ADVANCES IN IMMUNOLOGY

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PREFACE

The subjects reviewed in this volume range over the field of Immunology from basic experimental aspects to clinical considerations. The initial areas include the genetic basis of immunologic function and the substances involved in its control. Next is an overview of basic immunopathologic processes responsible for immunologic disease. Finally reviewed are two of the most common and important environmental stimuli, bacterial endotoxins and parasites, that interact with immunologic defenses. Characteristic of current thought in Immunology, the authors of this volume have emphasized the molecular and cellular aspects of their subjects, thereby providing fundamental explanations of the complicated events they discuss.

The extreme complexity of the regulatory mechanisms by which cells of the lymphoid system interact was not anticipated a few years ago. Now it is clear that both antigen-specific and nonspecific stimulation and suppression are part of the immunologic scheme. In the first article, Drs. Tada and Okumura draw extensively on their own research in presenting a clear and detailed account of antigen-specific T cell regulatory factors. Particularly helpful is the perspective in which the authors place the many and often conflicting published reports on this subject. Also, their consideration of the contributions of the genes from both the Ig V and the I regions in determining antigen-specific T cell regulatory function provides an attractive hypothesis and indicates the further experimentation necessary for confirmation or disproof.

It is becoming clear that immune complexes, the inevitable consequence of an antibody response, are important in immune processes far beyond their well-established role as mediators of tissue injury. In the second article, Drs. Theofilopoulos and Dixon describe the interaction of immune complexes with complement and with the cells of the immune system, the functions of which they profoundly influence. As might be anticipated in immunologic events, the effect of immune complexes depends to a great extent on their antigen–antibody ratio so that their influence, either stimulatory or suppressive, is itself modulated by quantitative aspects of the immune response. The development of numerous techniques for the detection and quantitation of immune complexes has stimulated clinically related research and expanded the list of diseases in which immune complexes appear to play an important role. An extension of this diagnostic technology is the ability to isolate immune complexes and, in turn, their antigenic com-

ponent, thereby making it possible to identify the antigens involved in immune processes of a great many diseases, including those of unknown etiology.

The human major histocompatibility complex-controlled equivalents of the murine Ia antigens are restricted in cellular distribution, related to differentiation, and strikingly associated with susceptibility to certain diseases. Drs. Winchester and Kunkel in the third article discuss conceptual and technical developments in the field of human Ia antigens by describing the chemical structure and immunologic relationship to their murine counterparts. The distribution of human Ia antigens is limited primarily to the B lymphocyte series, stimulated T cells, and stem and precursor hematopoietic cells, suggestive of both cell type and differentiation specificity. Finally, the association of certain Ia antigens with susceptibility or resistance to a variety of diseases, including rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, rheumatic fever, multiple sclerosis, and diabetes, provides both a valuable clinical predictive measure and a lead for the future study of pathogenetic factors possibly related to the particular Ia genes involved.

Exposure to bacterial endotoxins results in a prompt and profound immune reaction, a response of obvious survival value. The nature of endotoxins and their interactions with cells of the immune system are the subjects of the fourth article, by Drs. Morrison and Ryan, both prominent investigators in this field. The direct interaction of endotoxin with B lymphocytes, leading to the formation of antibodies to endotoxin as well as a spectrum of other immunoglobulin molecules, is considered in detail. In addition, the interactions of endotoxins with T cells and macrophages and the associated immunoregulatory effects are discussed. The central role of the lipid A portion of the endotoxin molecule in cellular activation and the molecular events occurring at the cell surface in the course of stimulation are presented. Finally, the potential of endotoxins as therapeutic manipulators of the immune response in man is evaluated.

Immunoparasitology, a field of great potential importance, is just beginning to attract attention. In the last article of this volume, Dr. Mitchell discusses this subject, emphasizing the general elements involved in resistance to parasitic infection and the characteristics of these infections that make them likely targets of immunodiagnosis, immunoprophylaxis, and/or immunotherapy. Consideration of work using current immunologic technology in the study of immune responses to parasitic infections is limited primarily to the mouse. The characteristic elements of these responses, such as hypergamma-

globulinemia and eosinophilia, are discussed in detail, as well as the less well-recognized immunosuppression, which accompanies a number of parasitic infections. This article should provide important background material for immunologists who might consider turning their attention to this new field.

We wish to thank the authors for their efforts in writing these reviews and the publishers for their painstaking preparation of this Volume.

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The Role of Antigen-Specific T Cell Factors in the Immune Response

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I. Introduction

We are now just beginning fully to realize that the immune response is achieved via a complex series of interconnected cellular events initiated by antigenic stimulation rather than the activation of a single isolated clone of lymphocytes. The initial concept of interaction of thymus (T)-derived cells and bone marrow (B)-derived cells has been considerably broadened to incorporate macrophage–T cell, T cell–T cell, and T cell–B cell interactions, which as a whole constitute the network of an immunoregulatory system.

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There has been a well known dichotomy in explaining the interconnectedness of the immunocompetent cells. The network concept originally formulated by Jerne (1972, 1975, 1976) explains it by idiotype–anti-idiotype interactions between lymphocytes. On the other hand, there have been abundant experimental data indicating that cell interactions are governed by genes in the major histocompatibility complex (MHC) (reviewed by Katz and Benacerraf, 1976; Benacerraf and Katz, 1975; Benacerraf and Germain, 1978; Rosenthal and Shevach, 1977; Miller and Vadas, 1977). One of the central issues in both concepts is the molecular mechanism whereby antigen-specific signals are conveyed from one component to the other. On the basis of the above dichotomy, it is predicted that if such mediators do exist, they should be able to recognize both antigen and target cells to which they make meaningful and unmistakable communications. Investigations performed in the last decade, in fact, resulted in the identification and characterization of such biologically active molecules produced by T cells, which we call antigen-specific T cell factors. They are able to bind to antigen (or perhaps to idiotype in some cases) and are capable of activating other cell types of the restricted nature (either B or T cells, syngeneic or allogeneic). Some of them enhance the antibody responses by B cells, whereas others suppress both humoral and cell-mediated immune responses. In certain experimental systems, there have been found strict genetic restrictions imposed by genes in MHC, whereas in other conditions only the antigen specificity is the restricting element. There are overt similarities and differences between the factors reported by different investigators, which may primarily be caused by the structure of molecules, experimental conditions, and even by artifactual observations. In addition, there are many other "factors" that nonspecifically modulate the immune response, whose participation in the observed net effect of antigen-specific factors is obvious. We shall, however, restrict ourselves to describing the T cell factors that unequivocally possess antigen specificity as determined by the criteria given below.

The term "factor" is by no means a clear definition of the molecules. It merely designates a heterogeneous group of molecules having distinctive immunologic functions whose molecular entity is still ambiguous. In fact, none of these antigen-specific factors has been either isolated or characterized chemically beyond localization of activity in column effluents. Nevertheless, these antigen-specific factors seem to provide important clues for solving most currently controversial problems in immunology. Since it is unlikely that T cells would release antigen-specific molecules other than T cell surface recognition struc-

tures, they are obviously the most likely candidates for the antigen receptor of T cells. Their definite functions in positive and negative immunoregulation also suggest that they represent physiologically legitimate mediators of specific T cell functions.

The purpose of this review is to bring together the available information on these T cell factors, albeit controversial and conflicting at the moment, and to examine their roles in cell interactions in the immune response. We shall, therefore, confine the discussion to considerations of those factors defined by their definite antigen specificity and functional characteristics rather than those separated and characterized by immunochemical means.

II. An Overview

Shortly after the discovery of T–B cell collaboration in the antibody response, several investigators predicated the antigen-specific receptor on helper T cells, which acts to focus antigen onto corresponding B cells (Mitchison and Rajewsky, 1974; Mitchison *et al.*, 1971; Taylor, 1969). Such a conceptual receptor of helper T cells is sometimes designated as IgX and has provided a basis for the development of the theory of "carrier antibody" (Bretscher and Cohn, 1968). By extension, Bretscher (1972) proposed a hypothesis in which he assumed the presence of a T cell immunoglobulin (Ig) or a factor that gives a second signal to B cells that have reacted with antigen (two-signal hypothesis). This hypothesis postulates that both the Ig receptor and some receptor for T cell-derived helper factor as part of B cell surface membrane are capable of signal transmission. These receptors differ from each other insofar as the Ig receptor conveys a tolerogenic signal by the binding of the haptenic determinants of the antigen, whereas the other receptor, being capable of interacting with the factor by virtue of the antigen-binding capacity of the factor, stimulates B cells toward differentiation and antibody synthesis. This hypothesis certainly stimulated the survey for the molecule relevant to the theory. A similar concept as a negative counterpart of IgX was presented by Gershon and Kondo (1971), when they first demonstrated the infectiousness of tolerance caused by suppressor T cells. As the suppression they observed was clearly antigen specific and mediated by T cells, they called the possible mediator for the suppression as IgY as opposed to IgX.

An actual demonstration of an antigen-specific factor having biologic activity was made by Feldmann and his co-workers (reviewed by Feldmann and Nossal, 1972; Feldmann, 1974a,b). They described

several features of an antigen-specific molecule of the immunoglobulin nature produced by T cells. The molecule termed IgT was capable of mediating specific helper activity in the *in vitro* antibody response, and almost ideally fit the idea of Bretscher and Cohn. It carries κ- and μ-chain determinants, and is considered to be the monomeric form of IgM. Coinciding with the detection and isolation of monomeric IgM from T cells of some animal species (Marchalonis *et al.*, 1972; Cone and Marchalonis, 1974; Szenberg *et al.*, 1974), the model of T–B cell collaboration and regulation by IgT attracted much attention from immunologists. The extensive studies by Feldmann and co-workers, indeed, gave very clear accounts of various facets of cell interactions involved in antibody formation, immunologic tolerance, antigenic competition, and specific suppression, as will be examined in Section IV. This attractive model, however, confronted serious difficulty as several other investigators failed to detect μ and κ chains on T cells by very sensitive methods. In addition, serum IgM antibody in a small quantity was also found to enhance the antibody response under certain circumstances (Henry and Jerne, 1968). However, we think that these do not constitute major objections to the presence of IgT, since other biologically active T cell factors discussed in this review are also undetectable by usual chemical means. It would be fair to state that there exist certain difficulties in concluding that IgT is the only or most important mediator from helper T cells. We should only remain alert for an alternative explanation of IgT, since there are a number of similarities between IgT and other helper factors, including that recently described by Feldmann and co-workers (1977b).

The second category of antigen-specific T cell factors comprises mostly, if not entirely, a small molecular weight protein of nonimmunoglobulin nature having either helper or suppressive activity. Such molecules are released or extracted from antigen-stimulated T cells of known biologic functions. One of the first demonstrations of such factors was made in the suppression of IgE antibody response in the rat (Tada *et al.*, 1973). This was followed by a demonstration of antigen-specific helper or cooperative factor by Taussig (1974) in a much better defined experimental system of mouse IgM antibody formation. Both suppressor and helper factors were first demonstrated in *in vivo* experiments, and later extensively analyzed in *in vitro* experimental systems. Thus the relevance of these factors in *in vivo* immune responses is obvious.

The most remarkable feature of this category of T cell factors is that they possess no known immunoglobulin constant region determinants despite their exquisite antigen specificity. They are able to bind anti-

gen with specificity and affinity comparable to those of serum antibodies and can be eluted similarly from antigen-coated columns to purify antibodies. Further analysis of these T cell factors by different investigators eventually led to the same conclusion—that the factors possess determinants coded for by genes mapped in the *I* region of the mouse major histocompatibility complex (MHC). Since several workers in this field have utilized well defined synthetic polypeptides to which immune responses are under the control of Ir genes, the argument concerning the relationship between these *I* region determinants (Ia) bearing T cell factors and functionally defined Ir genes has been reiterated during the last several years. However, this argument was unfortunately not very fruitful, since we now notice much more confusion than agreement on this issue (see Sections III,C and IV,D). Nevertheless, we have been considerably enlightened with respect to the genetics of immunoregulation by studies on these T cell factors, as the structural genes coding for the determinants on helper and suppressor factors have been unambiguously mapped in *I-A* and *I-J* subregions, respectively. Furthermore, there have been some important discoveries on the consequences of cellular events initiated by these T cell factors. We discuss diverse pathways of the action of T cell factors with respect to their cellular targets, subsequent activation processes, and genetic requirements in Section V.

Similar antigen-specific T cell factors have been demonstrated in the regulation of cell-mediated immune responses as well. These include factors involved in the delayed-type hypersensitivity to chemical compounds and in the cytotoxic T cell response to tumor antigens. These experiments allow us to envisage the widely diverse range of regulatory effects of very similar factors. It again stresses the possibility that T cell-derived factors represent the physiologically legitimate mediators in various types of cellular interactions.

Having established that the T cell factors discussed herein have exquisite antigen specificity, what picture emerges of the structural and genetic relationship between antigen-binding sites of the T cell factors and immunoglobulins? Recent studies from at least two major laboratories indicate that some of the T cell factors bear idiotype determinants identical or similar to those of antibodies against the same antigen (see Section VII). More recently, the presence of immunoglobulin *V* region structure has also been demonstrated. The chemical approach to purify and characterize these T cell factors has, however, long been hampered by the low recovery of the materials. One important strategy has recently been provided by making hybrid cell lines continuously producing some of the T cell factors in a quantity suffi-

cient for chemical analysis. The materials obtained from hybridomas would allow us far more precise analysis of their function, genetics, and chemistry.

There have been a number of reports on soluble antigen-specific T cell factors during the past 10 years other than those discussed in this review. We must confess that we have tried to put all the factors in order without any success, and therefore we have gathered information only on the factors that have been extensively characterized as to their biologic and immunologic properties. In addition, we are aware that it is still premature to draw any definite conclusions in this review. At this time, when absolutely revolutionary findings could appear at any moment and old erroneous experiments are being discredited every second, it is almost impossible to write a comprehensive and meaningful review on such a complex subject as this. We must be very careful, for erroneous rumors tend to spread rapidly throughout the scientific world. Thus, it is fair to reiterate that some of the presently empirical results should be reinterpreted in the future, old problems will disappear, and new ones will certainly emerge.

III. Antigen-Specific T Cell Factors That Augment the Antibody Response

A. Problems in T–B Cell Collaboration

One of the central functions of T cells is their collaborative or helper function in the synthesis of antibodies by B cells. Even with extensive studies performed during the last decade, the precise mechanism of T–B cell interaction is still unclear. However, we are now quite certain that the mode of T–B cell interaction for antibody synthesis is not a uniform process, but may have diverse pathways. In the antibody response against hapten carrier conjugates, it is generally accepted that helper T cells recognize carrier determinants and then assist in the stimulation of B cells by haptenic determinants. This is supported by a number of experimental studies both *in vivo* and *in vitro* and has created a number of theories, such as antigen-focusing (Mitchison *et al.*, 1971), carrier antibody (Bretscher and Cohn, 1968), two-signal and one-signal concepts (Bretscher, 1972; Coutinho and Möller, 1974). One important and widely accepted feature is that in order to induce optimal antihapten antibody response, haptenic and carrier determinants should be present on the same molecule, i.e., linked or cognate interaction (Mitchison, 1971a,b; Rajewsky *et al.*, 1969). This was shown not only in the hapten-carrier systems, but also in the response against multideterminant protein molecules. In such a situation, one

can imagine that antigen-specific helper factors, which recognize carrier determinants and interact with hapten-binding B cells, are ideal devices for envisaging the molecular bases of T–B cell interactions.

On the other hand, there are experimental data in which hapten-specific B cells can be triggered by T cells that are stimulated by unlinked carrier (Hamaoka *et al.*, 1973; Kishimoto and Ishizaka, 1973; Tada *et al.*, 1978a). Many antigen-nonspecific helper factors have also been reported. However, under physiologic conditions the stimulus by specific antigen is undoubtedly required for the induction of final antigen-nonspecific signals. Since the induction of helper T cells is also regulated by other T cells (Feldmann *et al.*, 1977a), there is no objection to the notion that antigen-specific factors are involved in the generation of antigen-nonspecific signals. Such an antigen-specific augmenting T cell factor mediating T–T cell interaction has been described by Tokuhisa *et al.* (1978). Furthermore, a number of recent reports suggest that there are more than two types of helper T cells with distinct functional and phenotypic expressions whose recognition structures are apparently different from each other (Marrack and Kappler, 1976; Janeway, 1975; Janeway *et al.*, 1977; Tada *et al.*, 1978b).

In general, helper T cells belong to the subclass of Lyt-1^+,2^-,3^- T cells (Cantor and Boyse, 1975). Accordingly, the source of antigen-specific augmenting T cell factors has been shown to be Lyt-1^+,2^-,3^- T cells (Feldmann *et al.*, 1977b; Tokuhisa *et al.*, 1978). One of the major controversies concerns the finding that these helper T cells do not express detectable immunoglobulins nor Ia antigens, which are possessed by antigen-specific helper factors derived from them. Although some helper T cells have been shown to possess Ia determinants detectable by cytotoxic treatment with anti-Ia and C (Okumura *et al.*, 1976; Tada *et al.*, 1978a), the determinants are apparently not related to those of helper T cell factors. Whether this is due to the rapid shedding of the helper molecules from cell surface membrane or to a quantity too small to be detected by serologic methods is presently unknown.

There is another theoretical difficulty in understanding the antigen specificity of existing T cell factors. Recent reports on the condition for T cell stimulation indicate that T cells recognize not only antigen itself but also self-antigens, namely the Ia antigens on macrophages (Rosenthal and Shevach, 1977; Paul *et al.*, 1977; Schwartz *et al.*, 1978; Rosenthal, 1978; Singer *et al.*, 1977; Kapplar and Marrack, 1976, 1977; Miller *et al.*, 1976a,b; Erb and Feldmann, 1975; Pierce and Kapp, 1976; Yamashita and Shevach, 1978). Whether helper T cells recognize antigen and macrophage Ia separately or in associated form has

not yet been settled. This problem should seriously be considered, if we admit the requirement of identities of certain critical genes in *I* region for effective T–B cell collaboration (Kindred and Shreffler, 1972; Katz *et al.*, 1975; Kappler and Marrack, 1977; Sprent, 1978a,b; Yamashita and Shevach, 1978). In addition, under certain experimental conditions, the identity of Ig allotype or idiotype genes between helper T cells and responding B cells is required (Herzenberg *et al.*, 1976; Woodland and Cantor, 1978). Thus, T–B cell collaboration is not merely a matter of bridging of T and B cells by antigen.

In cognizance of these problems, we should ask critically how the antigen-specific T cell factors described below can entertain the apparent complexity of the above phenomena. Although the activation of B cells by free molecules excreted from the membrane may be different from that induced by the T-B cell contact, the properties of helper T cell factors have to meet some of the requirements in addition to their antigen specificity.

B. Antigen-Specific T Cell Factor Bearing Immunoglobulin Determinants (IgT)

The first demonstration of the antigen-specific T cell product having immunoglobulin structure was made by Feldmann and Basten (1972) using Marbrook culture vessels with a double-chamber separated by a cell-impermeable nucleopore membrane. Activated T cells specific for a carrier, such as keyhole limpet hemocyanin (KLH) or fowl γ-globulin (FγG) were placed in one chamber and hapten, 2,4-dinitrophenyl (DNP)-primed B cells in the other, with antigen (DNP–KLH or DNP–FγG) in both compartments. The nucleopore membrane prevents the direct contact of B and T lymphocytes, while enabling the protein molecules to migrate freely from one compartment to the other. Feldmann and Basten described that activated T cells were capable of providing helper activity for the DNP-primed B cells to respond to DNP–KLH or DNP–FγG across the nucleopore membrane, depending on the specificity of activated T cells. Responses to unrelated antigens, such as donkey erythrocytes (DRBC), even if present in the same culture were either unaffected or only slightly increased. The marked specificity of the collaborative factor for both T and B cells mimics the rigid requirements for the "linked recognition" of determinants on the same antigen molecule by T and B cells as in the adoptive secondary response observed by Mitchison (1971a,b).

Feldmann and co-workers characterized the active principle in the supernatant of activated T cells. It was found that the specific coopera-

tive activity was removed by absorption with immunoadsorbents of anti-immunoglobulin reagents coupled to Sepharose beads, such as anti-Ig, anti-μ, and anti-κ (Feldmann, 1972a,b). Molecular weight was about 180,000, being very close to that of monomeric IgM (Feldmann *et al.*, 1973). The observation was strongly supported by the coinciding demonstration of monomeric IgM on T cells of certain species (Marchalonis *et al.*, 1972; reviewed by Marchalonis *et al.*, 1977).

Subsequent studies by Feldmann (1972a) revealed a very important property of IgT, i.e., the cytophilic nature of IgT. Activated T cells were incubated with antigen in one chamber and purified macrophages on the other side of the nucleopore membrane in double-chamber Marbrook vessels. After 1–2 days of cultivation the macrophages were harvested, extensively washed, and placed into a second stage of culture in which DNP-primed B cells were added to the macrophage without further addition of antigen. It was found that the cultured macrophages were able to help B cells to mount significant anti-DNP antibody response during the subsequent 4-day culture period in the absence of T cells and antigen. These results indicate that the cooperative factor is rapidly bound to macrophage after release from T cells, and trigger B cells without T cells. By using antisera to the haptenic (DNP) or to carrier (KLH) determinants, it was shown that "collaborating macrophages" carried DNP–KLH on their surface as the highly immunogenic entity (Feldmann, 1972a). Furthermore, it was demonstrated that treatment of KLH-primed T cells with monospecific antisera against mouse κ and μ chains before cultivation with macrophage inhibited their ability to sensitize macrophages. Trypsinization of sensitized macrophages abrogated their capacity to induce anti-DNP antibody response by B cells, suggesting that the collaborative material was bound to the macrophage surface. Macrophages incubated in the same concentration of antigen alone did not acquire the capacity to cooperate with purified B cells, and thus the product of activated T cells was found to be definitely needed. The cell type producing the active material was determined to be T cells, but not B cells. In addition, serum IgM antibody was found to be suppressive rather than enhancing in this *in vitro* culture system (Schrader, 1973).

Feldmann (1973) further studied the effect of IgT in the absence of macrophages on B cell response. Activated T cells specific for FγG were cultured with DNP-FγG. The supernatant was divided into two portions, and one half was incubated with a spleen cell suspension from mice primed with DNP-polymerized flagellin (POL), containing a normal number of macrophages for 16 hours. The other half was

incubated with macrophage-depleted spleen cells from the identical source. In the latter case, any IgT–antigen complexes that might have been generated would have the chance to go straight onto DNP-primed B cells. The cells were then cultured for further 4 days with or without challenge of appropriate dose of DNP–POL, a T cell-independent antigen. As expected from the previous experiment, the intact DNP–POL-primed spleen cells were able to mount anti-DNP antibody response in the presence of FγG-specific IgT even without further addition of antigen. The addition of DNP–POL enhanced the response due to its ability to stimulate B cells independent of the T cells' help. In sharp contrast, the very same supernatant containing IgT had an entirely different effect on macrophage-deprived spleen cells: Not only did it fail to cause antibody response in the absence of antigen, but also it affected the T cell-independent DNP-specific B cell response against DNP–POL. Only a negligible number of DNP-specific PFC were induced with the macrophage-depleted spleen cells when the cells were precultured with the putative IgT–antigen complexes. Feldmann concluded that the same collaborative factor from activated T cells in association with antigen would make B cells partially tolerant under the condition in which macrophages are depleted (Feldmann, 1974c).

This principle was further extended to explain the mechanism of antigenic competition (Feldmann and Schrader, 1974). In short, purified macrophages were incubated with supernatant of activated T cells specific for KLH and DNP-KLH. If such macrophages were used to reconstitute the macrophage-free spleen cells, which were subsequently stimulated with unrelated antigen, i.e., DRBC, there was a strong inhibition of antibody response to the latter antigen. Thus, it appears that macrophages saturated with DNP–KLH bound via the IgT simply could not accept the helper factor against unrelated antigen, namely, DRBC. In this context, IgT can explain at least three important cellular events, T–B collaboration, immunologic tolerance, and antigenic competition.

Besides these *in vitro* experiments, there have been some reports suggesting that IgT-like molecules manifest enhancing regulatory activity on *in vivo* antibody response. Taniguchi and Tada (1974) have shown that thymocyte extract from rats that had been hyperimmunized with carrier antigen (*Ascaris suum* extract, Asc) can reconstitute the IgE antibody response of neonatally thymectomized rats against DNP–Asc. The active molecule was shown to have Fab and μ chain as revealed by absorption with immunoadsorbents. The molecular weight was between 200,000 and 100,000, resembling IgT. How-

ever, even though the material can be obtained from washed thymocytes, there was no clear evidence that the molecule derived from antigen-primed T cells. Since a small amount of serum IgM antibody or its reduced material was found to have some enhancing activity in IgE antibody response, the possibility that contamination of B cell product in such an extracted material is not completely ruled out (Tada, 1975). In fact, injection of IgM but not IgG antibody against sheep red blood cells (SRBC) into mice was found to be enhancing the anti-SRBC antibody response (Henry and Jerne, 1968), and thus the formal proof for the role of IgT in *in vivo* antibody response is not yet established.

The model presented by Feldmann, which we have narratively described, can explain several important phenomena in the induction and suppression of the antibody response, T cell–macrophage–B cell interactions, antigenic competition, and tolerance induction. The model also explains the mechanism of B cell triggering by the single concept that IgT is capable of forming a matrix of antigenic determinants on the surface of macrophages that mimics T cell-independent antigen, such as DNP–POL, having a repeated cyclic structure of the antigenic determinants.

The validity of the above model is still to be established by future experimentation. In cognizance of the importance of findings by Feldmann and co-workers on IgT, we are also aware of several contradictory findings concerning T cell immunoglobulins. We do not, and need not, get into the hot debate about the presence of immunoglobulin on T cells, since such has been extensively discussed from both positive and negative sides (Marchalonis and Warr, 1979; Vitetta and Uhr, 1975). As we shall discuss later, at least some T cells have antigen-binding receptor, probably coded for by immunoglobulin V genes and having idiotypic determinants (see Section VII). The only reservation about drawing conclusions at the moment is based on the difficulty of defining μ and κ chain as the constant structure of T cell factors, as other laboratories were unable to detect them. The precise condition to rule out possible artifacts is still not established. If subtle differences in the reagents cause different results, there is no way to avoid erroneous conclusions. One should remember, however, that chicken antimurine IgM as well as anti-$F(ab')_2$ can stain T cells as well as B cells by immunofluorescence (Szenberg *et al.*, 1977; Hämmerling *et al.*, 1976). The same chicken anti-IgM was recently found to absorb an *in vitro*-induced helper factor bearing Ia antigen that is considered to be another class of antigen-specific T cell factor (Feldmann *et al.*, 1978). Thus, the critical argument would still de-

pend on the specificity of reagents used by different investigators. If the antisera made by lower vertebrates detect some highly cross-reactive determinants on T cell receptor rather than classical immunoglobulin isotypes, we should again return to examine the true nature of so-called IgT.

