reinfection both in research studies (Esrey *et al.*, 1991) and control programmes (Albonico *et al.*, 1996). Hookworm larvae are short-lived and most do not survive more than 1 month. The provision of adequate latrines would therefore be expected to have a more immediate impact on the control of hookworm infection than on *Ascaris* and *Trichuris* infections (Henry, 1988).

### 4.4. Footwear

Use of footwear to protect feet from contact with contaminated soil has been recognized as an important preventive measure against hookworm infection since the 1920s (Smillie, 1924). In a study in Dar es Salaam, Tanzania, only 2% of schoolchildren had hookworm infection compared with a prevalence of 12% in children not attending school. Socio-economic status was not a confounding factor, and the most plausible explanation for this difference was the requirement that all school pupils should wear shoes (Killewo *et al.*, 1991). A recent study in southern Thailand found that the only statistically significant protective measure for hookworm infection was the wearing of shoes, not past education, household income or latrine availability (Chongsuvivatwong *et al.*, 1996). However, there continues to be debate as to whether promotion of footwear use is a feasible and effective hookworm control measure given the difficulties inherent in any strategy attempting to change behaviour.

### 4.5. Approaching the Control of *S. stercoralis*

Compared with the four commonly studied intestinal nematode infections, much less is known about the public health significance of *S. stercoralis*. Morbidity is known to be substantial in individuals immunosuppressed by

chemotherapy, radiotherapy or genetic disorders (Genta, 1989). Furthermore, the possibility of autoinfection occurring with this helminth plays an important role in chronic disease, leading to increased morbidity among people living in endemic areas. Until recently, treatments for *S. stercoralis* invariably resulted in low CRs or were hampered by a high frequency of side-effects.

The introduction of ivermectin as a broad-spectrum, single-dose anthelminthic drug has presented a new option for dealing with a wide range of different intestinal nematode infections, especially for treatment in the community. There is increasing evidence that ivermectin is an effective treatment for some intestinal nematode infections. A recent WHO trial has shown a CR for *S. stercoralis* infection of 83% for ivermectin (200 $\mu g\ kg^{-1}$ single dose) compared with 45% for a course of albendazole (400 mg) for 3 days (Marti *et al.*, 1996). Treatment of infected children with ivermectin or albendazole resulted in CRs for *S. stercoralis* infection of 83 and 45% respectively. While *A. lumbricoides* was effectively eliminated by both drugs (100% and 99%), *T. trichiura* was eliminated in only 11% (ivermectin) and 43% (albendazole) of the subjects, although the mean egg counts were reduced by 59 and 92% respectively. Ivermectin was not found to be effective against hookworms, while albendazole resulted in a CR of 98%. No severe side-effects were recorded and any mild side-effects were of transient nature for both treatments. It was concluded that ivermectin provides a safe and highly effective single-dose treatment for *S. stercoralis* and *A. lumbricoides*, although it appears not to be an alternative for the treatment of *T. trichiura* and hookworm infections. The availability of this effective single-dose drug has reinforced the need to develop population-based studies on the real public health relevance of *S. stercoralis* and to evaluate the impact of treatment on the morbidity due to chronic infections.

### 4.6. Integrated Control

It has been increasingly recognized that for control of tropical diseases to be both effective and sustainable it will be necessary to adopt an integrated approach (Warren, 1990; WHO, 1997b). In many developing countries, primary health care may prove to be the best entry point. In 1996 WHO, in collaboration with the ministry of health representatives of five countries (Iran, Oman, Saudi Arabia, Zanzibar, Yemen), drafted plans for developing integrated disease control programmes (WHO, 1997b). Table 5 shows a possible approach for the use of available tools for the control of filariasis, intestinal helminths and schistosomiasis.

*Table 5* Recommended WHO strategy for the control of intestinal nematodes, urinary schistosomiasis and filariasis in endemic areas. (Adapted from WHO, 1997b)

| | Target group | Intervention | Drug | Schedule |
|---|---|---|---|---|
| Schistosomiasis | Schoolchildren | Targeted mass treatment at school in areas with prevalence of microhaematuria > 20%; selective treatment at school in areas with prevalence of microhaematuria < 20% | Praziquantel | Once or twice per year |
| Intestinal helminths | Pre-schoolchildren | Targeted mass treatment at creche or at maternal and child health (MCH) clinic when receiving vaccination | Albendazole, Levamisole, Mebendazole, or Pyrantel | Twice or three times per year |
| | Schoolchildren | Targeted mass treatment at school | | Twice or three times per year |
| | Pregnant women | Targeted mass treatment at MCH clinic when receiving vaccination | | Once after first trimester |
| Filariasis | Community, except children < 2 years | Universal treatment | Ivermectin and Albendazole | Once per year |

4.6.1. *Controlling Schistosomiasis*

The importance of treatment of school-age children with oral antischistosomal drugs has often been emphasized. Programmes to reduce morbidity should orientate their efforts to achieve complete coverage of the population of school-age children in endemic areas (WHO, 1995b). Retreatment of schistosomiasis is not recommended more than once a year and may be required only once every 5 years (WHO, 1993). Thus the epidemiological criteria for retreatment need to be established in every area, according to the available resources and the pattern of transmission. Both the presence of haematuria diagnosed by reagent strip (Savioli *et al.*, 1989) and the use of questionnaires (Lengeler *et al.*, 1991) have been recognized as reliable tools for identifying a high-transmission area and for ranking communities for treatment of urinary schistosomiasis.

4.6.2. *Controlling Lymphatic Filariasis*

The control of lymphatic filariasis has recently been enhanced by new treatment strategies that can effectively minimize the suffering of patients, interrupt transmission of infection in the community and be inexpensively integrated with other primary health care activities. The new strategy for controlling lymphatic filariasis has two major features: (i) a focus on treating the human population and (ii) use of community-wide treatment to replace other forms of drug delivery (WHO, 1996c; Ottesen *et al.*, in press). The best treatment options available are as follows.

1. One-day, once yearly treatment (for estimated 5–7 years) using any of the following:
   (a) Two-drug regimens (preferable): for 99% reduction in microfilaraemia for >1 year use ivermectin (200 $\mu g\ kg^{-1}$) plus DEC (diethylcarbamazine, 6 $mg\ kg^{-1}$) or ivermectin (200 $\mu g\ kg^{-1}$) plus albendazole (400 mg).
   (b) One-drug regimens: for 90% reduction in microfilaraemia for >1 year use DEC (6 $mg\ kg^{-1}$) or ivermectin (400 $mg\ kg^{-1}$).
2. DEC-fortified table/cooking salt as substitute for normal salt used for 9–12 months reduces microfilaraemia by 99% for at least 1 year.

4.6.3. *Integration of Schistosomiasis, Intestinal Nematode and Filarial Nematode Control*

Partnership between the health and education sectors must form the basis for future control of helminthic infections in school-age children. Schoolchildren are one of the most accessible groups for treatment and they generally belong also to the most heavily infected age group both for

schistosomiasis and intestinal nematode infections (WHO, 1993). High prevalences of mixed infections of intestinal helminths and schistosomes justify integrated control programmes including chemotherapy. Studies of concomitant administration of albendazole and praziquantel have shown the combination to be safe and effective (WHO, 1995b).

The tools and strategies recently developed for control of filariasis are similar to those used in controlling intestinal nematodes, the major difference being that the most effective approach to lymphatic filariasis involves the use of two-drug regimens. This means that in programmes using one drug to treat intestinal nematodes in regions with endemic filariasis, a greater public health impact can be achieved at minimally increased cost by adding a second drug, either ivermectin or DEC, to treat lymphatic filariasis as well. It is therefore clear that control of filariasis can be integrated with other on-going large-scale public health programmes, e.g. the African Programme for Onchocerciasis Control (APOC), where single-dose ivermectin is being administered once yearly in 16 countries in Africa (Ottesen *et al.*, 1997). There are also large-scale intestinal nematode control programmes using single-dose albendazole being undertaken in Ghana, India, Indonesia, Mexico, Tanzania and elsewhere (WHO, 1997b).

Better coordination and the combining of forces and resources would appreciably enhance the health impact of control efforts against these (and perhaps other) helminth infections. The major potential benefit of integrated helminth control is the strengthening of the health services by training the peripheral health care personnel, allowing coordination of diagnosis and treatment activities as part of a comprehensive control strategy. Such coordinated efforts should be more appealing to both health ministries and financial donors because of their greater cost-effectiveness for the afflicted communities.

## 5. PLANNING AN INTESTINAL HELMINTH CONTROL PROGRAMME

Different strategies and different timing are adopted according to the goal that is intended to be met. Most control programmes are designed to reduce morbidity in the short term. As a long-term goal, transmission of infection can be reduced by using periodic chemotherapy, promotion and implementation of sanitation, and health education. The main elements in planning control programmes are epidemiology, targets, chemotherapy, health education, sanitation, monitoring and evaluation. These elements should be integrated into the prevailing system of primary health care and must be based on multisectoral collaboration (WHO, 1985).

## 5.1. Epidemiology

Control programmes for intestinal nematode infections cannot be planned, implemented or sustained without recent and reliable information about the infections of interest, the people at risk and the associated morbidity. It will usually be necessary to carry out specific surveys to obtain the information needed for planning the programme. Advice on survey methodology is available in a WHO document (1990b) and a review by Thein Hlaing (1989); a manual for health planners is published by at WHO (*Intestinal Parasitic Infections Survey: a Basic Guide*).

Geographical distribution, seasonality and reinfection rates of helminth infection are crucial information for rational control. Diagnosis of the types of parasite found in the survey is equally important. Full details of how to process and examine human stool samples for evidence of protozoan and helminth infections are described in *Bench Aids for the Diagnosis of Intestinal Parasites* (WHO, 1994b). The Kato–Katz technique is strongly recommended for programmes dealing with intestinal helminths because it not only facilitates identification but also allows egg counts to be made so that a measure of intensity is obtained. Furthermore, the results can be reliably compared between regions.

## 5.2. Setting Targets

Health planners should set specific, realistic and achievable targets in order to quantify the long-term goal to reduce intestinal parasitic infections to a level that no longer constitutes a public health problem. Realistic targets should be identified according to the local epidemiological situation, the strategy adopted and the projected financial resources and available staff (WHO, 1987).

Before setting targets, a careful evaluation of the local epidemiology of intestinal nematode infections should be undertaken, by collecting data on prevalence and intensity of infections and various morbidity indicators (i.e. nutritional status) and by identifying specific target groups within the population (i.e. schoolchildren, pregnant women, farmers). In chemotherapy-based control programmes, which are aimed at reduction of morbidity, measurable targets could be percentage reduction in intensity of hookworm infections in specific target groups, or reduction in prevalence of related diseases in hospital records. Intensity of infection is an important indicator of morbidity for intestinal helminth infections, as worm burden is directly related to disease. A threshold of intensity-related disease could be estimated in different epidemiological situations (Bundy *et al.*, 1992). Alternatively, a simple and standardized indirect indicator of intensity is

the eggs released by female worms passed in faeces, counted by the Kato–Katz technique as eggs per gram of faeces. If improvement of the nutritional status of the population is one of the long-term goals, specific targets could be percentage reduction of anaemia or severe anaemia in the target population and improvement of anthropometry indicators (weight for age, height for age, weight for height) in children. If promotion of sanitation and safe water supply is implemented as the control strategy, then improvement of sanitation coverage, access to safe water supply, and reduction of infection rate to a given extent might be possible targets (WHO, 1987).

## 5.3. Monitoring and Evaluation

Monitoring and evaluation of intestinal helminth control programmes is essential to see if targets are being met and sustained. Monitoring is an important tool for health planners to adjust the control strategy according to the outcome achieved at regular intervals and so use resources effectively.

Indicators measured by simple, affordable and repeatable techniques should be adopted in monitoring activities. A carefully designed survey should be planned before intervention, assessing all possible useful indicators to be monitored after control activities have begun. A representative sample of the population should be selected in which standardized indicators are surveyed at regular intervals.

Monitoring and evaluation should be planned according to the targets and strategies adopted by each control programme. In chemotherapy-based control programmes, where the primary objective is reduction of morbidity, monitoring requires measurements of effective coverage, drug efficacy (i.e. reduction in prevalence and intensity of infections after treatment campaigns) and drug tolerance. Drug efficacy can be measured by the collection and microscopic examination of stool samples.

Several regional helminth control programmes have been evaluated for their impact on prevalence and intensity of helminth infection (Arfaa *et al.*, 1977; Asaolu *et al.*, 1991; Sorensen *et al.*, 1994; Albonico *et al.*, 1996) and several controlled trials have also demonstrated a positive impact of anthelminthic treatment on nutritional status and haemoglobin levels (Shield *et al.*, 1981; Robertson *et al.*, 1992; Stephenson *et al.*, 1993). However, evaluations of the effectiveness of large-scale helminth control programmes to improve growth and iron status have been lacking.

If nutritional status is taken as an indicator of morbidity, growth and iron status should be assessed by simple appropriate technology. If improvement of sanitation and safe water supply and promotion of health education are among the targets, monitoring and evaluation should include

also assessment of sanitation coverage, faecal contamination of the soil, reinfection rates and knowledge on preventive measures gained by the target population (through the use of questionnaires).

## 6. INTESTINAL HELMINTHS CONTROL PROGRAMMES: COUNTRY EXPERIENCE

### 6.1. Zanzibar: Control of Morbidity Due to Soil-transmitted Nematodes Through Chemotherapy in a High-transmission Area

Zanzibar comprises two main islands, Unguja and Pemba, lying in the Indian Ocean off the coast of mainland Tanzania. The population is about 700 000 most living in rural villages. Sanitation coverage is about 75% in urban areas but only 12% in rural areas. Only 3.2% of the rural population has access to piped water supply. The literacy rate on Pemba is approximately 70% and on average 60% of children attend schools. The infant and under-5 mortality rates are 120/1000 and 202/1000, respectively; the maternal mortality rate is 300/100 000. About 40% of children suffer from chronic malnutrition. The health budget per capita is about US$1 (Zanzibar Revolutionary Government, 1992).

#### 6.1.1. *Planning, Coordination and Management*

Helminth control began on Pemba Island in 1986, with the initial aim of controlling morbidity due to *Schistosoma haematobium*. That programme proved highly successful and is ongoing, with a yearly test, using reagent strips, and treatment with praziquantel based on surveys in schoolchildren (Savioli *et al.*, 1989). Within this programme, epidemiological surveys to assess the distribution and abundance of intestinal nematode infections were also undertaken. A community survey was performed in 1991 on 2200 individuals of all ages from eight different villages on the island. A total of 1120 schoolchildren from standard-5 classes in 35 schools were also examined for helminths in 1992. Intestinal helminth infections were confirmed to be a major public health problem among schoolchildren in Pemba, with a prevalence of 99.7% (Renganathan *et al.*, 1995). In the main island, Unguja, intestinal helminth infections have also increasingly come into focus as an important public health problem.

A trial for comparing the efficacy of single-dose albendazole 400 mg against single-dose mebendazole 500 mg was carried out in Pemba in 1993 (Albonico *et al.*, 1994a). A cohort of children was followed up at 4 and 6

months after treatment to evaluate the rate of reinfection. Results showed that for the common intestinal nematodes intensities of infection were back to pretreatment levels after 6 months (Albonico *et al.*, 1995). Pemba Island is a highly endemic area for intestinal nematodes and it seems likely that a high proportion of the children in the trial acquired new infections in the 4 months following treatment. These findings, suggesting high transmission rates, had significant implications for health planners both in the design of appropriate treatment schedules to control morbidity by mass treatment of schoolchildren and in the choice of the more cost-effective drug. Both drugs had similar efficacy in reducing intensity of infections and treatment with either drug did not prolong the interval of reinfection. Cost was the major determinant of the choice. Thus single-dose mebendazole 500 mg every 4 months was selected as standard treatment on Zanzibar, after ensuring the quality and *in vivo* efficacy of the generic mebendazole used (Albonico *et al.*, 1994a). An evaluation survey was also designed to assess the burden of morbidity due to intestinal nematode infection in children and to evaluate if periodic treatment, in addition to reducing egg counts, could improve other morbidity indicators like iron status, vitamin A status and growth of children (Stoltzfus et al., 1997).

The Government developed the National Plan for the Control of Helminth Infections in Zanzibar and control of intestinal nematodes was added to the established programme for the control of schistosomiasis. School-based control activities began in 1994 with a team of health officers working in close collaboration with teachers. Planning lasted over a period of 3 years. The aims of the programme were to reduce the intensities (measured as eggs per gram faeces) of *A. lumbricoides*, hookworm and *T. trichiura* infections by 60, 30 and 20%, respectively, in a target population of 70 000 schoolchildren. Recognizing the need for action to improve the health and well-being of Zanzibari children, the Government introduced the 5-year National Programme of Action (NPA), the main objectives of which are the survival, protection and development of children within the period 1992–2000. Three national goals to be achieved by the year 2000 have particular relevance to the helminth control programme (Zanzibar Revolutionary Government, 1992).

1. Reduction of moderate and severe malnutrition by half by the year 2000.
2. Universal access to basic education and completion of primary education by at least 80% of school-age children by the year 2000.
3. One-third reduction in iron-deficiency anaemia among women and children.

### 6.1.2. *Monitoring and Evaluation*

The evaluation study on Pemba Island measured the impact of a school-based deworming programme on helminth prevalence and intensity and on iron status, vitamin A status and growth of children (Stoltzfus *et al.*, 1997). Results from the first year of evaluation were encouraging: periodic treatment of schoolchildren with mebendazole 500 mg every 4 months substantially reduced helminth infection intensities. However, because hookworm transmission in this environment is very intense, even three treatments per year did not reduce hookworm intensity to low levels (Figure 2). Intestinal nematode infections were characterized by an important seasonally increasing trend, with a doubling of intensity after the rainy season. These findings suggest that (i) it is important to perform monitoring and evaluation in the same season, (ii) intensity (and not prevalence) is the reliable indicator for monitoring the impact of periodic chemotherapy and (iii) a more effective treatment might be necessary during the high-transmission season (M. Albonico, R.J. Stoltzfus, L. Savioli, H.M. Chwaya, E. d'Harcourt E. and J. Tielsch, unpublished observations).

On the nutritional side, deworming was associated with a significant (39%) reduction in the occurrence of new cases of severe anaemia detected during the 1-year follow-up period. The prevalence of depleted iron stores decreased by 20% in the group treated (Figure 3) (Stoltzfus *et al.*, in press a). Regarding growth, in non-stunted children the additional average weight gain attributable to deworming was 0.42 kg, about 20% of the control group weight gain. With regard to height, in children <10 years the entire benefit was in non-wasted children, in whom height improved 3.75 mm (9%) more than the gain in the control group. In children >10 years, the entire benefit was in boys, among whom height gain was

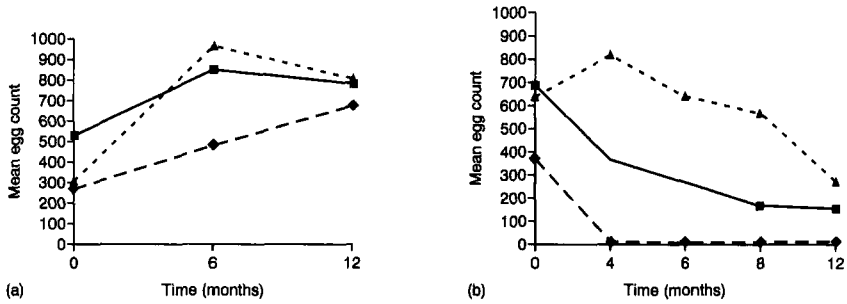


*Figure 2* Intensity of intestinal helminthiases in (a) the untreated cohort ($n$ = 1037) and (b) the cohort ($n$ = 1011) treated with mebendazole 500 mg every 4 months at (time 0, 4, 8), Pemba Island, 1995. ●, *Ascaris*; ■, *Trichuris*; ▲, hookworm. (M. Albonico, R.J. Stoltzfus, L. Savioli, H.M. Chwaya, E. d'Harcourt and J.M. Tielsch, unpublished observations.)

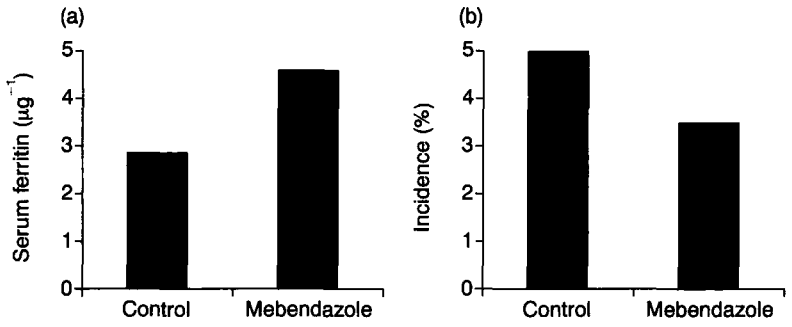


*Figure 3* (a) Changes in serum ferritin and (b) incidence of moderate–severe anaemia (haemoglobin < 9 g $dl^{-1}$) at 12 months in the untreated cohort ($n$ = 1002) and the cohort ($n$ = 970) treated with mebendazole 500 mg every 4 months, Pemba Island, 1995. (R.J. Stoltzfus, M. Albonico, H.M. Chwaya, J.M. Tielsch, K.J. Schulze and L. Savioli, unpublished observations.)

improved by 3.15 mm, about 7% of the control group (Stoltzfus *et al.*, in press b). Results from this evaluation study support the intensity-dependent relationship between hookworm infection and iron deficiency and indicate that reduction of infection intensity alone will help to control iron-deficiency anaemia in communities.