C. Antigen-Specific Helper Factors Bearing Ia Determinants

1. Cooperative T Cell Factor from Educated T Cells

Another entirely different type of antigen-specific helper T cell factor was first discovered by Taussig (1974). He obtained "educated T cells" from recipients of syngeneic thymocytes, who had been immunized with an appropriate dose of a synthetic branched copolymer, poly(L-Tyr, L-Glu)-poly(D,L-Ala)–poly(L-Lys) [(T,G)-A-L] in complete Freund's adjuvant. The spleen cells were cultured for a short period of time (6–8 hours) in the presence of antigen. The culture supernatant was tested for its capacity to replace the requirement of antigen-specific helper T cells by injecting it to irradiated mice that were concomitantly given syngeneic bone marrow cells from unprimed donors. The recipients were immediately immunized with 10 μg of (T,G)-A–L, and the IgM antibody response was measured by the PFC method.

Taussig's observation was that whereas adoptively transferred bone marrow cells alone could not mount (T,G)-A–L specific antibody response of IgM class, the concomitant transfer of the culture supernatant of antigen-stimulated educated T cells markedly increased the antibody response to this antigen. The response obtained with the cell-free supernatant was almost comparable to that with intact T cells. The response was clearly helper T cell-dependent, though composed entirely of IgM antibody. Thus the factor was found to be capable of replacing an otherwise absolute requirement for T cells in the primary antibody response to (T,G)-A–L by bone marrow cells. Furthermore, the factor was proved to be the T cell product, as the treatment of educated T cells with anti-Thy-1 antiserum and C completely inhibited the production of the factor. It was also shown that nylon wool-purified cells containing less than 0.1% B cells could produce an amount of T cell factor comparable to that in the unseparated preparation (Taussig *et al.*, 1976a).

The antigen specificity of this helper T cell factor was ascertained by two different criteria: First, the factor derived from (T,G)-A–L educated cells could help the response to (T,G)-A–L, but not to unrelated

antigen such as SRBC. Second, the helper activity was completely removed by absorption with immunoadsorbent composed of (T,G)-A–L, but not of other antigens (Munro *et al.*, 1974). Despite this exquisite antigen specificity, the helper activity of the supernatant was not absorbed with any of the anti-immunoglobulin columns, e.g., polyvalent anti-mouse Igs, anti-Fab, anti-γ, and anti-μ, indicating that the T cell factor has no immunoglobulin constant structures. The molecular weight of the factor was determined to be around 50,000, being much smaller than usual immunoglobulins (Taussig and Munro, 1974).

The most important feature of the antigen-specific helper factor is that it contains determinants coded for by genes in the *I* region of the *H-2* complex. Munro *et al.* (1974) attempted to absorb the factor with various alloantibodies against the products of *H-2* subregions. They found that the (T,G)-A–L specific helper activity was completely removed by passage through columns of immunoadsorbents composed of congenically raised alloantisera against the *H-2* complex of the strain in which the helper factor was produced. Mapping of the gene coding for the factor was accomplished by using alloantisera directed to different subregions of the *H-2* complex. It was found that an alloantiserum directed at *K*, *I-A*, and *I-B* subregions (B10.D2 anti-A/J) was capable of removing the helper activity, whereas those against *H-2K* (AQR anti-B10.A) and *I-B*, *I-C*, *S*, and *D* regions were ineffective. Hence, it is obvious that *I-A* subregion contains a locus controlling the determinants on the (T,G)-A–L specific helper factor (Taussig *et al.*, 1975).

Taussig *et al.* (1974) and Isac and Mozes (1977) were able to demonstrate similar antigen-specific T cell factors against other multichain synthetic polypeptides, poly(L-Phe, L-Glu)-poly(D,L-Ala)–poly(L-Lys) [(Phe,G)-A–L], poly(L-His,L-Glu)-poly(D,L-Ala)–poly(L-Lys) [(H,G)-A–L], and poly(L-Tyr, L-Glu)-poly(L-Pro)–poly(L-Lys) [(T,G)-Pro–L], to which responses in mice are controlled by Ir-1 genes with a notable exception of the response to (T,G)-Pro–L. Since these branched copolymers have a certain degree of cross-reactivity at the serum level, the T cell factors for these antigens give a unique opportunity to study the specificity of the putative T cell receptor for antigen as well as the site of expression of specific Ir genes. Although these problems are discussed in the following sections, we would point out at the moment that the helper factors for these antigens under the control of different Ir genes have very similar physicochemical and immunochemical properties (Mozes, 1976). In addition, Isac *et al.* (1977) showed that the factor specific for (T,G)-Pro–L, the response to which is clearly not under Ir-1 gene control (Mozes *et al.*, 1969), is also

a product of an *I-A* subregion gene. This raises an important question as to whether the factor itself is the molecular expression of Ir genes. Certainly very conflicting arguments concerning the relationship between the effect of Ia-bearing T cell factors and Ir gene control have arisen, as will be discussed later.

Mozes (1976) tried to isolate and characterize the (T,G)-A–L specific helper factor by several physicochemical and immunochemical techniques. Fractionation with ion exchange column chromatography using DEAE-cellulose with increasing NaCl concentration revealed that the active factor was eluted with a 0.25 *M* salt concentration, indicating a significant charge difference from known immunoglobulin molecules. An effort was also made to purify the (T,G)-A–L specific helper factor on the basis of binding to and elution from an affinity column. The active factor was eluted from the (T,G)-A–L column with 0.1 *M* ammonium hydroxide, which was capable of eluting antibodies against (T,G)-A–L. The purified material was subjected to the analysis of electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Two major peptide chains revealed on the gel had molecular weights of 45,000 and 70,000. It is not known, however, which of these bands is associated with the active helper factor. Similar methods employed in our laboratory to detect other antigen-specific factors also failed to obtain meaningful results. However, this most painstaking effort has revealed that at least the molecule has remarkable differences from known immunoglobulins and B cell Ia antigens.

Having established the fact that the cooperative factors hitherto mentioned are the products of *I* region genes, one may ask whether they are identical to or related to previously known Ia antigens detected mostly on B cells and macrophages. This question has partially been answered by Munro *et al.* (1974) using cross-reactive anti-Ia antisera. Ia specificities of various haplotype strains have been well established by cytotoxic killing of B cells by anti-Ia antisera, and such specificities are associated with at most two Ia molecules coded for by *I-A* and *I-E/C* subregions (Shreffler and David, 1975). A considerable cross-reactivity has been demonstrated between Ia antigens of different haplotypes; thus, if the determinants on helper factors are related to the conventional B cell Ia antigen, it is expected that anti-Ia antiserum raised against one strain of mice having one Ia specificity shared by another strain of mice should remove the helper factor derived from the latter. Munro *et al.* (1974), using anti-Ia antisera cross-reactive to one of the known Ia specificities, found that whereas anti-*H-2* antisera raised against the same haplotype in which T cell

factor was induced were always effective in removing the helper activity; none of the antisera produced against different haplotype strains having cross-reactive B cell Ia specificities were able to absorb it. This suggests that *I* region determinants on the helper factor are different from conventional B cell Ia antigens. On the other hand, Mozes (1978) found that (T,G)-Pro–L specific helper factor from $H\text{-}2^s$ mice was removed by anti-$H\text{-}2^r$ antiserum, suggesting that factors from $H\text{-}2^s$ and $H\text{-}2^r$ mice may have some cross-reactivity. The formal proof that different *I* region loci control Ia determinants on B cells and T cell-derived factors has not been made in this experimental system.

Another important observation made by Taussig and Munro (1976) is that the factor is capable of cooperating with allogeneic bone marrow cells acting across the major histocompatibility barrier. They produced (Phe,G)-A–L specific helper factor in B10 ($H\text{-}2^b$), B10.BR ($H\text{-}2^k$), and B10.D2 ($H\text{-}2^d$) mice, and performed crisscross experiments by cotransferring with bone marrow cells from either one of these strains into recipients syngeneic to bone marrow donors. There have been found no restrictions on the ability of the factor to cooperate with allogeneic B cells provided bone marrow cells are obtained from a responder strain. This is a notable difference from cooperation with viable T and B cells under physiologic conditions, where certain critical identities in *H-2* subregions have been required for the effective cooperation (Section III,A).

They have further shown that the cooperative factor probably acts directly on B cells (Munro and Taussig, 1975). The factor was found to be absorbed by bone marrow cells of responder haplotype even in the absence of antigen. Cells from nonresponder haplotype were unable to absorb the factor. The latter observation suggests that nonresponder B cells lack a device to accept T cell helper signal even though B cells themselves have immunoglobulin receptor for antigen.

Certainly a very interesting question can be asked on the basis of above observations as to the significance of the reaction between B cells and specific T cell factor. Taussig and Munro (1976) treated bone marrow cells *in vitro* with the factor and antigen, followed by centrifugation and resuspension in medium, and then transferred the cells into lethally irradiated recipients with antigen but without further addition of the factor. Such cells treated with the factor and antigen mounted a good antibody response. On the other hand, if bone marrow cells were treated with the factor alone without antigen they were incapable of mounting the antibody response even if antigen was added upon cell transfer. The results suggest that the factor is taken up by bone marrow cells in the absence of antigen, however, in order to

form an immunologically meaningful signal, B cells have to bind antigen which is to be "focused" by the T cell factor. Thus Taussig and Munro concluded that the process of B cell triggering is initiated by the antigen-Ig receptor interaction on B cell membrane followed by the factor–acceptor interaction by virtue of antigen-binding activity of the factor. The latter interaction gives the second signal for the B cell differentiation. This conclusion is apparently consistent with the two-signal hypothesis of Bretscher (1972).

The biochemical events after this signal transmission are almost entirely unknown. Recently, Mozes (1978) demonstrated that the addition of (T,G)-A–L specific helper factor to the suspension of (T,G)-A–L primed spleen cells significantly increased the cytoplasmic cyclic AMP (cAMP) level. Such an effect was not seen with spleen cells of low-responder mice or those from unprimed responder mice. The removal of (T,G)-A–L specific factor either with antigen-coated or anti-*H*-2 column abrogated this effect. Mozes also showed that the factor directly acts on splenic B cells free from Thy-1 bearing cells to increase their cAMP. Since it has been shown that some steps of B cell differentiation involve the activation of adenyl cyclase, causing the elevation of intracytoplasmic cAMP level (Watson, 1975; Bourne *et al.*, 1974), one important effect of helper factor appeared to be the activation of adenyl cyclase in B cell clones specific for the given antigen.

The above findings together indicate that the antigen-specific helper or cooperative factor acts on unprimed or primed B cells in the presence of antigen. The activation process in B cells perhaps involves three steps: (*a*) binding of antigen by Ig receptors; (*b*) receiving T helper factors at a special site (acceptor site); and (*c*) activation of certain intracellular biochemical processes including the increase of cAMP.

2. Helper Factors Induced in Vitro

A similar antigen-specific helper factor was recently demonstrated by Howie and Feldmann (1977). They were successful in inducing antigen-specific helper T cells by culturing normal mouse spleen cells with 1 μg of (T,G)-A–L per milliliter and random copolymers of L-glutamic acid–L-alanine–L-tyrosine (60 : 30 : 10) (GAT) for 4 days in Marbrook bottles (Howie *et al.*, 1977). Such cells if cocultured with unprimed B cells in the presence of DNP or 4-hydroxy-5-iodo-3-nitrophenyl (NIP) derivatives of homologous synthetic copolymers, were able to help to induce primary IgM PFC response by B cells. Both responder and nonresponder spleen cells can be triggered to induce specific helper T cells under appropriate

conditions, and thus the helper cell induction *in vitro* somehow bypasses a process on which the Ir gene effect is exerted in the *in vivo* condition.

They have then further cultured the *in vitro*-induced helper cells in Marbrook bottles for 24 hours with 1 μg of antigen per milliliter and tested the supernatant for its antigen-specific helper activity by adding to the naive spleen cells with antigen (Howie and Feldmann, 1977). The results are summarized as follows: The helper factor elaborated by the *in vitro*-induced helper cells to (T,G)-A–L could help both the (T,G)-A–L specific and DNP-specific primary antibody responses upon culturing with unprimed spleen cells in the presence of DNP–(T,G)-A–L. Both high-responder ($H\text{-}2^b$) and low-responder ($H\text{-}2^k$) spleen cells produced comparably efficient (T,G)-A–L specific helper factor, which was determined by limiting dilution of the factor. Amazingly, a 10^{-5} dilution of the supernatant was sufficient to induce a significant number of DNP-specific PFC.

Taussig *et al.* (1974) and Mozes *et al.* (1975) had previously reported that some but not all nonresponder strains are unable to produce their helper factor. $H\text{-}2^f$ and $H\text{-}2^s$ strains were known to be defective in producing the (T,G)-A–L specific helper factor. Howie and Feldmann (1977) confirmed their results by *in vitro* production of the helper factor; $H\text{-}2^q$ (B10.G) mice in addition to $H\text{-}2^f$ and $H\text{-}2^s$ mice did not produce the (T,G)-A–L specific helper factor *in vitro*. The reason why B10.G mice cannot produce the factor despite its ability to induce helper T cells (Howie *et al.*, 1977) is presently unknown.

The factor has been partially characterized by immunochemical and physicochemical means (Howie *et al.*, 1979). The molecular weight of the helper factor was shown to be around 50,000 to 60,000, being similar to that of Taussig and Munro's factor. The factor was stable after several cycles of freezing and thawing. The activity was solely adsorbed with a Sepharose immunoadsorbent column of homologous (T,G)-A–L, but not by cross-reactive (Phe,G)-A–L and GAT. The activity was recovered in acid eluate from the antigen column.

The target of the helper factor is apparently B cells, since the factor can help the response of spleen cells depleted of T cells with anti-Thy-1 antiserum (Howie and Feldmann, 1977). However, if macrophages were depleted by carbonyl iron, the activity of helper factors was not observed. The addition of a small number of peritoneal macrophages to the system repaired the deficit of helper activity. Thus, in order for the effect of the helper factor on B cells, macrophages are definitely required. Like cooperative T cell factors produced by *in vivo* educated T cells, the *in vitro* induced helper factors act across the

major histocompatibility barrier to help the responder type B cells of the different haplotype origin. The factor was also absorbed by anti-Ia antisera against the same haplotype of the donor of the T cell factor. The specificity of anti-Ia antisera that removed the (T,G)-A–L specific T cell factor was directed at the *I-A* subregion of the *H-2* complex (Howie *et al.*, 1979).

Interestingly, the factor was absorbed with a column of chicken anti-mouse Ig antibody but not with those of rabbit anti-mouse Igs (Howie and Feldmann, 1977). This raises an obvious question as to the relationship between the Ia-bearing T cell factor and previously reported IgT (see Section III,C). The chicken anti-mouse Ig antiserum has been shown to stain mouse thymocytes and T cells (Hämmerling *et al.*, 1976), although this does not mean that the antiserum detects T cell Ia antigen. However, if such phylogenically different fowl antiserum can absorb helper factors, the possibility should be considered that the T cell Ia antigen may be associated with a structure having a high degree of homology to Ig heavy chains. If this is the case, the problem of IgT should be reexamined to clear up some of the present controversies.

Howie *et al.* (1979) expanded their study to the *in vitro* induced helper factor specific for GAT, another synthetic polypeptide under the control of Is genes (see Section IV,D). They had already shown that, as far as the induction of helper T cells *in vitro* is concerned, both responder and nonresponder strains were equally capable of responding to GAT (Howie, 1977). The basic properties of GAT-specific helper factor are the same as those of (T,G)-A–L specific factor. However, an attempt to map the gene coding for the GAT-helper factor led to a rather surprising result. The same anti-Ia antiserum that detected the *I-A* subregion determinants of (T,G)-A–L-specific helper factor was ineffective in absorbing the GAT-helper factor. Instead, anti-*I-J* antisera, the very same alloantisera capable of absorbing the suppressor factors (see Section IV), could invariably absorb the GAT-helper factor without touching the helper activity of the (T,G)-A–L specific one. This was confirmed in the responses of both $H\text{-}2^k$ and $H\text{-}2^b$ haplotypes, in which appropriate anti-*I-J* antisera were available. The acid eluates from anti-*I-J* columns fully retained GAT-specific helper activity.

This conflicting result is to be considered in the context that multiple Ia-loci seem to be present in *I-J* subregion. Recent reports from our laboratory (Tada *et al.*, 1978a) indicated that some but not all helper T cells carry *I* region determinants controlled by genes mapped in *I-J* subregion. The relationship between *I-J* determinants on helper T

cells and GAT-specific factor remains to be determined, since the Ia-bearing helper T cells in our experimental system exert only an antigen-nonspecific polyclonal helper effect.

They further utilized a rabbit antimurine Ia antiserum to characterize the *in vitro* induced helper factors (Howie *et al.*, 1979). Parish *et al.* (1976a) have reported that there exist two classes of murine Ia antigens—one carrying carbohydrate-defined antigenic specificities, and the other carrying protein-defined determinants, although there has been no good agreement about it (David *et al.*, 1978). Heterologous anti-Ia antiserum has been claimed to react exclusively with carbohydrate-defined series of Ia antigens and to react with products of both *I-A* and *I-E/C* subregions. The absorption of (T,G)-A–L and GAT-specific helper factors resulted in the complete loss of their activities, suggesting that the factor has determinants defined by the cross-reactive carbohydrate moiety. Furthermore, the allogeneic anti-Ia antiserum after pretreatment with normal mouse serum, which is claimed to contain the glycolipid moiety of Ia molecules (Parish *et al.*, 1976b), led to the loss of its activity to absorb helper factors.

Based upon above findings, Howie *et al.* (1979) proposed a model of the antigen-specific helper factor where the polypeptide portion contains antigen-binding site (variable region) and a "constant region" responsible for biological activity as a helper, whereas an oligosaccharide side chain, attached either covalently by glycosylation or hydrophobically as a glycolipid, carries Ia antigenicity. Verification of this postulate should await further biochemical analysis of the factor, inasmuch as it remains to be elucidated whether the fine antigenic specificity of T cell Ia molecules would in fact be explained by carbohydrate determinants.

3. *Specificity of Ia-Bearing Helper Factors*

As already stated, the immunologic specificity of the T cell factor has been defined basically by two criteria. First, (T,G)-A–L factor can replace the helper T cell in the response to the same antigen, but not to *unrelated* antigens, such as SRBC (Taussig, 1974). Second, the (T,G)-A–L factor is removed by absorption with (T,G)-A–L–Sepharose column, but not those of *unrelated* antigens (Taussig and Munro, 1974). However, the question should be asked to what extent this specificity is conferred. If the factor is a shed-off or secreted T cell receptor for antigen, it is important to determine the degree of specificity in comparison with the specificity of antibodies. It has been well established that antibodies against (T,G)-A–L cross-react with other similar copolymers, such as (Phe,G)-A–L and (H,G)-A–L, as well as to

a lesser extent with (T,G)-Pro–L (McDevitt and Sela, 1967; Taussig *et al.*, 1974).

This problem was extensively analyzed by Isac and Mozes (1977) and Mozes *et al.* (1976) by two approaches: (*a*) study of the cooperative activity against these antigens; and (*b*) absorption of the T cell factor with cross-reactive polypeptides. The cooperative factors obtained from T cells educated with these cross-reactive copolymers were injected into irradiated recipients together with bone marrow cells, and the recipients were immunized with either (T,G)-A–L, (Phe,G)-A–L, (H,G)-A–L, or (T,G)-Pro–L. The cross-helper effect of the factors was estimated by the antibody titer of the recipient mice to the immunizing antigens. In addition, Mozes, Isac, and associates examined the ability of these synthetic copolymers to absorb the antigen-specific helper factors to other polypeptides.

Their results are summarized as follows:

1. The factors specific for (T,G)-A–L were injected together with either (T,G)-A–L, (Phe,G)-A–L, or (H,G)-A–L, and there were significant antibody responses against these antigens. In any of the combinations between these three antigens, there was found complete cross-helper activity, suggesting that the backbone of the polypeptide is important for the cross-reaction as in the case of antibodies against these molecules.
2. The (T,G)-A–L factor failed to cooperate with B cells to induce the antibody response to (T,G)-Pro–L, and the (T,G)-Pro–L factor could not help the response to (T,G)-A–L. It is noteworthy that antibodies to (T,G)-A–L cross-react with (Phe,G)-A–L, (H,G)-A–L and (T,G)-Pro–L, whereas antibodies to (T,G)-Pro–L do not cross-react with (T,G)-A–L. The latter antibodies are exclusively directed at the Pro-L moiety of the polypeptide.
3. The activity of the (T,G)-A–L specific factor was removed by absorption with (T,G)-A–L and (Phe,G)-A–L, but not with (T,G)-Pro–L. However, the immunoadsorbent of multichain poly-D,L-alanine (A–L) was unable to absorb the (T,G)-A–L specific factor. This indicated that the cross-reactivity of the factor is not due merely to the specificity for the backbone chain.
4. (T,G)-Pro–L specific factor can be removed by the immunoadsorbent of the homologous antigen, but not with those of polymers differing in amino acids in the backbone chain. However, the (T,G)-A–L factor can be absorbed with poly(Glu)-poly(D,L-Ala)–poly(Lys), G-A–L column, which indicates that the factor is looking not only at the branch moiety, but also at the backbone structure.

These results are not inconsistent with the rule of cross-reactivity obtained with antibodies against these antigens (McDevitt and Sela, 1967) except for a few points: (*a*) that antibodies against these polypeptides contain a substantial amount of anti-A–L antibodies whereas the factor is not able to bind A–L moiety; (*b*) that (T,G)-A–L and (T,G)-Pro–L cross-react at the level of antibody (Taussig *et al.*, 1974); (*c*) that proliferative response to (T,G)-A–L by T cells is not cross-reactive to (Phe,G)-A–L (Schwartz *et al.*, 1977).

The specificity of *in vitro*-induced helper factor as reported by Howie and Feldmann (1977) had somewhat different and more strict specificity to the immunizing antigen. The *in vitro*-induced (T,G)-A–L specific helper factor was completely absorbed with the homologous (T,G)-A–L but not with (Phe,G)-A–L or with GAT. There was no cross-reactive help of (T,G)-A–L specific factor in the *in vitro* responses to DNP-(Phe,G)-A–L and DNP-(T,G)-Pro–L. The specificity of the helper factor derived from high- and low-responder strains was the same. Thus it appears that the *in vitro*-induced helper factor has higher degree of antigen specificity than that obtained from *in vivo*-educated T cells. One possible explanation for this difference is that the different methods of education of T cells give rise to the products of different clones, as the primary contact with antigen *in vitro* may stimulate T cells having highly specific receptors for the given antigen.

The similarity and differences in the cross-reactivity between helper factors and antibodies again provoke a question whether the antigen-binding sites of these two entirely different classes of molecules are coded for by identical or different V gene pools. This problem will be discussed with regard to the expression of idiotypes on T cell factors (Section VII).

4. Antigen-Specific Helper Factor and Ir Gene Control

Since the antibody response of mice against these synthetic copolymers has been shown to be under the control of Ir-1 genes mapped in the *I* region of MHC, the obvious important question is whether the production of antigen-specific factor is under Ir-1 gene control. In other words, this question is concerned with the cellular nature of the expression of Ir-1 genes. If the production of antigen-specific factors exactly follows the Ir-1 gene control, one can predict that Ir-1 gene effect is expressed on antigen-specific helper T cells. Taussig *et al.* (1974) examined the ability to produce (T,G)-A–L specific helper factors in *H-2* congenic C3H.SW ($H\text{-}2^b$) and C3H/HeJ ($H\text{-}2^k$), high and low responders, respectively, to (T,G)-A–L; they found that T cells of

both C3H.SW and C3H/HeJ origins are equally capable of producing active cooperative factors in response to (T,G)-A–L. However, only responder type B cells could be stimulated by (T,G)-A–L and its specific T cell factor *in vivo*. They claimed that the genetically controlled differences between strains in the antibody response to given antigen is due not to the difference in the ability to produce T cell factors, but to the inability of B cells to respond to the T cell factor. Thus, as far as this experiment is concerned, the Ir-gene defect is not on T cells, but solely on B cells. The subsequent work by Mozes *et al.* (1975) revealed another type of genetic defect in the production of the T cell factor in nonresponder mice. When the educated T cells from SJL ($H\text{-}2^s$) strain, a nonresponder to (T,G)-A–L, were stimulated *in vitro* with the antigen, no (T,G)-A–L specific factor active on responder bone marrow cells was produced. B cells from SJL were also unable to respond to (T,G)-A–L and its specific factor, and therefore SJL strain has double defects in the production of and response to the (T,G)-A–L specific factor. However, T cells from the same SJL strain can produce (T,G)-Pro–L-specific helper factor that can trigger syngeneic B cells to produce antibody, suggesting that the observed T and B cell defects in SJL strain are antigen specific ones, not responsiveness in general. In addition, the responsiveness of animals to (T,G)-Pro–L had been shown not to be under the H-linked Ir-1 gene control (Mozes *et al.*, 1969, 1970). Nevertheless, the (T,G)-Pro–L specific factor was absorbed with anti-*I* region antisera (Isac *et al.*, 1977). This suggests that specific Ir gene control and the structural gene for the specific T cell factor have no correlation.

These results imply certain important points. First of all, the Ir gene control of the T helper factor effect is expressed by either B cells or T cells or both. It would be manifested as a failure to produce antigen-specific factors by T cells, or as a defect to receive the signal from T cells in B cells. Second, the inability to produce or receive the antigen-specific factors is not the inherent genetic property of the strain but is probably due to the defect in very specific recognition process of the antigen by T or B cells. Thus, it may be fair to conclude that the factors themselves are not Ir gene products, but there certainly exist some Ir gene-controlled processes that are bypassed in an *in vitro* condition (Howie *et al.*, 1977), most likely to be antigen-presentation by macrophages.

Munro and Taussig (1975) extended the above observations to demonstrate the nature of the molecular site on the B cell capable of accepting the T cell factor. By examining the ability of T and B cells from various strains of mice to produce or to accept the (T,G)-A–L specific

helper factor, they found that several low-responder strains have a B cell defect (B cell nonresponders), and one strain, B10.M (*H-2*^{f}), has a pure T cell defect, whereas SJL (*H-2*^{s}) lacks both T and B cell function. In fact, they showed that bone marrow cells and lymph node B cells can successfully absorb the T cell factor provided these cells are derived from responder animals. Low-responder type B cells were unable to absorb the factor. They further demonstrated that pretreatment of B cells with relevant anti-Ia antisera including cross-reactive anti-Ia can block the acceptor site to receive the T cell factor. Thus, it seems that two different gene products are expressed on T and B cells as the factor and its acceptor, and that the defect in the expression of either one could lead to the unresponsiveness.