For initial monitoring of the impact of periodic chemotherapy, about 1000 children from primary classes (standards 1–4) were treated with mebendazole every 4 months and then followed for 1 year. For future monitoring, 600 standard-4 children in the same schools will be selected. A sample of an equal number of standard-1 children just enrolled in school will be examined to account for secular changes in iron status not related to the deworming programme, and also to monitor any change in prevalence and intensity in the child population that was not treated as an indicator of transmission of the infection. This sample of children will be assessed for helminth infection (the quantitative Kato–Katz thick smear), iron status (haemoglobin, erythroporphyrin and serum ferritin) and growth (weight and height).

In conclusion, programme monitoring in Zanzibar has helped in identifying other target groups, defined the more cost-effective drug, established the reinfection rates and determined useful morbidity indicators. Short-term monitoring has proved that periodic treatment with mebendazole is a cheap and efficient short-term solution to control morbidity due to helminthic infections. However, long-term monitoring will be an essential tool to adjust the control strategy to the changes in morbidity and to see if targets set by the programme have been sustained.

## 6.2. Seychelles: Control of Morbidity Due to Soil-transmitted Nematodes Through Chemotherapy in a Low-transmission Area

The Seychelles archipelago comprises 115 islands with 73 000 inhabitants, although the vast majority of the population lives on the three main islands of Mahe, Praslin and La Digue. Conditions are markedly different from those in Zanzibar. The infant mortality rate is 9/1000, the under-5 mortality rate 4/1000, the maternal mortality rate 63/100 000 and 6.6% of children suffer from chronic malnutrition. The health budget per capita was $280 in 1994. Education covers 95% of the school-eligible age group. A school health programme is implemented by nurses from the nearby health centre and a health education programme (Social Education Programme) is also carried out by specially trained teachers. Treated water supply is utilized by 70% of the population and only 5% of the population lacks latrines (Albonico *et al.*, 1996).

### 6.2.1. *Planning, Coordination and Management*

In consultation with the Ministry of Health a plan of action was written, with the long-term objective of reducing intestinal parasitic infections to a level that no longer constitutes a public health problem. The specific objectives to be achieved within 3 years were (i) reduction in intensity (expressed in eggs per gram of faeces) of *A. lumbricoides* infections by 60% and *T. trichiura* and hookworm infections by 30% in school-age children, (ii) reduction in the target population of prevalence of *S. stercoralis* infections by 30% and (iii) reduction in the target population of prevalence of amoebiasis by 40% (Albonico *et al.*, 1996).

Schoolchildren and pregnant women represented the target groups. A survey to collect baseline data on prevalence and intensity of intestinal parasitic infections was necessary to better define the targets of the control programme and to establish a solid basis for monitoring and evaluation surveys. After having trained the health staff (school health nurses, environmental health officers, laboratory technicians) and teachers in the integrated approach, faecal samples from 5% of all schoolchildren were examined by the Kato–Katz technique to identify and count helminth eggs and by the formol ethylacetate concentration technique to detect protozoa and *S. stercoralis* larvae. Quality control was performed at a central level by examining randomly 10% of the total slides. Children were interviewed using a questionnaire to evaluate their knowledge of intestinal parasitic infections before intervention. Between June and July 1993, 338 pregnant women attending antenatal clinics had their stools examined for helminths and protozoa and their haemoglobin tested.

It was demonstrated that 60% of children were found to be infected with one or more parasites, with significant variation by region. *Trichuris* was the most common parasite with a prevalence of 53.3%, followed by *Ascaris* with a prevalence of 17.7%; hookworm infections were present with low intensity in 6.3% of schoolchildren and in 8.6% of pregnant women. Only 14% of women had a haemoglobin level below 11 g $dl^{-1}$, 1% had haemoglobin below 8 g $dl^{-1}$ and none had haemoglobin below 7 g $dl^{-1}$. No correlation was found between hookworm infections and anaemia in these pregnant women. Such prevalence and intensity of intestinal parasitic infections justified intervention with mass treatment in schoolchildren without prior screening (Albonico *et al.*, 1996).

The primary health care system is well established in the Seychelles, and the programme has been integrated from the beginning into the local health structure. The programme activities are coordinated by a national coordinator (programme manager) with technical support from the clinical and public health laboratories, health education unit and epidemiology unit. The school health nurses and environmental health officers are responsible for the control activities (health education, treatment campaigns, evaluation surveys) in their health centre catchment area, in close collaboration with teachers.

### 6.2.2. *Control Measures: Periodic Chemotherapy and Health Education*

All schoolchildren (about 20 000) were dewormed every 4 months in the first year, with a coverage rate of 99.4%. Mebendazole (500-mg tablets), given as a single dose, was the anthelminthic drug chosen by the Ministry of Health for periodic chemotherapy in schoolchildren. Treatment was delivered by teachers under the supervision of health staff from the nearest health centre. Due to the low prevalence in pregnant women, selective treatment was given to positive cases diagnosed by a routine stool examination performed during the first visit to the antenatal clinic. Treatment was administered after the first trimester of pregnancy.

It was recognized that long-term control of intestinal parasitic infections could not be achieved without a change in health behaviour. Print media (newspapers, posters, leaflets) and electronic media (radio, television, audiovisual aids) were used extensively to increase public information and awareness on intestinal parasite control. Since the start of the programme, preventive measures on intestinal parasites have been included in the school curriculum. Mobile health teams (environmental health officers, school health nurses), in collaboration with social education teachers, organized sessions and disseminated health messages in all schools. Radio advertised the programme's activities and general preventive methods, while television and national newspapers were also involved in advertising chemotherapy surveys. A video on prevention and control of intestinal parasitoses,

produced in the Seychelles, was widely distributed in the schools and health centres and broadcast on local television. Leaflets and posters on the prevention and control of intestinal parasitic infections were designed in Creole and printed locally (Albonico *et al.*, 1996; Crompton *et al.*, 1996).

### 6.2.3. *Monitoring and Sustainability*

The first parasitological evaluation after three chemotherapy campaigns was performed in a sample of about 1000 children from all schools. Results from the evaluation survey showed that the cumulative prevalence of intestinal parasites in the population of schoolchildren dropped from 60.5 to 33.8%. The mean eggs per gram of faeces was reduced by 85, 53 and 32% from the baseline mean for *Ascaris, Trichuris* and hookworm respectively (Albonico *et al.*, 1996). Children will be monitored at yearly intervals to assess the trend of prevalence and intensity of intestinal parasitic infections as the only indicator for morbidity. Pregnant women attending for the first visit at the antenatal clinic will be surveyed every 2 years, in order to monitor the possible reduction of infection in the adult non-treated population due to effective control in the school-age group. As part of the evaluation, a sample of schoolchildren will be interviewed with the same questionnaire used before the start of the programme, in order to assess the improvement in knowledge and awareness on prevention and treatment of intestinal parasitic infections.

The objectives of the programme were met after 1 year of periodic chemotherapy and there may even have been an impact on transmission. These results suggest that (i) in a low-transmission area, intensity and prevalence are reliable indicators of the impact of a control programme; (ii) health education has been a key tool to prevent infection in a country where facilities were available, and chemotherapy campaigns have been an excellent entry point for health education messages; (iii) the preventive impact of sanitation and safe water supply has been accelerated by the associated chemotherapy; (iv) the control of intestinal parasitic infections within a comprehensive primary health care approach has strengthened the technical capabilities of laboratory staff, increased management and epidemiological skills at the central level and promoted coordination among health centres, the central hospital and epidemiology unit; and (v) enhanced the collaboration between the Ministry of Health and the Ministry of Education and other ministries concerned (Environment, Finance and Communication, Community and Development). However, a long-term commitment is needed to sustain the outcome achieved. The keys for the sustainability will be political will, the participation of the community and the optimization of the available resources through the existing health facilities (Albonico *et al.*, in press).

## 6.3. Sri Lanka: Control of Morbidity Due to Soil-transmitted Nematodes in High-risk Groups

Nearly 1 million people live on tea, rubber and coconut plantations in Sri Lanka. Although substantial investments have been made in the plantations to improve living conditions, a large segment of the population still has to manage with poor housing, inadequate sanitary facilities and limited access to safe water. In the plantation sector, literacy levels are lower than in the rest of the country, especially among women. The health status of the estate population has improved during the last decade, but undernutrition and anaemia among women remain problems. A study of anaemia in pregnancy conducted in the plantation sector in 1990–1991 confirmed that 58.3% of subjects had a haemoglobin level below 11 g $dl^{-1}$ and that 23.4% were severely anaemic with haemoglobin levels below 8 g $dl^{-1}$ (Atukorala *et al.*, 1994). Iron deficiency is the most common cause of anaemia, with folate deficiency also playing a significant role.

Intestinal nematode infections are widespread in the estate communities. A survey done in 1922 revealed that 86.2% of women of child-bearing age and 89.7% of children aged 3–12 years were infected with at least one kind of nematode. Of the women, 41.4% were infected with hookworm (*N. americanus*) with a majority having light or moderate intensity of infection. Although the majority had light infections, it is likely that this is sufficient to cause iron-deficiency anaemia when the dietary intake of iron is also low. While at work as tea-pickers or rubber tappers, the women traditionally do not use footwear. Of the children, 23.2% were infected with hookworm with the prevalence increasing gradually with age during childhood and substantially so from 8 years of age and beyond. Hookworm infection was much more common in the low-country areas and is possibly related to warmer climatic conditions. There was a strong association of hookworm infection with the availability of sanitary facilities (Sorensen *et al.*, 1996).

### 6.3.1. *Measures to Control Intestinal Helminth Infections and Relieve Anaemia*

The control of intestinal parasitic infections in Sri Lanka has focused in recent years on the control of hookworm infection, with the aim of reducing iron-deficiency anaemia among women plantation workers (and their children) who live on tea and rubber estates. In the plantations, most pregnant women attend antenatal clinics conducted by medical assistants and public health midwives. In the absence of a simple field test, the detection of anaemia is based on clinical examination. All pregnant women receive one course of deworming treatment (mebendazole 200 mg for 3 days) after the first trimester, in addition to iron-folate supplements. The

study on anaemia in pregnant women revealed that one course of anthelminthic therapy significantly improved haemoglobin levels (Atukorala *et al.*, 1994). The iron supplement is begun early in pregnancy and is given with vitamin C to enhance absorption. The women are also given dietary advice and are specifically advised to avoid drinking tea for at least 1 hour after a meal, as tea reduces absorption of iron. These activities are part of the national strategy for control of anaemia in Sri Lanka.

In order to control the widespread infections with soil-transmitted nematodes in the plantations, a control programme was launched early in 1994, targeting more than 200 000 children 3–14 years of age. Biannual deworming with a single 500-mg dose of mebendazole is given to all children and to pregnant women after the first trimester. The deworming programme has been combined with health education conducted by the estate health workers. Efforts have also been made to include schoolchildren and teachers in health promotion activities. Leaflets with information about anaemia and its adverse effects and about the prevention of nematode infections have been produced and distributed to all health staff in the plantations (Crompton *et al.*, 1996).

Over several years, support for the construction of latrines on plantations has been available. Often the latrines have only lasted a few years because of poor construction and maintenance. During the last 2 years, latrines have been constructed on a self-help basis on estates. The provision of adequate latrines can be expected to have an immediate impact on hookworm infection. The control programme of soil-transmitted nematodes on the plantations includes an annual monitoring process, with collection of stool samples from a few selected communities where baseline data was available. After 2 years, an evaluation was carried out 5 months after the second round of mebendazole 500 mg. The results indicate that there has been a low to moderate decrease in both prevalence and intensity in all four estates. Intensities of infection were found to have decreased more in the two low-country estates compared with the up-country estates. This was not unexpected because in the up-country estates housing is more crowded and the soil tends to be more polluted than in the low-country estates where housing is more scattered. For ascariasis, the congested living conditions seem to be a determinant of infection and the provision of latrines and safe water does not change the situation in the short term (Sorensen *et al.*, 1994).

### 6.4. Kwa Zulu-Natal, Republic of South Africa: Helminth Control as Part of the Primary School Nutrition Programme

Following major political changes in South Africa, attempts are being made to redress the inequalities of the past through a Reconstruction

and Development Programme. One of the subprogrammes announced shortly after the change in government in 1994 was the Primary School Nutrition Programme, which has parasite control as one of its four initiatives. The overall aim of the Primary School Nutrition Programme is to improve the learning capacity of children through improvements in their nutritional status (Crompton *et al.*, 1996).

Climate and other geographical features play a part in the epidemiology of the various parasite infections, although endemic parasitic infections are particularly prevalent in those parts of the country that were designated as independent or non-independent 'homelands' and were under-resourced with respect to clean water and sanitation. However, recent studies have shown a high prevalence of *T. trichiura* and *A. lumbricoides* infection even in urban and in a transitional metropolitan area, with relatively efficient environmental and health services (Fincham and Jackson, 1997).

### 6.4.1. *Progress in Planning for the Control of Intestinal Parasitic Infections*

The province of Kwa Zulu-Natal probably has higher prevalence rates for intestinal helminths than any other province (Appleton and Gows, 1996). A computerized geographic information system is being used to estimate the number of primary schoolchildren who should be treated as part of the targeted mass treatment programme and the number of schools that will have to be visited (Crompton *et al.*, 1996). After a feasibility study in two schools, more extensive plans were made to pilot a control programme about 100 km south of Durban in an area containing some 40 schools and 20 pre-schools. In 1993 the National Nutrition Intervention Programme of the Medical Research Council reported that iron stores and growth improved significantly when children with a high prevalence of *T. trichiura* infection were treated with albendazole (Kruger *et al.*, 1996). Since then, a micronutrient intervention project in primary schools in Kwa Zulu-Natal, also routinely dewormed all the children (van Stuijvenberg *et al.*, 1997).

There is now the recognition of the need for community-based intervention to control intestinal parasites in South African children (Fincham and Jackson, 1997), as many South African communities are in dynamic transition and at increased risk of helminthic infections. Pre-school children have also been recognized as an important source of infection and treatment of this age group through local community-based organization has been addressed (Taylor *et al.*, 1995). Studies are ongoing to monitor the impact of periodic deworming in schoolchildren, record reinfection rates and to address the insufficient cure rates of *T. trichiura* infection with benzimidazole treatment (Evans *et al.*, 1997).

Future approaches aim to increase the understanding of the epidemiology of helminthic infections in other South African provinces using as a model the success of the experience in Kwa Zulu. There is also a call for systematic monitoring of the results of deworming interventions on parasitological and nutritional outcomes (Fincham *et al.*, 1996).

### 6.5. Mexico: Monitoring Large-scale Integrated Control Programmes

Since 1993, the National Health System in Mexico has organized periodical health campaigns, known as National Health Weeks (*Semana National de Salud*), which promote various interventions focused mainly on children, including distribution of oral rehydration packages, training of mothers in treatment of diarrhoea, administration of megadoses of vitamin A and EPI (expanded programme of immunization) vaccinations. Identification of the public health importance of infection by soil-transmitted nematodes in Mexico and the availability of low-toxicity drugs that can be administered in a single dose led to the inclusion of mass anthelminthic chemotherapy (albendazole) in National Health Weeks.

The principal aims and expected outcomes of the programme have been:

1. to provide anthelminthic chemotherapy for 95% of children aged 2–14 years in municipalities with high risk of intestinal infections;
2. to reduce the frequency of severe complications, including mortality associated with soil-transmitted nematodes;
3. to decrease the harmful impact of nematode infections on the growth, development and school achievement of children;
4. to decrease the egg output from soil-transmitted nematodes among groups with highest infection prevalence;
5. to decrease the prevalence of infection among school-age children living in specific areas (municipalities with high risk of intestinal nematode infections);
6. to decrease the reinfection rates among school-age children living in areas receiving mass anthelminthic chemotherapy.

The target groups for administration of albendazole were children aged 5–14 years (weeks 1 and 2) and 2–4 years (weeks 3, 4 and 5) (approximately 10 million children in total). A single dose of albendazole (400 mg), either tablet (5–14 years) or suspension (2–4 years), was administered every 4 months (October 1993, February 1994, June 1994, October 1994 and February 1995). Albendazole was purchased by the Ministry of Health through a consolidated contract at a cost of US$0.11 per treatment.

The social mobilization included a national, state and county consensus, the participation of private enterprise non-governmental orgnizations; participation of social and community leaders; television and radio (interviews and spots); graphic educational material (posters) and a 'health telegram' (10 million printed). The actions taken included informing and training decision-makers, training for state trainers (300 epidemiologists and health administrators), the design and production of 500 educational packages (videos, 60 slides, manuals), the training of operational personnel at local level and the training of teachers and volunteers (approximately 50 000) (INDRE, 1994).

### 6.5.1. *Surveys and Evaluation*

Study sites were selected for survey and evaluation from each of the 21 states that participated in this programme. A questionnaire was designed to record information on geographic and demographic variables of the community (climate, altitude, latitude, population size, literacy, characteristics of households, availability of piped water). Communities were stratified according to the degree of risk of infection (high, moderate and low). Evaluation of the intervention was accomplished through sentinel stool surveys from a sample of the population before and after each drug administration. Sentinel surveys are not fully representative of the total population, but offer information on groups and sites of interest.

Baseline data were obtained from 9337 children, with stools being examined by the Kato–Katz method. The results showed a prevalence for *A. lumbricoides* of 20.9% (range 0–84.9%), for *T. trichiura* of 17.4% (range 0–85.8%) and for hookworms of 0.9% (range 0–8.87%). Prevalences of single and multiple helminthiases were found to be associated with climate, latitude and altitude: the risk increased 17 times for residents in tropical locations below 500 m altitude and 16–20°N. During the 5 weeks, over 50 million doses of albendazole were administered and adverse effects due to the administration of the drug were minimal.

The evaluation demonstrated that mass administration of albendazole to children 2–14 years old living in high-risk municipalities produced a satisfactory CR of 83–93%. A decrease in infection intensity (99.9%) and cure of moderate and severe cases (92–93%) were observed with efficacy having a cumulative effect. Overall, infection with *T. trichiura* was the least responsive to treatment with a single dose of albendazole. From the perspective of individuals, subjects have rapid clinical improvement in health and well-being, coupled with a decrease in demand health services due to soil-transmitted nematodes. It is expected that nutritional status and school performance of individuals will have improved. From the community perspective there is an increase in interest and support for other national health programmes. As part of its preventive services, the Mexican

Ministry of Health has included in its National Health Program (1996–2000) the administration of mass anthelminthic chemotherapy for soil-transmitted nematodes to children living in high-risk areas (WHO, 1996c).

## 7. COST-EFFECTIVENESS AND SUSTAINABILITY

Techniques for the economic evaluation of health programmes in industrial countries are well developed, and include cost-minimization, cost-effectiveness, cost–utility and cost–benefit analyses. Such techniques, when applied to the control of intestinal helminth infection, attempt to measure the benefits derived from the programme compared with the costs involved. Studies have shown that chemotherapy is the widely preferred option for the control of morbidity due to intestinal helminths (Savioli *et al.*, 1992; WHO, 1996c) and is proved to be highly cost-effective (Evans and Guyatt, 1995).

The first question to be answered is whether chemotherapy-based helminth control is affordable given the many other health problems needing attention. In many endemic countries the annual health expenditure is only US$1–4 per capita (Fenwick, 1989). In cost analysis, both cost of the drug and its delivery system should be carefully taken into consideration. When cost comparisons between drugs are made before purchase, the cost of the total treatment, including such factors as storage, delivery to site, treatment on site and monitoring, should be estimated and not only the unit cost of the drug itself. Given the limited resources in developing countries, it is likely that vertical methods of drug delivery are not affordable on a large scale. More affordable, yet effective approaches include targeting treatment at high-risk communities rather than individuals. The use of the school system as a more effective and affordable delivery system and targeting schoolchildren for chemotherapeutic control of intestinal nematodes has been well publicized (Bundy *et al.*, 1990; Guyatt and Evans, 1992). Another option to reduce the cost of helminth control at the community level is to integrate strategies with existing health care services. This has proved successful where existing facilities and resources are already available (Albonico *et al.*, 1996). A similar integrated approach is to include helminth control in a larger package to control other endemic diseases, such as schistosomiasis, filariasis and, to a limited extent, malaria, involving management at the central, district and primary health care level. This integrated approach has been recently proposed by WHO (1997b) in a number of selected countries. An alternative means for identifying more cost-effective approaches to helminth control is to explore alternative ways of financing, including the possibility of cost recovery. This approach could

ensure the sustainability of control programmes, but the concept of equity and accessibility to health care should be carefully taken into consideration. People are willing to contribute to helminth control and studies have shown that people do spend a great deal in seeking treatment for intestinal helminths (Stephenson *et al.*, 1980). If drugs of proven efficacy were made available at health centres at acceptable prices, it would encourage people to self-medicate and could be an alternative option to active treatment in some communities.

Two types of cost-effectiveness analysis have been considered in assessing the feasibility of a chemotherapy-based anthelminthic programme: (i) determination of the most efficient delivery strategy and (ii) whether a helminth control programme is a cost-effective use of resources in public health, given the limited resources available in developing countries (Evans and Guyatt, 1995). The first analysis addresses the problem of choosing between universal or selective treatment and, beyond that, determines the frequency of treatment and coverage of the target population. It has been generally recognized that selective treatment for intestinal nematodes is a costly option, due to the lack of inexpensive and simple individual screening tests, compared with the low cost and safety of anthelminthic drugs (Anderson and Medley, 1985; Hall *et al.*, 1992). The only widely validated method is the search for parasite eggs in stool samples, which is relatively time-consuming and requires trained personnel. However, this method is highly recommended in limited screening of population samples for targeting the programme or monitoring its progress. Treatment targeted to children has been recognized as the most feasible option, because of the high coverage, the cheap cost involved due to the utilization of an infrastructure already available and the best return in terms of reduction of morbidity (children being the most heavily infected age group in the population) (Bundy *et al.*, 1990; Evans and Guyatt, 1995). In previous studies on effectiveness, misleading indicators of morbidity have been used, such as measures of coverage or cure rates, that do not reflect any long-term reduction in morbidity. More recently, the number of heavy infections prevented or cured or the total years of heavy infection prevented in a population have been used as outcome indicators, given the strong relationship between intensity of infection and morbidity. In particular, a recent study by Medley *et al.* (1993) has led to the development of a dynamic mathematical model for intestinal helminth transmission that determines the effectiveness of chemotherapy for disease prevention over time since the start of the programme, taking into consideration changes in the parasite population and reinfections. In this model, disease prevalence is represented by the prevalence of heavy infections and effectiveness is evaluated more on disease-prevented cases than on infection-prevented cases.

Economic studies have been undertaken only for *A. lumbricoides* (Guyatt *et al.*, 1993, 1995; Holland *et al.*, 1996). It has been shown that child-targeted treatment can be more cost-effective than population treatment in reducing the number of disease cases; in a high-transmission area, enhancing coverage is a more cost-effective strategy than increasing the frequency of treatment. However, there remain a number of questions to be answered. Firstly, economic considerations on the treatment of hookworm and *T. trichiura* infection and the impact on multiple infections require further in-depth research studies. Secondly, there is a lack of information on how to incorporate the economic benefit of treatment on growth and cognitive performance, even despite reinfection. Thirdly, in many developing countries, school enrolment and attendance rates could be very low; this implies that treatment would miss the children who are presumably poorer and most in need of health care. Indeed, how to develop methods on increasing the coverage of school-age children is an area in which much effort is actually made by the international agencies.

The second type of analysis has been widely neglected in the past and has only recently gained attention due to the development of new tools for morbidity control and the promising positive implication on the health status of infected children. The World Bank (1993) recommended anthelminthic treatment to be included in packages of essential public health intervention, but it is not yet clear on what basis the impact of treatment on morbidity is calculated. Little work has been done in this field and only one study has investigated a cost-effective way of investing funds in helminth control rather than in other competing public health intervention. A programme model such as described by Warren *et al.* (1993) would cost US$6–12 per disability adjusted life year gained. This would be an extremely efficient use of resources compared with other health interventions (such as childhood immunization) (Evans and Guyatt, 1995). However, there may be a tendency to underestimate the benefit of a school-based programme. In the first instance, it could be the entry point to health education and the control of other communicable diseases. In addition, the cost of anthelminthic drugs nowadays can be considerably lower than the cost estimated by Warren *et al.* (1993): a single dose of a benzimidazole costs less than US$0.10 per child (Albonico *et al.*, 1994a; WHO, 1996c).

An issue of paramount importance, closely related to the cost of anthelminthic drug, is the quality control of generic products. In view of the problems of ensuring satisfactory quality, it should be noted that the cheapest drug is not necessarily the most effective or the safest. In different preparations there may be variations in pharmaceutical formulation, bioavailability and therapeutic equivalence. Before purchasing any anthelminthic drug, especially if generic products, programme managers must take steps to avoid fake or counterfeit drugs. If drugs are not purchased

direct from a research-based pharmaceutical company, which will invariably guarantee the quality, managers should take action that quality has been assured by independent analysis. This issue emerged strongly as a recommendation in a recent informal consultation on the use of chemotherapy for the treatment of soil-transmitted nematodes held at WHO (1996c).

More research on the economic evaluation of helminth control is urgently needed to help health planners concentrate their efforts and resources on those activities that produce the greatest benefits for the least costs, ensuring the greatest efficiency of services. Such results will improve the process of making decisions about the allocation of resources between programmes within the health sector, and between sectors.

## 8. DRUG RESISTANCE

Drug resistance is defined as a genetically transmitted loss of sensitivity for a drug in a parasite population that was previously sensitive to the appropriate therapeutic dose.

### 8.1. The Extent of the Problem in Livestock

Widespread treatment of farm animals with anthelminthics has been widely practised to increase productivity and to prevent disease and death. Nowadays veterinary medicine is facing an important and increasing problem of drug resistance (i.e. infections persist in the presence of curative doses of the drugs) to the major anthelminthics. Resistance to benzimidazole is most commonly reported: in Australia, South America and South Africa, the prevalence of resistance to benzimidazoles varies between 40 and 90%. In addition, recent surveys in South American countries have shown increasing resistance against levamisole (24–84%) and ivermectin (73%) (Geerts *et al.*, 1997). Veterinary scientists warn of the potential danger of producing resistant helminths in humans through intensive widespread treatment (Coles, 1995).

### 8.2. The Mechanisms and Inducing Factors for Drug Resistance

The molecular nature of benzimidazole resistance is due to structural changes in the β-tubulin molecule of the nematode, resulting in reduced binding affinity of benzimidazoles. The mutant resistant gene is already

present in the nematode population and its distribution is enhanced by selection pressure (Roos, 1990). Will resistant strains be selected to become a significant proportion of the total population?

A theoretical approach to consideration of the problem of drug resistance in helminths is based on the following assumptions: (i) helminth populations are isolated, to the extent that there is no gene flow; (ii) universal anthelminthic treatment is applied; (iii) treatment is homogeneous; and (iv) continual selection pressure is applied. Selection pressure is enhanced by extremely frequent treatment, shorter than the generation time of the nematode, and by underdosing or treatment with partially effective drugs.

Under these conditions, it would be appear to be inevitable that drug resistance for soil-transmitted nematodes will appear, the most crucial factor being the generation time of the nematode species of interest. By extrapolation from the results of studies on the development of pesticide resistance in insects (Comins, 1984), it seems that 5–100 generations would be required under appropriate conditions before drug resistance would arise in a nematode population.

### 8.3. Monitoring and Management of Emerging Anthelminthic Drug Resistance in Humans

Although there have been recent reports of the development of anthelminthic drug resistance in human hookworms (De Clercq *et al.*, 1997) and schistosomes (Stelma *et al.*, 1995), convincing evidence for significant drug resistance in human helminths has yet to be documented (Brindley, 1994). In addition, drug resistance should be carefully distinguished from drug tolerance, an innate insusceptibility of a parasite to a drug even before the parasite has been exposed to the drug.

There is understandable concern that this potential problem might become manifest and threaten the use of anthelminthic drugs for the control of helminthic infections in humans (Savioli *et al.*, 1997). However, several measures can be taken to delay and even avoid the problem. For example, treatment of a proportion of the people in an infected population (e.g. drug targeted at schoolchildren, women of child-bearing age or preschool children) will ensure that some nematodes remain in the untreated population and serve as a reservoir of drug-susceptible genes, diluting the selection pressure. Treatment given at intervals greater than the generation time of the nematodes will act against drug resistance. Treatment frequencies of 10–15 per year, needed to control haemonchosis in sheep, will never be used in human intestinal nematode control programmes. Changing the drug of choice or using combinations of anthelminthic drugs in helminth

control programmes will also tend to reduce and delay the degree of selection pressure on the population of nematodes (WHO, 1996c).

The fundamental strategy is to apply chemotherapy so that the emergence of drug resistance is delayed or controlled and will not endanger the health benefits of any chemotherapy-based control programme. Measures to recognize and monitor drug resistance are necessary and steps are being taken in that direction. Only the faecal egg count reduction test has any application at present in detection of anthelminthic drug resistance in developing countries (WHO, 1996c). There is a need to develop a standardized protocol and to investigate reliable tests to confirm suspected drug resistance under the conditions occurring in developing countries. In addition, attempts should be made to isolate resistant worms from the field and reproduce them under laboratory conditions for research purposes. WHO is developing a protocol to investigate suspected drug resistance and scientists are setting up a network to investigate issues related to the use of praziquantel and to monitor the development of possible resistance towards other anthelminthics in humans (Savioli *et al.*, 1997).

## 9. FUTURE DIRECTIONS

Much progress has been achieved in the control of intestinal nematode infections in the last decades. Yet, some research questions remain to be answered and control strategies have to be refined accordingly. Are preschool children another group at high risk of morbidity and, if so, how can they be reached? What is the impact of controlling iron-deficiency anaemia by reducing hookworm infections on women's reproductive health and their pregnancy outcomes? How should a cost–benefit analysis be carried out to ensure comparability?

Guidelines should be developed to help health planners set targets for control programmes and determine the frequency of application of anthelminthic treatment for sustaining control programmes. In addition, information should be provided to health planners to facilitate the assessment of morbidity and mortality in relation to the intensity of soil-transmitted nematode infections. There should be vigilance for the expected arrival of drug resistance; a consensus should be achieved by experts in this field to establish postulates and criteria to define anthelminthic drug resistance both *in vivo* and *in vitro*.

Integration of intestinal nematode infection control within the primary health care and integrated control with other communicable diseases is an appealing approach but its feasibility should be investigated and tested in

the field. The challenge for the next century will be to develop sustainable approaches for controlling intestinal helminth infections and associated diseases, being aware of the growing demographic trends and increasing urbanization that will be occurring, particularly in developing countries.

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# DNA Vaccines: Technology and Application as Anti-parasite and Anti-microbial Agents

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## ABSTRACT

*DNA vaccines have been termed* The Third Generation of Vaccines. *The recent successful immunization of experimental animals against a range of infectious agents and several tumour models of disease with plasmid DNA testifies to the powerful nature of this revolutionary approach in vaccinology. Among numerous advantages, a major attraction of DNA vaccines over conventional vaccines is that they are able to induce protective cytotoxic T-cell responses as well as helper T-cell and humoral immunity. Here we review the current state of nucleic acid vaccines and cover a wide range of topics including delivery mechanisms, uptake and expression of plasmid DNA, and the types of immune responses generated. Further, we discuss safety issues, and document the use of nucleic acid vaccines against viral, bacterial and parasitic diseases, and cancer. The early potential promise of DNA vaccination has been fully substantiated with recent, exciting developments including the movement from testing DNA vaccines in laboratory models to non-human primates and initial human clinical trials. These advances and the emerging voluminous literature on DNA vaccines highlight the rapid progress that has been made in the DNA immunization field. It will be of considerable interest to see whether the progress and optimism currently prevailing can be maintained, and whether the approach can indeed fulfil the medical and commerical promise anticipated.*

## 1. INTRODUCTION

The 'Third generation of vaccines' was how we termed nucleic acid vaccines in a 1995 review and predicted that they would become the vaccines of the future (Waine and McManus, 1995). From the first report of a successful DNA vaccine in 1993 to a current total of well over 500 published articles by the beginning of 1998, and with protective immunity in animal models having been successfully obtained against a broad range of infectious agents including viruses, bacteria and parasites, that prediction has been rapidly realized. In the 1995 review we defined the first, second and third generations of vaccines primarily on the basis of how the antigenic material for vaccination was derived. First-generation vaccines are based on whole organisms, in either live attenuated or killed forms. Second-generation vaccines consist of defined native or recombinant protein components derived from the organism rather than the whole organism itself. These components are prepared by either biochemical purification or genetic engineering. The third generation of vaccines

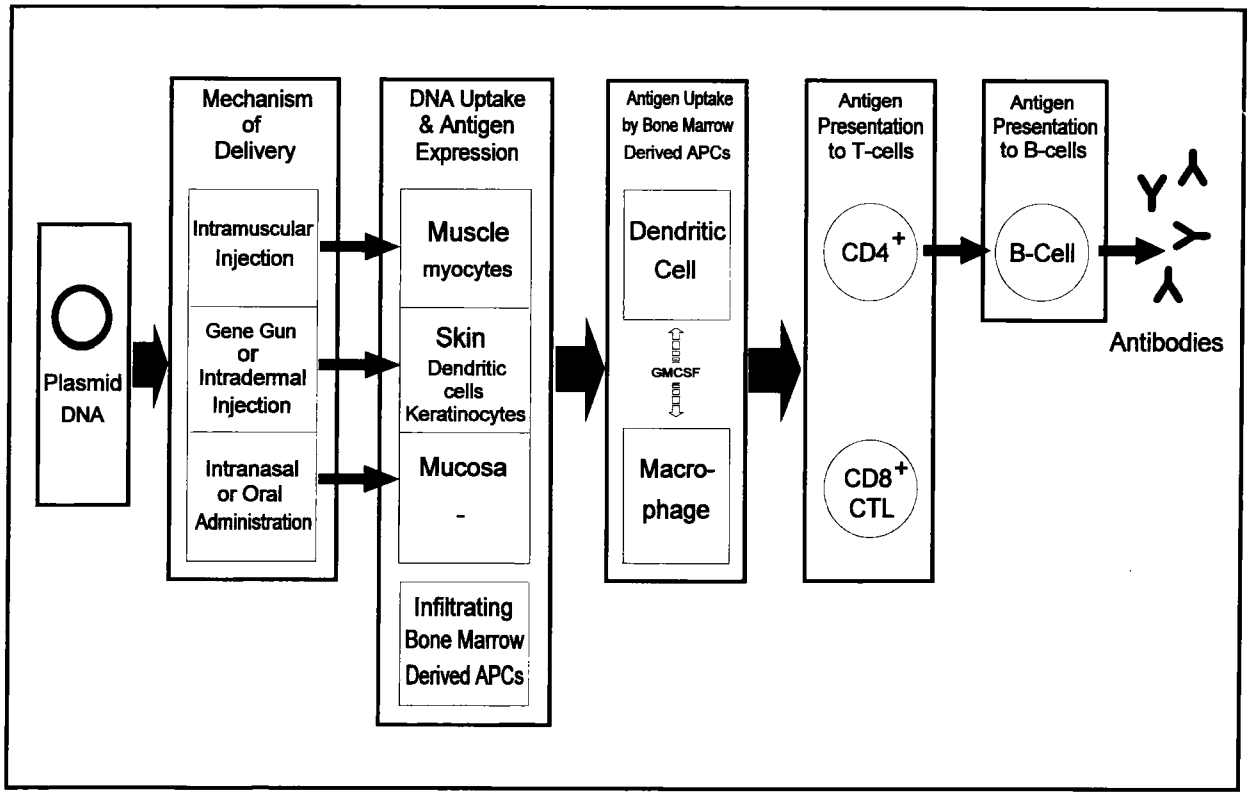
are the nucleic acid vaccines and are based on the genetic material of the infectious organism.

Live attenuated vaccines, such as the polio and smallpox vaccines, stimulate protective cytotoxic T lymphocyte (CTL) responses as well as T helper (Th) cell and humoral (antibody) immunity. A major concern with live vaccines is the intrinsic risk, however remote, of reversion to a pathogenic form (Ogra *et al.*, 1991; Weeks-Levy *et al.*, 1991). Killed vaccines do not carry this risk; however, while such vaccines can generate Th and humoral immune reponses against a pathogen, they are generally unable to generate specific CTL responses. Similarly, vaccination with defined protein components, such as tetanus or diphtheria toxoid or the recombinantly derived hepatitis B surface antigen, induces Th and humoral immune responses but generally not CTL responses. The attraction of nucleic acid vaccines is that they are able to induce all these responses, including specific CTL as well as Th and humoral responses, without the risks associated with live vaccines.

The first demonstration of protective immunity induced by DNA vaccination was in 1993 with the report of a vaccine providing heterologous protection against influenza virus (Ulmer *et al.*, 1993). In these pioneering experiments the DNA encoding influenza A nucleoprotein was injected into the quadriceps muscles of mice, which were shown to be protected from a subsequent challenge with a heterologous strain of influenza A virus. The precise mechanisms involved in DNA vaccination remain to be determined but the basic principle is simple, as illustrated in Figure 1. A DNA plasmid encoding the gene of interest is delivered directly to cells of the organism to be immunized; it is taken up and expressed by the host cells themselves and the endogenously expressed 'foreign' immunogen subsequently induces an immune response in the host. Thus, nucleic acid vaccines can, to a large extent, imitate or mimic features of vaccination with live attenuated viruses.

Nucleic acid vaccines have proved efficacious in generating protective immunity not only against infectious organisms but also, due to their specific CTL-inducing abilities, against several tumour models of disease as well. Here we review the current state of nucleic acid vaccines and cover a wide range of topics, including delivery mechanisms, uptake and expression of plasmid DNA, and the types of immune responses generated. Further, we discuss safety issues and document the use of nucleic acid vaccines against viral, bacterial and parasitic diseases, and cancer.

DNA vaccines offer many distinct advantages over first- and second-generation vaccines. Possibly the major advantages are that they obviate the need for peptide synthesis, expression and purification of recombinant proteins and the use of adjuvants. Moreover, plasmid DNA can be taken up by host cells *in vivo* and can persist long term in an extrachromosomal,


Plasmid DNA
Mechanism of Delivery
Intramuscular Injection
Gene Gun or Intradermal Injection
Intranasal or Oral Administration
DNA Uptake & Antigen Expression
Muscle myocytes
Skin Dendritic cells Keratinocytes
Mucosa
Infiltrating Bone Marrow Derived APCs
Antigen Uptake by Bone Marrow Derived APCs
Dendritic Cell
GMCSF
Macro-phage
Antigen Presentation to T-cells
CD4+
CD8+ CTL
Antigen Presentation to B-cells
B-Cell
Antibodies

non-replicative, circular form. Thus foreign genes can potentially be expressed for long periods from transfected host cells, making DNA vaccination a very promising way of programming cells *in vivo* to secrete proteins for immunization purposes. In addition, *in vivo* expression by the host (usually mammalian) cells ensures that the protein more closely resembles the normal eukaryotic structure, with the accompanying post-translation modifications of the native molecule, compared with a recombinant protein expressed in bacteria.

Throughout this review we generally use the term 'DNA' vaccines in reference to our discussion on nucleic acid vaccines. This is because the majority of research has focused on the production and use of DNA-based constructs (also called naked DNA). RNA vaccines have also been developed but to a very limited extent, as discussed below. As indicated above, the literature on nucleic acid vaccines is voluminous and there is, in fact, a very useful DNA Vaccine Web site (http://www.genweb.com/Dnavax), established by Dr Robert Whalen, Directeur de Recherche, Centre National de la Recherche Scientifique, Paris, France, which is frequently updated with new publications and other information on the topic.