To prove this two-gene model Munro and Taussig studied the response of F_1 hybrids between two different types of nonresponder mice to (T,G)-A–L, namely, B10.BR (*H-2*^{k}), which lacks an acceptor site for the T cell factor, and B10.M (*H-2*^{f}), which is unable to produce the T cell factor. They demonstrated that the (B10.BR × B10.M)F_1 is a high responder to (T,G)-A–L, which is predictable if two-gene complementation in fact occurs.

Two interesting problems worth considering arise from the above experiments. First, the acceptor site on B cells is blocked by anti-Ia antisera that are raised against a different haplotype having cross-reactive Ia specificities. Such antisera are by themselves not capable of absorbing T cell factors. Hence, acceptor sites are coded for by different genes from those for T cell factors. Second, there is a heterogeneity in the acceptor site for T cell factors of different antigen specificities. For example, B cells from *H-2*^{k} mice cannot absorb the (T,G)-A–L specific helper factor while being able to absorb the (Phe,G)-A–L specific helper factor, to which they are high responders. Nevertheless, acceptor sites for both (T,G)-A–L and (Phe,G)-A–L factors are equally blocked by the same anti-Ia antisera. From these results, Munro and Taussig concluded that there exist several "classes" of acceptor sites corresponding to different "classes" of T cell factors. Such acceptor sites are not clonally distributed on B cells, but can accept only one class of helper factor.

The question of whether or not this attractive hypothesis can account for the Ir gene effect is still an important challenge in cellular immunology. Unfortunately, the experiment of gene complementation between T cell and B cell low-responder strains has not been reproducible even by the authors themselves (Munro and Taussig, 1977). Another example of two Ir gene complementation in the response to GLϕ has turned out to be an entirely different mechanism, in which

two separate *I* region genes should be expressed on a single cell type, most probably on antigen-presenting cells (Dorf and Benacerraf, 1975; Dorf *et al.*, 1975; Schwartz *et al.*, 1976, 1978; Benacerraf and Germain, 1978). The contribution of non-*H-2* genes in the expression of acceptor site on B cells has also been reported (Mozes, 1978; Munro *et al.*, 1978). Thus, it would be reasonable to conclude that both helper factors and their acceptor sites per se cannot be defined as the direct products of functionally defined Ir genes, but the activation of helper T cell with macrophage, and the interaction of helper factor with B cells may be controlled by specific Ir genes.

5. *Augmenting T Cell Factors Specific for Protein Antigens*

The studies mentioned above were mostly performed in the antibody responses to synthetic polypeptides under the control of Ir genes; recent reports from two independent laboratories demonstrated antigen-specific T cell factors against protein antigens that augment the antibody response to a hapten coupled to these antigens (Shiozawa *et al.*, 1977; Tokuhisa *et al.*, 1978). In both experimental systems, the factors were obtained by physical extraction from antigen-primed T cells. The properties, as well as the inferences derived from experiments, differ from each other, but certainly provide new insights into other facets of antigen-specific helper effect. One factor reported by Shiozawa *et al.* (1977) is helper T cell replacing under certain *in vitro* conditions, but the other described by Tokuhisa *et al.* (1978) is not. The latter enhances the antibody response in the presence of specific helper T cell.

Shiozawa *et al.* (1977) demonstrated an interesting effect of carrier-specific helper factor obtained by physical extraction of educated T cells. Thymocytes were educated with rabbit γ-globulin (RGG), HGG, or BSA in the irradiated recipients. The sonicated extract was fractionated by isoelectric focusing to obtain semipurified helper factor having an isoelectric point of 4.5. The unique procedure they used was conjugation of trinitrophenylated RGG, HGG, or BSA to Ficoll (MW 400,000), which served as a backbone to present antigen epitopes to B cells. The stimulation of B cells with TNP-carrier–Ficoll complex alone did not induce TNP-specific primary antibody response, whereas the addition of helper factor specific for respective protein carriers induced significant numbers of TNP-specific IgM PFC. This helper effect was not observable when B cells were stimulated with TNP-RGG without a backbone molecule of Ficoll. Thus in this system, it was found that (*a*) antigens should be presented to B cells on a macromolecular backbone; (*b*) the specific helper factor was necessary

for triggering of B cells. The helper factor described in this experiment was shown to be specific for the respective carrier proteins, since RGG-specific factor was effective only when the B cells were stimulated with TNP-RGG–Ficoll. Even though RGG and HGG have extensive cross-reactivity at the level of serum antibody, there was no cross-reactive help. In addition, they showed that helper factor from one strain of animals could not help the response of B cells derived from allogeneic strains including *H-2* compatible but M locus incompatible mice. The target of the factor was apparently B cells, since T cell-depleted B cells were stimulated equally well in the presence of the factor. No further characterization of the factor has so far been reported.

Their results, though somewhat contradictory to others, give certain new insights into the role of T cell factor in direct triggering of B cells. The implication is that B cell triggering may primarily depend on the focusing of helper factor molecules rather than the focusing of antigen itself. On the contrary, antigen molecule having multiple carrier determinants would serve as a focusing device so as to have the factor molecules interact in sufficiently large numbers with cell surface membrane in the immediate vicinity of the Ig receptors. Thus, Diener *et al.* (1977) presented a working hypothesis that T cell-derived helper factor, which they propose to have amphipathic properties, triggers B cells by partially inserting itself into the phospholipid bilayer of the B cell membrane. Such a focusing effect may be enhanced by ligand-induced surface receptor aggregation due to the concentration of T cell factors in a restricted part on B cell surface membrane. This implication is a reverse version of so-called "matrix hypothesis" in which B cell triggering occurs by cross-linking of Ig receptors by haptenic determinants presented in a linear sequence on macrophages (Diener and Feldmann, 1972). Their theory instead stresses the requirement for a sequence of closely spaced carrier determinants to allow for the focusing of helper molecules on the B cell surface. The interaction between the helper factor and the site to react with the factor on B cell membrane is apparently restricted by unknown genes. Whether this phenomenon can be interpreted as recognition of antigen by T cells in association with self-antigens on macrophage and B cell is still not formally proved.

Another antigen-specific T cell factor that augments the antibody response was described by Tokuhisa *et al.* (1978). They utilized extracts of thymocytes and spleen cells of mice that had been immunized with KLH or EA without adjuvant. As will be discussed later, such extracts generally contain antigen-specific suppressor factor, which is

detected by addition of the extracts to Marbrook cultures of DNP–KLH-primed spleen cells at the onset of cultivation. However, if the same extracts were added to the culture 2–3 days after the start of cultivation, significant enhancement of antibody response up to two to three times higher than the control response was observed (Tada *et al.*, 1977a). Furthermore, the response to DNP–KLH by certain strains of mice (B10 congenic lines and D2.GD) were found not to be suppressible by the syngeneic KLH-specific suppressor factor, but always enhanced by the KLH-primed spleen cell extracts (Taniguchi *et al.*, 1976b; Tada *et al.*, 1977a). The activity was antigen specific, and the molecule capable of inducing this effect was absorbed with antigen, but not with anti-immunoglobulin columns. The question was raised whether the augmentation of antibody response is induced by the same suppressor molecule under different conditions. Obviously, the augmenting T cell factor is different from the suppressor factor, since a nonsuppressor strain (A/J) could produce the augmenting T cell factor (Taniguchi *et al.*, 1976b).

By absorption with various alloantisera having different subregion specificities, Tokuhisa *et al.* (1978) unambiguously mapped the gene coding for the augmenting T cell factor in the *I-A* subregion. However, the Ia determinants expressed on the augmenting T cell factor were shown to be distinct from B cell Ia antigens, since cross-reactive alloantisera raised against different haplotype strains were unable to absorb the T cell factor regardless of their cytotoxicity for B cells. Furthermore, if an anti-Ia antiserum (A.TH anti-A.TL) was preabsorbed once with T cells of $H\text{-}2^k$ mice, its ability to remove the augmenting T cell factor produced by $H\text{-}2^k$ mice was completely abrogated, leaving the B cell cytotoxic anti-Ia activity intact. To the contrary, even after extensive absorption of the antiserum with purified B cells, the ability to absorb the T cell factor was well retained. These results, in keeping with the results of Taussig and Munro (1976), suggest that the *I-A* subregion contains two or more loci, one controlling B cell Ia antigen (Ia-1) and the other the family of antigen-specific augmenting and helper factors. The cellular origin of the KLH-specific augmenting T cell factor was determined to be the Lyt-1^+,2^-,3^- subclass, a finding similar to the results of Feldmann *et al.* (1975).

A notable difference of this augmenting T cell factor from those discussed in previous sections is that this factor is not T cell replacing. The factor had no effect on T cell-deprived DNP-primed spleen cells even in the presence of relevant hapten-carrier conjugate (DNP–KLH). In addition, for the KLH-specific augmenting factor to function,

the coexistence of KLH-primed helper T cells was definitely necessary, since the addition of KLH-specific factor to the cultured DNP-EA-primed spleen cells did not result in the augmentation. In analogy to the effect of antigen-specific suppressor factor, Tada (1977) presented a hypothesis that the factor would act on primed Lyt-1^+,2^+,3^+ cells to initiate a multistep cellular process that finally augments the effect of preexisting helper T cell.

A second important characteristic of the KLH-specific augmenting factor is a very strict *H-2* histocompatibility requirement for the augmentation of the antibody response of other strains. The critical identity of genes required for this effect was determined to reside in the *I-A* subregion: The factor derived from one strain of mice could augment the response of other strains only if the haplotype origin of the *I-A* subregion is identical regardless of other subregion as well as background genes. Thus here again, factor–acceptor complementation appeared to be a prerequisite for the augmentation of the antibody response (Tokuhisa *et al.*, 1978; reviewed by Tada *et al.*, 1976a).

KLH-specific helper factor has also been demonstrated in the system of Howie and Feldmann (1977). By the same procedure used to make helper factor against synthetic polypeptide, they were able to produce helper factor to KLH that specifically augments the B cell response to DNP–KLH *in vitro*. As has been discussed before, the factor has physicochemical and immunochemical properties identical to those of (T,G)-A–L specific T cell factor, and therefore we do not discuss the point here.

The observations on the carrier-specific augmenting factors described by Howie and Feldmann (1977), Tokuhisa *et al.* (1978), and Shiozawa *et al.* (1977) present several contradictory problems, as we still see some overlaps in phenomenology. The factor reported by Tokuhisa *et al.* (1978) is by no means T cell replacing, whereas the factor reported by Shiozawa *et al.* (1977) acts on B cells only when the antigens are cross-linked on the B cell surface. Both factors are extracted materials from primed T cells and have strict genetic restrictions. On the other hand, the KLH-specific helper factor of Howie and Feldmann is clearly T cell-replacing and acts across the MHC barrier. These differences, however, do not constitute too much conceptual contradiction at the moment, since the observed effect of T cell factors, regardless of whether they are similar molecules or not, is exerted on different processes of B cell triggering, and the question of interrelationship between different mechanisms is left entirely open.

IV. Antigen-Specific T Cell Factors That Suppress the Immune Response

A. Problems concerning Antigen-Specific Suppressor T Cells

Since the original discovery of the antigen-specific suppressor T cell, the cell types that actively suppress both humoral and cell-mediated immune responses have been phenomenologically studied in different experimental systems (reviewed by Gershon, 1974). The mechanisms whereby T cells suppress the immune response are, however, not fully analyzed. Obviously the suppressor mechanism is now becoming more and more complicated by the discovery of multiple cell types that play different roles in the whole pathway of immunosuppression. The original naive concept that the suppressor T cell, being a negative counterpart of the helper T cell, would directly suppress B cell differentiation is no more valid at the present time. We are sure that the suppression of antibody response is a consequence of a series of cellular events in which both positive and negative feedback mechanisms are involved (Gershon, 1974; Gershon *et al.*, 1977; Eardley *et al.*, 1978; Tada *et al.*, 1977b; Benacerraf and Germain, 1978). Thus, the suppressor T cell is a definition based on the ultimate suppressive effect we observe, but in many cases it only initiates a consequence of cellular interactions that finally lead to the suppression. The antigen-specific suppressor factors that we discuss here are in many cases also not the final effector molecules, but ones that determine the suppressor pathway.

Antigen-specific suppressor T cells found in many of the experimental systems have a surface phenotype of Lyt-1^-,2^+,3^+ (Cantor *et al.*, 1976; Tada *et al.*, 1977b; Feldmann *et al.*, 1975). In addition, they are positive for Ia antigen, especially that coded for by genes in *I-J* subregion (Murphy *et al.*, 1976; Tada *et al.*, 1976b; Hämmerling and Eichman, 1978; Feldmann *et al.*, 1977b; Fujimoto *et al.*, 1978; Thèze *et al.*, 1977a; Greene *et al.*, 1977c). However, these have been determined by treating the whole spleen cells at different steps in the suppressor pathway; it has to be determined what steps of T cells express these characteristic cell surface markers. As will be discussed later, there are several cell types involved in the total consequences of the suppressor pathway, and the locus of the effect of suppressor T cells is apparently not uniform. In addition, the induction of suppressor T cells requires interactions between different T cell subsets (Feldmann *et al.*, 1977c; Gershon *et al.*, 1977; Eardley *et al.*, 1978) and is controlled by macrophages (Howie and Feldmann, 1977; Kapp *et al.*, 1975; Pierres and Germain, 1978).

However, the cell type on which we would focus our special attention is the suppressor T cell, which potentially elaborates the antigen-specific suppressor factor that initiates the whole course of the suppressor pathway. An effort to separate such suppressor T cells was partially successful on the basis of adsorption to and elution from insolubilized antigen. Okumura *et al.* (1977) took splenic T cells from KLH-immunized mice. The T cells that were able to bind KLH were successfully obtained by adsorption to a KLH-coated Sephadex column at 37°C followed by elution at 4°C. A very similar procedure was found to be useful for obtaining HGG-specific suppressor T cells by adsorption to and elution from HGG-coated Petri dishes by shifting temperature (Taniguchi and Miller, 1977). The yield of cells after these procedures was less than 0.5% of original splenic T cells, but the specific suppressor activity recovered from semipurified antigen-binding T cells was enriched by more than 100-fold. Both these groups demonstrated that the purified population contained 40–50% of Lyt-2^+,3^+ (including Lyt-1^+,2^+,3^+) T cells and 30–40% of *I-J* positive T cells. By serial killing with anti-Lyt and anti-*I-J* antisera, they found that *I-J* coded antigens were mostly associated with Lyt-2^+,3^+ T cells. Okumura *et al.* (1977) further showed that Fc receptor-negative T cells in this antigen-binding cell population were the producers of the antigen-specific suppressor factor.

The question of whether these *I-J* positive T cells can in fact bind antigen was recently examined by Matsuzawa and Tada (1979) by scanning electron microscopy. The purified antigen-binding T cells were incubated with KLH at 37°C. The cells were washed and then subjected to analysis by scanning electron microscopy. About 30% of cells were found to bind KLH molecules on their flat surface of lymphocyte hemisphere. They are small to medium-sized lymphocytes with scanty microvilli. Interestingly, the binding of KLH was inhibited by pretreatment of the cells with anti-*I-J* antiserum without C, suggesting that *I-J* determinants are closely associated with antigen-binding sites. These results indicate that the cells that produce antigen-specific factors have antigen-binding sites and belong to Lyt-1^-,2^+,3^+, Ia (I-J)$^+$ type of T cells.

How then, are such special T cells selectively generated under conditions employed by different investigators? It has been reported that the injection of relatively large doses of protein antigens without adjuvants frequently induces antigen-specific suppressor T cells, but in certain cases hyperimmunization with adjuvant has been found to be quite effective. In the experiments performed by ourselves of suppression of IgE antibody response in the rat, hyperimmunization with car-

rier antigen in complete Freund's adjuvant was routinely used to obtain the suppressor T cell and the factor from it (Okumura and Tada, 1971; Tada *et al.*, 1973). The method was utilized because of the experience that the immunization of rats with antigen in Freund's complete adjuvant was ineffective in producing IgE antibody, but rather suppressed the response induced by subsequent immunization with the immunogenic form of antigen with a potent adjuvant for IgE production, i.e., *Bordetella pertussis* vaccine.

In the suppression of IgG antibody response in mice against haptenated KLH, we found that adjuvant was not necessary, but rather inadequate to obtain consistently high levels of suppressor T cells to KLH. Thus, we routinely immunized animals with a relatively high dose of soluble KLH (100 μg each) twice with a 2-week interval. The suppressor activity is already detected 3–5 days after immunization with KLH, but a consistent activity is obtained 10–14 days after the second injection of KLH. This method was found to be effective in producing suppressor T cell and suppressor factors specific for many other protein antigens, such as BGG and EA (Tada and Takemori, 1974). Other investigators took very active suppressor T cells 3–5 days after a single injection of a high dose of antigen. Basten *et al.* (1975) were successful in obtaining potent suppressor T cells by injecting a high dose of tolerogen, deaggregated HGG. Cells were usually taken 7 days after a single injection of a large dose of HGG. Taniguchi and Miller (1978a) used the same method to obtain splenic T cells to prepare the HGG-specific suppressor factor. Thus, it is generally true that immunization of animals requires a higher dose of soluble antigen to induce suppressor T cells than the optimal dose required for the effective antibody formation. As will be discussed later, such high doses of antigen would bypass the antigen-presentation by macrophages, which is required to stimulate helper T cells, probably resulting in the direct contact of suppressor T cells with antigen.

The induction of suppressor T cell is under genetic control (reviewed by Benacerraf and Dorf, 1977) and, therefore, some antigens, such as synthetic copolymers, can selectively stimulate suppressor T cells in certain strains of mice but not in others. Kapp *et al.* (1974a) and Debré *et al.* (1975, 1976a) determined strains of suppressor genotype for specific antigens, GAT, and a random copolymer of L-glutamic acid–L-tyrosine (50 : 50) (GT). In order to induce strong suppression, antigens have to be given at relatively high doses in a soluble form or in alum. However, if the same antigens were coupled to immunogenic carriers, such as methylated BSA (MBSA), strains of

suppressor genotype do not generate the cells producing antigen-specific suppressor factors. In the case of genetically determined suppression, the suppressor T cells have to be taken 3–5 days after the administration of antigen, the time when the potent suppressor factor is obtained. However, the suppressor activity is found up to 4 weeks after the immunization with GAT, but not at 6 weeks (Kapp *et al.*, 1976). Thus, the suppressor T cell producing the suppressor factor seems to be short-lived, and is active only for a short period of time after immunization. The same was true for the tumor-specific suppressor T cells. Fujimoto *et al.* (1975, 1976) observed that tumor-specific suppressor T cells were found in animals carrying a growing tumor. The surgical extirpation of the solid tumor resulted in the rapid disappearance of splenic suppressor T cells within 3–5 days.

How then, can we envisage the long-term suppression by a single injection of suppressor T cells or suppressor factors? Apparently there is an amplification or maintenance pathway of immunosuppression. Gershon *et al.* (1977) and Cantor and Boyse (1977) recently proposed a dynamic process of suppressor cell induction via the interactions between different subsets of T cells. The key role of Lyt-1^+,2^+,3^+ T cells in the amplifying process has been presented. Experimental evidence indicating the presence of amplification and maintenance loops in the suppressor mechanism will be discussed in Section V.

The role of macrophage in the induction of suppressor factor has been studied by Howie and Feldmann (1977) in an *in vitro* culture system. They were able to induce suppressor T cells of high potency by culturing normal spleen cells with high dose of a variety of antigens. A concentration about 100 times higher than for induction of helper T cells was needed to induce suppressor T cells *in vitro*. If macrophages were depleted from spleen cells, a much lower dose of antigen was capable of preferentially inducing suppressor T cells. In addition, in this *in vitro* system even the spleen cells of high-responder strain origin were turned on to produce suppressor T cells to synthetic copolymer GAT. This also suggests that the *in vitro* condition of suppressor cell induction occurs, bypassing the Is gene control, which may be dependent on the macrophage function in *in vivo*.

Similar observation was recently made by Thèze *et al.* (1977c) and Pierres and Germain (1978), who showed that GAT- and GT-specific suppressor T cells can be triggered *in vitro* even in high-responder spleen cells if they are incubated only with a high concentration of antigen. The depletion of macrophage from high-responder spleen cells led to the preferential induction of suppressor T cells with a

normally immunogenic concentration of GAT. Similar results concerning the suppressor cell induction after macrophage depletion have been reported by Ishizaka and Adachi (1976) using protein antigens. Furthermore, Pierce and Kapp (1978a) recently showed that (responder × nonresponder)F_1 mice primed to soluble GAT give secondary antibody response only to GAT presented on responder, but not nonresponder, type macrophage. This differential responsiveness to macrophage-bound GAT is associated with suppressor T cell function, in that radiosensitive antigen-specific suppressor T cells for GAT was preferentially induced when the primed F_1 cells were secondarily stimulated with GAT on macrophages allogeneic to the priming macrophages.

These results raise several important issues about macrophage function and the induction of suppressor T cells. First, the preferential induction of suppressor T cells in genetically nonresponder animals is partially due to the inability of macrophage to present antigen efficiently to helper T cells. On the basis of this lack of macrophage function, suppressor T cells are predominantly induced. Accordingly, the depletion of macrophage causes the induction of suppressor cells in high-responder animals. Second, the excess of immunogen which bypassed the step of macrophage processing-presentation of antigen would make a direct contact with suppressor precursor cells to make them proliferate and produce the factor. These results are in general agreement with the recent concept that Ir gene functions at the macrophage level to induce or prevent the effective stimulation of helper and proliferating T cells as well as those involved in delayed-type hypersensitivity (Pierce and Kapp, 1976; Singer *et al.*, 1977; Schwartz *et al.*, 1978; Miller and Vadas, 1977). However, since the treatment of nonresponder mice with anti-*I-J* antiserum or cyclophosphamide leads to the induction of primary anti-GAT response *in vivo*, the suppressor cell induction is due not solely to the lack of antigen-presentation of macrophages, but partially to the animals' inherent ability to develop suppressor T cells. In fact, more recent results by Pierce and Kapp (1978b) suggest that after immunization of (responder × nonresponder)F_1 mice with soluble GAT a segregated suppressor T cell subset behaving phenotypically as nonresponder type is stimulated. If this is the case, functional allelic exclusion of the Ir/Is gene function may have occurred at the T cell level in addition to macrophages. As the T cell factors as well as suppressor T cells express *I-J* subregion gene products as the possible functional sites, the interplay between Ir/Is genes and *I-J* structural genes may be a crucial event for the genetic regulation of immunosuppression.

B. Antigen-Specific Suppressor Factor Controlling IgE Antibody Response in the Rat

Probably the first demonstration of the antigen-specific suppressor molecule from T cells was made by us in the suppression of IgE antibody response (Tada *et al.*, 1973). We had been able to demonstrate the suppression of anti-hapten (DNP) IgE antibody response by a passive transfer of thymocytes or spleen cells of rats that were hyperimmunized with carrier antigen, *Ascaris suum* extract (Asc) (Okumura and Tada, 1971). This system had certain advantages to detect the suppressive effect of T cells, since IgE antibody response in the rat was very susceptible to various maneuvers that change T cell activities (reviewed by Tada, 1975; Ishizaka, 1976), and the consistent and sustained IgE antibody response was induced only by immunization with potent immunogens, such as DNP–Asc together with sublethal X-irradiation, which was shown to diminish suppressor T cells (Tada *et al.*, 1971).

Tada *et al.* (1973) utilized this system to detect antigen-specific suppressor molecules. Wistar strain rats were sublethally irradiated and immunized with DNP–Asc plus pertussis vaccine so as to produce sufficiently high titers of anti-DNP IgE antibodies, which persisted for a long period of time. The thymocytes and spleen cells of syngeneic rats that had been hyperimmunized with *Ascaris* antigen were disrupted by sonication followed by ultracentrifugation to obtain cell-free extracts. The extracts were injected intravenously into recipient animals that had been producing high titers of anti-DNP IgE antibody. By this simple procedure Tada *et al.* were able to show that the cell-free extracts of both thymocytes and spleen cells dramatically suppressed ongoing IgE response in the recipient. This suppression of IgE antibody response *in vivo* was clearly antigen-specific, since the extracts from normal rats as well as from those immunized with unrelated antigens were unable to suppress the response, whereas those immunized with *Ascaris* extract reproducibly suppressed the antibody response. The results suggested that sonicated extracts from carrier-immunized spleen and thymus cells contain subcellular components that specifically suppress the IgE antibody response against a hapten on the same carrier by which suppressor T cells were generated.

The specificity of the suppressor factor was further warranted by its affinity to the carrier determinants. The activity was completely removed by absorption with *Ascaris* antigen but not other antigens. In spite of this definite specificity, the suppressive component contained no immunoglobulin determinants as revealed by a similar absorption

procedure using immunoadsorbents composed of anti-Fab, anti-μ, anti-γ, and polyvalent anti-rat Igs. Heterologous antisera raised against rat thymocytes were in many cases capable of removing the suppressor activity, suggesting that the activity component possesses T cell membrane antigens. The suppressor activity was not affected by treatments with DNase and RNase, but was completely destroyed by digestion with trypsin and Pronase (Okumura and Tada, 1974). In sucrose density gradient ultracentrifugation, the activity component sedimented more slowly than rat IgG; and in gel filtration with Sephadex G-200, the molecular weight of the factor was estimated to be between 35,000 and 60,000. The electrophoretic mobility was β to α. If spleen cells were treated with antithymocyte antiserum and C, there was no recovery of suppressor activity. These findings simply indicated that an antigen-specific suppressor molecule of the protein nature can be obtained from thymic and splenic T cells by physical extraction, and that such a molecule is discrete from conventional immunoglobulins.

Unfortunately, similar experiments performed in the mouse in an attempt to suppress IgE antibody response were not successful, partially because many of the strains of mice produce high titers of IgE antibody against DNP–*Ascaris*, which is much more difficult to manipulate than in the rat. Probably, a more complicated regulation of class distribution of produced antibodies is operative as discussed by Ishizaka (1976).

C. Carrier-Specific Suppressor Factor and Its Properties

Around 1973 to 1974, a molecule of less than 60,000 MW with antigen-specific activity was hardly welcomed widely in the immunologic community, inasmuch as the factor was found in the rat, and was observed exclusively in IgE antibody response. Thus, our laboratory made an attempt to develop a similar experimental model to demonstrate antigen-specific suppression by primed T cells and the factor in more general immune responses, namely primary and secondary IgG antibody responses to defined protein antigens in the mouse. The donor of the suppressor factor was primed with a relatively high dose of carrier antigen (KLH), and the factor was obtained by sonication of the thymus and/or spleen cells. In an *in vivo* experiment, the injection of the sonicated material suppressed the anti-DNP IgG antibody response of the syngeneic recipients that were subsequently immunized with DNP–KLH. The same extract was ineffective in suppressing the antibody response against DNP-coupled to unrelated antigens, i.e., DNP–BGG, DNP–EA (Takemori and Tada, 1975). It was also found that the extract could suppress the adoptive secondary anti-

body response against DNP–KLH. As in the rat IgE system, the active factor was not absorbed with any of the anti-immunoglobulin antibodies, such as anti-Igs, anti-Fab, anti-μ, and anti-γ. Nevertheless, the factor was absorbed onto the column of specific antigen (KLH) and could be eluted by acid from the column. The molecular weight was estimated to be between 35,000 and 55,000. The most important property of the factor is that the whole suppressive activity is absorbed with anti-*H-2* antisera, more specifically with antisera directed to the *I* region of the *H-2* complex. By using antisera directed at different subregions of the *H-2* complex, it was determined that the molecule is at least a product of the *I* region, but not of the *K* or *D* regions. Furthermore, the factor obtained from BALB/c ($H\text{-}2^d$) mice could suppress the responses of syngeneic BALB/c and *H-2* compatible DBA/2, but not that of C57BL/6 ($H\text{-}2^b$) mice, suggesting that there is a genetic restriction of the effect of the suppressor factor *in vivo* (Takemori and Tada, 1975).

A very similar effect of T cell extract from animals tolerized with human γ-globulin (HGG) was reported by Taniguchi and Miller (1978a). Animals were given a high dose (1 mg) of deaggregated HGG. After 7–14 days, spleens and thymuses were removed and cell-free extracts were obtained by sonication. The injection of the extracted material into syngeneic mice greatly suppressed the primary antibody response against native HGG. Physicochemical and immunochemical properties of the active molecule were identical to those of KLH-specific T cell factor.

The more precise characterization of the suppressive T cell factor was made possible in an *in vitro* secondary antibody response (Taniguchi *et al.*, 1976a). The experimental system in brief is as follows: The donor of the suppressor factor was immunized with high dose of KLH, and cell-free extracts of their thymuses and spleens were prepared as stated above. *In vitro* secondary antibody response was elicited in Marbrook culture bottles containing 1×10^7 spleen cells from mice primed 4 weeks earlier with DNP–KLH and pertussis vaccine with 0.1 μg of homologous antigen per milliliter. Different doses of splenic and thymic extracts were added to the culture at the start of cultivation. Usually, the dose corresponding to 1×10^7 of original spleen cells gave maximal suppression of up to 95% as measured by DNP-specific plaque-forming cells.

By this simple procedure, it was possible to confirm the results obtained in the *in vivo* experimental system. The factor was found to be antigen-specific by adsorption with KLH-coated immunoadsorbent. Anti-*H-2* and anti-Ia columns could remove the activity, whereas none

of the anti-mouse immunoglobulin isotypes could. Physicochemical properties were the same as those determined *in vivo*. The cellular origin of the factor was determined by treating the spleen cells with anti-Ly antisera and C (Tada, 1977). Unlike the source of the augmenting T cell factor, which was Lyt-1^+,2^-,3^- (Tokuhisa *et al.*, 1978), the suppressor factor was found to be the product of Lyt-1^-,2^+,3^+ T cells.

One interesting point is that only little activity was released from primed spleen cells upon culturing with antigen for 6 hours, whereas the residual cells after cultivation retained very strong suppressive activity. The extract of the cultured cells exerted a striking suppressive activity, which was also removed by an antigen-coated column (Taniguchi *et al.*, 1976a). Although they were later able to obtain somewhat stronger suppressor material after 24 hours of culture, it appeared that the suppressor molecules were not easily secreted by short-term cultivation with antigen.

An attempt to map the gene that codes for the determinants of the KLH-specific suppressor factor was made by absorption studies with various anti-Ia antisera directed at restricted *I* subregions (Tada and Taniguchi, 1976). At the time of the experiment, only three subregions, *I-A*, *I-B*, and *I-C*, had been defined by the existing recombinant strains. Thus, the initial experiment could not map unambiguously the gene in a single *I* subregion. Taniguchi *et al.* (1976a), however, had already stated that the putative locus controlling the KLH-suppressor factor is present to the right of *I-B* and to the left of *I-C*, suggesting the presence of a new subregion intercalated between the *I-B* and *I-C* subregions. This observation coincided with the discovery by Murphy *et al.* (1976) of the Ia-4 locus, which controls cell surface determinants on allotype-specific suppressor T cells in (SJL × BALB/c) F_1 mice prenatally exposed to anti-Ig-1b allotype. The subregion marked by the Ia-4 locus was designated the *I-J* subregion. By the occurrence of two pairs of recombinants, B10.A(3R) and B10.A(5R), and B10.S(9R) and B10.HTT, which differ in the *I-J* subregion, antisera directed at *I-J* subregion gene products were made available. Tada *et al.* (1976b, 1977a) were able to map unambiguously the gene coding for the KLH-suppressor factor in the *I-J* subregion, although it was still unclear whether or not the antigenic determinants on the factor are identical to those controlled by the Ia-4 locus.

Taniguchi *et al.* (1976b) found that the factor obtained from one strain of mice could effectively suppress the responses of syngeneic and semisyngeneic F_1 mice. The KLH-specific suppressor factor obtained from F_1 mice of two different *H-2* haplotypes, e.g., $H\text{-}2^{k/d}$, could suppress the response of F_1 as well as those of either parental strain

($H\text{-}2^k$ and $H\text{-}2^d$). Thus, in general, the ability to produce *I* region-related suppressor factor is codominant, and the putative acceptor sites for T cell factors of a given haplotype is also codominantly expressed on F_1 lymphoid cells (see below). Subsequent experiments by Tada *et al.* (1977a) indicated that the factor obtained from *H-2* recombinant mice could suppress the responses of other strains of mice only if the *I-J* subregion of the donor and recipient strains is of the same haplotype origin. Thus, the factors from B10.A(3R) and B10.S(9R) could suppress the response of $H\text{-}2^b$ but not $H\text{-}2^k$ mice, since both 3R and 9R have *I-J* subregion of $H\text{-}2^b$ mouse origin. Similarly, the factors from B10.A(5R) and B10.HTT, whose *I-J* subregion is of $H\text{-}2^k$ origin, could suppress only the response of $H\text{-}2^k$ mice. So far no exceptions have been found with respect to this rigid genetic restriction. These results suggest that the factors encoded by *I-J* subregion genes are recognizing the complementary structure (acceptor sites) also coded for by *I-J* subregion genes to confer the suppressor signal.

Such a suppressor–acceptor model was further supported by the presence of two types of defects in the production and acceptance of the KLH-specific suppressor factor. Taniguchi *et al.* (1976b) found that A/J ($H\text{-}2^a$) mice were unable to produce the KLH-specific suppressor factor even with a modified immunization regimen. The extracts similarly obtained from A/J could not suppress the responses of syngeneic A/J and *I-J* subregion-compatible C3H and CBA ($H\text{-}2^k$) mice. Conversely, the suppressor factor from $H\text{-}2^k$ mice was able to suppress the response of A/J, indicating that A/J has a normal expression of acceptor site. Interestingly, the extract from (A/J × BALB/c)F_1 was able to suppress the response of BALB/c, but not of A/J. Since an acceptor site is normally present on responding A/J spleen cells, the above result indicates that the inability of A/J to produce the suppressor factor is inherited by (A/J × BALB/c)F_1 and associated inherently with the *H-2* segment originally carried by A/J. A similar genetic defect of A/J in producing GT-specific suppressor was also demonstrated by Waltenbough *et al.* (1977b). Lack of the ability to produce suppressor factors may account for the extremely high responsiveness of A/J to KLH (Cerottini and Unanue, 1971). A/Sn, being closely related to A/J, also was found to be a suppressor–nonproducer. However, this does not appear to be a general defect of A/J in suppressing immune responses, since idiotype-specific as well as tumor-specific suppressor T cells have been demonstrated in the A/J strain (Eichmann, 1974, 1975; Fujimoto *et al.*, 1975, 1976).

Another type of defect in the expression of the suppressor function as an exact counterpart of A/J has been observed in B10 congenic lines

and the D2.GD strain (Taniguchi *et al.*, 1976b). These strains are good producers of *H-2* restricted suppressor factors when the effect is tested in other strains of non-B10 congenic mice. However, the secondary antibody responses against DNP–KLH mounted by spleen cells obtained from any of the B10 congenic strains were not suppressed by the KLH-specific factors produced by syngeneic donors as well as by *H-2* compatible noncongenic strains. Thus, the defect observed in B10 congenic lines is the inability to accept the T cell factors produced by themselves. Interestingly, the response of C57BL/6 spleen cells was suppressible by the $H\text{-}2^b$ T cell factor, even though the genetic differences between C57BL/6 and C57BL/10 (B10) are very small. Recently, Germain *et al.* (1978b) found that C57BL/6 is a nonacceptor strain in the GT suppressor system (see Section V). Also, B10.A mice whose *H-2* complex was introduced from a suppressor–nonproducer strain, A/Sn, were capable of producing the KLH-specific suppressor factor. Complementation of suppressor and acceptor genes was demonstrated in $(A/J \times B10.A)F_1$, which could both produce and accept the KLH-specific factor (Taniguchi *et al.*, 1976b; Tada *et al.*, 1976a).

Such a putative "acceptor" site was actually demonstrated by absorption of the KLH-specific suppressor factor with syngeneic lymphoid cells. Unprimed thymocytes and splenic T cells were able to remove the suppressor activity, whereas B cells, macrophages, and bone marrow cells were unable to do so. Twice as many thymocytes as unfractionate spleen cells were necessary to absorb the suppressor factor. Results indicate that the acceptor site for the T cell factor is expressed on T cells, but not B cells, in nonclonal distribution. As will be discussed later, such acceptor sites are blocked by anti-*I-J* antisera, indicating that the acceptor sites are also coded for by genes in the *I-J* subregion (Tada *et al.*, 1978b).

Taken together, it is conceivable that in the KLH-specific suppressor system two complementing genes closely linked and located in *I-J* subregion are expressed on different subsets of T cells. The complementary interaction between these products, the suppressor factor and its acceptor site, would allow meaningful and unmistakable interactions between T cell subsets to lead to the final suppression of the antibody response. The cellular consequences of suppression will be discussed later.

Kontiainen and Feldmann (1977) reported that a similar type of antigen-specific suppressor factor is secreted in the culture supernatant of antigen-stimulated T cells. They were successful in generating antigen-specific suppressor T cells by culturing unprimed spleen cells with a relatively high dose of antigen (Kontiainen and Feldmann,

(1976, 1977). The suppressor factor thus generated is a metabolic product of Lyt-1^-,2^+,3^+ suppressor T cells, and is capable of suppressing primary IgM antibody response to hapten (TNP) coupled to corresponding carrier. In the case of the KLH-specific suppressor factor, it could suppress the response to DNP–KLH, but not to other unrelated antigens. No suppression was observed in the T cell-independent antibody response. The properties of the KLH-specific suppressor factor thus far examined are very similar to those of the extracted factor except their relative stability and the lack of genetic restriction. The factor had a molecular weight of about 80,000 and was absorbable by anti-*H*-2, but not by anti-Igs (Kontiainen and Feldmann, 1978).

The structure of the *in vitro*-induced KLH-specific suppressor factor was analyzed using serological means. As already discussed in Section III,C, rabbit antiserum raised against suppressor factor that had been purified by adsorption to and elution from antigen-coated column could absorb the suppressor factors against any antigens regardless of the strains from which the suppressor factors were obtained. This antiserum could not absorb the helper factor. On the other hand, the syngeneic mouse antisera against KLH-specific suppressor factor were capable of absorbing both suppressor and helper factors against KLH, but not those generated with unrelated antigen. From these results, Kontiainen and Feldmann (1979) claimed that rabbit antiserum to the suppressor factor is reactive to the "constant region" of the family of suppressor molecules, whereas the mouse anti-suppressor factor would recognize variable-portion or idiotypic determinants of factors directed to the same antigen. Thus, they proposed a hypothetical model of suppressor T cell factors, consisting of at least three determinants, i.e., *I* region determinants (*I-J* product for suppressor factors), constant region determinants detectable by rabbit anti-suppressor factor, and variable region determinants associated with idiotypic determinants detectable by mouse anti-suppressor factor. They suggested that such idiotypic determinants are not *H*-2 linked, but have some relationship with Ig allotype.

This hypothetical model certainly entertains the presently most controversial problem about the structure of T cell factors. However, the verification of this postulate entirely depends on the future experimental evidence, as the cross-reactive idiotype of KLH-specific factors was not formally proved in the mouse strains used by the authors. To date, no convincing linkage studies between KLH-idiotypic determinants and Ig allotype have been performed. These points will be further discussed in Section VII.

More recently, Chaouat (1978) reported that a very similar antigen-

specific suppressive T cell factor can be obtained from spleen cells taken from mice tolerized to a high or low dose of HGG by a short-term *in vitro* culture without antigen. The factor was released in the culture supernatant during 10–18 hours in serum-free medium. When the supernatant was mixed with HGG-primed spleen cells and injected into X-irradiated recipients, the adoptive secondary antibody response was greatly suppressed. The specificity was confirmed by the selective suppression of the response to HGG, but not to HSA. It was adsorbable by relevant anti-*H*-2 and antigen (HGG), and the molecular weight was about 45,000 and 50,000. The cellular site of action of the factor was suggested to be helper or amplifier T cells, since a large number of immune T cells, but not B cells, could overcome the suppressor effect. A strict genetic restriction in the effect of this factor was claimed, as the factor from CBA ($H\text{-}2^k$) suppressed the response of B10.S(9R) but not B10.HTT, and the former has the same $I\text{-}J^k$ as CBA (G. Chaouat, personal communication).

D. Suppressor T Cell Factors Specific for Synthetic Polypeptides

Besides well known Ir gene effects controlling the ability of animals to respond to various antigens, it has become increasingly clear that the specific unresponsiveness of animals to certain synthetic copolymers is also controlled by autosomal dominant genes, designated as immunosuppressor (Is) genes (Debré *et al.*, 1975, 1976a; reviewed by Benacerraf *et al.*, 1977; Benacerraf and Dorf, 1977). The observed effect of Is genes is to control the capacity of animals to develop suppressor T cells specific for given antigen. Two complementing Is genes have been mapped in *I-A/I-B* and *I-C* subregions, respectively (Debré *et al.*, 1976a). This discovery brought about very important approaches to document the genetic regulation of antigen-specific T cell factors.

Kapp *et al.* (1974a,b, 1975) presented evidence that an injection of small quantities of GAT into nonresponder strains of $H\text{-}2^p$, $H\text{-}2^q$, and $H\text{-}2^s$ haplotypes makes these animals refractory to the subsequent challenge with GAT complexed with immunogenic carrier, methylated BSA (MBSA), to which all nonresponder strains can mount a substantial anti-GAT PFC response. This suppressive effect of preimmunization with GAT was found to be due to the the stimulation of suppressor T cells specific for GAT. Later, this experimental system was applied to demonstrate Is genes that control the specific immunosuppression to another polypeptide, GT, which by itself is nonimmunogenic in all strains of mice, but is capable of eliciting antibody response when coupled to MBSA (Debré *et al.*, 1975, 1976a). The suppressor hap-

lotypes to GT were $H\text{-}2^{d,b,k,s}$. The third related copolymer utilized here is L-glutamic acid-L-alanine (50:50) (GA) to which $H\text{-}2^{s}$ mice were able to respond in addition to all the responder strains to GAT, even though these two polypeptides were highly cross-reactive to each other (Merryman and Maurer, 1976). By utilizing these three synthetic copolymers, Benacerraf and co-workers have performed very extensive studies on the molecular basis of specific immunosuppression, as briefly described below.

1. Suppressor T Cell Factor Specific for GAT

Kapp *et al.* (1976) obtained a GAT-specific suppressor factor by a method similar to that of Tada *et al.* (1973). They immunized nonresponder DBA/1 ($H\text{-}2^{q}$) and A.SW ($H\text{-}2^{s}$) mice with 10 μg of GAT in alum and took the spleen, thymus, and lymph node cells 3–5 days later. The cells were disrupted by sonication, and the cell-free extracts were obtained by ultracentrifugation.

The antigen-specific suppressor activity of the extracts was demonstrated both *in vivo* and *in vitro*. In *in vivo* studies, Kapp *et al.* injected the extracts corresponding to 1.5×10^{8} cells intravenously into syngeneic or allogeneic nonresponder mice that were subsequently immunized with GAT coupled to MBSA. Whereas the comparable dose of extracts from normal spleen or thymus cells did not significantly suppress the GAT-specific PFC response to GAT-MBSA, the extracts from GAT-primed nonresponder mice suppressed the GAT-specific IgG response from 60 to 82%. An interesting observation is that if the donor animals were primed with GAT-MBSA, an immunogenic form of GAT, the splenic extract from them had no suppressive activity. Similarly, the extracts obtained from responder strains, e.g., C57BL/6 ($H\text{-}2^{b}$), produced no suppressor activity when transferred to syngeneic mice challenged with GAT-MBSA.

One of the noteworthy observations made by these authors is that the extracts obtained from one nonresponder strain are capable of suppressing the anti-GAT responses of other nonresponder strains having different *H-2* haplotypes. Thus, the extract from GAT-primed DBA/1 ($H\text{-}2^{q}$) mice suppressed the responses to GAT-MBSA of syngeneic DBA/1 and allogeneic A.SW equally well (and vice versa). However, the same extract was unable to suppress the response mounted by responder strains to GAT-MBSA *in vivo* (Kapp *et al.*, 1977). This indicates that there is a genetic restriction in the effect of suppressor factor, which is perhaps controlled by Is genes, but the identity of *H-2* complex is not required (Kapp, 1978).

The similar GAT-specific suppressor activity of the extracts from

GAT-primed lymphoid cells was also demonstrable in an *in vitro* culture system in which spleen cells from unprimed nonresponder mice were stimulated *in vitro* with an immunogenic form of GAT-MBSA to induce a primary IgG antibody response (Kapp *et al.*, 1977; Waltenbough *et al.*, 1977a). The GAT-specific PFC response was significantly suppressed by the addition of extract from GAT-primed nonresponder mice at a dilution of 1 : 200 to 1 : 1000.

Specificity of the GAT specific suppressor factor was also confirmed by both *in vivo* and *in vitro* studies. Since nonresponder strains can respond to GAT if it is coupled to immunogenic carriers such as MBSA and pigeon red blood cells (PRBC), they injected the extract from GAT-primed DBA/1 spleen cells into syngeneic animals that were then immunized with GAT-PRBC or GAT-MBSA. The response to GAT was suppressed regardless of which carrier was used, but the response to PRBC was not suppressed. The specific binding of the GAT-primed factor from nonresponder mice to antigen was well established by adsorption to and elution of the factor from a GAT-Sepharose column. The active moiety was eluted from the column by elution with 0.6 *M* KCl, which is precisely the concentration of KCl that causes the elution of anti-GAT serum antibodies of DBA/1 mice immunized with GAT-MBSA (Thèze *et al.*, 1977a). Thus, the GAT-specific suppressor factor and GAT antibodies should have roughly similar affinity for the antigen. The fine specificity of the GAT-specific factor was tested by absorption with immunoadsorbents composed of cross-reactive copolymers, e.g., GT and GA. The absorption of the GAT-specific factor with GT but not GA columns significantly reduced the specific suppressor activity for the response to GAT-MBSA, but substantial activity remained in the absorbed material (Thèze *et al.*, 1977a).

Other immunochemical and physicochemical properties of the GAT-specific factor are quite the same as those of the KLH-specific suppressor factor. The molecular weight estimated by filtration with an Amicon tube was between 10,000 and 50,000 (Kapp *et al.*, 1976), and the activity was removed by absorption with an anti-*H-2* column (Thèze *et al.*, 1977a). The GAT-specific suppressor factor was absorbed with anti-$H\text{-}2^q$ (D2.LP anti-DBA/1J) and anti-K^q + I^q, (DBA/2J × B10.BR)F_1 anti-B10.T(6R), but not with anti-K^q, (C57BL/10 × A/WY)F_1 anti-B10.AQR. However, the determination of the more accurate specificity of the factor as well as the subregion assignment of the gene encoding this factor should await the analysis of the suppressor factor specific for a cross-reactive copolymer, GT, which provides abundant information for the problems arising (see below).

Thèze *et al.* (1977a) further examined properties of semipurified GAT suppressor factor. They found that the factor in the crude extract was partially removed by rabbit anti-GAT antibody, indicating that the factor is associated with GAT or its fragment. This finding turned out to be of great significance in considering the cellular consequence of actual suppression of the GAT-specific antibody response (see Section V). After partial purification by adsorption to and elution from a GAT-coated column, the rabbit anti-GAT antibody could no longer remove the factor activity. This observation rules out the possibility that the apparent antigen specificity is due to the antigen bound to the factor, since the purified material devoid of antigen fragments fully retained the specific suppressive activity, which in fact was considerably greater than the crude extracts on a weight basis. The purified material was also absorbable by anti-$H\text{-}2^q$ column, indicating that both GAT-binding site and *I* region determinants are present on the same molecule. The molecular weight of the purified material was 40,000 to 55,000.

Kontiainen and Feldmann (1977) could also detect GAT-specific suppressor factor in the culture supernatant of unprimed spleen cells that had been preincubated with a high concentration (100 μg/ml) of GAT, followed by cultivation without antigen for 4 days. Unlike the results of *in vivo* generation of the GAT-specific suppressor T cells, spleen cells of both responder and nonresponder mice were capable of inducing GAT-specific suppressor factor. The factor is secreted from Lyt-$1^-,2^+,3^+$ T cells and acts on B cells of both responder and nonresponder origins. In all likelihood, GAT-specific suppressor factors from *in vivo*-primed T cells and *in vitro*-stimulated T cells are the same, and only minor differences in their action are noted (see below).

2. *Suppressor Factor Specific for GT*

The studies on the GAT-specific factor were extended to the analysis of the factor specific for another synthetic polypeptide, GT. As mentioned before, GT is nonimmunogenic by itself, but is capable of inducing suppressor T cells upon immunization of certain selected strains of mice having GT-suppressor genotypes. These strains include mice of $H\text{-}2^{d,f,k}$ and $H\text{-}2^s$, and the suppression is controlled by two complementing *H*-2-linked Is genes (Debré *et al.*, 1975, 1976a). GT-specific suppressive material which suppresses the response to immunogenic form of GT-MBSA, is extracted from spleen and thymus cells of BALB/c ($H\text{-}2^d$) and B10.BR ($H\text{-}2^k$) mice primed 3 days earlier with 10–100 μg of GT intraperitoneally. The extracted material upon injection into normal syngeneic as well as allogeneic mice very effec-

tively suppressed the primary anti-GT IgG antibody response to GT-MBSA (Waltenbough *et al.*, 1977a). No indication of *I* region restriction was observed. On the other hand, the extracts obtained from strains with nonsuppressor genotype ($H\text{-}2^a$, $H\text{-}2^b$, and $H\text{-}2^q$) had no suppressive activity in the responses both of suppressor and of nonsuppressor strains. Furthermore, if the extracts were taken from suppressor strains preimmunized with GT-MBSA, an immunogenic form of GT, they contained no suppressive activity.

The intriguing observation made by Waltenbough *et al.* (1977b) is that the factor from a suppressor strain, e.g., BALB/c, was able to suppress the response of GT-nonsuppressor strains, e.g., A/J ($H\text{-}2^a$), which themselves can neither be suppressed by GT-preimmunization nor produce a GT-suppressor T cells (Debré *et al.*, 1976a). This indicates that A/J mice do not have the ability to produce GT-specific suppressor factor, but can accept the suppressor factor of other strains to manifest final suppression. The results are in agreement with the observation made by Taniguchi *et al.* (1976b), who also showed the same defect in A/J strain in producing of KLH-specific suppressor factor while capable of accepting the factor derived from *I-J* compatible strains. The fact that GT-specific suppressor factor can suppress the response of GT-nonsuppressor strain, A/J, suggests that the factor stimulates the production of new suppressor T cells in the recipient. The cellular consequence of the factor-induced suppression is discussed in Section V.

The properties of the GT-specific factor are very similar to those of the GAT-specific factor except for their distinct specificity for the respective copolymers. Taking advantage of the availability of alloantisera directed at restricted subregion of $H\text{-}2^k$ haplotype, Thèze *et al.* (1977b) determined that the factor from B10.BR can be absorbed by an immunoadsorbent prepared with the globulin fraction of an anti-$I\text{-}J^k$ antiserum (3R anti-5R), but not by the immunoadsorbent specific for $H\text{-}2K^k$ and $I\text{-}A^k$ subregions. The suppressive moiety from B10.BR that is effective in suppressing the response of BALB/c was also abrogated by absorption with anti-$I\text{-}J^k$, and thus the same $I\text{-}J^k$-coded factor was shown to suppress the responses of both $H\text{-}2^k$ and $H\text{-}2^d$ mice without notable hindrances caused by *H-2* incompatibility.

Thèze *et al.* (1977d) further demonstrated that B10.BR ($H\text{-}2^k$) GT-specific suppressor factor can be purified on the basis of its affinity for GT-Sepharose by the process of adsorption to and elution from it. The purified material thus obtained had much higher suppressive activity on the weight basis of both *in vivo* and *in vitro* antibody responses to GT-MBSA. The eluted material after neutralization was then absorbed

with anti-$I\text{-}J^k$ to see whether or not the purified material has *H-2*-coded determinants. The suppressor activity was completely removed by passage through an anti-*I-J* immunoadsorbent column, which conclusively established that the active molecule has both antigen-binding site and *I-J*-coded determinants on the very same molecule.

3. *Fine Specificity of Suppressor Factors*

Cross-reactivity between three related copolymers, GAT, GT, and GA, has been well established at the level of serum antibody (Pinchuck and Maurer, 1965; Dorf *et al.*, 1974). In fact, Thèze *et al.* (1977d) showed that GT-specific PFC response can be accurately measured by using GAT-coated SRBC without notable difference from using GT-coated SRBC. In addition, GAT-specific suppressor factor can be significantly absorbed with GT-Sepharose (Thèze *et al.*, 1977a). However, the strain distribution of both responders and suppressors to these two antigens are entirely different. Hence, the examination of fine antigen specificity of T cell factors would provide a unique opportunity to determine the epitope of antigen that preferentially induces suppressor T cells in the mouse of suppressor genotype.