## 2. PLASMID VECTORS AVAILABLE FOR DNA VACCINE USE

DNA vaccines consist of a plasmid with a strong viral promotor, the gene or complementary DNA (cDNA) of interest and a polyadenylation/transcriptional termination sequence. The plasmid is usually propagated in *Escherichia coli*, purified and then delivered into the host by various methods that are discussed later. As the plasmid vector is the vehicle from which the cDNA is expressed, vector design is crucial to allow for

*Figure 1* (opposite) A proposed model for induction of protective immunity by nucleic acid vaccines. Plasmid DNA can be delivered to various host tissues using a variety of mechanisms, including injection (usually into muscle), particle bombardment using a gene gun (usually into skin) and intranasal or oral administration. The DNA is taken up and expressed by host cells within the tissue (e.g. myocytes in muscle). The expressed antigen is then transferred to bone marrow-derived antigen-presenting cells (APCs) which, in turn, process and present the antigen to T-helper ($CD4^+$) cells or $CD8^+$ cytotoxic T lymphocytes (CTLs). The former cells are subsequently involved in antigen presentation to B cells, resulting in antibody production. An alternative pathway, which may occur concurrently, is that APCs such as dendritic cells, which infiltrate the target tissue, may themselves be directly transfected with plasmid DNA. These cells may express the antigen and present it directly to $CD8^+$ CTLs via their class I major histocompatibility complex molecules. GM-CSF, granulocyte–macrophage colony-stimulating factor. (Modified from Waine and McManus, 1997.)

maximal expression. To express within mammalian cells the plasmid vector is required to be driven by a eukaryotic promoter, the choice of which appears to be particularly important. Early DNA vaccine plasmid vectors were most commonly driven by the Rous sarcoma virus (RSV) promoter, shown to produce a 1000-fold higher rate of expression compared with the conventional SV40 promoter (Davies *et al.*, 1993a). More recent plasmid vectors incorporate a cytomegalovirus (CMV) immediate early promotor, providing even greater rates of expression. Thus, using the chloramphenicol acetyltransferase (CAT) gene as an indicator, plasmids containing the CMV promoter expressed greater levels of CAT than plasmids containing RSV promoters (Lee *et al.*, 1997). Furthermore, constructs expressing hepatitis B surface antigen (HBsAg) stimulated significantly higher levels of HBsAg-specific serum antibody titres when driven by a CMV promoter compared with an SV40 promoter (Bohm *et al.*, 1996). Various other modifications to vector design have also contributed to greater rates of expression. The insertion of enhancer sequences, synthetic introns, adenovirus tripartite leader (TPL) sequences and modifications to the polyadenylation and transcriptional termination sequences have all been shown to increase plasmid vector expression (Hartikka *et al.*, 1996; Lee *et al.*, 1997; Norman *et al.*, 1997).

## 3. RNA VACCINES

Theoretical concerns regarding the use of DNA vaccines relate to their ability to persist, the effect of long-term expression and the possibility of their integration into host chromosomal DNA. These issues are discussed later, but one possibility for circumventing many of these concerns is to use RNA instead of DNA for genetic vaccination purposes. RNA is transient in nature, does not persist, will not integrate into chromosomal DNA and does not cause insertional mutagenesis. RNA can be introduced into host tissues by direct injection or with the gene gun (see below) or by alphavirus-based expression vectors.

Alphaviruses are positive-strand RNA viruses that have had their replication machinery engineered for high-level expression of heterologous RNAs and proteins within insect and vertebrate cells (Frolov *et al.*, 1996). Alphaviruses such as sindbis (Johanning *et al.*, 1995) and SFV (Semliki Forest virus) (Zhou *et al.*, 1994) have been shown capable of expressing reporter molecules such as β-galactosidase and CAT in cell culture, as well as other proteins and RNA including the hepatitis B virus pregenome RNA, the papillomavirus 16 capsid protein, the neurokinin receptor, human immunodeficiency virus (HIV) glycoproteins and hepatitis

C virus glycoproteins (Frolov *et al.*, 1996). Strong CTL and humoral responses and, in some cases, protection induced by alphaviruses indicate their potential as nucleic acid vaccines (Zhou *et al.*, 1994; Dalemans *et al.*, 1995; Tubulekas *et al.*, 1997). Safety issues concerning the pathogenicity of alphaviruses remain, although attenuation, mutations and additional safeguards have been implemented and tested (Frolov *et al.*, 1996).

The intramuscular injection of luciferase-encoding mRNA into mice has mediated high, yet transient *in vivo* expression (Conry *et al.*, 1995a). Expression has also been obtained following gene-gun delivery of mRNA coding for reporter molecules, including luciferase and human growth hormone, while gene-gun delivery of human α-antitrypsin mRNA also resulted in strong antibody responses (Qiu *et al.*, 1996). The transient protein expression provided by RNA vaccines may be useful for inducing an immune response to a proto-oncogene product or growth factor that poses a risk of inducing malignant transformation consequent to prolonged protein expression (Conry *et al.*, 1995a; Strong *et al.*, 1997).

A major disadvantage with mRNA is that it is far less stable than DNA and the costs associated with its production, storage and transportation are likely to be much higher. As a consequence, far less work has been undertaken on developing RNA vaccines compared with their DNA counterparts. Furthermore, transient expression is less likely to induce long-term expression and, therefore, one dose may not be sufficient to generate long-term immunity.

## 4. METHODS OF DELIVERY

A number of different methods have been employed to introduce nucleic acid vaccines into animal cells and tissues and a description of each follows.

### 4.1. Intramuscular Immunization

Intramuscular injection, the most common form of DNA vaccine delivery, involves the injection of plasmid DNA suspended in an aqueous solution into the muscle of an individual host. While this technique has been predominantly used to obtain gene expression in mammals, especially mice, gene expression following intramuscular injection has also been demonstrated in fish (Hansen *et al.*, 1991; Anderson, E.D. *et al.*, 1996; Leong *et al.*, 1997). Skeletal muscle is the most common muscle injected due to its size and accessibility. In experimental mice, most reports have

utilized injection into the quadriceps muscle. Recently, however, one report has shown the immunity induced by DNA injection of the anterior tibial muscle in mice significantly exceeded that induced following injection of the quadriceps muscle (Yokoyama *et al.*, 1997). Cardiac muscle and muscle of the diaphragm have also been found to express plasmid DNA introduced by injection (Acsadi *et al.*, 1991a; David and Hasmin, 1993). The fact that this technique does not require any special delivery mechanism and leads to the permanent expression of foreign genes of cDNAs within muscle makes intramuscular injection an invaluable tool for vaccination as well as gene therapy (Davies *et al.*, 1993b; Danko and Wolff, 1994).

DNA injected intramuscularly has been found to distribute throughout muscle, with the DNA diffusing through the extracellular matrix, crossing the external lamina and entering individual myofibres (Wolff *et al.*, 1992a). The exact mechanisms involved in the uptake of DNA by individual muscle cells are unknown, but uptake by some type of cell membrane transporter has been proposed (Wolff *et al.*, 1992a). The transport of plasmid DNA into the cell nucleus appears to occur through the nuclear pore by a process common to other large karyophilic macromolecules (Dowty *et al.*, 1995).

Certain morphological characteristics of muscle make it an inefficient site for DNA uptake. The large amounts of connective tissue surrounding each muscle fibre and each group of fibres as well as the basal lamina form a physical barrier that could interfere with transfection of these cells. It is perhaps for this reason that only a small proportion of targeted muscle fibres (1–2%) are actually transfected (Davies *et al.*, 1994; Levy *et al.*, 1996). Several strategies have been employed to increase transfection of muscle cells. Regenerating muscle fibres that have been damaged by the myotoxic local anaesthetic bupivacaine are able to take up more DNA than muscle injected with DNA alone (Wells, 1993; Coney *et al.*, 1994; Davies *et al.*, 1995a). Muscle treated with bupivacaine is able to recover from the degeneration caused by its injection with no long-term histological effects (Danko *et al.*, 1994). Injection of DNA in conjunction with hypertonic solutions such as sucrose or saline has also been shown to increase the uptake of DNA by increasing the osmotic pressure around the injection site, improving distribution and resulting in less variability of expression (Davies *et al.*, 1993a; Feltquate *et al.*, 1997). Further, the needle alignment during injection can influence gene expression, with longitudinal injection parallel to the muscle fibres resulting in greater levels of gene expression (Levy *et al.*, 1996). Other factors, such as needle type, speed of injection, volume of injection fluid, type of muscle, physiological condition of the muscle and age as well as sex of the animal injected, can also influence plasmid DNA uptake and expression (Wolff *et al.*, 1991; Wells and Goldspink, 1992).

### 4.2. Intradermal Immunization

Another site suitable for DNA vaccination is the skin. The skin is one of the most frequent sites at which foreign antigen is encountered and therefore must be considered an important site for antigen presentation. Throughout evolution, the skin has developed its own immunological environment known as the skin immune system (SIS) (Bos, 1997). As a result, the skin and underlying dermis contain a high concentration of professional antigen-presenting cells (APCs) (dendritic and Langerhans' cells), which can take up foreign antigen and present it to lymphocytes in the draining lymph (Raz *et al.*, 1994; Condon *et al.*, 1996; Bos, 1997). Consequently, smaller amounts of DNA than those used in intramuscular injection are required to induce an immune response of the same magnitude (Fynan *et al.*, 1993a; Raz *et al.*, 1994; Webster *et al.*, 1994).

Although injection has been used to transfer DNA intradermally, the same results can be achieved following abrasion of the skin using a tuberculin-testing device coated with DNA; as long as the DNA penetrates the cornified epithelium it can be exposed to the skin-associated lymphoid tissues and other viable cells (Ciernik *et al.*, 1996).

### 4.3. Particle-mediated Immunization

Particle-mediated DNA vaccination involves the acceleration of plasmid DNA complexed to carrier particles into the tissues of an individual. Gold is the popular carrier particle of choice due to its chemical inertness and ease of manipulation and production. This form of gene transfer was originally developed for the transfection of plant cells using gunpowder acceleration (Klein *et al.*, 1987). Further development and refinement of the system for use on animal models by Auragen Inc. (formerly Agracetus) led to the development of a hand-held device, the gene gun, using compressed helium as the accelerant. The gene gun is now commercially available, with one of the most commonly used models being marketed by BIORAD (in collaboration with Auragen). Oxford Biosciences Ltd has developed a similar but smaller-size system (Powderject) that also uses a helium gas accelerant (Degano *et al.*, 1998).

While most commonly used on skin surfaces, particle-mediated DNA vaccination has also been applied to various other tissues such as liver (Williams *et al.*, 1991) and brain (Jiao *et al.*, 1993). By using the gene gun, cells are directly transfected with DNA and therefore less DNA is needed compared with intramuscular and intradermal injection, where uptake of DNA is from extracellular spaces (Williams *et al.*, 1991; Johnston and Tang, 1994). As the skin has been found to contain a dense network of

APCs, a potentially high number of these APCs could be transfected and express the DNA. The gene gun therefore requires as little as 16 ng of DNA to achieve potent humoral and cytotoxic cellular immune responses, demonstrating the significant potential in the use of this device in genetic vaccination (Eisenbraun *et al.*, 1993; Pertmer *et al.*, 1995; Haynes *et al.*, 1996).

While most commonly used on murine models, the gene gun has also been used in trials on larger animals. For example, antibodies were generated in rabbits after gene-gun delivery of plasmid expressing β-galactosidase (Sundaram *et al.*, 1996). In dermis, sustained luciferase activity was observed for over 1.5 years after gene delivery. *In vivo* gene expression was also detected in rhesus monkey tissues following gene-gun delivery of luciferase DNA (Cheng *et al.*, 1993).

Plasmid delivery by gene gun had been found to elicit different immune responses compared with intramuscular injection. Feltquate *et al.* (1997) found that intramuscular injection of influenza haemagglutinin (H1)-expressing plasmid DNA suspended in saline raised a predominantly Th1 response with mostly IgG2a anti-H1 antibodies while gene-gun DNA immunization produced a predominantly Th2 response with mostly IgG1 anti-H1 antibodies. While this could be due to the method of delivery, the difference could also be attributed to the different cell types transfected at each site. Torres *et al.* (1997) found that the excision of an injected muscle bundle in mice within 10 min of DNA vaccination did not affect the magnitude or longevity of the antigen-specific antibody response. In contract, biopsy of the skin target site up to 24 hours after gene-gun delivery completely abrogated the antibody response in the majority of mice. In another experiment, Winegar *et al.* (1996), using the polymerase chain reaction (PCR), found a dramatic decrease in intramuscularly injected plasmid DNA over a short period of time. From this, these authors concluded that cells transfected in gene gun-bombarded skin, but not needle-injected muscle, play a central role in DNA-initiated antibody and CTL responses. Therefore, it appears that the method of delivery and the different cell types transfected as a result contribute to the type of immune response generated.

### 4.4. Pneumatic (Jet) Injection

Another form of injection, pneumatic or jet injection, involves the use of air propulsion to deliver DNA suspended in an aqueous solution into tissues (Furth *et al.*, 1992; Davies *et al.*, 1994; Vahlsing *et al.*, 1994). No particles need to be used as a carrier for the DNA, as is necessary in gene-gun delivery. Moreover, pneumatic injection can be used to deliver DNA to

cells millimetres to centimetres below the skin surface (Furth *et al.*, 1995). Serum and colostrum antibody responses have been induced by jet injection of sheep with DNA encoding a *Cryptosporidium parvum* antigen (Jenkins *et al.*, 1995). This form of injection is also currently in use and under trial for the delivery of pharmaceuticals such as anaesthetics (Greenberg *et al.*, 1995; Queralt *et al.*, 1995) and insulin (Logwin *et al.*, 1996).

Although this is a promising technique, studies have shown significant shearing of the DNA after the high-pressure expulsion from the pneumatic gun (Furth *et al.*, 1992, 1995). This may explain the 10-fold lower expression of DNA using this method compared with conventional intramuscular injection, as well as lower levels of antibody and CTL generation (Vahlsing *et al.*, 1994). Also, up to 300 μg of DNA per pneumatic injection are required to elicit an immune response, which is even higher than that generally used in intramuscular injection into tissues (Furth *et al.*, 1992).

### 4.5. Use of Liposomes

Liposomes have long been used to transfect cell cultures *in vitro* with DNA; more recently, liposomes have also been used to transfect cells *in vivo*. Stronger CTL and humoral responses have been achieved following vaccination with plasmid DNA in combination with cationic liposomes compared with DNA alone (Ishii *et al.*, 1997a). Plasmid DNA encoding HBsAg, complexed to cationic liposomes and injected intramuscularly, generated IgG1 antibody titres 100-fold higher than those generated by the plasmid DNA alone (Gregoriadis *et al.*, 1997). It has been proposed that DNA–liposome complexes circumvent the need for muscle involvement in DNA vaccination and, instead, facilitate DNA uptake by APCs present at the site of injection or in the lymphatics (Gregoriadis *et al.*, 1997). The proposed mechanism of uptake of liposomes is via endocytosis (Friend *et al.*, 1996).

The use of liposomes also extends to facilitating DNA vaccination of mucosal membranes such as the nose and lung. Aerosolized cationic lipid gene-delivery vehicles have been demonstrated to increase the expression of CAT in mouse lung to levels 25-fold higher than those achieved by naked DNA (Felgner *et al.*, 1995). The incorporation of small amounts of a polyethylene glycol-containing lipid to the liposome complexes protects plasmid DNA from nebulizer-induced shearing and maintains its stability over long periods of time, helping to maintain full biological activity of the DNA both *in vitro* and *in vivo* (Eastman *et al.*, 1997; Hong *et al.*, 1997).

Liposomes have also been shown to increase the transfection of plasmid DNA injected intravenously. A single intravenous injection of plasmid

DNA–cationic liposome complexes into adult mice efficiently transfected virtually all tissues, including the lung, spleen, lymph nodes and bone marrow (Zhu *et al.*, 1993; Liu *et al.*, 1995). DNA vaccination by this technique produces levels of antibodies and CTL comparable to those seen following intramuscular injection of DNA in saline (Yokoyama *et al.*, 1996). By incorporating cholesterol as the neutral lipid and preparing them as multilamellar vesicles, the efficiency of DNA-liposome complexes has been increased due to prolonged circulation time following intravenous injection which produces even higher levels of expression (Liu *et al.*, 1997).

Intraperitoneal injection of cationic liposomes complexed to CAT can transfect splenic Thy $1.2^+$ T lymphocytes, as well as bone marrow-derived haematopoietic cells in adult mice (Philip *et al.*, 1993). Cationic lipid formulations have also been used to transfect luciferase genes into fetal brain cells in culture, resulting in luciferase expression when transplanted into adult host brains (Jiao *et al.*, 1992a).

Disadvantages of cationic liposomes include toxicity, ineffectiveness in serum and risk of disease or immune reactions (Alpar *et al.*, 1997). The development of novel liposomes, including those incorporating single cysteine, tryptophan and lysine repeats (Wadhwa *et al.*, 1997), metallo-nucleoliposomes (Kovalenko *et al.*, 1996) and polyamines (Longley *et al.*, 1995; Thierry *et al.*, 1997), aims to overcome these disadvantages while promoting greater DNA vaccine efficiency.

### 4.6. Immunization via Mucosal Membranes

Mucosal membranes, which include the oral, respiratory, synovial, gut, urinary and reproductive epithelia, are often the first barriers to invasion by viruses, bacteria and parasites. IgA, the predominant antibody isotype found along mucosal surfaces, has generally been viewed as an immune barrier to prevent the adherence and absorption of antigens. Recent studies have identified the ability of IgA to neutralize intracellular microbial pathogens such as viruses directly within epithelial cells, as well as the ability to remove antigens within the mucosal lamina propria, excreting them through the adjacent mucosal epithelium (Mazanec *et al.*, 1993; Lamm *et al.*, 1995). The development of vaccines that induce protective mucosal immunity, including IgA, would have the potential to prevent the establishment of these pathogens, including those that are sexually transmitted (Brown, 1996; Reyes *et al.*, 1997; Wang, B. *et al.*, 1997).

DNA vaccines targeted at mucosal membranes have been shown to generate cell-mediated and humoral responses (Langermann, 1996; Etchart *et al.*, 1997; Hinkula *et al.*, 1997a; Sizemore *et al.*, 1997). Recently, researchers demonstrated that local mucosal vaccination with DNA

vaccines induced not only an immune response at the local mucosa but also at distal mucosal sites as well as systemically (Asakura *et al.*, 1997). Intranasal vaccination of mice with plasmid DNA encoding various antigens, complexed to cationic liposomes, resulted in the expression of those antigens in various other tissues, including the trachea, lung, cervical and mesenteric lymph nodes and gastrointestinal tract (Kuklin *et al.*, 1997). DNA vaccination of nasal mucosa has been subsequently shown to induce a humoral response in serum and vaginal fluids and also a proliferative and CTL response in the spleen and iliac nodes draining the genital and rectal mucosa (Klavinskis *et al.*, 1997; Okada *et al.*, 1997). The intranasal immunization route provides good mucosal reactivity by inducing IgG, IgA and T-cell proliferative responses (Hinkula *et al.*, 1997a). Furthermore, complexing DNA with cationic lipids increases the efficiency of gene delivery and protein expression in nasal epithelium (Barnfield *et al.*, 1997).

The induction of mucosal immune responses by oral delivery of DNA vaccines has also been investigated. To prevent the degradation of plasmid DNA within the gut, various delivery systems have been developed including live and attenuated bacterial and viral vectors (Sizemore *et al.*, 1995; Estes *et al.*, 1997; Levine *et al.*, 1997; Pascual *et al.*, 1997; Toebe *et al.*, 1997). The oral delivery of plasmid DNA encapsulated within biodegradable polymer particles stimulated specific systemic and mucosal humoral responses as well as cell-mediated responses (Jones *et al.*, 1997a,b). Topical ocular and intravaginal application of DNA encoding foreign proteins has also been shown to be an effective means of inducing systemic and mucosal immune responses (Daheshia *et al.*, 1997; Wang, B. *et al.*, 1997).

A major advance in this area is the work of Darji and colleagues (1997) who used an attenuated strain of the bacterium *Salmonella typhimurium* as a vehicle for oral genetic immunization. They engineered the *S. typhimurium* aroA autotrophic mutant to carry either of two genes (ActA and listeriolysin) that encode virulence factors of *Listeria monocytogenes* and then delivered the DNA vaccine orally to mice. The salmonellae crossed the gut wall, presumably via the lymph nodes, and invaded phagocytic cells, their normal host cells. Excellent systemic immune responses to the DNA-encoded antigens expressed by the phagocytes resulted and protective immunity was induced with the listeriolysin transformants. This study suggests that safe and effective oral DNA vaccination against a range of phathogens and diseases is an achievable goal.

### 4.7. Expression Library Immunization

Expression library immunization (ELI) is a novel approach to genetic immunization in that potentially all of the genes present in a pathogen

can be delivered as a single vaccination. ELI is a rapid method for isolating protective genes from a pathogen, and may prove especially useful for pathogens that are difficult to grow or attenuate. In some cases, ELI may provide the only path for developing an effective vaccine (Barry *et al.*, 1995; Ulmer and Liu, 1996; Johnston and Barry, 1997). This technique was first tested using expression libraries made from the DNA of *Mycoplasma pulmonis*, a lung pathogen of rodents that was chosen due to its relatively small genome (Barry *et al.*, 1995). The approach showed that even partial expression libraries made from the DNA of *M. pulmonis* can provide protection against challenge from the pathogen, and it is somewhat surprising that it has not been more widely used.

## 5. IMMUNE RESPONSES GENERATED BY NUCLEIC ACID VACCINES

One of the most attractive features of DNA vaccines is their ability to induce a broad range of immune responses, including antibody protection, T-cell proliferation, lymphokine release, generation of CTL responses, and delayed hypersensitivity reactions. In this section we review the current state of knowledge associated with the induction of these responses by DNA vaccination. An excellent overview of this area has also been provided by Tighe *et al.* (1998).

### 5.1. Humoral Responses

A wide range of different nucleic acid constructs have successfully elicited the production of a humoral (antibody) response. However, the kinetics and magnitude of the response and the type of antibodies predominantly produced are influenced by numerous variables. These include the type of antigen encoded; whether or not the antigen is normally located intracellularly or secreted; the number, frequency and dose of immunizations; and perhaps most importantly, the site and method of delivery. The following sections demonstrate the characteristics and variation in humoral immune responsiveness that can be generated following DNA vaccination.

#### 5.1.1. *Longevity of the Humoral Response*

DNA vaccination can induce a particularly long-lived humoral response. For example, intramuscular injection of plasmid DNA encoding hepatitis B virus envelope protein (HBsAg) induced a strong humoral response to

the antigen that was sustained for up to 74 weeks without boost (Davies *et al.*, 1996a). Antibody levels did not diminish over time, and these could be further increased 10–200-fold by boosting with a second injection of DNA or with recombinant protein. A single injection of DNA resulted in a longer-lasting humoral response compared with a single injection of recombinant protein.

A single injection of 10–100 μg of plasmid DNA encoding influenza virus nucleoprotein produced IgA antibodies that first appeared 4–6 weeks after injection and peaked at 6–8 weeks, after which they remained stable for months; at early stages this response was dose dependent (Rhodes *et al.*, 1994). The nucleoprotein DNA vaccine, delivered as a single intramuscular injection, can generate nucleoprotein specific cellular responses that last at least 1 year (Yankauckas *et al.*, 1993). Further, mice injected into the skin with free plasmid DNA encoding the nucleoprotein gene had discrete foci of epidermal and dermal cells, including cells with dendritic morphology, that contained immunoreactive nucleoprotein antigen and induced both anti-nucleoprotein-specific antibodies and CTL (Raz *et al.*, 1994).

A study in which mice were inoculated with DNA encoding influenza haemagglutinin using a gene gun demonstrated that life-long maintenance of protective response could be generated. Further, utilization of a sensitive single-cell enzyme-linked immunospot assay showed that all of the haemagglutinin-specific plasma cells were localized in the bone marrow and spleen 1 year after immunization (Justewicz *et al.*, 1996).

### 5.1.2. *Kinetics of the Humoral Response*

Analysis of the antibody responses induced in mice by immunization with either soluble recombinant CD4 (rCD4) or plasmid DNA encoding CD4 (DNA-CD4) demonstrated that although specific antibody titres in mice receiving DNA-CD4 were lower than in those receiving rCD4, the DNA immunization induced antibodies reactive with the native cell surface molecule in all mice whereas only two of five mice immunized with rCD4 produced antibodies reactive with the native cell surface molecule, suggesting that the DNA approach may lead to a qualitatively superior response (Attanasio *et al.*, 1997). Differences in the kinetics of antibody production were also observed; antibodies reacting with cell-surface CD4 were present in the DNA-CD4-vaccinated mice 28 days after the first vaccination, whereas mice immunized with rCD4 produced specific antibodies only following a booster injection.

Mice immunized three times by intramuscular injection with a DNA construct encoding the herpes simplex virus (HSV) type 1 glycoprotein D gene under control of a CMV promoter developed an HSV-specific IgG but not IgA antibody response detectable in both serum and vaginal secretions

(Bourne *et al.*, 1996a). Intramuscular injection of plasmid DNA, expressing the three envelope proteins of the hepatitis B virus (HBV), induced IgM antibodies within the first 1–2 weeks (Michel *et al.*, 1995). Over the next few weeks, an IgM to IgG class switch occurred with peak IgG titres achieved by 4–8 weeks; these titres were maintained for a least 6 months without further injections.

Three intranasal doses of plasmid DNA encoding glycoprotein B of HSV-1 over a 3-week period was reported to induce a distal mucosal IgA response, which could be enhanced by co-administration of cholera toxin (Kuklin *et al.*, 1997). In contrast, intramuscular injection of the same construct induced a strong IgG response in serum, but was inefficient in generating a mucosal IgA response. In addition, mucosal immunization with glycoprotein B DNA induced anti-HSV cell-mediated immunity *in vivo* as measured by delayed-type hypersensitivity.

Immunization of mice with the regulatory genes *nef*, *rev* and *tat* from HIV-1 generated both humoral and cellular immune responses to the corresponding gene products (Hinkula *et al.*, 1997a). Humoral responses were seen after boosts, while potent T-cell proliferative responses were noted after a single immunization. A Th1 response was demonstrated early after immunization, while a 3–75-fold stronger T-cell response was observed in animals receiving DNA epidermally compared with those receiving intramuscular injections.

### 5.1.3. *Antibody Isotypes Generated*

Several studies have examined the isotype of antibodies generated following DNA immunization. Characterization of humoral responses induced by influenza haemagglutinin DNA (Hinkula *et al.*, 1997b) showed that the antibody response was maintained for at least 1.5 years and that the immunoglobulin isotype profile of the antibodies was predominantly IgG2a. This is similar to that induced by live virus infection but contrasts with the relative abundance of IgG1 antibodies observed after inoculation with formalin-inactivated whole virus (Deck *et al.*, 1997).

We have demonstrated that IgG2a antibodies are the predominant isotype induced when mice are immunized intramuscularly with plasmid DNA encoding paramyosin (Sj-97) from *Schistosoma japonicum* (Yang *et al.*, 1995; Waine *et al.*, 1997). However, we also observed that a number of other *S. japonicum* cDNAs encoding different antigens were unable to induce any humoral response in mice, rats or rabbits, even when fused to paramyosin (Waine *et al.*, 1997). This is exemplified in Figure 2 where a DNA construct comprising Sj-GST26 (previously shown unable to induce antibodies in either of the two different vectors, pRSV-BL or pBK-RSV) was fused in frame to the end of a cDNA encoding a fragment of paramyosin (PMY6). We had shown (Yang, W. and McManus, D.P.,

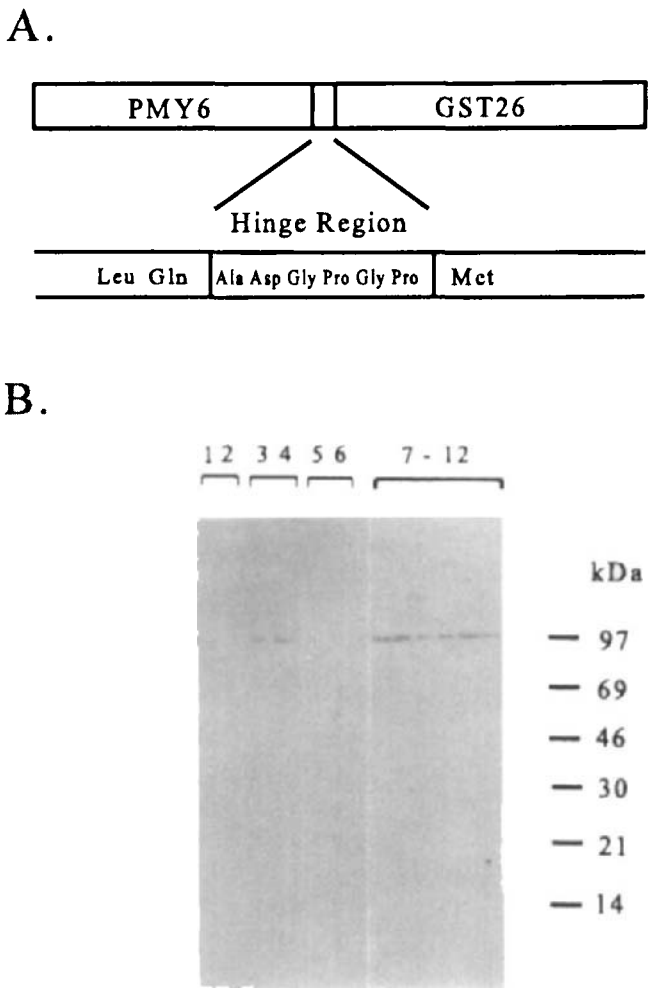



*Figure 2* (A) Design of a fusion construct constituting a paramyosin fragment (PMY6) and full-length 26-kDa glutathione S-transferase (GST26) of *Schistosoma japonicum*. The hinge region is enlarged and amino acids are indicated by the standard three-letter code. (B) Qualitative Western blot analysis (performed using standard procedures) of the antibody response in mice vaccinated with the PMY6/GST26 fusion construct, showing the reaction of sera from mice vaccinated with non-recombinant plasmid pRSV-BL (lanes 1 and 2), PMY6 (lanes 3 and 4), GST 26 (lanes 5 and 6) and the PMY6/GST26 fusion construct (lanes 7–12). Mouse sera were used at a 1:100 dilution. BALB/C/H-$2^d$ mice were immunized with the DNA constructs (or vector only as negative control) by injection of 50 μg plasmid DNA into each quadriceps muscle (100 μg per mouse) on weeks 0, 3 and 6 using a 26-gauge needle. Blood for sera was collected from the tail vein of immunized mice prior to, during and following the immunization regime and analysed by Western blotting using soluble adult *S. japonicum* extract as antigen.

unpublished data) that Sj-GST26 recombinant protein produced in *E. coli* is able to induce, in BALB/B mice, a strong antibody response detectable in Western blot. Sera from mice immunized with the fusion construct or appropriate controls (PMY6 or non-recombinant vector) were analysed for specific antibodies against native paramyosin (97 kDa) or GST26 (26 kDa), using whole parasite extracts as antigen. Western analysis showed that all the mice immunized with the PMY6 construct generated antibodies to a 97-kDa protein. Similarly, all the mice immunized with the fusion construct recognized a protein of 97 kDa. However, none recognized a protein at 26 kDa. Even when high serum concentrations (1:10) were tested, no anti-Sj-GST26 antibodies were detectable.

Administration of plasmid DNA encoding influenza virus nucleoprotein by intramuscular (100 μg) or gene gun (1 μg) vaccination detected differences in the relative levels of two IgG subclasses; intramuscular inoculation yielded predominantly IgG2a antibodies, whereas gene-gun delivery yielded predominantly IgG1 antibodies (Pertmer *et al.*, 1996). The production of IgG1 antibodies (a Th2-like response) by gene gun was accompanied by increasing interleukin (IL)-4 production with successive immunizations. In contrast, similar levels of IL-4 production were not observed following intramuscular immunization.

Comparison of antibody isotypes and lymphokine secretion following intradermal immunization of mice with either β-galactosidase protein or plasmid DNA encoding β-galactosidase in a CMV-based expression vector identified preferential induction of a Th1 immune response and inhibition of specific antibody formation by plasmid DNA immunization (Raz *et al.*, 1996). Specifically, the DNA immunization induced mainly IgG2a antibodies, whereas β-galactosidase protein in saline or alum induced IgG1 and IgE β-galactosidase specific antibodies.

Following intramuscular injection of mice, a plasmid containing a synthetic gene encoding the non-toxic C-terminal domain of tetanus toxin induced predominantly IgG2a, while the stimulated splenocytes secreted high levels of interferon (IFN)-γ (Anderson, R. *et al.*, 1996). In another study, comparison of the immune response induced by saline injection or gene-gun inoculation of plasmid DNA encoding influenza haemagglutinin (H1) demonstrated that injection into skin or muscle raised a predominantly Th1 response with mostly IgG2a anti-H1 antibodies produced, while gene-gun immunization into skin or muscle induced a predominantly Th2 response with mostly IgG1 anti-H1 antibodies (Feltquate *et al.*, 1997). These distinct types of immune responses were generated by the method not the route of DNA immunization, and were not due to differences in the doses of DNA. The initial immunization established the cell-type of the immune response, which did not change with further DNA immunizations using either method or after viral challenge.

When mice were immunized and boosted with plasmid DNA encoding the circumsporozoite protein of the malarial parasite *Plasmodium yoelii* (PyCSP), the initial humoral response was localized to the draining lymph nodes and was characterized by production of IgG1 anti-PyCSP antibodies and the Th2 cytokine Il-4 (Mor *et al.*, 1995). In contrast the secondary response was dominated by IFN-γ production (a Th1 cytokine) and the secretion of IgG2a anti-PyCSP antibodies in the spleen. PyCSP DNA and mRNA were detected only in the quadriceps muscles, the sites of injection. Yet these sites lacked either cytokine or antibody-secreting cells, indicating that circulating lymphocytes encounter plasmid-encoded antigen in the muscle bed, initiate a humoral response in the draining lymph nodes and then seed distal lymphoid organs.

Immunization with plasmid DNA encoding the measles virus haemagglutinin and nucleoprotein induced both humoral and cell-mediated immunity (Cardoso *et al.*, 1996). The major antibody isotype induced with both haemagglutinin and nucleoprotein DNA was IgG2a, consistent with a Th1 response. In contrast, immunization with a plasmid that directed the synthesis of a partially secreted form of haemagglutinin gave mainly IgG1 antibodies. When the amount of haemagglutinin plasmid DNA was reduced, no antibody was induced but a CTL response was observed.

However, predominance of one particular isotype does not always occur, as demonstrated by the analysis of the antibody induced in mice by immunization with either rCD4 or DNA-CD4 (Attanasio *et al.*, 1997). The two groups produced antibodies of different isotype distribution, with no clear predominance of specific IgG subclass observed for the DNA immunization compared with a predominantly IgG1 isotype response, indicating a Th2-like response, in the mice immunized with the recombinant protein.

### 5.1.4. *Other Features of the Humoral Response*

Another factor that may influence the immune response is whether or not the expressed protein is secreted. Mice immunized intramuscularly with DNA plasmids expressing a secreted or non-secreted form of hepatitis C virus (HCV) nucleocapsid induced IgG2a antibodies associated with a Th1-like profile characterized by the *in vitro* splenic production of IL-2 and IFN-γ (Inchauspe *et al.*, 1997a). Interestingly, the secreted form induced antibody titres three to five times higher than the non-secreted form. In contrast, mice immunized with recombinant protein (HCV nucleocapsid) induced a predominant, but not exclusive, Th2-like profile as characterized by the splenic production of IL-4 and IL-10.

Other evidence indicates that the context in which an antigen is expressed may also affect its ability to induce humoral immunity. In one study, vectors expressing the first 58 amino acids of the HCV nucleocapsid alone or as a fusion protein with the middle (pre-S2 and S) or major (S) surface antigens of HBV were constructed (Major *et al.*, 1995). Intramuscular immunizations of mice with the chimeric constructs in the form of naked DNA elicited humoral responses to antigens from both viruses within 2–6 weeks after injection. However, no anti-HCV responses were obtained in mice immunized with the vector expressing the HCV sequence in the non-fusion construct.

### 5.2. Anti-allergy Responses

DNA vaccination inhibits IgE antibody and associated allergy-type responses. For example, intramuscular injection of rats with plasmid DNA encoding a house-dust mite allergen resulted in long-term expression and specific immune responses (Hsu *et al.*, 1996). However, this approach prevented the induction of IgE synthesis, histamine release in bronchoalveolar fluids and airway hyperresponsiveness in rats challenged with aerosolized allergen. Furthermore, this suppression was persistent and could be transferred into naive rats by $CD8^+$ T cells from gene-immunized rats. In another study (Raz *et al.*, 1996), mice were immunized intradermally with either β-galactosidase protein or plasmid DNA encoding β-galactosidase in a CMV-based expression vector. DNA immunization induced mainly IgG2a antibodies, whereas β-galactosidase protein in saline or alum induced IgG1 and IgE β-galactosidase specific antibodies. Further, priming of the mice with the plasmid form prevented IgE antibody formation to a subsequent intraperitoneal injection of β-galactosidase with alum; this effect was antigen specific because priming with the *lac*Z plasmid did not inhibit IgE anti-ovalbumin antibody formation. This study demonstrated that DNA immunization induced a Th1 response that could dominate an ongoing protein-induced Th2 response in an antigen-specific manner, since the DNA immunization was able to reduce the antigen-specific IgE production generated in the antigen/alum-primed mice.

Passive transfer of $CD4^+$ or $CD8^+$ cells from DNA-immunized mice in which the IgE response is suppressed has demonstrated that the inhibition of IgE antibody formation by plasmid DNA immunization is mediated by both $CD4^+$ and $CD8^+$ T cells (Lee, D.J. *et al.*, 1997). The reason for the down-regulation or lack of IgE production may be explained as a result of recent observations published by Holmes *et al.* (1997), who have systematically investigated the role of $CD8^+$ cells in the regulation of IgE and $CD4^+$ T-cell responses to ovalbumin by $CD8^+$ T-cell depletion *in vivo*. In a

series of elaborate experiments, this group showed that depletion of $CD8^+$ cells at a critical time after immunization enchanced the magnitude and duration of the IgE response and that adoptive transfer of antigen-specific $CD8^+$ T cells resulted in profound inhibition of the IgE response. Furthermore, depletion of $CD8^+$ T cells led to immune deviation of antigen-specific $CD4^+$ T cells away from the normal Th1-like response, characterized by high IFN-$\gamma$ and low IL-4, to up-regulation of IgE production and a Th2-like, IL-4-dominated response. These results demonstrate that antigen-specific $CD8^+$ T cells inhibit IgE in the immune response to non-replicating antigens. The data indicate two possible mechanisms; first, $CD8^+$ T cells have direct inhibitory effects on switching to IgE in B cells; and second, they inhibit ovalbumin-specific IL-4 production but enhance IFN-$\alpha$ production by $CD4^+$ T cells (Holmes *et al.*, 1997).

We have observed a similar inhibition of IgE production following DNA immunization. The 22.6-kDa tegumental membrane-associated antigen of schistosomes is known to be recognized by specific IgE antibodies following human infection with schistosomiasis (Webster *et al.*, 1997). Moreover, we have recently demonstrated that the recombinant protein encoding the 22.6-kDa antigen of *S. japonicum* can itself induce IgE when injected into mice without any adjuvant (Waine *et al.*, 1997). However, when delivered as a DNA vaccine using the gene gun, the same cDNA induced specific IgG but not IgE antibodies (G.W. Waine *et al.*, unpublished results). Presumably, as discussed previously, immunization with the recombinant protein does not induce a $CD8^+$ T cell response, thus permitting IgE production; however, immunization using the cDNA presumably induces a $CD8^+$ T cell response that suppresses specific IgE production.

### 5.3. CTL Responses

One of the greatest attributes of DNA vaccines is their ability to induce $CD8^+$ CTL responses without the risk associated with live vaccines. This ability has been well documented in several reviews (see for example Donnelly *et al.*, 1997) and is therefore not reiterated here. Instead, we introduce a few recently published studies relating to CTL responses and the mechanism of CTL priming. DNA constructs encoding influenza virus nucleoprotein have demonstrated that DNA immunization is capable of eliciting protective cellular immunity against both immunodominant and immunorecessive CTL epitopes (Fu *et al.*, 1997). Moreover, when plasmids expressing influenza nucleoprotein were delivered in tandem with the co-stimulatory molecule B7-1 (but not B7-2), antigen-specific CTL responses were shown to be significantly enhanced. Antigen-specific CTL responses were also enhanced when the nucleoprotein plasmids were co-injected with

granulocyte–macrophage colony-stimulating factor (GM-CSF)- and/or IL-12-expressing plasmids (Iwasaki *et al.*, 1997). Enhancement of cell-mediated immunity has also been demonstrated recently by co-inoculation of an expression plasmid encoding TCA3. CTL assays demonstrated that cell-mediated immunity, elicited by co-inoculation of a DNA vaccine encoding a HIV antigen together with the TCA3 expression plasmid, was markedly enhanced compared with that obtained using the DNA vaccine alone (Tsuji *et al.*, 1997a).

$CD8^+$T lymphocytes recognize major histocompatibility complex (MHC) class I molecules complexed to peptides of 8–10 amino acid residues derived largely from cytosolic proteins. Such proteins are generally thought to be fragmented in the cytoplasm, and delivered to nascent class I molecules in the endoplasmic reticulum. At least one study (Restifo *et al.*, 1995) has indicated that targeting proteins to the endoplasmic reticulum by addition of an $NH_2$-terminal insertion sequence can, in some cases, greatly enhance the $CD8^+$ immune response. Although this study used vaccinia virus constructs, the approach should be transferable to naked DNA.

In other work, epitope-specific CTL responses have also been obtained by inserting a CTL epitope (from influenza A virus nucleoprotein) into the *lac*Z gene (Normura *et al.*, 1996); the latter successfully expressed the nucleoprotein epitope and primed epitope-specific CTLs. It has recently been shown that targeting of HIV-1 antigens for rapid intracellular degradation enhances CTL recognition and the induction of *de novo* responses *in vivo* after immunization (Tobery and Siliciano, 1997). Specifically, targeting a type I transmembrane protein, the HIV-1 envelope protein, for expression in the cytoplasm, rather than allowing its normal co-translational translocation into the endoplasmic reticulum, sensitized target cells expressing this mutant more rapidly for lysis by an env-specific CTL clone. Additionally, a greatly enhanced *de novo* env-specific CTL response was induced *in vivo* after immunization of mice with recombinant vaccinia vectors expressing the cytoplasmic env mutant. Similarly, targeting a cytoplasmic protein, HIV-1 nef, to undergo rapid cytoplasmic degradation induced a greatly enhanced *de novo* nef-specific $CD8^+$ CTL response *in vivo* after immunization of mice with either recombinant vaccinia vectors or DNA plasmids expressing the degradation-targeted nef mutant.