This was performed by Waltenbough *et al.* (1976) at the cellular level by examining the effect of preimmunization with one of the cross-reactive polypeptides on the subsequent development of immunosuppression of the other. They showed that preimmunization of DBA/1 ($H\text{-}2^q$) mice with GA suppressed the response to GA- and GT-MBSA, but not to GAT-MBSA. The pretreatment of SJL ($H\text{-}2^s$) with GAT suppressed the response to GAT-MBSA, but not to GT-MBSA. GAT was able to suppress the response of DBA/1 to GA-MBSA without any effect on the response to GT-MBSA. GT-preimmunization caused suppressed responses to GA, GAT-MBSA, and GT-MBSA in SJL, but not in DBA/1. These results point out basically two important findings about the recognition of antigenic determinants by suppressor T cells: (*a*) that the cross-reactive immunosuppression does not follow predictable patterns based upon the cross-reactivity established with serum antibody; and (*b*) that cross-immunosuppression is sometimes one way and not always reciprocal.

This difference in the specificity between serum antibody and T cell factor is also corroborated by an observation by Thèze *et al.* (1977a) showing that GAT and GT suppressor factors were selectively absorbed with immunoadsorbents of respective antigens, although some cross-reactive absorption was observed between these two adsorbents. Germain and Benacerraf (1978) further substantiated this observation by testing the GT- and GAT-specific T cell factors in the suppression of

antibody responses to the homologous and cross-reactive polypeptides. They found that GAT suppressor factor is not effective in suppressing the *in vitro* PFC response to GT-MBSA, and vice versa. These observations provide some important insights into the nature of antigen specificity carried by T cell factors: (*a*) the recognition specificity by T cell factor of closely related polypeptide is narrower than that of conventional serum antibody; (*b*) the determinants on these related polypeptides, which are responsible for the induction of suppression in different haplotypes, may be different. It is worth noting that such a discriminatory ability of T cells and T cell factors is determined by genes linked to the *H-2* complex, and thus the Ir/Is genes appear to control the fine specificity of produced suppressor T cell factors.

Since GAT is a heterogeneous molecule with respect to its molecular size, Thèze *et al.* (1977c) separated it into three fractions by gel filtration. They found that a small molecular weight fraction (MW <10,000) exhibited only suppressive, but not immunogenic, properties in high-responder BALB/c mice. Whether or not such a small molecule represents only the suppressor epitope is to be clarified, since in other experimental systems determinants that preferentially induce suppressor T cells have been demonstrated (Sercarz *et al.*, 1978).

The observed very fine discriminative ability of the T cell factors for these related copolymers can be attributed to at least two independent factors; *V* region-like structure on these molecules and specific Ir/Is gene controls. If the antigen-binding structure on the T cell factors is a product of V genes, one should imagine that there are different regulatory mechanisms in the expression of V genes on B and T cells, and that the latter is less diversified, retaining original finer specificity than those on B cells. Alternatively, specific Ir/Is genes may contribute to the fine specificity by altering the way of presentation of antigen epitopes to B and T cells. These problems will be discussed in connection with the rapidly disclosed serologic structures of antigen-binding sites on T cell factors (see Section VII).

E. T Cell Factors Suppressing Delayed Hypersensitivity

Since the original discovery by Chase (1946), it has been well documented that the contact hypersensitivity to chemical compounds can be suppressed by a prior feeding or by injecting a relatively large dose of the same or closely related chemicals (Frey *et al.*, 1971; Asherson and Ptak, 1970; Polak, 1976). In recent years, it has been shown that the apparent unresponsiveness observed in above systems is at

least in part due to the selective stimulation of antigen-specific suppressor T cells (Zembala and Asherson, 1973; Phanuphak *et al.*, 1974b; Claman *et al.*, 1974).

Zembala and Asherson (1973) reported that mice injected intravenously with picrylsulfonic acid failed to show contact hypersensitivity upon subsequent immunization with a potent sensitizer picryl chloride by skin painting. They further demonstrated that the passive transfer of lymph node cells from animals treated with picrylsulfonic acid into syngeneic recipients prevented the development of contact sensitivity to picrylchloride. The suppressive effect of lymph node cells was specific for trinitrochlorobenzoate group, since the sensitization by unrelated compound oxazolone was unaffected. A similar observation was made by Claman *et al.* (1974), who showed that administration of dinitrobenzene sulfonic acid ($DNBSO_3$) shortly before skin painting with dinitrofluorobenzene (DNFB). They were able to show that lymph node cells from $DNBSO_3$-treated mice could suppress specifically the development of contact sensitivity to DNFB upon transfer into normal or lightly irradiated recipients (Phanuphak *et al.*, 1974b; Moorhead, 1976). The cells responsible for this suppression were found to be T cells by their sensitivity to anti-Thy-1 antiserum and C. However, subsequent studies by both of these groups indicated that the suppression of contact sensitivity is a multistep process in which various cell types as well as different mechanisms are involved. One step on which we focus our attention is the efferent phase of development of contact sensitivity where antigen-specific suppressor factors play a substantial role.

Zembala and Asherson (1974) took lymph node cells from picryl sulfonic acid-treated mice a few hours after challenge by skin painting with immunogenic picrylchloride and cultured them *in vitro* without further addition of antigen for 24–48 hours. The culture supernatant was injected intravenously into irradiated hosts together with picryl chloride-sensitized lymph node cells. By this experimental procedure, they demonstrated that the lymph node cells from unresponsive animals elaborate a factor that specifically suppresses the ability of sensitized lymph node cells to transfer skin reactivity to irradiated recipients. The source of the factor was found to be present in the lymph node and spleen, but not in the thymus. They are short-lived T cells that disappear 3 weeks after adult thymectomy (Asherson *et al.*, 1976).

The intriguing point with the suppressor factor in this system is that the factor acts on macrophages, but not on T cells. Normal peritoneal exudate cells can absorb the activity of the suppressor factor, and the macrophages pretreated with the factor were endowed with the ability

to suppress the skin reactivity of transferred sensitized lymph node cells. The factor was also shown to suppress the production of migration inhibitory factor (MIF) (Zembala and Asherson, 1974) and, under certain conditions, prevents anti-DNP IgG antibody response (Thomas *et al.*, 1979).

The factor has a definite antigen-specificity, as confirmed by adsorption to the picryl–albumin–Sepharose column and by elution from it with picryl-6-aminocaproic acid (Zembala *et al.*, 1975). Furthermore, the specificity of suppression was so strict that the factor produced with picryl chloride did not suppress the sensitivity to TNCB, a cross-reactive sensitizer differing only on a single NO_2 group on benzene ring. The molecular weight of the factor was estimated to be around 50,000.

However, there are several differences from antigen-specific factors discussed previously and below: The factor was shown to be resistant to trypsin digestion and heating at 56°C, and was absorbed by a Con A–Sepharose column exhibiting the glycoprotein nature of the molecule (Zembala and Asherson, 1974). The factor is absorbed by peritoneal macrophages, but not by T cells. Even heat-killed macrophages can absorb the factor, although the killed macrophages do not mediate the final suppression. The suppressive effect of macrophages pretreated with the factor ("armed" macrophages) was entirely antigen nonspecific, as the response of mice challenged simultaneously with picryl chloride and oxazolone responded to neither antigen (Asherson and Zembala, 1974). Finally, the factor is effective only at the final effector phase; i.e., it suppresses the passive transfer of preestablished contact sensitivity into the irradiated host, but is unable to prevent the sensitization itself. These points distinguish their factor from others.

Work by Frey *et al.* (1971), which showed that prior parenteral injection of guinea pigs with $DNBSO_3$ induces tolerance to contact sensitization with DNFB, was adopted by Claman and his colleagues to study the mechanism of tolerance in the mouse. They showed by a series of experiments that pretreatment of animals with a hapten $DNBSO_3$ or with haptenated syngeneic lymph node cells induced a tolerance to contact sensitization with DNFB (Phanuphak *et al.*, 1974a; Claman *et al.*, 1974; Moorhead, 1976), and that this tolerance can be transferred to normal recipients by injecting lymph node cells before or simultaneously with sensitization of the recipient with DNFB. Moorhead (1977a) tested the possibility that this passive transfer of tolerance is mediated by the factor similar to that of Asherson's group. Lymph node cells from animals pretreated with $DNBSO_3$ and

painted with DNFB 5 days later were cultured *in vitro* for 6 hours, and the ability of supernatants to prevent the adoptive transfer of contact sensitivity was studied by the following method. The lymph node cells from sensitized mice were incubated with the culture supernatants of the normal or DNFB-sensitized mice. The cells were passively transferred into normal recipients that were subsequently challenged with DNFB in the ear. Moorhead (1977a) found that the ability to transfer DNFB contact sensitivity of the sensitized lymph node cells was greatly diminished by incubation with supernatants from tolerant lymph node cells but not those of the control. In order to release the active material from tolerant cells, it was found necessary to paint the tolerant animals with DNFB 16–20 hours before sacrifice, suggesting that suppressor factor was made in response to antigenic stimulation. The specificity of the factor was established by the criteria that the factor from $DNBSO_3$-suppressed mice inhibited the sensitivity to DNFB but not to TNCB, and that the suppressor activity in the supernatant was absorbed with a column of DNP-KLH.

The most remarkable finding in his study is that the factor derived from CBA ($H\text{-}2^k$) could suppress the contact sensitivity of syngeneic mice but not allogeneic BALB/c ($H\text{-}2^d$) or C57BL/6 ($H\text{-}2^b$) mice (Moorhead, 1977a). The factor from BALB/c mice could suppress the reactivity of sensitized lymph node cells of semiallogeneic (BALB/c × A/J) F_1, and that from F_1 mice also effectively suppressed the expression of sensitivity by both parental strains, BALB/c and A/J. Thus, certain identities of genes in *H-2* complex between the donor of the factor and immune lymph node cells are required. Furthermore, the factor was absorbed by anti-*H-2* antibodies but not by anti-immunoglobulin and anti-DNP antibodies.

Moorhead (1977b) further expanded his study to solve the genetic relations of his antigen-specific factor. Using *H-2* congenic and recombinant mice, he observed an interesting relationship between genes coding for the factor molecule and the putative acceptor site for the factor. He found that the suppressor factors from both $H\text{-}2^k$ and $H\text{-}2^d$ mice equally suppressed the reactivity of immune lymph node cells from A/J, which shares left side (*K* end) or right side (*D* end) of the *H-2* complex with the donor strains. The factor from A/J could suppress immune lymph node cells of both $H\text{-}2^k$ and $H\text{-}2^d$ mice. If the supernatant from tolerant A.TL lymph node cells was tested in sensitized lymph node cells of CBA (*I* region identical), A.TH (*H-2K* and *H-2D* identical) or BALB/c (*H-2D* identical), only the responses by the latter two strains were suppressed, while that of CBA was unaffected. These results indicate that in order for the suppression of contact sensitivity

to take place the identity of *K* or *D* region (or their vicinity) of *H-2* complex between the donor of the factor and responding T cells is required, whereas the *I* region identity is unnecessary. This interaction between the *H-2* products expressed on the factor and the responding cells was further strengthened by an experiment in which the factor derived from A/J mice after absorption with BALB/c lymph node cells (*D* region identical) still retained the ability to suppress the response of CBA lymph node cells (*K* region identical) while being unable to suppress BALB/c response. These results indicate that Moorhead's factor(s) is heterogeneous with respect to the restriction specificity in its action. The factor would consist of bifunctional molecules that recognize products of either the *H-2K* or *H-2D* locus together with the identical haptenic determinant.

The results at first glance are a bit conflicting since Moorhead later found that the factor was a product of gene or genes mapped in the *I* region of the *H-2* complex (Moorhead, 1977b). However, if antigen-specific factors are recognition molecules of T cells for antigen, they would have recognized antigen associated with *K* or *D* region products (Zinkernagel and Doherty, 1977; Shearer *et al.*, 1977; Miller *et al.*, 1976a). The resultant T cell factor may have been educated to react with such complexes. On the other hand, if T cells have not seen antigen associated with *K/D* products of their own haplotype, effector molecules liberated from them have no ability to recognize them. Moorhead (1977b) has further shown that the $DNBSO_3$ specific factor is adsorbable by lymph node cells from DNFB-sensitized mice but not from normal mice, which suggests that the factor is reactive with *H-2* products associated with antigen. These results are probably in accordance with the fact that contact sensitivity to DNFB can be successfully transferred to the irradiated recipients sharing only the *H-2K* or *H-2D* region of *H-2* complex but not to those sharing the *I* region (Vadas *et al.*, 1977). If this is the case, suppressor T cells are recognizing the same antigenic complex as that recognized by effector T cells.

Greene *et al.* (1977c) found that the supernatant factor obtained by the same procedure by Zembala and Asherson (1974) was capable of suppressing the primary development of contact sensitivity to picryl chloride in normal mice if injected for 3 consecutive days after sensitization. The physicochemical and immunochemical properties of the factor were very similar to those reported in the suppression of antibody responses to protein and synthetic antigens as it was absorbed to and eluted from the TNP column. Greene *et al.* (1977c) further characterized the genetic relation of the suppressor molecules which limit the contact sensitivity to picryl chloride. The supernatants obtained

from tolerant lymph node cells of CBA/J (H-2^k) mice were absorbed with immunoadsorbents of alloantisera having I subregion specificities. They demonstrated that the suppressive moiety in the supernatant was removed with alloantisera containing anti-I-J^k specificity, whereas those lacking I-J subregion specificity were unable to absorb it. The results suggest that the I-J subregion genes regulate a wide, diverse range of specific suppressor response including delayed-type hypersensitivity (DTH) to simple chemicals.

One of the most exciting applications of this model to analyze the nature of antigen-specific suppressor factor limiting DTH was recently introduced by the same group (Bach *et al.*, 1978). They adopted a method originally developed by Miller and Claman (1976) to induce hapten-specific tolerance by intravenous administration of hapten directly coupled to syngeneic spleen cells. The subcutaneous injection of the same hapten-coupled spleen cells caused development of DTH upon challenge with the same hapten or hapten–spleen cell conjugate. Bach *et al.* (1978) utilized hapten *p*-azobenzenearsonate (ABA) and A/J strain, a combination that apparently has a great advantage in detecting ABA-specific major cross-reactive idiotype on antigen-specific molecules. They were successful in developing ABA-specific DTH as well as tolerance. Furthermore, the spleen and thymus cells from tolerant mice were capable of inhibiting the development of DTH in syngeneic recipients that were subcutaneously sensitized by ABA-spleen cells.

Greene *et al.* (1979) subsequently obtained ABA-specific suppressor factor by extraction from ABA tolerant spleen and thymus cells. The factor was capable of abolishing the DTH response if injected for 5 consecutive days after sensitization with ABA-spleen cells. The factor was obtainable also by culturing spleen cells of mice given an intravenous injection of ABA-spleen cells followed by challenge with ABA diazonium salt 24 hours before sacrifice. The factor was specific for ABA group, since the DTH response to TNP-spleen cells was unaffected, and the suppressor activity was absorbed to and eluted from ABA-coated Sepharose column. The estimated molecular weight was in the range of 33,000 to 68,000, although some suppressive activity was found in smaller and larger fractions. The factor was absorbed with anti-H-2 column but not anti-Igs. The similar treatment of animals with syngeneic anti-ABA antibodies did not affect the DTH response.

Certainly a very interesting question can be asked as to whether the ABA-specific suppressor factor carries the cross-reactive idiotype shared by serum antibodies to ABA. Since A/J mice when immunized

with ABA-KLH produce a restricted spectrum of anti-ABA antibodies, 20–70% of which bear a cross-reactive idiotype (Tung and Nisonoff, 1975; Nisonoff *et al.*, 1977), it is now possible to answer this question. Subsequent studies by Bach *et al.* (1979) indeed demonstrated the presence of the cross-reactive idiotype as will be discussed in Section VII.

Liew and Chan-Liew (1978) also found a factor that suppresses both the induction and effector phases of DTH to SRBC. Cultured spleen cell supernatant from mice given intravenous injection of a large dose of SRBC specifically suppressed the development of DTH to SRBC but not to chicken RBC. The factor was absorbable by SRBC. The molecular weight was less than 35,000 and stable for heating at 56°C for 30 minutes. The source of the factor was found to be cyclophosphamide-sensitive T cells (Gill and Liew, 1978).

F. Antigen-Specific T Cell Factors Detected in Tumor Immunity

A number of recent reports indicate that antigen-specific suppressor T cells are playing a substantial role in the failure of animals to develop immunity to certain tumors which are growing in them. One of the most straightforward demonstrations of suppressor T cells was made by Fujimoto *et al.* (1976). They utilized two methylcholanthrene-induced sarcomas of the A/J mouse origin, S1509a and SaI. A notable advantage in their system is that these two tumor cell lines, although established and maintained at different laboratories have a considerable cross-reactivity at the level of cytotoxicity; the cytotoxic T cells generated by either one of them could kill both cell lines as evidenced by *in vitro* cytotoxicity and by cross-resistance to secondary transplantation *in vivo* (Fujimoto *et al.*, 1975). If syngeneic A/J mice were immunized with one of these tumors by transplantation and subsequent surgical removal of the tumor, they acquired the immunologic resistance to reject both the secondarily transplanted S1509a and SaI.

They utilized this system to study the specificity of immunosuppressor T cells obtained from mice that are carrying growing tumors (Fujimoto *et al.*, 1976). The thymocytes and spleen cells of tumor-bearing mice were transferred into mice that had been immunized either with S1509a or SaI. What they have found is apparently important to interpret the recognition system of suppressor T cell and suppressive T cell factor: The cells obtained from animals carrying S1509a significantly retarded the rejection of S1509a by syngeneic

mice immune to the homologous S1509a as well as to cross-reactive SaI. But the same cells from S1509a-bearing mice exerted no effect on the rejection of the cross-reactive SaI regardless of whether either tumor was used to induce the immunity. The same is true for the cells from SaI-bearing animals, which suppressed the rejection of SaI but not S1509a, even though animals were equally resistant to these two cross-reactive tumors. This was later confirmed in an *in vitro* experiment, where cytotoxic T cells generated by either one of the tumors could equally kill both tumor cells, while suppressor T cells could suppress only the cytotoxic killing of the target by which suppressor T cells were generated (Fujimoto *et al.*, 1978). It should be stressed that cytotoxic T cells recognize common antigenic determinants shared by these two chemically induced sarcoma lines, whereas suppressor T cells are effective in inhibiting only the cytotoxic response to the homologous tumor, but not to the cross-reactive tumor. This suggests that the suppressor T cell and its antigen-specific component recognize the individual tumor antigens uniquely expressed by these two tumor cell lines. The nature of the immunosuppressor cell in their system was very similar to the antigen-specific suppressor T cell in the humoral antibody response; it is a T cell of Lyt-2^+,3^+ carrying Ia determinants encoded by an *I-J* subregion gene (Fujimoto *et al.*, 1978). As discussed later, the *I-J* expression of this suppressor T cell provides important clues for studying the regulatory mechanism of tumor immunity.

Greene *et al.* (1977a, 1978) extended their work to study the nature and properties of the molecule involved in the suppression of tumor immunity. They prepared soluble extracts from suppressor T cells of tumor-bearing animals. The thymic and splenic cells of tumor-bearing animals were subjected to sonication, and the resultant cell-free supernatant was injected into immunized animals that had gained specific resistance to S1509a and SaI. As expected, the sonicated material from animals carrying either one of the tumors could suppress the rejective reaction to the homologous tumor when injected into immune recipients. The factor was not absorbed by anti-mouse Fab, but was adsorbable by anti-*H-2* antiserum. By using anti-$H\text{-}2^k$ and anti-$H\text{-}2^d$ antisera, the genes controlling this antigen-specific factor of A/J mice ($H\text{-}2^a$) were mapped to the left of *I-C* subregion (Greene *et al.*, 1977a). It is a protein of molecular weight less than 70,000 and is absorbed with the homologous, but not the cross-reactive tumors.

Perry *et al.* (1978a) further obtained evidence that S1509a-specific suppressor factor bears an *I-J* subregion gene product. The suppressor extract after absorption with anti-*I-J* was incapable of suppressing the

tumor rejection in S1509a immune host, and the activity was fully recovered in acid eluates from the column. Furthermore, they showed that in normal animals injected with the S1509a-specific suppressor factor, new suppressor T cell (Ts_2) is induced. The specificity of Ts_2 was identical to that of the factor. This "infectiousness" of suppressor mechanism by the factor may be important for the sustained suppressed state in tumor-bearing animals (see Section V).

A tumor-specific soluble factor was demonstrated by Takei *et al.* (1978) in another syngeneic tumor system. They found that the thymus of DBA/2 ($H\text{-}2^d$) mice injected with syngeneic P815 mastocytoma cells 8 days previously contained a high number of tumor-specific suppressor T cells, which were capable of suppressing the generation of cytotoxic T cells *in vitro* (Takei *et al.*, 1976, 1977). The experiment in brief is as follows. Takei *et al.* took spleen cells of DBA/2 mice immunized 12 days earlier with P815 tumor cells and cultured *in vitro* with mitomycin C-treated homologous tumor for 4 days. By this procedure they were able to demonstrate the generation of highly efficient cytotoxic T cells specific for P815. To demonstrate the suppressor activity, thymocytes obtained from animals inoculated 8 days previously with P815 were added to the cultured immune spleen cells at the onset of *in vitro* activation of cytotoxic T cells. They were able to show that the coexistence of thymocytes from P815-bearing animals with the immune spleen cells strongly inhibited the generation of cytotoxic T cells from the latter. The suppression was specific for the tumor carried by the donor of thymocytes, since the addition of thymocytes from animals bearing heterologous tumor, i.e., L1210 leukemia cells, was unable to suppress the generation of P815-specific cytotoxic T cells (Takei *et al.*, 1977).

Takei *et al.* (1978) have further shown that these suppressor T cells can be replaced by a sonicated extract of thymocytes obtained from P815-bearing animals. The soluble extract, when added to the culture of immune spleen cells within the first 30 hours of culture with mitomycin C-treated tumor cells, was capable of suppressing the *in vitro* generation of cytotoxic T cells specific for P815. The suppressive factor in this system also had a molecular weight between 40,000 and 60,000 and was absorbed with an immunoadsorbent column prepared from membrane proteins of P815, but not by analogous columns prepared by L1210 membrane proteins. The suppressive material was not removed by its passage through columns of anti-mouse immunoglobulins. Preparative isoelectric focusing of the extract established that the active material has an isoelectric point in the range of 4.6 to 4.9. They have observed that the factor is absorbed with an anti-$H\text{-}2^d$ column.

The factors obtained from thymocytes of other strains having different *H-2* haplotypes were equally effective in the suppression of generation of cytotoxic T cells in DBA/2 spleen cells (J. Levy, personal communication).

These *in vivo* and *in vitro* observations indicate that similar suppressive T cell factors encoded by *H-2* genes are involved in the suppression of tumor immunity. The results are consistent with the recent reports by Greene *et al.* (1977b), Perry *et al.* (1978b), and Fujimoto *et al.* (1979), who showed that injections of minute quantities of anti-*I-J* antiserum significantly retarded the growth of syngeneic tumor transplanted into naive or immunized hosts. It is thus apparent that the generation of *I-J* bearing tumor-specific suppressor T cells is one of the major factors that limits the specific tumor resistance, and that studies on the nature and functions of suppressor factors would give important clues for understanding the apparent immunologic unresponsiveness to the syngeneic tumor in tumor-bearing hosts.

G. T Cell Factors Produced by Hybridoma Cell Lines

The application of cell hybridization to immunology was initiated by Köhler and Milstein (1976), who showed that myeloma cell lines could be fused with specific antibody-producing cells to make continuous cell lines secreting antibodies that were originally produced by normal spleen cells. The cloning of such hybrid cells resulted in the establishment of permanent cell lines producing homogeneous monoclonal antibodies both *in vivo* and *in vitro*. A similar principle has been applied to make T cell hybrids that retain certain functions of the parental T cells. Thus, if one can select the cells producing antigen-specific T cell factors, it is theoretically possible to make hybrid cell lines by fusion with certain thymomas, which would continuously produce biologically active T cell factors. Such cell lines have already been produced in various laboratories and provide useful means to study the functional and chemical aspects of T cell factors. This section will summarize data obtained with T cell hybridomas in different laboratories up to this time. However, as readers are aware, such experiments are relatively new, and several controversies over the function as well as chemical relationship are not yet resolved. Nevertheless, this summary of findings would be a prologue for reports of an important area that will be extensively studied in the near future.

Although stable T cell hybrids expressing membrane markers of both parental thymoma and normal T cells have been established (Goldsby *et al.*, 1977; Hämmerling, 1977), three groups of investigators have reported the derivation of hybridomas having antigen-

specific suppressor activity. Taniguchi and Miller (1978b) made hybridomas fused between a T cell lymphoma, EL-4 of C57BL/6 origin, and HGG-specific suppressor T cells prepared from spleen cells of HGG-tolerant CBA ($H\text{-}2^k$) mice. They used two advantageous procedures: preparation of antigen-binding T cells by specific adsorption to and elution from HGG-coated Petri dishes (Taniguchi and Miller, 1977), and separation of *I-J* positive hybrids by a fluorescence-activated cell sorter (FACS). They were able to establish a number of clones expressing $I\text{-}J^k$ determinants as determined by analysis with FACS, although many of them were unstable during the subsequent 3 months of maintenance.

The suppressor factors were extracted by sonication of the hybrid cells and tested for their effect on the *in vivo* adoptive secondary antibody responses to DNP-HGG and to horse erythrocytes in irradiated recipients. They found that extracts from 6 out of 18 such hybrid cell lines had specific suppressive activity in the response to DNP–HGG, and 5 of them had nonspecific activity suppressing both antibody responses to DNP–HGG and horse erythrocytes. Extracts prepared from the rest of 7 hybridomas had no suppressive activity. Nevertheless, all these hybridomas were stained with anti-$I\text{-}J^k$ antiserum. These findings are of importance in considering the heterogeneity of *I-J* subregion gene products, as will be discussed later.

Kontiainen *et al.* (1978) demonstrated the derivation of KLH-specific hybridoma cell lines continuously secreting specific suppressor factors in the culture supernatant. They utilized *in vitro*-induced KLH-specific suppressor T cells to fuse with enzyme-deficient thymoma cells (BW5147) of AKR origin. After selection by assaying the suppressive activity of the culture supernatants, they established two stable cell lines, one having antigen-specific and the other antigen-nonspecific suppressor activities. The cell line upon which attention will be focused secreted a material that could suppress the *in vitro* antibody responses to TNP–KLH but not to either DNP–GAT or TNP–polyacrylamide beads (a T cell-independent antigen). The active factor secreted by the hybridoma was removed by absorption with KLH, anti-Ia^k, rabbit anti-mouse suppressor factor, and syngeneic anti-suppressor factor putatively reactive with idiotype-like determinants of antigen-specific factors (see Section V). Thus, the suppressor factor secreted by the hybridoma had very similar properties to those of normal *in vitro*-induced suppressor factors. The surface phenotype of the suppressor hybridoma fully expressed the genotype possessed by both parental T cells, i.e., Thy-1.1, Thy-1.2, Lyt-1.1, Lyt-1.2, even though BW5147 had no Lyt expressions. This hybridoma has been stable for over 14 months without changing its surface phenotypes.

Taniguchi *et al.* (1979) expanded their study to produce hybrid cell lines fused between purified KLH-specific suppressor T cells of C57BL/6 (*H-2*^{b}) mice and BW5147 by the same procedure described above. They were able to establish a number of hybrid cell lines with constant *I-J*^{b} expressions. As already stated, *I-J*$^+$ hybrids are divided into three groups: those having KLH-specific suppressor activity, nonspecific suppressor activity, or no detectable suppressor activity. Such functional heterogeneity was partially explained by the fact that *I-J* subregion contains at least two or more loci that are selectively expressed on different subsets of T cells (Tada *et al.*, 1979b). In fact, some of these cell lines were killed by anti-*I-J* antiserum preabsorbed with Lyt-1$^+$,2$^-$,3$^-$ splenic T cells, but not with that preabsorbed with Lyt-1$^-$,2$^+$,3$^+$ T cells. The reverse was true for some other hybridomas.

They have reported a case of hybridoma with intriguing properties (Taniguchi *et al.*, 1979). This cell line was found to be transplantable to (C57BL/6 × AKR)F_1 mice, but not to either parental strain. It grows rapidly in F_1 as a solid tumor when transplanted subcutaneously, and produces a large quantity of ascitic fluid if injected intraperitoneally. The ascites contained extremely strong antigen-specific suppressive activity as revealed by the complete suppression of *in vitro* antibody response against DNP–KLH, but not DNP–EA, with a minute quantity. The factor was absorbable by KLH, anti-*H-2*^{b}, and anti-*I-J*^{b}, but not with anti-Igs. They also demonstrated that suppressor factor derived from their hybridomas could suppress the responses mounted by spleen cells of *H-2*^{b} and *H-2*$^{k/b}$ F_1 mice but not by *H-2*^{k} mice. Thus, the strict genetic restriction observed with normal KLH-specific suppressor factor was also envisaged by the theoretically homogeneous material derived from hybridomas. Some biochemical studies are now in progress with the hybridoma-derived suppressor factors.

More recently, Taussig *et al.* (1978) reported a T cell hybridoma excreting an antigen-specific suppressor factor having somewhat different properties from those described above. They took spleen cells of C57BL/10 (B10) mice primed 5 days earlier with SRBC and fused them with BW5147, using polyethylene glycol. They were fortunate to obtain a cell line that continuously secretes the material suppressing the *in vitro* primary antibody response to SRBC. The suppression was antigen-specific, since the primary IgM antibody response to SRBC was suppressed, but the response to DRBC was unaffected. Furthermore, the suppressor activity of the supernatant was completely absorbed with SRBC, but not by DRBC. They also found the supernatant to be equally effective on B10 (*H-2*^{b}) and CBA (*H-2*^{k}) responding cells. The activity was not removed by anti-Ig antisera, whereas anti-*H-2*^{b}, but not anti-*H-2*^{k}, antiserum was capable of removing the activity.

They also showed the direct binding of the radioiodinated anti-$H\text{-}2^b$ antibodies to SRBC that had been allowed to react with the supernatant of the hybridoma.

These properties are, at first glance, very similar to those of the KLH-specific suppressor supernatant of the hybridoma reported by Kontiainen *et al.* (1978); however, there have been revealed some very unique properties associated with the factor derived from the SRBC-specific hybridoma (Taussig and Holliman, 1978). First of all, the molecular weight of the suppressor factor was estimated to be about 200,000, being much higher than the values obtained by other investigators. No activity was found in fractions with molecular weights of less than 150,000. This was confirmed with the semipurified material obtained by adsorption to and elution from SRBC. Second, although the factor was absorbed with an anti-$H\text{-}2^b$ antiserum, anti-*I-J* antisera were unable to remove it. The locus controlling the suppressor factor was mapped to the right of the *I-J* subregion (M. Taussig, personal communication).

Taussig and Holliman (1978) further studied some chemical properties of the suppressor factor derived from their hybridoma. Internal labeling of the factor was performed by incorporation of [^{3}H]-leucine into the hybridoma cells. The labeled supernatant was obtained and allowed to react with SRBC. Significant radioactivity was bound to SRBC. After washing, SRBC was lysed in SDS under reducing conditions, and the lysate was electrophoresed on 10% and 15% polyacrylamide gels (SDS-PAGE). This revealed two polypeptide chains, a large chain with a molecular weight of about 85,000 and a small chain of about 25,000. Even in the absence of reducing agents, these two chains were separated, indicating that they are not linked by disulfide bonds. If the supernatant was pretreated with anti-$H\text{-}2^b$ antiserum, both chains were removed. Taussig and Holliman further attempted to identify the SRBC-binding site on these two chains. The hybridoma cells were labeled with [^{3}H]-leucine, and the membrane was dissolved in the nonionic detergent NP-40. The extract was allowed to react with SRBC, and the radioactive material specifically bound to SRBC was likewise analyzed by SDS-PAGE. It was found that only the large chain of 85,000 molecular weight was specifically bound to SRBC. This peak persisted even after the lysate was precipitated with anti-$H\text{-}2^b$. These findings imply that the supernatant factor secreted by the hybridoma is composed of two chains, the large chain having a specific antigen-binding site and the small chain with *H-2* determinants, and that they are assembled when they are secreted from the hybridoma.

Taussig *et al.* (1979) determined the cellular site of action of the above hybridoma-derived suppressor factor. The factor was absorbed with both syngeneic and allogeneic B cells, but not with other cell types. In addition, they found the factor to be effective at any stage of B cell differentiation. In this regard, their suppressor factor is analogous to the (T,G)-A–L specific helper factor described by Taussig *et al.* (1976a) and to the suppressor factor described by Kontiainen and Feldmann (1978). Hence, they suggested that B cells are equipped with nonclonally distributed acceptors sites where both suppressor and helper factors may exert their effects directly without requirement for an intermediary T cell and macrophage.

So far, this is the first biochemical characterization of the antigen-specific suppressor factor derived from a hybridoma cell line, and provided valuable information for further chemical analysis. Although there are several discrepancies between the suppressor molecules described by Taussig *et al.* (1978a) and by others with respect to their physicochemical and immunochemical properties, these hybrid T cell lines with suppressor function promise to be of great value in the study of the structure and function of T cell products and receptors. Indeed, there are some similarities between the hybridoma-derived suppressor factor of Taussig *et al.* (1979) and antigen-specific receptor molecules of T cells described by Binz and Wigzell (1977b), Rajewsky and Eichmann (1977), and Krawinkel *et al.* (1977a,b), at least in their physicochemical properties and molecular structure. We should await forthcoming reports, which will rapidly reveal whether the T cell factors described herein belong to the same class of T cell receptor or to different subclasses on the basis of biochemistry.

V. Cellular Consequences of Suppression Induced by Antigen-Specific Suppressor Factors

In cognizance of the diversity in the effect of the suppressor factors, the question is asked what in reality these suppressor factors do. We no longer think that they are always effector molecules that actually suppress the antibody synthesis by B cells or proliferation-differentiation steps of other cell types. Conversely, it is becoming clear that the factors initiate a complex series of activation of other cell types that finally lead to suppressed immune reactivity. Therefore, the suppressor factors are sometimes not "inhibitors," but activators or initiators of certain active processes. Major efforts to solve this problem have been made in two different experimental systems, KLH-specific suppressor and GAT- and GT-specific suppressor systems.

In the KLH-specific suppressor system it has been demonstrated

that the *I-J*-bearing factor derives from Lyt-1^-,2^+,3^+ suppressor T cells (Tada *et al.*, 1977b) (see Section IV,A). The cell type that directly "accepts" the suppressor factor was found to be neither B cells nor macrophages. A novel cell type of T cell lineage was found to be the direct target cell for the suppressor factor, which in turn initiates a complex series of suppressive cell interactions. As stated before, the suppressor factor was adsorbable to splenic T cells, but not to B cells and macrophages (Taniguchi *et al.*, 1976b). An interesting property of acceptor T cells is that they tend to stick to a nylon-wool column, unlike other functional T cell subsets. They have a high density of Thy-1 antigen and are extremely sensitive to X-irradiation. It has been shown that the mixture of nylon-purified KLH-primed T cells and DNP-primed B cells can give a good *in vitro* secondary antibody response to stimulation with DNP–KLH. However, this antibody response was not suppressed by the addition of KLH-specific suppressor factor. If a small number of nylon-wool-adherent T cells were added to this reaction mixture, there was a strong suppression of anti-DNP antibody response (Tada *et al.*, 1977b). Subsequent studies indicated that the adherent cell type that accepts the suppressor factor has surface phenotype of Lyt-1^+,2^+,3^+: If the adherent cells were pretreated with either anti-Lyt-1 or anti-Lyt-2,3 alloantisera and C, they could no longer convey active suppression to the mixture of helper T and B cells. Thus, this was clear-cut evidence that Lyt-1^+,2^+,3^+ cells were necessary to induce antigen-specific suppression (Tada *et al.*, 1977b). Another remarkable property is that this cell type has an Ia antigen coded for by the *I-J* subregion and utilizable as the acceptor site for the suppressor factor. The treatment of the adherent T cells with anti-*I-J* blocked acceptance of the T cell factor derived from the same haplotype strains.

Tada *et al.* (1977b) subsequently found that the KLH-primed nylon-adherent T cells upon cultivation with the suppressor factor and antigen for 48 hours are activated to become a second type of suppressor T cells, which can suppress the antibody response mounted by B cells and nylon-purified helper T cells without further addition of the suppressor factor. The newly induced suppressor T cell, which we call Ts_2, was able to suppress the antibody response against unrelated antigens, e.g., DNP-EA, provided specific antigen (KLH) coexisted. Furthermore, the effect of Ts_2 was found to have no *H-2* restriction in that Ts_2 derived from BALB/c ($H\text{-}2^d$) mice could suppress the response of C3H ($H\text{-}2^k$) and vice versa. The newly induced suppressor effector cells were found to be Lyt-1^-,2^+,3^+, $I\text{-}J^+$. Thus, the suppressor factor was shown to turn on a second step of suppressor mechanism by acting on the Lyt-1^+,2^+,3^+ acceptor cell type.

The question whether the Lyt-1^+,2^+,3^+ acceptor T cells would directly become actual suppressor T cells (Ts_2) was further examined by a complex series of experiments. In short, we utilized Lyt-congenic mice of C57BL/6 background. The KLH-primed nylon wool-adherent T cells from Lyt-1 congenic and Lyt-2,3 congenic mice were mixed, treated with anti-Lyt-1 of either one genotype (Lyt-1.1 or Lyt-1.2). The resultant mixture contains Lyt-1^+,2^+,3^+ cells of only one genotype origin, whereas Lyt-2^+,3^+ cells of both genotypes coexist. The cells were cultured for 48 hours with suppressor factor of C57BL/6 mice plus antigen to generate Ts_2. The Lyt phenotype of the induced Ts_2 was examined by treating them with anti-Lyt-2.1 or anti-Lyt-2.2. If Ts_2 derives from Lyt-1^+,2^+,3^+ cells, its activity should be removed by treatment with anti-Lyt-2 antiserum against the allelic product originally possessed by preexisting Lyt-1^+,2^+,3^+ cells. On the other hand, if Ts_2 differentiated from Lyt-2^+,3^+ precursor cells, the induced suppressor activity should be partially abrogated by antisera against either Lyt-2.1 or Lyt-2.2. The result clearly demonstrated that the latter is the case. Hence, the conclusion is that Ts_2 derives from preexisting Lyt-2^+,3^+ precursor cells and the presence of Lyt-1^+,2^+,3^+ cells is definitely necessary.

These results imply that a small number of Lyt-2^+,3^+ antigen-specific suppressor T cells elaborate a factor that acts on Lyt-1^+,2^+,3^+ acceptor T cells. In the presence of the latter cell type, Lyt-2^+,3^+ suppressor precursor cells are turned to become newly induced suppressor effector T cells (Ts_2) that act across the MHC barrier in an antigen-nonspecific fashion. By this circuit loop involving Lyt-1^+,2^+,3^+ and Lyt-2^+,3^+ cells, the initial antigen-specific suppressor signal is amplified to make long-lasting antigen-nonspecific signals. The hypothetical scheme of this two-step suppression is illustrated in Fig. 1, in theoretical comparison with the effect of antigen-specific augmenting T cell factor (see Section III,C). These implications are partially in agreement with the concept of the "circuit suppression" recently presented by Eardley *et al.* (1978). The mechanism as well as the target of the final suppression by Ts_2 is not yet known, but may have some bearing on some of the well known antigen-nonspecific suppressor mechanisms, as partially discussed by Waksman and Tada (1977). Another two-step suppressor mechanism studied by Benacerraf and co-workers (reviewed by Benacerraf and Germain, 1978) will be discussed below.

Although the above observations can explain many of the facets of suppressive immunoregulation by T cell factors, there has recently been found to exist another pathway of suppression by the T cell factor. We have described that two types of helper T cells exist in the anti-

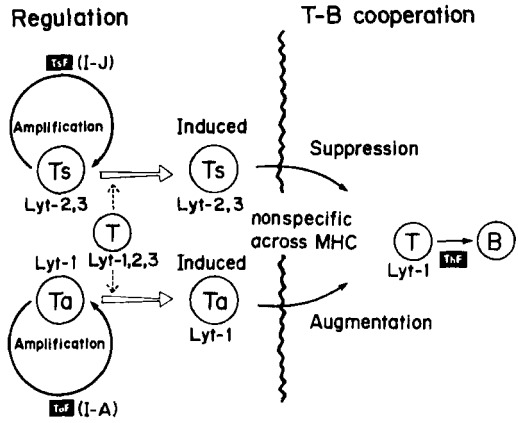

FIG. 1. Possible amplification loops in the suppression and augmentation of antibody response initiated by antigen-specific suppressor and augmenting T cell factors. Antigen-specific factors derived from different subsets of T cells stimulate self-amplicating loops in which small numbers of suppressor (Ts) and augmenting (Ta) T cells induce larger numbers of "induced" suppressor or augmenting T cells, which subsequently act nonspecifically across the major histocompatibility complex (MHC) barrier to regulate the final T-B cell cooperation. Different antigen-specific T cell factors (TsF, suppressor factor; TaF, augmenting T cell factor; ThF, helper factor) are involved in different types of cell interactions. Lyt-1^+,2^+,3^+ T cells play important roles in the regulatory compartment (see text).

body response to hapten-carrier conjugates (Tada *et al.*, 1978a). As mentioned in Section III,A, one type of helper T cells is a conventional one that can help the response of B cells by linked or cognate interaction with antigen (Th_1). It can be purified by passage through a nylon wool column and thus corresponds to the helper T cell used in the experiment mentioned in the preceding paragraph. The other cell type was found to stick to the tightly packed nylon wool column (Th_2). Both of them are carrier specific and belong to Lyt-1^+,2^-,3^- subclass. The notable difference between them is that nylon-nonadherent helper T cells carry no detectable Ia antigen, whereas the adherent helper T cells are killed by anti-Ia antisera and C. The subregion to which the Ia locus of Th_2 was assigned is the *I-J* subregion, which was originally defined by the presence of Ia-4 locus controlling determinants selectively expressed on suppressor T cells and suppressor factors (Murphy *et al.*, 1976; Tada *et al.*, 1976b).

There are a number of other differences in the helper activity of Th_1 and Th_2. Th_1 can help B cells only if the carrier and haptenic determinants are present on the same molecule (cognate of linked recogni-

tion), whereas Th_2-B cell interaction can take place even with unlinked carrier and hapten(polyclonal help) provided the free carrier corresponding to Th_2 coexists. Thus, our prediction is that Th_1 is the source of the antigen-specific helper factor, although we have not been successful in obtaining it.

The obvious question at this moment is whether the *I-J* subregion products expressed on the suppressor T cells (and suppressor factors) and Th_2 are the same or different. Recent studies in our laboratory clearly indicated that they are different molecules controlled by two different loci in *I-J* subregion (Tada *et al.*, 1979a,b). In view of the fact that the KLH-specific suppressor factor acts only on *I-J* compatible spleen cells contained in nylon-adherent T cells, we have examined whether the suppressor factor can directly suppress the helper effect of Th_2. The KLH-specific suppressor factor was added to DNP-primed B cells which were cocultured with KLH-primed Th_1 or Th_2. As mentioned above, the secondary antibody response mounted by B cells and nylon-purified helper T cells (Th_1) was not suppressed by the KLH-specific factor, whereas the response mounted by B cells and Th_2 was greatly suppressed. Participation of Lyt-1^+,2^+,3^+ intermediary T cells was excluded by the treatment of nylon-adherent T cell population with anti-Ly-2 antiserum before testing the effect of the suppressor factor. The results indicate that the KLH-specific suppressor factor turns off helper activity of some (Th_2) but not all helper T cells, and that there is another pathway of the suppression of antibody response by the antigen-specific suppressor factor. Figure 2 illustrates our hypothetical scheme, in which suppressor factor on the one hand activates a pathway of induction of new suppressor T cells, and on the other hand directly suppresses some of the helper T cells. The induced suppressor T cell (Ts_2) may participate in the self-amplifying maintenance of suppression, and the direct suppression of Th_2 may be responsible for an acute and short-term suppression. In both pathways nylon wool-adherent T cells play substantial roles.

Independently from above studies, another two-step mechanism of T cell-mediated suppression has been extensively studied by Benacerraf's group using GT suppressor system (reviewed by Benacerraf *et al.*, 1977; Benacerraf and Germain, 1977). They have already shown that GAT and GT suppressor factors can act across the MHC barrier in the suppression of primary antibody response to GAT- and GT-MBSA (Kapp *et al.*, 1976; Kapp, 1978; Waltenbough *et al.*, 1977a), and thus suggesting that a different suppressor pathway exists in the suppression of primary antibody response. A noteworthy observation is that GT-suppressor factor obtained from BALB/c (*H-2*^{d}) mice could effec-

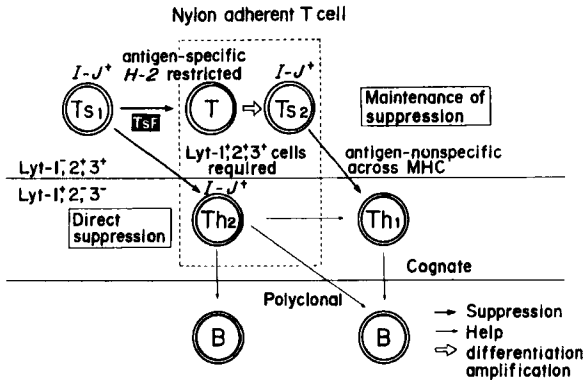

FIG. 2. Two distinct pathways of the suppressor factor-mediated immunosuppression. The initial suppressor T cell (Ts_1) produces an antigen-specific suppressor factor(s) that on the one hand induces a new suppressor T cell (Ts_2) for the amplification and maintenance of suppression. On the other hand, the factor acts directly on some, but not all, helper T cells (Th_2) to diminish sharply the helper effect. Conventional helper T cell (Th_1) and Th_2 are distinguished by the expression of *I-J* determinants. In both pathways, nylon wool-adherent T cells play important roles (for details see text).

tively suppress the response to GT-MBSA of A/J strain, which does not produce GT-specific suppressor cells by pretreatment with GT either intravenously or intraperitoneally in a very wide dose range (Germain *et al.*, 1978b). Furthermore, A/J mice treated with BALB/c suppressor extract was able to transfer the acquired suppression into normal A/J recipients that were subsequently challenged with GT-MBSA. The results indicate that BALB/c suppressor factor turns on otherwise uninducible suppressor cells in A/J, which then specifically suppress the response to GT-MBSA. This also suggests that the genetic defect in A/J mice resides in their inability to produce the GT-suppressor factor by the usual technique of GT-pretreatment, which is always successful for the production of such factors in other strains. Therefore, A/J mice were found to be capable of responding to produce active specific suppressor cells, if they were given GT-specific suppressor factor produced by other allogeneic strains together with GT.

Another interesting observation made by the same group (Debré *et al.*, 1976b) is that pretreatment of BALB/c mice with cyclophosphamide abolishes their ability to develop GT-specific suppression by the usual intraperitoneal administration of GT. This indicates that the initial suppressor T cell or the producer of suppressor factor is sensitive to cyclophosphamide. However, administration of GT-suppressor factor from normal BALB/c mice into cyclophosphamide-treated BALB/c

mice was capable of inducing specific suppression to GT in the recipients. The induced suppressor T cells are apparently resistant to the cyclophosphamide treatment (Germain *et al.*, 1978a).

The above two observations indicate that there is a two-step mechanism in the suppression of GT-specific antibody response: The first step is initiated by antigen in suppressor genotype mice, and the second step is mediated by the factor generated in the first step. Cyclophosphamide treatment abolishes cells (Ts_1) concerned in the first step without affecting those involved in the second step (Ts_2), and A/J mice have a selective defect in the antigen-initiated step with normal ability to manifest the second step of immunosuppression.

To prove this two-step model of immunosuppression, Waltenbough *et al.* (1977b) further investigated the long-term effect of suppressor extract of GT- and GAT-treated spleen cells. They demonstrated that even 5 weeks after the administration of GT- or GAT-specific suppressor factors of BALB/c or DBA/1 mice, the syngeneic recipients manifest a strong suppression to GT- or GAT-MBSA. Such a long-lasting effect of the factor is only ascribed to the generation of new suppressor cells (Ts_2). In addition, much greater suppressive activity of BALB/c GT suppressor factor was observed when the extract was injected 1 week before immunization with GT-MBSA rather than the administration of the extract on the day of immunization. They also showed that such "induced" suppressed state by Ts_2 was transferrable to normal syngeneic recipients (Waltenbaugh and Benacerraf, 1978).

This work was further extended by Germain *et al.* (1978a,b) and Germain and Benacerraf (1978) to prove that the GT- and GAT-specific suppressor factors do, in fact, generate suppressor cells from normal syngeneic and allogeneic cells *in vitro*. They cultured normal spleen cells with crude extracts from GT- or GAT-treated suppressor spleen cells for 2 days with or without antigen. The cultured spleen cells were then transferred into syngeneic recipients that were subsequently immunized with GT- or GAT-MBSA. By this experimental procedure, they were unequivocally able to demonstrate that the suppressor factor derived from Ts_1 stimulates the induction of Ts_2 from naive spleen cells, whose activity can be measured by passive transfer of antigen-specific suppression. As mentioned before, the crude spleen cells extracts from GAT- or GT-treated mice contained a small amount of antigens or their fragments associated with the suppressor factor, and thus were capable of inducing Ts_2 without further addition of antigen. However, if the active materials were semipurified on the basis of adsorption to and elution from an antigen-coated column, supplementation with antigen was definitely needed to induce Ts_2. In

addition, they confirmed that coexistence of *I-J*$^+$ suppressor factors and antigen was prerequisite for the induction of Ts_2 from normal spleen cells (Germain *et al.*, 1978a). The suppressor T cell (Ts_2) thus induced with antigen plus suppressor factors was killed by the treatment with anti-*I-J* for the second haplotype and C, although the precursors of Ts_2 do not express Ia antigens (Germain *et al.*, 1978b). Unlike the results of Tada *et al.* (1977b), the induced Ts_2 had an exquisite specificity to the antigen by which initial Ts_1 was generated. Such an "infectiousness" of specific immunosuppression is important for understanding the long-term suppression that we observe in many instances, where a minute quantity of antigen can induce a sustained suppressed state as in certain types of immunologic tolerance.

The two-step model of suppressor phenomena, though different from one system to the other in details, suggests that activation of a limited number of factor-producing cells would result in the generation of greater number of effector suppressor cells. The first step appears to be controlled by an Ir or Is gene, but apparently the factor from suppressor strain can initiate the induction of Ts_2 in responder strains (Germain and Benacerraf, 1978). The interplay between Ir/Is genes and the *I-J* subregion genes that code for suppressor factors is one of the important problems to be clarified. In addition, these phenomena suggest the presence of dynamic negative and positive feedback systems in regulation, as proposed by Gershon *et al.* (1977).

Another aspect of the factor-induced suppression of antibody response has been presented by Kontiainen and Feldmann (1978). They were able to obtain antigen-specific factor to KLH in the supernatant of cultured normal spleen cells with antigen. They examined the cellular target of the *in vitro*-induced suppressor factor by adding it to the culture of DNP-primed B cells and KLH-primed nylon column-purified T cells. Unlike the results of Tada *et al.* (1977b) and Benacerraf *et al.* (1977) the factor was able to suppress directly the activity of helper T cells. Helper T cells obtained after treatment of spleen cells with anti-Lyt-2,3 antiserum were also suppressed, and hence the participation of Lyt-1$^+$,2$^+$,3$^+$ and Lyt-2$^+$,3$^+$ cells was excluded. It is not known, however, whether or not the helper T cell involved in this *in vitro* primary IgM antibody response corresponds to Th_2 of Tada *et al.* (1979b). They also showed that there is no restriction for the effect of suppressor factors on allogeneic helper T cells. Thus, in this experimental system the secreted suppressor factor is shown to be an effector molecule directly suppressing the helper T cells.

The mechanism of suppression of cell-mediated immunity by T cell factors is less extensively investigated. The picryl chloride-specific

suppressor factor of Zembala and Asherson (1974) utilizes macrophages as the effector vehicle as discussed in Section IV, E. The mode of action of tumor-specific suppressor factors has not been fully analyzed, but a recent study by Perry *et al.* (1978a) indicated that the *I-J* coded factor stimulates a population of new suppressor T cells in non-tumor-bearing hosts. The newly induced suppressor T cell (Ts_2) had identical fine discriminative activity as Ts_1, obtained from tumor-bearing animals. Thus the suppressor circuit is obviously operative in limiting the host resistance to tumors (Section IV,F).

These widely diverse effects of suppressor factors observed in different experimental systems indicate that there exist multiple loci at which T cell factors act, resulting in the activation or inactivation of different cell types. As we notice sufficiently overt differences between experimental systems, the effects on primary and secondary, as well as on IgM and IgG antibody responses may be influenced by the cell types involved in these responses, which have learned a different discipline of antigen-recognition. The identity of suppressor molecules detected in different experimental systems has yet to be determined by the chemical and serologic analysis in the future. Also, how the teleology of the immune system can accommodate these diverse pathways in the action of basically very similar suppressor factors should be asked by future theoretical considerations.