Research on the kinetics of the CTL response has shown that DNA plasmid vectors encoding the HBV envelope protein induced a highly specific CTL response, initiated between 3 and 6 days after DNA injection (Davies *et al.*, 1995b). By 6–12 days after a single DNA injection, *ex vivo* cytolytic activity was nearly maximal; similar high levels of activity were still detected 4 months after injection. In other work, Condon *et al.* (1996) demonstrated that cutaneous immunization with DNA resulted in potent, antigen-specific, CTL-mediated protective tumour immunity

resulting from the transfection of skin-derived dendritic cells, which localized in the draining lymph nodes.

## 5.4. Mechanism of CTL Priming

Until recently, the mechanism of CTL priming has been unknown. However, recent research has begun to shed some light on this question, providing evidence that induction of CTL responses by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells and that a form of 'antigen transfer' may be involved. Parent–$F_1$ bone marrow chimeric mice were generated in which the H-2b×d recipient mice received bone marrow-derived APCs that expressed only H-2b or H-2d MHC molecules (Corr *et al.*, 1996). These mice were injected intramuscularly with a single antigen containing epitopes for both H-2Db and H-2Kd. The resulting CTL responses were restricted to the MHC haplotype of the bone marrow alone and not to the haplotype expressed by the recipients' myocytes, indicating that the role of somatic tissues expressing protein from injected plasmids may be to act as a reservoir for that antigen, which is then transferred to the APC. These experiments show that the method of priming in DNA vaccination uses the MHC from bone marrow-derived APCs, which are efficient at providing all the necessary signals for priming the T cell.

In another elaborate study, Fu *et al.* (1997) demonstrated conclusively that antigen transfer from muscle cells to professional APCs can occur. Again, parent–$F_1$ bone marrow chimeric mice were generated whose somatic cells included muscle cells bearing both parental MHC haplotypes, while their professional APCs expressed only the donor MHC haplotypes. Upon injection of nucleoprotein DNA, CTL responses generated in the chimeras were restricted to the donor MHC haplotype, demonstrating that cells of the bone marrow lineage were responsible for priming CTL responses after DNA immunization. Moreover, expression of antigen by muscle cells in bone marrow chimeric mice after myoblast transplantation was sufficent to induce CTL responses restricted only by the MHC haplotype of the donor bone marrow, indicating that transfer of antigen from myocytes to professional APCs can occur, thus obviating a requirement for direct transfection of bone marrow-derived cells. These authors also were of the opinion that since immunization with soluble recombinant nucleoprotein protein did not induce MHC class I-restricted CTL responses or protection against cross-strain virus challenge, the molecular form of nucleoprotein or the means by which nucleoprotein is transferred might be different from the soluble nucleoprotein when injected. The antigen transfer observed in these experiments after myoblast transplantation provides further evidence for MHC class I-associated antigen processing and

presentation, such as the proposed mechanism known as cross-priming (Brossart and Beven, 1997). These studies demonstrate that bone marrow-derived professional APCs are required for CTL priming and that they can provide all the necessary signals for this process.

### 5.5. Th Cell Responses

Immunization with DNA induces a range of Th responses including lymphoproliferation and the generation of various cytokine profiles. A number of studies have been undertaken to examine these responses to a range of antigens. In many cases, but not always, a Th1-like cytokine profile is induced following DNA immunization. Immunization of mice and monkeys with expression vectors encoding HIV proteins was shown to elicit long-lived (7 months) Th cell proliferative responses *in vitro* following antigen stimulation (Shiver *et al.*, 1995, 1996). Moreover, analysis of cytokines showed that approximately 100-fold more IFN-γ than IL-4 was secreted, indicating that the immunization generated a Th1-like response. Humoral responses were also produced to most of the encoded antigens, but not to rev, an intracellular protein.

In another study, mice immunized intradermally with plasmid DNA encoding β-galactosidase in a CMV-based expression vector induced splenic $CD4^+$ Th cells to secrete IFN-α but not IL-4 and IL-5; this was accompanied by the production of mainly IgG2a β-galactosidase-specific antibodies (Raz *et al.*, 1996). This was in contrast to results obtained when β-galactosidase protein was administered in saline or alum, which resulted in the secretion of IL-4 and IL-5 but not IFN-α after *in vitro* stimulation with antigen and was accompanied by the induction of IgG1 and IgE β-galactosidase-specific antibodies. Similarly, immunization with plasmid vectors expressing HBsAg has been shown to induce strong CTL responses as well as a dominant Th1 phenotype among the splenic lymphocytes of immunized mice (Whalen *et al.*, 1995). The Th1 cytokine profile was obtained in two different strains of mice and with two types of proteins, HBsAg and β-galactosidase.

In contrast, other studies have observed Th2-like or mixed Th responses following DNA immunization. For example, a plasmid encoding human carcinoembryonic antigen (CEA) was shown to induce both humoral and cellular immune responses specific for CEA in immunized mice (Conry *et al.*, 1994). CEA-specific memory T cells were detected in three of five mice, while lymphocytes from two of five mice had IL-2/IL-4 release in response to CEA.

A study by Waisman *et al.* (1996) investigated cytokine release in H-2u mice after immunization with myelin basic protein. In these mice, a variable

region gene of the T-cell receptor, *Vβ8.2*, is rearranged and its product expressed on pathogenic T cells that induce experimental autoimmune encephalomyelitis in H-2u mice after immunization with myelin basic protein. Vaccination of these mice with naked DNA encoding *Vβ8.2* protected mice from the disease. Cytokine analysis of T cells reacting to the pathogenic portion of the myelin basic protein molecule indicated that, in the DNA-vaccinated mice, there was a reduction in the Th1 cytokines IL-2 and IFN-α accompanied by an increase in IL-4, a Th2 cytokine associated with suppression of the disease.

In an important advance, Boyle *et al.* (1998) used a different approach, targeting DNA-encoding antigen-ligand fusion proteins to APCs and lymph nodes directly to increase cellular and humoral immune responses. The results indicated that by targeting different lymphoid compartments, some control over the magnitude and form of the immune response could be gained. This generic approach may prove useful in the development of both genetic and more conventional vaccines so that the immune response most likely to confer protection can be generated.

## 5.6. Modulation of the Immune Response

Extensive work has been undertaken to investigate ways of modifying the immune response, in many cases to enhance the response, at other times to alter the Th1/Th2 balance. In the majority of research undertaken to date, this work has involved the delivery of DNA encoding various cytokines.

Early studies demonstrated that direct injection into mouse skeletal muscle of plasmid DNA encoding IL-2, IL-4 or transforming growth factor (TGF) β1 could induce biological effects characteristic of these cytokines, including the regulation of both humoral and cellular immune responses (Raz *et al.*, 1993). Later it was shown that co-inoculation of a plasmid expressing the glycoprotein of rabies virus with plasmids encoding murine GM-CSF enhanced B and Th cell activity to rabies virus, while co-inoculation with a plasmid expressing IFN-α resulted in a decrease of the immune response to the viral antigen (Xiang and Ertl, 1995).

Immunization of mice using HCV core (a non-secreted viral structural protein) DNA expression plasmids had demonstrated an efficient CTL response but relatively weak humoral and Th cell proliferative responses (Geissler *et al.*, 1997a). $CD4^+$ inflammatory T-cell proliferative responses as well as $CD8^+$ CTL activity to HCV core protein were enhanced substantially after co-immunization with IL-2 and GM-CSF DNA expression constructs. In contrast, co-immunization with an IL-4 producing construct induced differentiation of Th cells towards a Th0 subtype and suppressed HCV core-specific CTL activity.

Co-delivery of DNA plasmids encoding IL-12 or GM-CSF with a plasmid encoding HIV-1 antigen has also been shown to modulate the immune response (Kim *et al.*, 1997a). Co-delivery with IL-12 resulted in a reduction of specific antibody response compared with controls, while co-injection with GM-CSF genes resulted in enchancement of the antibody response. Moreover, a dramatic increase in specific CTL response was observed in the group co-immunized with the HIV-1 DNA vaccine and IL-12 genes.

In a recent study the ability of cytokine DNA to modulate disease, herpetic stromal keratitis, an inflammatory lesion in the cornea orchestrated by $CD4^+$ Th1 lymphocytes following ocular infection with HSV, was demonstrated following a single topical adminstration of DNA encoding IL-10 (Daheshia *et al.*, 1997). The IL-10 DNA was beneficial to the majority (75%) of treated animals, while 50% (vs. 10% in controls) resolved their lesions. Further evidence of the potential of cytokines delivered as naked DNA to modulate disease activity was demonstrated in murine systemic lupus erythematosus when expression vectors encoding the genes for TGF-β or IL-2 were delivered (Raz *et al.*, 1995).

HIV-1 specific cell-mediated immunity was enhanced by co-inoculation of an expression plasmid encoding TCA3 with a DNA vaccine (Tsuji *et al.*, 1997a). TCA3, a member of the β-chemokine family, has chemotactic activity for monocytes/macrophages and neutrophils. In another study, a fusion construct containing a nine-amino-acid peptide from IL-1β was shown to augment anti-tumour immune responses induced by a DNA vaccine (Hakim *et al.*, 1996).

In addition to the use of cytokines, other approaches have also proved effective at modulating the immune response to DNA vaccines. For example, co-delivery of B7-1, a ligand present on APCs that interacts with the T-cell co-stimulatory molecules CD28 and CTLA-4 during induction of the immune response to many antigens, was shown to enhance the antibody and anti-tumour effects of a human CEA DNA vaccine when B7-1 and CEA were encoded in the same plasmid (Conry *et al.*, 1996). Of note, however, was that co-delivery of cDNAs encoding B7-1 and CEA in the form of two separate plasmids produced no augmentation, suggesting that the positive effect was contingent upon co-expression of B7-1 and CEA within the same cell.

## 5.7. Influence of the Source of DNA and CpG Motifs on Immunogenicity

An accumulating body of evidence indicates that DNA itself has immunological properties that are dependent on both its source and sequence-specific determinants (Sato *et al.*, 1996). Early work indicated that DNA

from *E. coli*, but not mammalian DNA, could stimulate lymphocytes *in vitro* generating a dose-dependent proliferative response (Messina *et al.*, 1991, 1993); further, whereas DNA from three different bacterial sources induced proliferation, DNAs from three mammalian species were non-mitogenic. Depletion of T cells from lymphocytes did not reduce proliferation, suggesting that bacterial DNA directly triggered B-cell proliferation.

More recently, CpG motifs in bacterial DNA were shown to trigger direct B-cell activation (Krieg *et al.*, 1995). Bacterial and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B-cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo*. Optimal B-cell activation required a DNA motif in which an unmethylated CpG dinucleotide was flanked by two 5′ purines and two 3′ pyrimidines. These data suggest a possible innate immune defence mechanism against microbial DNA based on the recognition of CpG dinucleotides that are present at the expected frequency in prokaryotic DNA but which are under-represented (CpG suppression) and unmethylated in vertebrate DNA (Krieg, 1996a,b).

CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN-α (Halpern *et al.*, 1996). A six-base DNA motif consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines was shown to contribute to the immune response by inducing polyclonal B-cell activation (Klinman *et al.*, 1996). This stimulatory motif is 20 times more common in the DNA of bacteria than higher vertebrates. This study further showed that the same motif induced the rapid and co-ordinated secretion of IL-6, IL-12 and IFN-γ but not IL-2, IL-4, IL-4, IL-5 or IL-10 *in vivo* and *in vitro*.

It has been demonstrated that CpG motifs present in DNA vaccines (which are constructed from plasmids of bacterial DNA) induce the same cytokines as those stimulated by bacterial DNA. Moreover, the immunogenicity of a DNA vaccine was shown to be significantly reduced by methylating its CpG motifs and significantly increased by co-administering exogenous CpG-containing DNA, indicating that the CpG motifs in the plasmid backbone of DNA vaccines play an important role in induction of antigen-specific immunity (Klinman *et al.*, 1997).

Work by Yi *et al.* (1996a, b) has shown that treatment of a B-cell line with CpG DNA led to an increase in the transcriptional activity of the IL-6 promoter. This CpG DNA-induced IL-6 production was not mediated by a protein kinase C-, protein kinase A- or nitric oxide-dependent pathway, but was inhibited by an anti-oxidant. In addition, the level of intracellular reactive oxygen species was increased within 20 min after CpG DNA treatment. These results suggest that CpG DNA-induced IL-6 production is mediated through a reactive oxygen intermediate-dependent pathway and, moreover, that this induction in response to CpG motifs in bacterial

DNA may be an important immune defence mechanism that facilitiates a rapid response to microbial infection.

Other research has demonstrated that bacterial DNA or certain synthetic oligonucleotides displaying unmethylated CpG motifs can trigger macrophages *in vitro* to induce nuclear translocation of nuclear factor κB, accumulate tumour nercrosis factor α mRNA and release large amounts of tumour necrosis factor α, which *in vivo* leads to pro-inflammatory cytokine release (Sparwasser *et al.*, 1997).

In addition to bacterial DNA, strong stimulation of B-cells has been shown using DNA from various other organisms that display little or no methylation of CpG motifs, including yeast, nematodes, molluscs and insects (Sun *et al.*, 1997). For these organisms, DNA induced B-cells to up-regulate the activation marker CD69, and caused B-cells to enter the cell cycle, indicative of polyclonal activation. Methylation of CpG motifs inhibited the effect. In contrast, no stimulation of B-cells was observed with DNA from organisms whose CpG motifs are heavily methylated, including vertebrates such as mammals, fish and frogs, and also plants (corn). Despite this correlation, DNA prepared from two murine cell lines exhibiting hypomethylation of CpG motifs caused little or no stimulation of B cells. Thus, the authors of this study (Sun *et al.*, 1997) suggested that the idea that the stimulatory properties of DNA correlate solely with the presence of unmethylated CpG motifs may be an oversimplification.

## 6. USE OF DNA VACCINES AGAINST INFECTIOUS AND PARASITIC DISEASES

### 6.1 Viral Diseases

#### 6.1.1. *Influenza*

Much of the pioneering research of DNA vaccines has been undertaken on viral pathogens. Indeed, as referred to earlier, the first report describing the protective efficacy of plasmid DNA in an animal challenge model involved the influenza virus (Ulmer *et al.*, 1993). The plasmid DNA construct, encoding the internal conserved nucleoprotein of influenza A virus, was injected directly into the quadriceps muscle of mice. This vaccine successfully protected 90% of the mice against a subsequent lethal viral challenge, while only 20% of the controls survived. Both strong nucleoprotein-specific cell-mediated immunity, including CTL responses, and high-titre nucleoprotein-specific IgG antibodies were detected in these experiments and by others (Yankauckas *et al.*, 1993; Rhodes *et al.*, 1994; Pertmer *et al.*, 1996).

The CTLs generated were shown to be functional *in vivo*, since nucleoprotein DNA-vaccinated mice were significantly protected from cross-strain challenge whereas neither immunization with recombinant nucleoprotein nor passive transfer of nucleoprotein-specific antibodies mediated cross-strain protection (Ulmer *et al.*, 1993). Importantly, it should be emphasized that conventional inactivated influenza vaccines do not induce CTL responses but rely instead upon generating antibodies against the polymorphic surface antigens. A number of subsequent publications have emphasized the efficacy of DNA vaccines for stimulating CTLs capable of controlling viral infections (see, for example, Fu *et al.*, 1997; Sarzotti *et al.*, 1997).

With another construct, namely a DNA plasmid encoding haemagglutinin, a variant surface glycoprotein of influenza virus, high titres of anti-haemagglutinin murine antibodies were detected following intramuscular injection and the mice were protected against challenge with the homologous viral isolate (Ulmer *et al.*, 1994). The level of anti-haemagglutinin antibodies was shown to be maintained for 1.5 years, the immunoglobulin isotype profile of the antibodies was predominantly IgG2a and complete protection was conferred by as little as 1 μg of DNA (given twice) (Deck *et al.*, 1997). Mice vaccinated with haemagglutinin DNA by the gene gun generated both IgG- and IgA-producing plasma cells (Justewicz *et al.*, 1995) and, again, the protective B-cell responses were shown to be long term (Justewicz and Webster, 1996). Indeed, single gene inoculations of as little as 0.04 μg and booster inoculations of 0.004 μg haemagglutinin DNA raised complete protection in BALB/c mice against lethal viral challenge, although, at the high lethal dose of challenge virus used, no protection was elicited in mice immunized with nucleoprotein DNA (Robinson *et al.*, 1997).

In an important advance, immunization of ferrets with DNA encoding haemagglutinin, nucleoprotein and the conserved internal matix protein (M1) was shown to induce protective immune responses against human clinical strains (as opposed to mouse-adapted strains) of influenza as the challenge virus (Donnelly *et al.*, 1995). Protective immune responses have also been generated by DNA vaccines encoding haemagglutinin in ferrets (Webster *et al.*, 1994) and in chickens (Fynan *et al.*, 1993a,b; Robinson *et al.*, 1993). More recently a DNA vaccine mixture composed of a plasmid encoding haemagglutinin from a 1991 strain and plasmids encoding nucleoprotein and M1 from a 1989 strain protected ferrets against challenge with an antigenic-drift 1994 strain, as assessed by the intensity and duration of virus shedding (Donnelly *et al.*, 1997). Significantly, the DNA vaccine provided equivalent protection to a licensed inactivated virus vaccine that exactly matched the challenge strain used in these studies. In similar vein, gene-gun delivery of haemagglutinin DNA provided high

levels of protection in chickens as good as, if not better than, those achieved with a conventional whole virus vaccine, with fewer instances of morbidity and death (Kodihalli *et al.*, 1997).

While DNA immunization for influenza virus has been demonstrated to be effective in pre-clinical models, recent studies have shown that a vaccine mixture of DNA plasmids encoding haemagglutinin of each of three relevant strains (A/H3N2, A/H1N1 and B) as well as nucleoprotein and M1 from the A/H3N2 and B strains could generate effective levels of functional (haemagglutination-inhibiting) antibody in African green monekys (Liu, M.A. *et al.*, 1997). The monkeys were immunized twice, as is recommended for immunization of humans without a prior exposure to influenza virus, and doses as low as 10 μg per construct were efficacious. After the second immunization the resulting high-titre antibody was as good as, or better than, licensed vaccines for influenza and was reactive against both the homologous and a drift variant of the virus. Influenza epidemics tend to occur every 2–3 years and result in considerable morbidity, mainly in children and young adults, and significant mortality in the elderly. Antigenic variation of the major surface proteins, especially haemagglutinin is a major contributing factor to epidemic influenza. The recent results obtained with pre-clinical models, notably the ferret, and with monkeys provide optimistic signs that DNA vaccination can elicit broad protection against emergent viral strains by eliciting both cell-mediated immune responses against conserved internal proteins and antibodies against the surface proteins.

### 6.1.2. *Hepatitis*

(a) *HBV.* This virus remains an important health problem worldwide. The currently available antigen-based vaccines, comprising empty subviral particles composed of HBsAg, are safe and effective but expensive. This limits their widespread use and there is no effective treatment against the chronic carrier state, which can lead to cirrhosis and hepatocellular carcinoma. The available evidence suggests that DNA-based immunization against HBV is potentially useful for both prophylactic and therapeutic applications.

It is well recognized that antibodies against HBsAg alone are sufficient to confer protection. Encouragingly, immunization of mice by parenteral injection or by particle bombardment with HBsAg-expressing plasmid DNA results in rapid induction of strong and long-lasting humoral as well as CTL responses (Davis *et al.*, 1993c, 1994, 1995b, 1996b, 1997a; Fuller *et al.*, 1995; Whalen and Davies, 1995; Whalen *et al.*, 1995; Mancini *et al.*, 1996). DNA immunization of H-2B mice with HBV core antigen-expressing plasmids similarly induced antibody and CTL responses (Kuhober *et al.*, 1996). In general, the HBsAg expression vectors induced murine

humoral responses mimicking those observed in humans during the course of natural HBV infection (Michel *et al.*, 1995). Recent work has shown that even stronger humoral and cellular responses can be elicited against HBsAg if the DNA is injected into regenerating mouse muscle (initially damaged by necrotizing agents) by co-injection of DNA encoding cytokines such as IL-2 or one of the B7 co-stimulatory molecules such as pCMV-B7 (Chow *et al.*, 1997). As a prophylactic vaccine, therefore, the DNA approach may prove superior to the use of recombinant HBsAg, especially for the induction of a very rapid humoral response in neonates born to chronic HBV carrier mothers (Davies *et al.*, 1997a). Importantly in the clinical context, intramuscular vaccination of two chimpanzees with HBsAg plasmid DNA induced detectable levels of anti-HBV antibodies; in one animal, extremely high titres were attained and these were maintained for a least 1 year (Davies *et al.*, 1996b). Subsequently, it was shown that two newborn chimpanzees immunized intramuscularly with HBsAg plasmid DNA, could be successfully protected against HBV challenge (Prince *et al.*, 1997).

Transgenic mice expressing the HbsAg-encoding sequences in the liver have been used as a model of chronic HBV carriers, with the aim of studying the potential of genetic immunization to induce an immune response that could break tolerance to the viral antigens and thus control the infection (Mancini *et al.*, 1996; Davis *et al.*, 1997b). Remarkably, the immune response induced after a single intramuscular injection of DNA resulted in the complete clearance of circulating HBsAg and in long-term control of transgene expression in hepatocytes. Adoptive transfer of fractionated primed spleen cells from the DNA-immunized mice showed that T cells were responsible for the down-regulation of HBV mRNA in the liver of the transgenic animals. These highly encouraging results and the fact that there was no evidence of liver pathology in the immunized transgenic mice suggest that a DNA vaccine may be effective, possibly because of the induction of a strong CTL response, for therapeutic immunization of HBV chronic carriers.

(b) *HCV.* This virus is a major cause worldwide of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Vaccine development against HCV is complicated by the high degree of variability of the envelope region, the lack of a suitable animal model other than the chimpanzee and the lack of relevant *in vitro* replication assays. A further hindrance to progress is that whereas both humoral and cellular immune responses are likely to be important for controlling HCV infection, neither response has been definitively implicated in such control (Inchauspe *et al.*, 1997a). In terms of antigens likely to stimulate appropriate protective immune responses, the viral nucleocapsid (C), the non-structural protein 3 (NS3) and the envelope glycoproteins E1 and/or E2 have been most studied. DNA vaccination offers a rapid method for studying immune responses to HCV and evidence to date, using the experimental mouse model, indicates that it

is possible to induce humoral and cellular immune, including CTL, responses to HCV antigens using this approach (Lagging *et al.*, 1995; Major *et al.*, 1995; Tokushige *et al.*, 1996; Davis *et al.*, 1997a; Inchauspe *et al.*, 1997a,b; Saito *et al.*, 1997; Tedeschi *et al.*, 1997). In general, however, in contrast to the situation with DNA-derived HBV immunogens, induction of immune responses with a number of HCV DNA-derived formulations, including chimeras, is weak and possibly transient. The cellular and humoral immune responses can be enhanced using HCV DNA-based vaccines augmented with cytokine-expressing plasmids (Geissler *et al.*, 1997a); however, at present it appears that it will be necessary to improve the presentation of DNA plasmids encoding HCV determinants in order to achieve results similar to those reported in other systems.

(c) *Hepatitis E.* Hepatitis E virus (HEV) is recognised as a major cause of water-borne enteric disease, particularly in developing countries where a vaccine would be extremely valuable for reducing the impact of the infection. The poor yields of virus obtained under currently available cell culture conditions preclude the development of vaccines based on killed or live-attenuated HEV but subunit vaccines are considered feasible. There has, to date, been only one report of the use of DNA-based immunization for the generation of an immune response to an HEV protein, namely the structural protein ORF-2, which elicited a long-term humoral response in mice (He *et al.*, 1997).

### 6.1.3. *Genital herpes*

(a) Herpes simplex virus (HSV). Genital infections caused by HSV types 1 and 2 continue to present serious public health problems on a global scale. In the USA alone, over 100 million individuals are infected by HSV-1 and at least 40–60 million people with HSV-2. As with other viral vaccines, the development of an HSV vaccine has attracted great interest but success has so far not been achieved because of the unique properties of the virus to become latent and reactivated and its complicated biology in the human host. Vaccines composed of recombinantly expressed proteins are the furthest advanced and immunization with DNA encoding these proteins offers an exciting, additional approach. HSV envelope glycoproteins D and B have been the focus of efforts to develop subunit vaccines.

Immunization with DNA constructs expressing HSV-1 glycoprotein or glycoprotein B induced protective immunity in mice (Manickan *et al.*, 1995) and nucleic acid vaccines encoding full-length HSV-2 glycoprotein D or a truncated form of HSV-2 glycoprotein B were shown to protect mice from HSV-2 (Kriesel *et al.*, 1996; McClements *et al.*, 1996). Subsequently, induction of mucosal immunity against HSV-1 was shown in mice following intranasal immunization with HSV-1 glycoprotein B plasmid DNA but

the immunity elicited was inferior to intramuscular DNA delivery in providing protection against lethal HSV challenge via the vaginal route (Kuklin *et al.*, 1997). In contrast to HSV murine models, where the relevance to human disease is uncertain, the guinea-pig vaginal infection model closely resembles human genital herpes and is now commonly used to test HSV vaccines. Indeed, guinea-pigs vaccinated with nucleic acid vaccines expressing HSV-1 glycoprotein D (Bourne *et al.*, 1996a) and HSV-2 glycoprotein D (Bourne *et al.*, 1996b) were protected against challenge with HSV-2. McClements *et al.* (1997) further examined the potential of DNA vaccination for human use by assaying a DNA plasmid expressing HSV-2 glycoprotein B by itself and in combination with another DNA plasmid expressing HSV-2 glycoprotein D for protective efficacy in the guinea-pig vaginal infection model. The HSV-2 glycoprotein B DNA and the DNA mixture induced humoral immune responses to the encoded proteins, and these responses were associated with the reduction of primary and recurrent HSV-2-induced disease. These results indicate a potential role for DNA immunization in development of a human genital herpes vaccine.

(b) *Bovine herpesvirus.* There are also encouraging signs that DNA vaccination against bovine herpesvirus (BHV) type 1 may be achievable. BHV-1 is a significant pathogen of cattle responsible for a range of disease states that include respiratory and genital infections, abortions in pregnant animals amd meningoencephalitis. Intramuscular immunization of cattle with a polynucleotide construct encoding the BHV-1 major viral envelope component, glycoprotein D, was shown to induce an immune response capable of abrogating clinical disease following challenge with a virulent strain of BHV-1 (Cox *et al.*, 1993). These cattle trials were important because they indicated that large species could be immunized with DNA vaccines. Follow-up studies using a murine model indicated the immunomodulatory relevance of co-administering plasmid-encoding cytokines with glycoprotein D DNA and, importantly demonstrated that the glycoprotein D plasmid could circumvent pre-existing passively transferred, glycoprotein D-specific, polyclonal antisera and lead to the development of an active immune response (Lewis *et al.*, 1997). This latter observation adds to the growing evidence that DNA-based vaccines can provide a novel method to circumvent maternal antibody-mediated immunosuppression and allow successful and safe vaccination of neonates.

### 6.1.4. *HIV*

Vaccine development against HIV-1 is complicated by the diversity of the virus and by the latency typical of lentiviruses. No immunization strategy has yet been demonstrated to be completely effective in preventing HIV infection *in vivo*, although a recent study in chimpanzees, described more

fully below, provides preliminary but encouraging evidence that DNA vaccines may protect against experimental infection with HIV-1. Of various vaccines tested against the related simian immunodeficiency virus (SIV), only the live attenuated virus may be effective in protecting against *in vivo* infection (Kim *et al.*, 1997a). A live attenuated vaccine runs the risk of the emergence of pathogenic revertant virus and, in any case, this approach has not yet proved successful in controlling HIV-1 infection *in vivo.*

No single measure of immunity is predictive of *in vivo* control of viral replication but there is mounting evidence that a successful HIV-1 vaccine will need to induce both cellular and humoral immune responses to control infection. A large number of groups have thus reported on efforts to engineer a DNA-based vaccine that can induce a broad spectrum of responses and fulfil the safety criteria crucial for future human use. Humoral and cellular responses have been induced to a number of HIV-1 and SIV antigens, notably the envelope (gp120, gp160) and rev regulatory proteins, through various applications of DNA vaccination technology (intramuscular/intradermal vaccination, gene gun-based immunization, single gene constructs, combinations of genes, DNA multiple epitope construct, co-delivery or consecutive delivery with co-stimulatory molecules, peptides or viral vectors) in rodents and non-human primates (Wang *et al.*, 1993, 1994, 1995a; Coney *et al.*, 1994; Lu *et al.*, 1995, 1997; Okuda *et al.*, 1995, 1997; Shiver *et al.*, 1995, 1996, 1997; Asakura *et al.*, 1996; Boyer *et al.*, 1996; Yasutomi *et al.*, 1996; Barnett *et al.*, 1997; Fuller *et al.*, 1997; Heydenberg Fuller *et al.*, 1997; Hinkula *et al.*, 1997a,b; Ishii *et al.*, 1997b; Kim *et al.*, 1997a,b; Letvin *et al.*, 1997; Prayaga *et al.*, 1997; Richmond *et al.*, 1997; Suhrbier, 1997; Tobery and Siliciano, 1997; Tsuji *et al.*, 1997a,b; Ugen *et al.*, 1997; Hanke *et al.*, 1998).

Recently, Boyer *et al.* (1997a), in a pioneering study, analysed the immunogenicity and efficacy of an HIV-1 DNA vaccine encoding gp160, r*ev* and the *gag* and *pol* structural genes in the chimpanzee (*Pan troglodytes*), the most relevant model for HIV-1 infection in humans. The animals were challenged with a heterologous HIV-1 chimpanzee isolate (SF2) and subsequently followed for 48 weeks after challenge. PCR coupled with reverse transcription results indicated infection in the control animal whereas the vaccinated animals were protected from the establishment of infection 1 year after challenge. Although a small number of animals (two vaccines and 1 control) were used in this experiment and a number of technical issues (e.g. eight injections of the vaccine were used) and many questions remain to be addressed, this study serves as an important benchmark in the use of DNA vaccine technology for the development of a successful HIV-1 vaccine for use in humans. In a further advance, the same group were able to use DNA vaccination as anti-HIV-1 immunotherapy in infected chimpanzees (Boyer *et al.*, 1997b).

### 6.1.5. *Measles*

The DNA vaccination strategy has been used with some success against measles virus. Intramuscular immunization with plasmid DNAs encoding envelope proteins (haemagglutinin, fusion) or nucleoprotein has led, depending on the construct used, to humoral (neutralizing antibodies) and cell-mediated immunity in mice and rabbits (Cardoso *et al.*, 1996; Fooks *et al.*, 1996; Yang *et al.*, 1997). CTL responses were also generated in mice immunized mucosally with a DNA plasmid encoding the virus haemagglutinin (Etchart *et al.*, 1997). Future challenges will be to evaluate the potential of these protein-expressing plasmids to raise protection in non-human primates and to evaluate whether the use of DNA will support successful immunization in the presence of maternal antibody.

### 6.1.6. *Rabies*

Xiang and colleagues (Xiang *et al.*, 1994, 1995; Xiang and Ertl, 1995; Ertl and Xiang, 1996) showed that a plasmid expressing the full-length rabies virus glycoprotein injected intramuscularly was able to induce B- and T-cell-mediated immune responses that fully protected mice against lethal challenge. Co-inoculation with a vector expressing mouse GM-CSF enhanced B- and T-cell activity while co-inoculation with a plasmid expressing IFN-$\gamma$ resulted in a decrease in immune response to the viral antigen (Xiang and Ertl, 1995). Another group obtained very similar results targeting the same protein (Ray *et al.*, 1997) and also were able to obtain high levels of neutralizing antibody and protection against lethal rabies virus challenge using gene-gun particle-mediated vaccination (Lodmell *et al.*, 1998) Noteworthy also are the results of genetic immunization of 1-day old piglets with the gd glycoprotein of pseudorabies virus (Monteil *et al.*, 1996). Neutralizing antibodies were produced against the virus in immunized animals born from non-immune sows but the piglets were not protected following challenge. Further, the vaccine was shown to be ineffective in piglets from immune dams where colostral antibodies had presumably inhibited the immune response.

### 6.1.7. *Other Viral Infections*

Genetic immunization, generally with protective immune responses being elicited, has proved successful in a number of preclinical animal models against a range of other viruses, including rotavirus (mouse, Herrmann *et al.*, 1996a,b; Choi *et al.*, 1997), papillomavirus (cottontail rabbit, Donnelly *et al.*, 1996), bovine viral diarrhoea virus (mouse, Harpin *et al.*, 1997), human T-cell leukemia virus type 1 (rat, Kazanji *et al.*, 1997), dengue virus

(mouse, Kochel *et al.*, 1997), human CMV (mouse, Pande *et al.*, 1995), St Louis encephalitis virus (mouse, Phillpotts *et al.*, 1996), Ebola virus (guinea-pig, Xu *et al.*, 1998), lyssaviruses (including rabies virus) (mouse, Bahloul *et al.*, 1998) and lymphocytic choriomeningitis virus (mouse, Yokayama *et al.*, 1995). Other viral infections, against which DNA vaccination has induced immune responses and/or encouraging levels of protective efficacy, include Newcastle disease in chickens (Sakaguchi *et al.*, 1996), feline immunodeficiency virus (entire genome used) in cats (Rigby *et al.*, 1997), foot-and-mouth disease virus in pigs (Ward *et al.*, 1997) and infectious haematopoietic necrosis in rainbow trout (*Oncorhycus mykiss*) (Anderson, E.D. *et al.*, 1996).

## 6.2. Bacterial Diseases

### 6.2.1. *Tuberculosis*

Tuberculosis (cause by *Mycobacterium tuberculosis*) remains a major health problem, affecting millions of people worldwide and killing about 3 million, more that any other infectious agent. The attenuated *Mycobacterium bovis* BCG vaccine continues to be widely used, although its efficacy appears variable. A new approach for developing a more effective vaccine against tuberculosis is urgently required and recent results of genetic immunization have provided encouraging signs, albeit in mice, that a single or a few protein antigens given as DNA can protect as effectively as BCG. Plasmids expressing *Mycobacterium leprae* hsp 65 (Silva *et al.*, 1995; Tascon *et al.*, 1996) and other mycobacterial antigens, the 30–32 kDa antigen 85 complex (Huygen *et al.*, 1996; Lozes *et al.*, 1997; Montgomery *et al.*, 1997), 38 kDa glycoprotein (Zhu *et al.*, 1997), hsp70, 36 kDa and 6 kDa (Tascon *et al.*, 1996; Lowrie *et al.*, 1997) have provided persistent protection in vaccinated mice challenged with live BCG or *M. tuberculosis.* The major challenge will be to obtain equivalent levels of efficacy in humans.

### 6.6.2. *Pneumonia*

Pneumonia caused by the pneumococcus, *Streptococcus pneumoniae*, is the most common infectious cause of death in non-malarious areas. A vaccine based on the capsular polysaccharide is available but is not widely used because it is poorly immunogenic, particularly in children, and immunological memory is poor. As with a number of pathogens, DNA immunization might prove an invaluable approach to the development of a more reliable vaccine. Indeed, immunization of mice with a plasmid expressing

pneumococcal surface protein A has been shown to elicit protection against fatal infection with the pathogen (McDaniel *et al.*, 1997), which provides encouragement to workers in this difficult area.

### 6.2.3. *Mycoplasmosis*

Some success has been achieved with genetic immunization against *Mycoplasma pulmonis*, a natural pathogen of rodents, in that vaccination with expression libraries of *M. pulmonis* DNA (Barry *et al.*, 1995) or DNA encoding one or two individual antigens (A7-1, A8-1) (Lai *et al.*, 1995, 1997) has been shown to provide protection from challenge infection with the pathogen.

### 6.2.4. *Lyme Disease*

Lyme disease is a zoonosis of rodents caused by the spirochaete *Borrelia burgdorferi*. Spread to humans by the bites of infected ixodid ticks, Lyme disease is now recognised to be cosmopolitan in distribution and a serious public health problem. Vaccination with plasmid DNA encoding the outer surface lipoprotein A (OspA) of *B. burgdorferi* has been shown to provide protective responses to the pathogen (Simon *et al.*, 1996; Zhong *et al.*, 1996; Luke *et al.*, 1997). Immunized mice produced OspA-specific and protective IgG antibodies and the mice, where challenged, were protected against subsequent experimental infection with virulent *B. burgdorferi*.

### 6.2.5. Salmonella typhi

Killed and live attenuated vaccines against *S. typhi* provide varying degrees of protection, mainly due to longevity of protective responses. DNA vaccination may provide more long-lasting immunity and, accordingly a DNA vaccine has been developed for *S. typhi* (Lopez-Macias *et al.*, 1995). Based on the ompC porin protein, detectable levels of specific antibody were induced in mice, although it remains to be seen whether these antibodies can provide suitable protection.

### 6.2.6. Clostridium tetani

A successful DNA vaccine, based on the protective non-toxic portion of tetanus toxin, fragment C, has been developed (Anderson *et al.*, 1996). Although the antibody response generated was low compared with conventional tetanus toxoid vaccination, it was still protective.

## 6.3. Parasitic Infections

As will be evident, most reports on nucleic acid vaccines deal with the application of this technology to viral infections. This is due, in part, to the fact that CTL responses can be readily generated to direct the immune response against infected cells; this makes the approach a potentially safe alternative to live attenuated or killed viral vaccines. At the same time, however, humoral and Th responses are also generated, which makes it feasible to use nucleic acid vaccination against the more complex parasitic organisms. Nevertheless, despite the ease of construction of DNA plasmids for vaccine delivery, there are relatively few reports on the application of nucleic acid vaccination for parasitic infections, the majority being on parasitic protozoa.

### 6.3.1. *Protozoal Infections*

(a) *Malaria.* The majority of work involving the development of genetic vaccines effective against malaria has been undertaken by Hoffman and his group (Naval Medical Research Institute, Bethesda, USA) and they have contributed several recent reviews in this area (Doolan and Hoffman, 1997; Hoffman *et al.*, 1997a,b). Indeed, the first attempts to immunize against a parasitic infection were reported by this team for the rodent malaria model, *Plasmodium yoelii* (Py), and incorporated a plasmid encoding the circumsporozoite protein (CSP), a proven target of protective immunity in malaria (Sedegah *et al.*, 1994). Mice were immunized by intramuscular injection and the antibody titres after immunization were relatively high, although the sera showed low inhibitory activity against sporozoites. Enzyme-linked immunosorbent assay (ELISA) studies revealed that only a fraction of the antibodies produced recognized the important B-cell epitopes of CSP within the sequence ([QGPGAP]2) which could explain these findings. A genetically restricted $CD8^+$ T-cell-dependent cytolytic activity was observed after immunization with PyCSP DNA that was significantly greater than in mice immunized with irradiated sporozoites, clearly demonstrating the efficiency of DNA vaccination for inducing CTL responses. After challenge, 86% of the mice were protected against liver-stage infection and 54% against blood-stage infection. The authors further showed that the protective effect was completely dependent on the elimination of infected hepatocytes by CTLs. A subsequent study examined the nature and complexity of the cytokine and antibody response elicited by immunization with PyCSP (Mor *et al.*, 1995). Further DNA vaccine work by this group included study of other *P. yoellii* proteins, such as the hepatocyte erythrocyte 17-kDa protein (PyHEP17), sporozoite surface protein 2 (SSP2) and merozoite surface protein (Doolan *et al.*, 1996; Gardner *et al.*, 1996).

As also discussed elsewhere in this review, it is possible to overcome genetic restriction of protection by combining protective antigen plasmid constructs that individually show different patterns of genetic restriction. This is an important concept because it suggests that it may be possible to circumvent genetic restriction of immune responses in an outbred population. Thus, by combining the PyHEP17 DNA with the PyCSP DNA, 85% protection against challenge was obtained whereas significantly less protection was achieved when the constructs were used individually (Doolan *et al.*, 1996). Furthermore, the individual constructs were only protective in mice of certain genetic backrounds, whereas the mixture was able to circumvent this restriction, protecting mice of different genetic make-up.

The success in the *P. yoellii* model provided the basis for developing DNA vaccines against *P. falciparum* aimed at inducing protective $CD8^+$ T-cell responses against *P. falciparum*-infected human hepatocytes. The *P. falciparum* homologues of CSP (PfCSP), SSP2 (PfSSP2/TRAP), HEP17 (PfExp-1) and *P. falciparum* liver-stage antigen 1 (PfLSA-1) were selected for initial study by Hoffman's team. Having established, for all four plasmids, *in vitro* expression and *in vivo* immunogenicity in the rodent model and suitable immunogenicity in rhesus macaques, it was decided to proceed, in mid-1997, to human clinical trial using a DNA vaccine encoding a single gene, PfCSP, to assess safety and immunogenicity; this clinical trial is ongoing. Future plans by the group involve testing (including sporozoite challenge of vaccinated volunteers) a multi-gene liver-stage DNA vaccine comprising the four plasmids already selected and at least one other protein-encoding cDNA in the mixture. As an optimally effective malaria vaccine will need to induce protective immune responses against all stages of the parasite life cycle (Hoffman *et al.*, 1997a), the likely long-term approach will require the construction of DNA plasmids that optimally express proteins from all stages of the life cycle and induce the appropriate antibody and $CD4^+$ and $CD8^+$ T-cell responses necessary for protection.