VI. Antigen-Specific T Cell Factors Derived from or Acting on Human Lymphocytes

Antigen-specific T cell factors described herein have mostly been detected in the mouse. Although these studies considerably broadened our knowledge of the molecular basis of multicellular responses to specific antigen, we should also ask the relevance of such information to human immune response and its abnormalities. If such molecules do exist in the human, they would be of great value in estimating the ability of human lymphocytes to respond specifically to antigen, and even in future therapeutic applications to allergic and autoimmune diseases as well as to certain immunodeficiencies.

One such approach was made by Taussig (1978) to map Ir genes in human beings, using antigen-specific helper factors produced by mouse T cells acting on human peripheral blood B cells. As discussed in Section III, C, Taussig *et al.* (1975) demonstrated that the helper factors produced by mouse T cells educated with (T,G)-A–L and (Phe,G)-A–L were able to help allogeneic B cells to make antibodies, provided B cells were derived from genetically responder strains. They extended their study to demonstrate the effect of mouse T cell

factors on xenogeneic lymphocytes across the species barrier (Taussig and Finch, 1977; Luzzati *et al.*, 1976). An analogous molecule made in the rabbit, in fact, reacted well with mouse lymphocytes (Taussig *et al.*, 1976b). Thus, they claim that the vital sites on the acceptor molecules to which the factor combines have been preserved during evolution and specification. If we admit the assumptions that B cell acceptor site is an Ir gene product and that such a product is preserved among species, it may be possible to envisage human Ir genes to certain antigens.

Taussig (1978) tested the ability of human peripheral blood lymphocytes to absorb mouse T cell factors specific for (T,G)-A–L and (Phe,G)-A–L. They found that lymphocytes from about 65% of the donors could absorb the (T,G)-A–L-specific helper factor and the rest of 35% was unable to absorb it. For (Phe,G)-A–L-specific factor, about 27% were found to be low absorbers, and 15% of population so far tested could not absorb both (Phe,G)-A–L- and (T,G)-A–L-specific factors. Furthermore, they identified some individuals whose lymphocytes were able to absorb (T,G)-A–L specific factor but not (Phe,G)-A–L specific factor and vice versa.

By examining HLA haplotypes of high and low absorbers in a few families, they concluded that (*a*) acceptor sites for (T,G)-A–L specific and (Phe,G)-A–L specific factors are controlled by separate genes both dominantly inherited by children linked to HLA haplotype; (*b*) there are some cases in these families where crossing-over has occurred between these two possible loci; and (*c*) a possible human Ir (T,G)-A–L gene is mapped to the right of the *HLA-C* locus, while Ir(Phe,G)-A–L gene is at least to the left of *HLA-A* probably associated with *HLA-D*. In addition, the high-absorber lymphocytes were found to respond well to the antigen in the *in vitro* PFC response (Taussig, 1978).

This observation is phenomenologically intriguing, but certainly requires further extensive studies in order to establish that they are really defining the human Ir genes. As discussed in Section III, C, it is no longer valid to state that the site and function of Ir gene effect is only on B cells. Also, it has been shown that the expression of acceptor site is controlled by at least two genes, one of which is not linked to the *H-2* complex.

More recently, some reinforcement of these results was made by Kantor and Feldmann (1979), who showed the production of the antigen-specific T cell factor by human peripheral blood T lymphocytes, which acts to augment the antibody response of mouse spleen

cells in culture. They cultured human peripheral lymphocytes in the presence of an optimal concentration of (T,G)-A–L, OVA or KLH in the same manner to obtain *in vitro*-induced mouse helper factors (Howie and Feldmann, 1977). The factors were added to the cultured unprimed spleen cells of the mouse, which were concomitantly stimulated with given antigen. It was found that even though the supernatant of cultured human lymphocytes had some nonspecific augmenting effects on the response of mouse spleen cells, it always increased the response to the homologous antigen more than that to heterologous antigen. The human helper factor was effective in the absence of T cells in the responding mouse spleen cells, and thus it is a helper rather than an amplifier. Furthermore, such a specific augmenting effect of culture supernatants of human lymphocytes was removed by absorption with relevant antigen but not other antigens verifying the antigen-specificity of the *in vitro*-induced human helper factors. This experiment, obviously a mirror image of Taussig's finding, again suggests that helper T-cell factors are effective across the species barrier when they directly act on B cells.

Based on the above findings Zvaifler *et al.* (1979) examined the ability of peripheral lymphocytes from seven healthy donors to respond to produce (T,G)-A–L- and GAT-specific helper factors *in vitro*. Unfortunately, the number of donors is too small for study of the association between the ability to make T cell factors and the *HLA* haplotype; however, there was some selective responsiveness to one of the antigens. We should await further results before judging whether this method is suitable for studying human Ir genes.

Although the above studies were performed in xenogeneic combinations between the mouse factor and human lymphocyte or vice versa, Cobi *et al.* (1979a,b) were successful in demonstrating human helper factor acting on human B cells. They had established a system to induce *in vitro* antibody responses to SRBC and OVA with human peripheral blood lymphocytes (Cobi *et al.*, 1979a,c; UytdeHaag *et al.*, 1978; Gmelig-Meyling *et al.*, 1977). By addition of antigen-primed T cells having Fc receptor for IgM (Tμ) or IgG (Tγ), they were successful in modulating this *in vitro*-induced antibody response. One notable point in their experimental system is that both enhancement and suppression were found to be antigen specific, since Tμ and Tγ cells precultured with SRBC were selectively effective in the response to SRBC, but not to OVA. Thus, the antigen-specific helper and suppressor activities reside in different T cell subpopulations similar to antigen-nonspecific activities found in the pokeweed mitogen-

induced immunoglobulin synthesis by B cells (Moretta *et al.*, 1977). The helper factors specific for these antigens were induced *in vitro* from peripheral T cells, essentially by the same method as described by Kantor and Feldmann (1979). The factor was added to isolated B cells together with antigen, and the IgM PFC response was measured after 6 days. Cobi *et al.* were able to show that SRBC-induced helper factor was capable of replacing the helper T cells for the response to SRBC, and OVA-specific factor specifically enhanced the B cell response to OVA. There were no genetic restrictions between the donor of the T cell factor and responding B cells. The same group has recently demonstrated the antigen-specific effect of the suppressor factor obtained *in vitro* with a higher dose of antigen acting on human B cells (Cobi *et al.*, 1979a). No information about the physicochemical and immunochemical properties of these factors is as yet available.

Although we must say that above experiments on antigen-specific factors in the human immune response are still in the very soft part of current cellular immunology, such techniques if improved will facilitate the analysis of immunologic disorders in humans. Since numerous immunologic diseases are associated with HLA haplotype, the characterization of human T cell factors and the analysis of the mechanism of their action are important approaches in understanding the immunologic background of such diseases. In view of the ethical constraints of immunizing humans with antigen, assessing specific lymphocyte functions *in vitro* by measuring antigen-specific factors is a suitable way for studying immune responsiveness of humans. However, the studies in humans are still very limited owing to the difficulty in obtaining reliable culture systems, and thus no conclusion may be drawn at the moment with a precision analogous to studies with experimental animals.

VII. The Nature of Antigen-Binding Site on T Cell Factors

Most T cell factors discussed above have no known immunoglobulin isotypic determinants, and their molecular weight is less than 60,000. No structural information is so far reported as to whether the factor consists of one single chain or double chains. A definitive characteristic common to these factors is that they carry determinants coded for by genes in the *I* region of MHC. Even though MHC products have considerable polymorphism, it is difficult to explain by the known heterogeneity of Ia antigens the exquisite specificity and affinity for antigens possessed by these small molecular weight proteins. The question has long been asked whether the T cell factors carry an un-

identified variable part comparable to *V* region of immunoglobulin chains. It now seems very likely that this is the case.

Mozes (1976, 1978) challenged this question by examining the idiotype on the (T,G)-A–L specific T cell factor. She utilized a guinea pig antiserum raised against purified anti-(T,G)-A–L antibodies of C3H.SW high-responder mice. After appropriate absorption with normal mouse Ig of the same allotype, this antiserum specifically bound 20–30% of ^{125}I-labeled anti-(T,G)-A–L antibodies carrying cross-reactive idiotype (Mozes, 1978). This anti-idiotype antiserum was used as a probe for the idiotype of (T,G)-A–L specific helper factor. The factor was passed through a column consisting of the anti-idiotype antibodies, and the residual helper activity was assayed by mixing the effluent with C3H.SW bone marrow cells, which were subsequently transferred into irradiated recipients. The results clearly demonstrated that the (T,G)-A–L specific factor was completely removed by passage through the column of anti-idiotype antibodies. The concurrent absorption experiment with guinea pig anti-Fab antibodies did not remove the helper activity. These results suggest that the (T,G)-A–L specific T cell factor shares identical or cross-reactive idiotypes with serum antibodies directed to (T,G)-A–L. Since the same anti-idiotypic antibody can remove only 20–30% of anti-(T,G)-A–L antibodies, it is suggested that the T cell factor has less heterogeneous idiotype than hyperimmune antibodies, the fact being consistent with the results of Krawinkel *et al.* (1977a,b), who showed that T cell receptor for a hapten, NIP, has an idiotype that is preserved for a long time after repeated immunization, while the same idiotype on B cells declines.

A similar approach has been made by the group of Benacerraf with GAT-specific suppressor factor. Thèze and Sommé (1979) were able to produce rabbit antiserum against purified anti-GAT antibodies of GAT-responder (BALB/c) mice. After appropriate absorption, the antiserum was capable of removing anti-GAT antibodies carrying a cross-reactive idiotype. Ju *et al.* (1978a,b) were also able to produce guinea pig antiserum detecting a common idiotype possessed by a majority of anti-GAT antibodies of the mouse. An interesting point with the anti-GAT idiotype is that both responder and nonresponder to GAT produce antibody carrying the same idiotype after immunization with GAT-MBSA regardless their Ig allotypes. In addition, the cross-reactive idiotypes were found in the serum of several other animal species, i.e., rat, rabbit, and guinea pig (J. Thèze, personal communication). There was no positive or negative linkage to Ig allotype genes, since this idiotype is produced in all strains tested and is considered to be preserved among species. This in turn imposes a certain limitation

in studying the genetic linkage between GAT V gene and Ig allotype genes.

Germain *et al.* (1979) demonstrated that the anti-GAT-idiotype antiserum can remove GAT-specific suppressor factor produced by GAT nonresponder strains. They took GAT-specific suppressor factors from DBA/1 (*H-2*^{q}, Ig-1^c) and SJL (*H-2*^{s}, Ig-1^b) strains, and passed them through a column of guinea pig antibody specific for the major cross-reactive idiotype of mouse anti-GAT antibodies. The GAT-specific suppressive activity was completely removed by this absorption, and was recovered from the column by elution with acid. The eluted material indeed contained the antigen-specific suppressor factor heretofore described, since the activity of eluate was again completely removed by a passage through a second column of anti-*I-J* antiserum. This clearly established the fact that the GAT-specific factor has the GAT binding site possessing a cross-reactive idiotype and *I-J* determinants on the same single molecule.

One interesting question emerges from their experiment: Since the antibodies against GAT and GT, but not GA, cross-react extensively, does the GT-specific factor, in fact, react with anti-idiotype GAT? One possible prediction is that GT-specific factor has a similar or cross-reactive idiotype possessed by GAT-specific factor. However, it has been demonstrated that GT-specific factor does not suppress the response to GAT and vice versa (Germain and Benacerraf, 1978). Experiments to reconcile this apparent discrepancy would finally lead to the better understanding of the functional fine specificity of T cell factors on structural basis. Another important question is whether the idiotype detected on the GAT-specific T cell factor is, in fact, coded for by the same Ig V genes, or whether the configuration similar to the Ig V region is determined by convergent evolution of genetic units. This problem is not answered, since all mouse strains have the same cross-reactive GAT idiotype. However, it is not likely that two separate structural genes code for Ig V region and combining region of T cell factor with the same specificity and idiotypic structure.

The latter problem was recently solved by extensive works by Greene *et al.* (1979) and Bach *et al.* (1979). They were able to obtain the suppressor factor specific for ABA, which suppresses the DTH reaction to ABA in the A/J strain of mice (see Section IV, E). It has been shown that A/J mice produce a large quantity of anti-ABA antibodies having cross-reactive idiotype, the gene for which is unambiguously linked to the immunoglobulin C_H locus (Nisonoff *et al.*, 1977). Obviously, it is possible to perform genetic analysis of the ABA-specific suppressor factor utilizing appropriate mouse strains of known allotype genes. It has already been shown that ABA-specific suppressor

factor can be obtained in a variety of strains having different *H-2* and allotype alleles (Greene *et al.*, 1979).

Bach *et al.* (1979) prepared an immunoadsorbent column composed of $F(ab')_2$ fragments of rabbit antibodies specific for cross-reactive ABA idiotype. The spleen cell extract containing ABA-specific suppressor activity was passed through the column to see whether the anti-idiotype can remove the suppressor activity. The results with the A/J mouse-derived factor clearly showed a significant reduction of suppressor activity after absorption with the anti-idiotype column. The suppressor activity was recovered in the acid eluate from the column. The ability of $F(ab')_2$ rabbit anti-idiotype to remove the suppressor factor was abrogated by pretreatment with purified anti-arsonate antibody from A/J mice, which had been shown to contain a high concentration of antibodies carrying a cross-reactive idiotype (Tung and Nisonoff, 1975). It was therefore concluded that a significant fraction of ABA-specific suppressor factor carries a cross-reactive idiotype shared with serum anti-arsonate antibodies.

Bach *et al.* further showed evidence that idiotype-bearing molecules in the suppressor supernatant were also absorbed by ABA-FγG column and anti-*H-2* column by successive adsorption to and elution from one immunoadsorbent followed by the other. Thus, it is clear that the suppressor molecule has both *H-2* coded determinants and idiotypic determinants, the latter of which is associated with the ABA-binding site.

The genetic analysis of the ABA-specific suppressor factor derived from different mouse strains clarified the relationship between the influence of MHC and the heavy-chain allotype linkage group in the expression of idiotypic determinants on ABA suppressor factors. It was found that in addition to A/J ($H\text{-}2^a$, Ig-1^e), C.AL ($H\text{-}2^d$, Ig-1^d), produced the ABA specific suppressor factor carrying cross-reactive idiotype, whereas the ABA suppressor factors from B10.A ($H\text{-}2^a$, Ig-1^b), C57BL/6 ($H\text{-}2^b$, Ig-1^b), and BALB/c ($H\text{-}2^d$, Ig-1^a) were negative for the idiotype defined in A/J anti-arsonate antibodies. Since A/J and B10.A have the same *H-2* haplotype, and C.AL-20 and BALB/c are congenic except for the Ig heavy-chain allotype locus, it is apparent that the idiotype expression on ABA-suppressor factors is linked to the C_H allotype gene, but not to *H-2* locus. It has been demonstrated that cross-reactive idiotype of anti-ABA antibodies defined in A/J mice was also produced in AL/N ($H\text{-}2^a$, Ig-1^d) mice (Pawlak and Nisonoff, 1973), from which IgC genes were introduced into the C.AL-20 strain. Thus, it is seemingly clear that idiotypic determinants on suppressor factors are coded for by identical V genes of the immunoglobulin linkage group.

Kontiainen *et al.* (1978) used a somewhat different approach to

characterize the antigen-binding site of *in vitro*-induced helper and suppressor factors. They purified the *in vitro*-induced KLH-specific suppressor factor from CBA mice over a column of KLH-immunoadsorbent. The acid eluate from the column was used to immunize normal CBA mice. This antiserum is expected to react with the structure on the factor recognizable by syngeneic mice, which is postulated to be an idiotype-like determinant. This putative anti-idiotypic antiserum was used to absorb suppressor or helper factors specific for KLH or GAT obtained from different mouse strains having different Ig allotype. They reported that this CBA anti-KLH suppressor factor could absorb only the syngeneic KLH-specific suppressor factor, but not those from B10, A/J, or B10.HTT. The antiserum was incapable of removing the GAT-specific suppressor factor of the CBA origin, and therefore the specificity of this antiserum is directed at KLH-specific structure possessed only by CBA mice. No linkage studies were performed on Ig allotype or *H-2*. However, since CBA and A/J or B10.HTT share a part of *H-2* subregions but differ in Ig allotype alleles, these authors imply that the antibody might have recognized "idiotype-like" determinants on the suppressor factor.

They have further studied the effect of CBA anti-suppressor factor on *in vitro*-induced KLH-specific helper factor. They showed that the same anti-suppressor factor could absorb the KLH-specific helper factor of CBA origin, but not the GAT-specific CBA helper factor. These results at first glance suggest that both suppressor and helper factors carry the same antigenic structure related to the antigen specificity, and that such structure is likely to be an idiotype(s) determined by genes linked to Ig allotype loci. However, in order to verify this postulate, exact linkage studies as well as the definitive probe for idiotype may be required. Since cross-reactive idiotype in anti-KLH antibodies is at most only 4% in A/J (Ig-1^e allotype), which tends to produce KLH-specific idiotype, and no cross-reactive idiotype has so far been detected in other strains (Nisonoff, 1975), it may not be easy to produce anti-idiotype antibodies by immunization with a minute amount of the KLH-specific suppressor factor in syngeneic mice. We should probably await further genetic and chemical studies to reach the conclusion.

One opposite experiment arguing against the presence of Ig variable region on helper-T cell factor was presented by Taussig *et al.* (1976b). They obtained the antigen-specific helper factor from peripheral lymphocytes of rabbits immunized with SRBC. The factor was released *in vitro* by culturing the primed peripheral lymphocyte with SRBC for 6–8 hours. The supernatant was tested for the ability to collaborate with mouse bone marrow cells in the irradiated host. Since rabbit

allotype *a* locus is expressed in *V* region of heavy chain, the SRBC-specific helper factor of rabbit was passed through a Sepharose immunoadsorbent carrying anti-*a* allotype antibody to see if the helper activity was absorbed. The result was completely negative, suggesting that the rabbit helper factors do not carry the *V* region *a* allotype. Concurrent experiments using anti-*b* allotype (expressed on C region of κ chain) and heterologous anti-Ig antibodies showed that none of the anti-Ig adsorbent, both allogeneic and xenogeneic, were capable of absorbing the T cell factor. They concluded that the binding site of antigen-specific T cell factor, which they assume to be a T cell antigen receptor, would be different from the *V* region of immunoglobulins. Apparently, there are many arguments about the implication, as the experiment was done in a xenogeneic combination of the factor and responding cells in which no definitive proof for the identity of the T cell factor had been established. The contribution of *V* region of light chain is also not excluded. Thus, this problem should be reinforced by further experiments, since the expression of *a* allotype in the *V* region is certainly advantageous to resolve this issue.

One important clue was recently provided by the availability of xenogeneic antibody against the framework structure of mouse heavy-chain *V* region (anti-V_H). The rabbit antiserum was made against purified V_H fragment of MOPC-315 myeloma protein (IgA) and was specifically purified by adsorption to and elution from the myeloma protein column (Ben-Neriah *et al.*, 1978). This antiserum was recently shown to react with many of the myeloma immunoglobulins of different classes, and was proved to absorb idiotype-bearing T cell receptor molecules (K. Rajewsky, personal communication). This antiserum was applied to study whether or not the extracted KLH-specific suppressor factors carry V_H structure (Tada *et al.*, 1979c). As a preliminary experiment, antigen-specific suppressor T cells obtained by adsorption to and elution from an antigen-coated column were stained with this anti-V_H antiserum. As mentioned in Section IV,A, the purified T cell fraction contained 30–40% $I\text{-}J^+$ T cells, whereas the fraction that did not bind to a KLH-coated Sepharose column had a negligible number of $I\text{-}J^+$ T cells. The staining with anti-V_H revealed that a comparable number of cells were found to express V_H, which were proved to be T cells by their sensitivity to anti-Thy-1 antiserum.

The KLH-specific suppressor factor extracted from purified suppressor T cells was passed over the anti-V_H immunoadsorbent column. This procedure was found to reduce significantly the KLH-specific suppressor activity, indicating that the suppressor molecule in this system also contains the V_H expression. The acid eluate from the anti-V_H col-

umn contained most of the applied suppressor activity (Tada *et al.*, 1979c). This again suggests that most, if not all, suppressor molecules possess an antigen-binding region coded for by Ig V_H genes. However, some of the hybridoma-derived factors having antigen-specificity were not always absorbed by anti-V_H antibodies. Therefore, we must be alert for additional information as to whether V_L genes in some cases contribute to the antigen-binding site of T cell factors. In this respect, it is of interest that the *VK-1* locus controlling the κ-chain *V* region determinant was found to be closely linked to the locus controlling Lyt-2 antigen, which is expressed on suppressor and killer T cells (Laskin *et al.*, 1977).

VIII. Minimal Models for Biologically Active T Cell Receptors

The phenomenology of antigen-specific T cell factors heretofore discussed now collectively suggests that the molecules contain at least two well defined moieties, i.e., *I* region determinants and Ig *V* region structure sometimes carrying idiotypes. If factors are composed of one polypeptide chain, we would be confronted with a serious difficulty in explaining the apparent genetic discrepancies. It requires highly improbable mechanisms, such as translocation and insertion of genes from one chromosome to the other, or reorganization of two separate messenger RNAs on ribosomes. If factors are composed of two chains, we should ask again what structural moieties are located in either chain and how they are associated to become active molecules. We would put aside this otherwise unfruitful question until the definitive biochemical data are available, probably through the use of hybridoma-derived factors.

One may then ask what picture emerges from the above-mentioned phenomenology. We should examine the other side of the coin in relation to this question. There is growing evidence that at least some T cells carry idiotypic specificities shared with B cells (Binz and Wigzell, 1977a; Cosenza *et al.*, 1977; Eichmann, 1978; Pincus *et al.*, 1979; Rajewsky and Eichmann, 1977). Soluble T cell products carrying idiotypes have also been demonstrated (Binz and Wigzell, 1976, 1977a,b; Krawinkel *et al.*, 1977a,b,c). These molecules have been chemically isolated and characterized mostly by their idiotypic and antigen-binding properties, while the definitive regulatory roles in cell interactions have not been established. On the other hand, T cell factors heretofore discussed were originally defined by their regulatory activities, while the structural basis relating to their antigen-binding

capacity has poorly been analyzed. There are a number of differences between isolated T cell receptors and antigen-specific T cell factors as to physicochemical and immunochemical properties. The putative T cell receptors are most probably multichain molecules that are broken down into smaller subunits in detergents. The variable portion, which is responsible for antigen-binding capacity, is clearly a V_H gene product, while the constant portions is only negatively defined at the moment. No MHC determinants nor the contribution of *H-2* genes to the antigen recognition are found to be associated with these molecules. On the other hand, Krammer and Eichmann (1977) have recently reported that the idiotypic pattern of alloreceptors on mouse T lymphocytes are controlled by genes both in the heavy-chain linkage group and MHC. One should also remember that helper T cells, in general, recognize not only antigen but self Ia antigens on macrophages for their activation, and that the induction of suppressor T cells is influenced by the mode of antigen-presentation by macrophages. Thus, one obvious prediction is that, for the *normal* functioning of T cells, at least two restricting elements are required, i.e., the antigen specificity and the "restriction specificity" for self MHC. It is not very important in this argument whether such a requirement is explained by the "dual recognition" or the "altered self" model.

Germain *et al.* (1979) recently presented a hypothesis that T cell factors have both Ig V region determinants and *I* region-coded determinants either on single or separate chains. The former would recognize antigen, while the latter is involved in biologic effector functions (e.g., helper, suppressor, and *H-2* restricted cell-to-cell communications) in a manner similar to Fc portion of immunoglobulins. This model would provide a unifying concept for the Ir gene effect in terms of both cell interaction and antigen-specific activity, in that the lack of the formation of the complex of *I* region and V region products would preclude the T cell activity for given antigen. The particular function of the factor, i.e., helper or suppressor, would be determined by *I* region-coded structures.

We agree mostly with their hypothetical model, as we have also presented a similar hypothesis that *I*-region determinants on the suppressor T cell factor serve as the restricting element in the suppressive cell interaction (Taniguchi *et al.*, 1976b; Tada, 1977). Thus, T cell factors are considered to be bifunctional molecules in that they recognize antigen by one end and interact with other cell types with the second end, which is determined by *I* region genes. The first end is the product of an Ig V gene, and the second end is the product of an *I*

region gene that determines the restriction specificity. The function of the factor is determined by the second end, which correctly finds the next cell type to interact with. Our prediction extends to the multiplicity of *I* region genes that code for complementary structures on different subsets of T cells with which they can make unmistakable and meaningful cell interactions. Probably, this restriction specificity may be learned in the thymus by mutation and selection during the differentiation of T cells, as we notice that the restrictive element for cell interaction is not rigid in certain circumstances as in the bone marrow chimera (von Boemer and Sprent, 1976; Zinkernagel and Doherty, 1977; Katz, 1977).

Such a requirement of restriction specificity may differ from one experimental model to the other depending on the type of cell interactions we observe (primary vs. secondary, T–T vs. T–B, macrophage dependent vs. independent). The most important function of the *I* region-coded second end of the factor molecules would be to determine the subsequent cellular consequences leading to suppression or augmentation by simply selecting the second cell type with its restriction specificity. Such a restriction specificity is analogous to that of helper and proliferating T cell in recognition of antigen together with self-Ia antigen on macrophages. By endowing this type of restriction specificities to T cell, *I* region genes would control the initial activation of T cells, as well as the subsequent cell interactions. This model would accommodate both Ir/Is gene effects and double recognition of T cells.

The final assumption we would propose is that antigen-specific T cell factors and idiotypically isolated T cell receptors are related to each other in some way. Since idiotypes are detected on functionally active helper and suppressor T cells, it is highly unlikely that Ia-bearing T cell factors are independently synthesized from other T cell receptors. The separation procedures used for T cell factors do not exclude the possibility that some structures present on T cell receptors are destroyed by degradation with proteolytic enzymes and physical disruption. On the other hand, isolation of idiotype-bearing T cell receptors may result in a dissociation of *I* region-coded moiety from idiotype-bearing polypeptides. The most probable explanation is that both the idiotype-defined and *I* region-defined molecules from T cells do not conform to the complete structure of T cell receptor, and we are looking at different sides of a coin. How these theoretical models fit actual experimental observations is one of the important challenges of the future.