Another group (Hanke *et al.*, 1998) have investigated the potential of building multi-CTL epitope (polyepitope) antigens in combination with genetic immunization technology for development of malaria (and HIV-1) vaccines.

Further, Grifantini *et al.* (1998) were able to show that DNA vaccination with four plasmids encoding malarial antigens did not induce any antigenic competition but enhanced the immunogenicity of a poorly immunogenic plasmid, indicating an adjuvant effect of DNA. In another advance, Schneider *et al.* (1998) were able to show enhanced immunogenicity for $CD8^+$ T cell induction and unprecedented protective efficacy of malaria (*P. berghei*) DNA vaccination by boosting with modified vaccinia virus Ankara. This approach may provide a generic procedure for induction of high levels of CD8+ T cells.

(b) *Theileriasis.* Species of *Theileria*, spread by ticks, are the cause of theileriasis (theileriosis) in cattle and other animals. *Theileria annulata* is the cause of tropical theileriasis or Mediterranean coast fever in cattle and is widely distributed across southern Europe, North Africa and Central Asia. Active research on the development of vaccines, including DNA vaccines, against the disease is in progress. Plasmid DNA encoding allelic forms (Tams1-1 and Tams1-2) of the major merozoite surface antigen of *T. annulata* has been shown to confer protection in cattle; although no specific antibodies were detectable, two of the three calves were protected when challenged with a parasite stabilate generated from blood of an infected animal with 30% piroplasm parasitaemina (d'Oliveira *et al.*, 1997).

(c) *Cryptospiridiosis.* This is a zoonotic intestinal parasitic disease caused by *Cryptosporidium parvum*. Most prevalent in young animals and children, immunocompromised individuals, particularly AIDS patients, are also at risk of infection by this opportunistic organism. Construction of a recombinant plasmid encoding epitopes of 15 and 60 kDa surface antigens of *C. parvum* sporozoites has been described (Jenkins *et al.*, 1995). The plasmid was used to immunize sheep either intramuscularly or in the mammary tissue. High antibody titres were achieved by either route of immunization, although intra-mammary immunization elicited higher titres in the colostrum and longer-lasting serum IgG levels. In immunofluorescence studies the authors additionally showed that the antibodies from colostrum and serum were able to recognize surface antigens of the sporozoites, suggesting that those antibodies might be used to neutralize parasites in the gut.

(d) *Leishmaniasis.* Caused by intracellular (macrophage) parasites of the genus *Leishmania*, this disease occurs in most parts of the world with an incidence of approximately 400 000 and a prevalence of around 12 million. Currently, treatment is based on antimony compounds but these are not always effective against the disease and vaccination seems to be a logical, alternative means of control. Experimental data support the feasibility of developing a vaccine; indeed, whole killed parasites, isolated parasite antigens and recombinant antigens such as the glycoproteins gp63 and gp64 have been shown to induce protection in mice.

In terms of DNA vaccination, DNA encoding gp63 has been tested in highly susceptible mice which, when challenged with *Leishmania major*, developed significantly smaller lesions with a markedly delayed onset of infection compared with controls; immunized animals also had two to three orders of magnitude lower numbers of parasites than mice immunized with control plasmid (Xu and Liew, 1995a,b). More recently, subcutaneous vaccination with DNA encoding the immunodominant *Leishmania* homolog of receptors for activated C Kinase (LACK) parasite antigen has been shown also to confer protective immunity to susceptible BALB/c mice infected with *L. major* (Gurunathan *et al.*, 1997). Protection

induced by LACK DNA was similar to that achieved by LACK protein and recombinant IL-12 protein, but superior to LACK protein without the recombinant cytokine.

(e) *Trypanosomiasis cruzi. Trypanosoma cruzi* is the causative agent of Chaga's disease, afflicting between 16–18 million people in Latin America. Drug treatment is rather ineffective and vaccination could provide an additional approach for patients who do not respond to conventional chemotherapy. One potential target for immunotherapy is an unique enzyme expressed by *T. cruzi* called *trans-sialidase* (TS). This enzyme is required for the parasite to obtain sialic acid from host glycoconjugates and several lines of evidence suggest TS is a virulence factor implicated in the establishment of infection. A team led by Fabio Costa (Costa *et al.*, 1998) constructed a DNA plasmid containing a gene encoding the catalytic domain of TS. They were able to show the generation in mice of T-cell mediated immune responses and antibodies which recognised the native enzyme and inhibited its activity *in vitro*. Upon challenge with bloodstream trypomastigotes, the immunized animals displayed reduced parasitaemia, and mortality which provides encouragement that development of a TS-DNA plasmid-based vaccine may be achievable for human use.

(f) *Toxoplasmosis. Toxoplasma gondii* is a major problem in immunosuppressed patients, especially in individuals with AIDS. Treatment of toxoplasmosis is difficult due to the toxicity of available drugs, and reinfection occurs rapidly. As with other protozoan infections, vaccination may prove to be a useful adjunct to control. To date, vaccine development has focused on the tachyzoite surface antigen P30, which can elicit protection either as the native protein or as a result of passive transfer of monoclonal antibodies against P30. P30 has also been targeted for DNA vaccine purposes and a recombinant plasmid, comprising the cDNA of P30 cloned in frame with tissue plasminogen activator leader sequence cDNA into a CMV promotor-driven plasmid, has been constructed (Angus *et al.*, 1996). Mice were immunized with the recombinant plasmid and all vaccinated animals exhibited a strong antibody response to the P30 antigen compared with animals injected with control plasmid. Cytokine and other cellular responses were not evaluated and whether the P30 DNA vaccine is protective remains to be established.

### 6.3.2. *Helminth Infections*

There are few published reports on helminth DNA vaccines (Kalinna, 1997), although the potential benefits of genetic immunization for use against multicellular parasites, whose life cycles are either predominantly or completely extracellular, have been fully emphasized (Waine and McManus,

1995). Much of the work in this area has been undertaken on the schistosomes, particularly *Schistosoma japonicum*, the Asian or oriental schistosome. The case for developing anti-schistosome vaccines, including DNA vaccines, and progress towards that goal have been discussed in a number of recent reviews (Bergquist, 1995; Waine and McManus, 1997).

A panel of *S. japonicum* cDNAs has been cloned into eukaryotic expression vectors, injected into animals and tested for immunogenicity (Yang *et al.*, 1995; Waine *et al.*, 1997). These cDNAs included full-length paramyosin, several paramyosin fragments, glyceraldehyde 3-phosphate dehydrogenase, 22-kDa tegumental antigen, a 14-kDa fatty acid-binding protein, 26-kDa glutathione S-transferase (GS7–26), 28-kDa glutathione S-transferase (GST-28), calreticulin, calpain and the novel fusion cDNA construct, described above, comprising GST-26 fused to a fragment of paramyosin cDNA. Only constructs encoding parts of or the full-length paramyosin, which included the sequence of a protective IgE antibody B-cell epitope, were able to produce specific antibodies in mice after intramuscular injection. Characterization of the titre and isotypes of the generated antibodies (IgG1, IgG2a, and IgG2b) revealed that a Th1-like response occurred and titres of approximately 1/6000 were obtained. However, subsequent challenge experiments did not yield protection in terms of reduced worm burden. Specific anti-paramyosin antibodies (IgG1 was the predominant isotype subclass) were also produced in mice immunized with full-length paramyosin DNA using the gene gun (Figure 3).

Experiments have also been carried out using plasmid DNA encoding the protective GST-28 of *Schistosoma mansoni* (Dupre *et al.*, 1997). Following plasmid DNA administration into the skin of rats, the parasite antigen was detected in skin cells by immunohistochemistry. Long-lasting specific IgG antibodies, with a predominance of IgG2a and IgG2b subclasses, were detectable in the sera of immunized animals. Further, these sera were able to mediate antibody-dependent cellular cytotoxicity *in vitro*, leading to killing of schistosomula, and a parasite challenge performed on the plasmid DNA-immunized animals induced a strong and rapid boosting of the specific IgG antibody response. Whether the *S. mansoni* GST28 plasmid DNA can protect immunized animals awaits further investigation.

Some success has been achieved with nucleic acid vaccination using DNA encoding a host-protective antigen (45W) against the sheep tapeworm *Taenia ovis*. Recombinant 45W plasmid in combination with conventional adjuvanted vaccine (Rothel *et al.*, 1997a) or recombinant ovine adenovirus (Rothel *et al.*, 1997b) resulted in specific antibodies, predominantly of the IgG1 subclass, being generated; with the latter delivery system, host protection from experimental challenge with *T. ovis* was obtained.

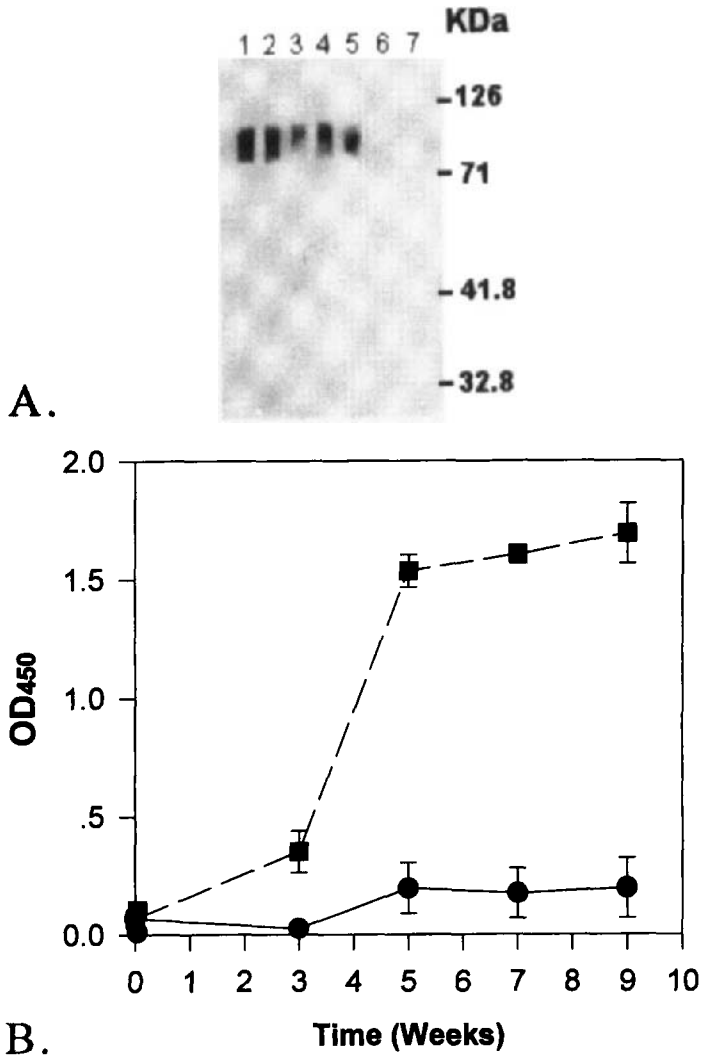



*Figure 3* Qualitative and quantitative analysis of the antibody response in mice vaccinated with a plasmid encoding full-length *Schistosoma japonicum* paramyosin (FLPMY). The mice were immunized with FLPMY DNA (five mice) or non-recombinant VR1012 plasmid DNA (two mice) at week 0 (2 μg DNA), week 3 (1 μg DNA) and week 7 (2 μg DNA). Mice were bled for sera at these times and at week 9 of the experiment. (A) Western blot showing specific antibody recognition in murine sera (at week 9) of a 97-kDa (equivalent to the known molecular mass of *S. japonicum* paramyosin) antigen in a soluble adult *S. japonicum* extract. Lanes 1–5, FLPMY DNA-vaccinated mice; lanes 6 and 7, VR1012 DNA-vaccinated mice (serum dilution, 1:150). (B) Results of enzyme-linked immunosorbent assays (wells coated with soluble adult *S. japonicum* extract) detecting total anti-*S. japonicum* serum IgG (serum dilution, 1:150). ■, FLPMY; ●, VR1012 control.

## 7. NUCLEIC ACIDS AS ANTI-CANCER VACCINES

DNA encoding tumour antigens, foreign MHC class I or II antigens or cytokines have been tested as potential DNA cancer vaccines (Restifo, 1996). Immunization with genes encoding tumour-specific antigens may be an effective method of inducing cell-mediated mechanisms against tumours (Bright *et al.*, 1995, 1996; Nabel *et al.*, 1995; Spooner *et al.*, 1995; Wang *et al.*, 1995b). The models and antigens that this technique has been applied to include B-cell lymphoma (Stevenson *et al.*, 1995; Abe *et al.*, 1996; Hakim *et al.*, 1996; Syrengelas *et al.*, 1996), human chorionic gonadotrophin (produced and secreted by human lung, bladder and pancreatic tumours) (Geissler *et al.*, 1997b), MAGE-1 and MAGE-3 (expressed in many human melanomas and other tumours) (Bueler and Mulligan, 1996), erbB2/neu (oncoprotein of breast cancer) (Concetti *et al.*, 1996) and CEA (Conry *et al.*, 1994, 1995b).

The transfection of target tumour cells with DNA encoding foreign MHC class I or II antigens creates an immunostimulatory environment that might facilitate an immune response not only to the transfected cells but also to non-transfected tumour cells. Targeting an anti-viral T-cell response to a growing tumour facilitates the priming of a tumour-associated antigen-specific T-cell response (Bohm *et al.*, 1997).

The transfection of tumour cells *ex vivo* with plasmid DNA encoding cytokines, followed by the vaccination of an individual with the cytokine-expressing tumour cells, aims to induce or augment the individual's immune response to other, non-transfected tumours. Preclinical studies have demonstrated protection to challenges with non-transfected tumour cells after vaccination with GM-CSF-transfected melanoma cells (Mahvi *et al.*, 1997). Clinical trails, currently underway, aim to assess the acute and long-term toxicities of intradermal vaccination of cancer patients with lethally irradiated tumour cells that have been transfected by particle-mediated gene transfer with gold particles coated with human GM-CSF DNA in a plasmid expression vector (Mahvi *et al.*, 1996, 1997). Alternatively, the direct transfection of tumour cells *in vivo* with DNA encoding GM-CSF, or other cytokines such as IL-12 and IL-2, by gene gun or injection offers a more efficient alternative without the requirement for *ex vivo* transfection (Nicolet *et al.*, 1995; Sun *et al.*, 1995; Keller *et al.*, 1996; Rakhmilevich *et al.*, 1996).

## 8. DNA VACCINES IN GENE THERAPY

The use of genetic vaccines to treat genetic disorder and autoimmune diseases is another area of growing research. This form of vaccination has tremendous potential in muscle-based gene therapy (Svensson *et al.*, 1996). One such example is Duchennes muscular dystrophy, which causes progressive myopathy of skeletal and cardiac muscles and premature death. The intramuscular injection of normal human dystrophin expression plasmids into dystrophin-deficient mice results in the expression of human dystrophin protein in 1% of the injected mouse muscle fibres (Acsadi *et al.*, 1991b). Augmentation of this procedure aims to increase the number of muscle fibres transfected, and the method would then have the potential to replace or supplement defective or absent genes in individuals with genetic disorders.

Gene therapy by genetic immunization may provide an effective, long-term control mechanism for autoimmune diseases. The intramuscular injection of mice with plasmids encoding TGFβ prolonged the survival of mice with systemic lupus erythematosus (Raz *et al.*, 1995). Similarly, the intramuscular injection of mice with a plasmid encoding for hsp65 (a mycobacterial heat shock protein), an agent known to induce adjuvant arthritis, resulted in significant protection of mice from the disease (Ragno *et al.*, 1997). Work also continues into the use of genetic vaccination in other autoimmune disease, including central nervous system inflammatory diseases (Ramshaw *et al.*, 1997), and in the gene therapy of brain disorders (Jiao *et al.*, 1992a).

## 9. SAFETY ISSUES

A number of theoretical concerns exist regarding the safety of DNA vaccines. These include the potential for chromosomal integration and the effects of long-term plasmid persistence and long-term antigen expression (Robertson, 1994).

There is no evidence to indicate that introduced DNA can be integrated into the host genome (Wolff *et al.*, 1992b; Wahren, 1996; Mucke *et al.*, 1997). This is mainly due to the fact that the most common type of DNA vaccine is in the form of a self-expressing plasmid that remains extrachromosomal. The length of time that a plasmid expresses *in vivo* is an area not well researched. DNA delivered into mouse muscle was found to be stably expressed for 19 months but as this is limited to the length of a mouse's lifespan, it is not yet known how long DNA will continue to express with a

human host and whether long-term expression has any saftey implications (Wolff *et al.*, 1992b).

The induction of tolerance and autoimmunity are possibilities resulting from the long-term expression of a foreign antigen (Robertson, 1994). Research into the potential induction of tolerance in DNA-vaccinated individuals showed that although a plasmid DNA vaccine encoding the malaria circumsporozoite protein initiated an immune response in adult mice, it induced tolerance in neonatal mice that were 2–5 days old (Mor *et al.*, 1996). This is in contrast to the results of Wang, Y. *et al.* (1997) who could not distinguish the immune response between neonatal and adult mice after vaccination with a plasmid expressing the rabies glycoprotein. In this series of experiments, mice inoculated within 24 hours after birth with the plasmid DNA developed antibodies as well as Th cells to the rabies virus glycoprotein. The response could not be distinguished from that observed in DNA-vaccinated adult mice, indicating that the immune system, known to be prone to induction of immunological tolerance to some antigens applied during the early neonatal period, can readily respond to rabies virus glycoprotein induced by a plasmid vector (Wang, Y. *et al.*, 1997).

Addressing the potential generation of autoimmunity using DNA vaccines, studies have been conducted to determine if any immune response is mounted against the foreign DNA and if this response has the potential to lead to autoimmunity, i.e. an immune response against host DNA. Until recently, all DNA from all organisms was considered to be the same and immunologically benign. However, recent research has shown the bacterial DNA in itself is unique and can provoke the immune system into a response (Pisetsky, 1996). As DNA vaccines are in the form of a plasmid, manufactured from bacteria, they too possess the potential of provoking an immune response. It has been found that although some autoantibodies are created in response to the DNA, the levels are insufficient to induce active autoimmune disease (Mor *et al.*, 1997). Non-human primates, closely related immunologically to humans, are essential models in evaluating the immunogenicity and safety of DNA vaccines (Kennedy *et al.*, 1997). Encouragingly, sera obtained from non-human primates previously immunized with a DNA vaccines failed to produce any anti-DNA antibodies (Liu, M.A. *et al.*, 1997).

## 10. FINAL COMMENTS

There can be little doubt that vaccination is one of the most cost-effective measures to control and help prevent the spread of infectious and other diseases, including cancer. *Nature Medicine* (volume 4, number 5, May

1998, pp. 475–534) was devoted to this important field and highlighted the potential impact that new vaccines, including genetic vaccines, are likely to have on human health. Being easy to construct and produce, nucleic acids provide arguably one of the most novel approaches available to develop and administer vaccines, especially against pathogens where effective vaccine tools do not yet exist. They represent one of the closest, if not the closest, approach to a natural infection without problematical (as far as we are currently aware) side-effects and provide a high probability of inducing effective and long-term protective immunity. In just 5 years, remarkable progress has been achieved in the use of DNA immunization strategies for disease prevention and therapy. There is a clear commercial element to the genetic development of DNA-based vaccines; indeed, various groups have laid claims for rights to the procedure of genetic immunization, although the current patent position remains unclear. VICAL (San Diego, CA) was the first to be issued with a US patent on 3 December 1996, claiming rights to the delivery of exogenous DNA sequences into mammals. Soon after, on 31 December 1996, VICAL was issued with another US patent claiming rights to the induction of a protective immune response in mammals following injection of a DNA sequence. Since then, no less than three other groups have been issued with US patents claiming rights not only to genetic immunization as a whole (Wistar Institute, Philadelphia, PA) but also to the use of DNA vaccines against rotavirus infections (University of Massachusetts Medical Center, Worcester, MA) and the use of cationic liposomes for gene therapy (GENEMEDICINE Inc., The Woodlands, TX).

To conclude, the immunogenicity and protective efficacy of DNA vaccines have now been demonstrated against a spectrum of infectious and non-infectious diseases. The early potential promise of DNA vaccination has been fully substantiated, with recent exciting developments including the move from testing DNA vaccines in laboratory models to non-human primates and initial human clinical trials. In this regard, the first human trial of a DNA-based vaccine for treatment of HIV-1 infection was recently completed (MacGregor *et al.*, 1998). These advances highlight the rapid progress that has been made in the DNA immunization field and it will be of considerable interest to see whether the progress and optimism currently prevailing can be maintained, and whether the approach can indeed fulfil the medical and commercial promise anticipated.

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