IX. Conclusion

We have already examined several facets of antigen-specific T cell factors found independently by different investigators. There exist both overt similarities and differences between these T cell factors with respect to their immunochemical properties, genetic control, and roles in the immune response. We are impressed by the voluminous phenomenology accumulated during the past almost 10 years. Although this information undoubtedly provides new insights into the most important problems concerning the antigen-recognition and functional properties of T cells, the validity of these molecules in the actual regulatory cell interactions should await forthcoming immunochemical and biochemical characterization of T cell receptors.

How, then, can we reconcile the diverse and apparently contradictory observations made by different investigators? We are now aware that there are multiple pathways in T–B cell collaboration and suppression. But unfortunately, we are always inclined to see only one process among many alternative ones as being dictated by our own experimental system. Owing to our blindness in other experimental conditions, we are sometimes unable to integrate other mechanisms properly. In fact, if we examine carefully the different experimental systems and data obtained under these conditions, we would be surprised by the differences in the language by which they express their experimental results. It is certainly possible that under a selected condition we might visualize only one of the diverse effects of T cell factors, bypassing others. In this regard, we must admit that this area of "factorology" is unfortunately "dusty," since many laboratories cannot reproduce the very same experiments, mostly owing to subtle differences in technology, and rumors spread not infrequently throughout the immunologic community without any substantiation. This, however, should not in itself impose major objections to the models proposed by investigators. We should remain alert for the way to reach some agreement between different laboratories by exchanging materials and information. We are indeed much more impressed by similarities than by differences in the properties of T cell factors independently determined by different laboratories.

At this time, our knowledge on the structure of T cell factors is still very limited. It is, however, becoming more feasible that the antigen-binding site has a striking similarity to the *V* region of immunoglobulin heavy chains. On the other hand, it is almost certain that structures encoded by *I* region genes are associated with the functional site. The major question is how these two discrete gene products are assembled

on a single molecule, and how they determine the regulatory pathways. We do not even know whether the molecule consists of one or two chains, or whether the observed molecule is a degraded product of a T cell receptor of higher molecular weight. The answer to this question would depend on the biochemical analysis of T cell factors and isolated T cell receptor molecules that is in progress in various laboratories. Such studies will, it is hoped, solve the currently most controversial problems concerning both the T cell receptor for antigen and the mechanism of cell interactions.

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ADVANCES IN IMMUNOLOGY, VOL. 28

The Biology and Detection of Immune Complexes[1]

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I. Introduction

Upon exposure to most antigens, an individual responds by synthesizing specific antibodies that subsequently may interact with the inciting antigens and unite noncovalently with them to form immune complexes (ICs). This course of the normal immune response is designed to eliminate and/or neutralize the antigens, thus benefiting the host. However, there is no doubt that formation of ICs under some circumstances is detrimental to the host.

The harmful effects of ICs were first suggested at the beginning of this century by von Pirquet (1911), who proposed that the onset and course of serum sickness were determined by toxic factors produced by the interaction of host antibody and antigen in the circulation. Thereafter, others made similar observations (Longcope, 1915; Rich

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and Gregory, 1943; Hawn and Janeway, 1947). The definite pathogenic role of the ICs was shown during the 1950s by the work of Germuth (1953), Germuth and McKinnon (1957), Germuth *et al.* (1957), Dixon and associates (1958, 1961), and Dixon (1963), who confirmed and amplified von Pirquet's original theory using the experimental rabbit model of "one shot" serum sickness. It was demonstrated that the onset of glomerulonephritis and generalized vasculitis coincided with the appearance of soluble ICs in rabbits' circulations, a decrease in serum complement (C) activity, and a deposition of ICs in the sites of injury (Dixon, 1963; Dixon *et al.*, 1958, 1961).

In vivo and *in vitro* experiments have more recently clarified many factors involved in IC formation, removal, and localization as well as the mechanisms of IC-induced inflammatory reactions. Moreover, ICs are now viewed, apart from their potential phlogogenicity, as regulators of both cellular and humoral immune responses by virtue of their capacities to interact with antigen receptor-bearing lymphocytes and subpopulations of T and B cells, as well as with unclassified lymphocytes and macrophages having Fc and C receptors. With the recognition of the immunopathologic consequences of ICs and the development of new techniques for demonstrating ICs in tissues and biological fluids, considerable evidence has accumulated substantiating the primary pathogenic significance of ICs in a variety of animal and human diseases.

In the following report we will summarize studies related to the formation, localization, and fate of ICs and their biologic activities. Moreover, we will describe in considerable detail the currently available methods for detecting circulating ICs, the application of these methods to studying IC-associated human diseases, and the state of present attempts to isolate and characterize circulating ICs.

II. Formation and Fate of Immune Complexes

There are three general anatomical sites of antigen–antibody interactions.

1. Antibody can react with structural antigens that form part of the cell surface membrane or with fixed intercellular structures. Antibody originally bound to a cell membrane component or to structural antigens may be shed secondarily either free or complexed with the antigen into the extracellular milieu (Unanue and Dixon, 1967; Robert and Revillard, 1976; Robert *et al.*, 1976; Perrin and Oldstone, 1977). One structural antigen to which autologous and heterologous immune responses are made is the basement membrane, and such responses

can lead to a variety of anti-basement membrane antibody-induced diseases, including anti-glomerular basement membrane (GBM) antibody glomerulonephritis, anti-basement membrane antibody Goodpasture's syndrome (glomerulonephritis and pulmonary hemorrhage), and antitubular basement membrane antibody tubulointerstitial nephritis (Wilson and Dixon, 1976).

2. Antibody can react with antigen secreted or injected into the interstitial fluids. The classical experimental example of this form of ICs is the Arthus reaction, wherein antigen injected in an intradermal site interacts with antibody in and about the blood vessels that carry the antibody to the site of injection. Such ICs can also form around thyroid follicles as in thyroiditis (Clagett *et al.*, 1974), around spermatogenic tubules following vasectomy (Derrick *et al.*, 1974; Gupta *et al.*, 1975), and around parasites in tissues.

3. Antibody can also react with soluble antigens in the circulation. While most circulating ICs are taken up by circulating or fixed phagocytes, some may be trapped in one or more of the vascular or filtering structures of the body, apparently for anatomic and physiologic reasons, and induce IC disease (Dixon, 1963; Cochrane and Koffler, 1973; Cochrane and Dixon, 1978; Wilson and Dixon, 1976). This review will primarily focus on the diseases and the mechanisms related to circulating ICs.

The mechanisms of antigen–antibody interactions and the forces involved in such interactions have been reviewed (Eisen, 1973) and mathematical models devised (Steensgaard *et al.*, 1977). The antigen–antibody interaction is reversible, especially with monovalent antigens, and involves hydrophobic, Coulombic, and hydrogen bonds and van der Waals forces. Antigens that are sparingly soluble in water (e.g., dinitrophenyl haptens) tend to form particularly stable ICs with antibodies, whereas antigens that are highly soluble in water (e.g., sugars) tend to form more dissociable ICs since the binding of an antibody molecule with antigen may be regarded as a competitive partition of antigen between water and antibody-binding sites, which are relatively hydrophobic (Kabat, 1968).

Earlier reviews of this subject by Weigle (1961), Unanue and Dixon (1967), and Cochrane and Koffler (1973) and more recently by Haakenstad and Mannik (1978), show that formation, fate, biologic activities, and phlogogenic potential of circulating ICs depend on the nature of the antibodies (reactivity with phlogogenic mediators, valence, affinity) and antigens (size, valence, chemical composition) involved as well as on the molar ratio of the two reactants (Table I).

Features of the antibody that are important to the formation and func-

TABLE I
PARAMETERS THAT DETERMINE THE SIZE, FATE, AND BIOLOGIC ACTIVITIES OF IMMUNE COMPLEXES

Size of antigen
Valence of antigen
Chemical composition of antigen
Amount of antigen and rate of production
Class and subclass of antibody
Valence of antibody
Amount of antibody
Affinity of antibody
Ratio of antigen to antibody
State of the C system
State of the phagocytic system

tion of ICs are, first, the immunoglobulin (Ig) class, which determines the antibody's valence for a specific antigen as well as its ability to bind to cellular Fc receptors and its ability to activate the C system; and second, the association constant (avidity) for the union of specific antibody and antigen. IgG, monomeric IgA, IgD, and IgE all have a valence of 2, whereas that of IgM is 10 with small haptenic groups and 5 with large antigens, and that of dimeric secretory IgA is 4 (Metzger, 1970; Spiegelberg, 1974; Haakenstad and Mannik, 1978). In inbred mice, antibodies to soluble protein antigens vary in amount and avidity according to the strain tested (Soothill and Steward, 1971; Petty *et al.*, 1972). Mice producing high amounts of antibody of high avidity or affinity clear the antigen from the circulation at a much faster rate than do mice producing low amounts of low-affinity antibodies (Alpers *et al.*, 1972). Soothill and Steward (1971) found that murine strains with low-affinity antibodies after neonatal infection with lymphocytic choriomeningitis virus develop glomerulonephritis, whereas strains with high-affinity antibodies remain free of nephritis. The same investigators (Steward *et al.*, 1975) noted that the sera of New Zealand mice with glomerulonephritis contain low-affinity antibodies to DNA. On the basis of their findings, these authors suggested that susceptibility to IC disease is associated with a genetic predisposition to the production of low-affinity antibody, which fails to eliminate the antigen concerned, favoring the persistence in the circulation of antigen excess ICs that are then deposited in the tissues. However, their findings may be misleading, since antibody of whatever affinity found in the serum may not be representative of the antigen-complexed antibody that is deposited in the tissues. In fact, when Winfield *et al.* (1977a) evaluated the anti-native DNA antibodies in patients with systemic lupus

erythematosus (SLE) with and without active nephritis, sera from the patients with active nephritis contained antibodies of lower affinity. However, anti-native DNA antibody of uniformly high affinity was found by these investigators in the glomerular eluates of SLE patients with nephritis.

The nature of the antigen can influence ICs in several ways. Thus, when univalent antigens such as haptens are injected into the circulation of an animal whose serum contains specific antibodies, Ag_1Ab_1 or Ag_2Ab_1 complexes are formed, where each antigen molecule forms a separate antigen–antibody bond with one of the two antigen binding sites on the bivalent antibody molecule. Owing to the absence of lattice formation, such ICs remain in the circulation for a long period without tissue deposition (Schmidt *et al.*, 1974). In contrast to univalent antigens, multivalent antigens, such as polysaccharides and proteins, when combined with their specific antibodies that are directed against the multiple and frequently different antigenic determinants, do form lattices and ICs of varying composition depending on the molar ratio of the reactants (Heidelberger, 1939; Pedersen, 1936; Heidelberger and Pedersen, 1937; Marrack, 1938; Pauling *et al.*, 1944; Metzger, 1970; Arend *et al.*, 1972; Haakenstad and Mannik, 1974). Sometimes, owing to "monogamous" interactions, large antigens with multiple repetitive antigenic determinants do not form lattices upon complexing with bivalent antibody molecules (Klinman and Karush, 1967).

The ratio of antigen and antibody is the most important factor in determining the fate and the biologic activities of ICs. Thus, in antigen excess, polyvalent antigens form soluble ICs the size of which vary inversely with the amount of excess antigen (Arend *et al.*, 1972). In general, the larger the IC, the more rapidly it is removed from the circulation and even complexes of 11 S show rapid elimination (Lightfoot *et al.*, 1970; Arend and Mannik, 1971; Mannik and Arend, 1971; Mannik *et al.*, 1971; Haakenstad and Mannik, 1974). Very small ICs formed at large antigen excess typically contain a single antibody molecule divalently attached to two antigen molecules (Ag_2Ab_1). Such ICs usually will not fix C and by-and-large cannot initiate inflammatory processes. On the other hand, ICs formed at very large excess of antibody over antigen are large and insoluble, and they are rapidly phagocytized. These ICs do fix C and therefore have phlogogenicity, but, since they are phagocytized and catabolized very quickly, their phlogogenic potential is limited. The greatest pathologic potential seems to lie between these two extremes of complex size, i.e., in ICs formed when antigen excess is modest. These ICs are of intermediate

size and soluble but are not rapidly phagocytized. They are widely disseminated as they circulate and are large enough to fix C; consequently, they can exert their phlogogenic effects widely (see Section III, A). The important role of the size of the ICs and of the molar ratio of antigen and antibody is shown very clearly in the experimental chronic serum sickness model, in which the disease can be turned off or on by changing the dose of antigen. If the dose is either lowered to allow a continual antibody excess or raised to give a very large antigen excess, progression of disease is stopped. If the dose of antigen is again returned to a level giving rise to soluble ICs, the disease is progressive (Dixon, 1963).

The magnitude and duration of antigen exposure is also very important in determining the development of an IC disease. Thus, if antigen exposure is of limited duration, tissue injury and clinical manifestations that may occur after complexing with antibody are transient as in classical "one shot" serum sickness (Germuth and Rodriguez, 1973; Unanue and Dixon, 1967; Wilson and Dixon, 1970, 1971, 1976; Cochrane and Koffler, 1973). In contrast, if the antigen remains for a long time in the circulation or if there is a continuous supply of antigenic material for protracted periods of time, as in chronic infections or with autoantigens, the potential exists for continuing IC formation. The closest experimental approximation to such chronic IC disease in humans is "chronic" serum sickness (Dixon, 1971; Cochrane and Dixon, 1978; Wilson and Dixon, 1976; Andres *et al.*, 1963; Germuth and Rodriquez, 1973).

Apart from the nature of the antigen and of the antibody as well as their molar ratio in the complexes, the fate of circulating ICs is dependent on the state of the host's phagocytes, both circulating leukocytes and reticuloendothelial system. In experimental animals with serum sickness, more than 99% of the ICs are eliminated by phagocytes, predominantly by the liver's Kupffer cells, leaving less than 1% of the ICs capable of producing disease (Wilson and Dixon, 1971). Overload of this system (Benacerraf *et al.*, 1959a; Cochrane and Koffler, 1973; Kijlstra *et al.*, 1978; Haakenstad and Mannik, 1974) or possible blockade of Fc and C receptors on mononuclear phagocytic cells by anticellular autoantibodies may be important factors in precipitating an IC disease. It is known that attachment of ICs to macrophages is promoted by C, but ingestion requires an intact Fc region of the IgG in the complex (Mantovani *et al.*, 1972). If the class or subclass of antibody in the IC is such that it does not fix C or does not interact with cellular Fc receptors (see Section III, B), then ICs may not be eliminated by the phagocytic system and thus may be deposited in tissues

and cause injury. However, it should be noted that the rate of removal of large-latticed ICs made *in vitro* and injected into rabbits is not dependent on an intact C system, since animals depleted of C by cobra venom factor clear the injected ICs at a rate similar to that in animals normal in this respect (Arend and Mannik, 1971; Mannik *et al.*, 1971).

Mechanisms for the deposition of circulating ICs in tissues and the ensuing inflammatory reactions have been the subject of research for many years and they have been reviewed previously (Dixon, 1963, 1971; Cochrane and Koffler, 1973). Cochrane and associates (Cochrane, 1963; Kniker and Cochrane, 1968) as well as others (Benacerraf *et al.*, 1959b; Waalkes and Coburn, 1959) demonstrated in experimental animals that release of vasoactive amines and increased vascular permeability are prerequisites for IC deposition and that ICs of 19 S or larger are necessary for the induction of vasculitis (Cochrane and Hawkins, 1968; Wilson and Dixon, 1971). In acute experimental serum sickness of the rabbit, a leukocyte-dependent, C-independent mechanism helps initiate the deposition of ICs in tissues (Henson and Cochrane, 1971). This mechanism comprises the degranulation of basophils sensitized with IgE antibody and antigen, subsequent release of a platelet-activating factor (PAF), and aggregation of and release of mediators such as vasoactive amines from platelets (Benveniste *et al.*, 1972; Henson, 1976a; Henson and Oades, 1976). PAF is a low molecular weight phospholipid (Benveniste *et al.*, 1977; Kater *et al.*, 1975). While similar evidence is not yet forthcoming for man, the detection of PAF in human basophils (Benveniste, 1974; Benveniste *et al.*, 1975) implies that similar mechanisms may be implicated in the deposition of ICs in humans. Increased permeability may also be mediated by the anaphylatoxins C3a and C5a produced after C activation by ICs or by C-activating lysosomal enzymes released from neutrophils after their interaction with ICs. Vascular permeability induced by C3a and C5a may be mediated secondarily by their direct interaction with basophils and mast cells followed by release of PAF (Camussi *et al.*, 1977). Release of vasoactive amines from platelets may also occur after the direct interaction of ICs with the platelets' Fc or C receptors as well as after direct or indirect lysis of platelets by IC-induced C activation (Henson and Cochrane, 1971). Depletion of platelets, the principal reservoir of vasoactive amines, prevents the development of IC disease in the rabbit (Kniker and Cochrane, 1968).

The inflammatory response evoked by ICs deposited along vascular basement membranes is dependent in large part on the biologic activities produced by C activation, in particular, by fragments of C3 and

C5. These activities include the phenomena of chemotaxis and immune adherence. Immune adherence is that process in which ICs containing C3b bind to specific receptors on various cell types, among them neutrophils (see Section III,B), resulting in phagocytosis. The influx of inflammatory cells is promoted both by chemotactic factors (C5a) and by anaphylatoxins (C3a, C5a) that directly attract neutrophils and degranulate mast cells. The resultant tissue injury is induced by the action of lysosomal enzymes released from neutrophils (Dixon, 1971; Cochrane and Dixon, 1978). Although this sequence seems to be the general mechanism of IC-induced tissue injury in experimental animals, it should be noted that, while depletion of C by cobra venom factor or depletion of neutrophils from these animals averts the development of necrotizing arteritis, glomerulonephritis can still develop (Henson and Cochrane, 1971).

IC deposition seems to depend greatly on the anatomy of particular sites of circulation. The glomerulus, choroid plexus, synovium, skin, and uveal tract have in common a high degree of blood flow per unit mass of tissue and the related potential to trap large quantities of ICs in their vascular walls. As a consequence, these sites are at high risk in IC-associated disorders (Cochrane and Koffler, 1973; Wilson and Dixon, 1976).

III. Biologic Properties of Immune Complexes

Upon specific combination with antigens, antibody molecules usually take on new biologic functions that they do not have in the free state—for example, an increased ability to interact with serum components such as C proteins and with cellular Fc and C receptors.

A. Interaction of Immune Complexes with Complement

Uncomplexed monomeric human IgG and IgM may bind the first component of C (C1) although the reaction is weak and reversible (Augener *et al.*, 1971; MacKenzie *et al.*, 1971; Müller-Eberhard and Calcott, 1966). It is generally agreed that efficient activation of C requires nonspecific aggregation of Ig molecules or complexing of antibody with antigen. However, there is little agreement in the literature about the molecular composition of the smallest IgG-type ICs able to activate C; some advocate the doublet Ab_2Ag_1 (Cohen, 1968), others claim no less than a tetramer of antibody (Hyslop *et al.*, 1970), whereas still others have shown activation of the classical pathway of C by a single IgG molecule binding a monovalent hapten (Goers *et al.*, 1977). In general, it would appear that fixation of C by soluble ICs made with

IgG antibody requires a lattice structure containing more than two or three antibody molecules (Mannik *et al.*, 1971). Dose-response curves indicate that single molecules of pentameric IgM are capable of binding C, but nonspecific aggregates or IgM-antigen complexes bind C1 more avidly (Borsos and Rapp, 1965; Ishizaka *et al.*, 1968; Metzger, 1970, 1974; MacKenzie *et al.*, 1971; Augener *et al.*, 1971; Plaut *et al.*, 1972.

ICs or Ig aggregates of appropriate size can activate both the classical and the alternative C pathway (Müller-Eberhard, 1975; Götze and Müller-Eberhard, 1976; Austen, 1974; Ruddy, 1974; Spiegelberg, 1974; Osler and Sandberg, 1973; McLean *et al.*, 1975; Gigli, 1976; Rodrick *et al.*, 1978; Porter and Reid, 1978). Aggregates or ICs containing antibodies of the IgG and IgM variety activate the classical C pathway by combining with the recognition unit, C1q, of this pathway's first component, C1. Activation of the classical C pathway may be followed by activation of the alternative C pathway secondarily via the C3b amplification loop (Götze and Müller-Eberhard, 1976). Human myeloma antibodies of the IgG_1, IgG_2, and IgG_3 subclasses can efficiently activate C via their Fc portion, but IgG_4 is a very poor activator (Ishizaka *et al.*, 1967; Spiegelberg, 1974; Ruddy, 1974; Gigli, 1976). Unfortunately, no one has identified the amino acid sequences that are present in IgG subclasses capable of interacting with C1 but absent in those incapable of such interaction. The primary structure of the C_H2 region (N terminal half of the Fc) of human IgG_1, which is known to carry the binding site for C1q in this subclass (Ellerson *et al.*, 1972; Kehoe *et al.*, 1974; Yasmeen *et al.*, 1976) is more than 90% homologous with the corresponding region of human IgG_4, which does not fix C. One might suppose, therefore, that the few differences would be related to the ability to interact with C1q. This has not proved to be the case (Kehoe *et al.*, 1974). Schur and Christian (1964) and Isenman *et al.* (1975) observed that reduction of the inter-heavy chain disulfides in rabbit antibody destroyed its ability to interact with C. Furthermore, Isenman *et al.* (1975) found that Fc fragments from IgG_1 and IgG_4 (in contrast to the intact IgG_4 molecule) fixed C1q with a comparable affinity, and that Fc-mediated C fixation was insensitive to the presence of intact interchain disulfides. These data taken together suggested to the investigators that quaternary interactions with other regions of the molecule (i.e., Fab) may modulate the activity of the C1q-binding site. In other words, although certain Ig subclasses or other substances may be endowed with C1q binding sites, these sites are prevented from reacting by local folding patterns or interaction with a one-dimensionally distant region of the molecule. The C_H4 do-

main appears to represent the major portion of the C1q binding site in IgM (Hurst *et al.*, 1974; Shimizu *et al.*, 1971). Johnson and Thames (1976), who used chemically modified human IgG_1, suggested that the amino acid residues, tryptophan, tyrosine, and arginine, are important for C fixation. Furthermore, by the use of synthetic peptides, they showed that the area surrounding the tryptophan and tyrosine at positions 277 and 278 probably constitutes the C-fixing site of human IgG_1 and that the tryptophan at position 488 may be the C-fixing site of human IgM. However, a synthetic peptide mimicking the sequence 487–491 of human IgM was 200 times less effective in C-fixation than human IgG1.

C1q occurs in serum as a weak association of C1q with a tetrameric complex of $C1r_2$-$C1s_2$ that is formed in the presence of Ca^{2+} (Lepow *et al.*, 1963; Gigli *et al.*, 1976). As stated above, interaction of C1 with aggregated antibody is through C1q (Müller-Eberhard and Kunkel, 1961), which binds in the absence of $C1r_2$-Ca^{2+}-$C1s_2$ to the second constant domain of IgG. The $C1r_2$-$C1s_2$ complex binds to C1q on the antibody through C1r (Ziccardi and Cooper, 1976a,b). The C1r serves both as a physical link between C1q and C1s and as the activator of C1s. However, C1s alone does not interact with antibody (Ziccardi and Cooper, 1976a,b). C1q is a 400,000 molecular weight glycoprotein with an unusual amino acid composition, similar to that of collagen (Calcott and Müller-Eberhard, 1972; Yonemasu *et al.*, 1971; Porter and Reid, 1978). The C1q molecule, as seen by electron microscopy, resembles a bunch of six tulips with the lower half of the stems held together and the upper half branching into six separate stems and ending in a globular head (Svehag *et al.*, 1972; Shelton *et al.*, 1972; Knobel *et al.*, 1975). Binding studies have shown that the valence of C1q for IgG and IgM is either 5 or 6 (Valet and Cooper, 1974; Gigli, 1976; Ruddy, 1974; Müller-Eberhard, 1969; MacKenzie *et al.*, 1971). The binding sites of C1q for Ig may be located in the noncollagen-like portion of the molecule, which corresponds to the peripheral globular structures (Reid and Porter, 1975; Knobel *et al.*, 1975; Hughes-Jones and Gardner, 1979). Once ICs or Ig aggregates bind to these globules of C1q, and possibly to the central subunit of C1 (Goers *et al.*, 1977), activation of the full C cascade ensues as described by Müller-Eberhard and associates (Müller-Eberhard, 1975; Müller-Eberhard, 1976). Basically, C1q interacts via its collagen tails (Reid *et al.*, 1977) with the $C1r_2$-Ca^{2+}-$C1s_2$ complex resulting in the activation of C1r, perhaps by cleavage of a peptide bond, and then $C\bar{1}r$ proteolytically cleaves and activates the C1s. Subsequently, the $C\bar{1}s$ acquires the ability to activate the next components in the classical pathway, C4

and C2 (Müller-Eberhard, 1975). Via the action of the $C\bar{1}$ inactivator, the $C\bar{1}r$ and $C\bar{1}s$ are released whereas the C1q remains attached to the complex (Ziccardi and Cooper, 1979). The activated C4 binds to the Fab portion of the antibody (Chan and Cebra, 1968) and interacts with activated C2 molecules to form the C3 convertase, $C\overline{42}$. The $C\overline{42}$ convertase then splits C3 into C3a and C3b, and C3b associates to give $C\overline{423b}$, which changes in specificity from C3 activator to C5 activator.

Quantitatively, C consumption per molecule of antibody added is 1.5–2 times higher for antibody-coated cells than for Ig aggregates (Porter and Reid, 1978). The binding of C1 to the complexed antibody is extremely efficient (>95% in antibody excess) and activation is complete. However, it has been reported (Füst *et al.*, 1978) that ICs made *in vitro* or aggregates may behave differently as far as C1 fixation and ensuing classical C pathway activation is concerned, i.e., various complexes or aggregates may (*a*) fix C1 but not activate C; (*b*) activate C without much C1 fixation, or (*c*) fix C1 well and efficiently activate the C system. C4 is rapidly activated by Cls, but only a small fraction (<10%) is bound to aggregates or antibody-coated cells, the rest becoming inactivated in the serum (Müller-Eberhard *et al.*, 1966; Cooper and Müller-Eberhard, 1968). Binding of C4 to antibody-coated cells is 10–15 times more efficient than to Ig aggregates, but the number of C4 molecules that become effective in forming C3-convertase with C2 is only about twice as great (Porter and Reid, 1978). C3 binds to immune precipitates covalently via disulfide bonds (Czop, 1979), and polysaccharides present on Ig are the acceptor sites for C3b (Capel *et al.*, 1978). Although the mode of assembly is well characterized for C proteins on antibody-sensitized cells (Porter and Reid, 1978; Kolb and Müller-Eberhard, 1976), on soluble Ig aggregates or ICs the assembly sequence is far from clear.

In addition to activating the classical C pathway, certain IgG antibodies complexed with antigens on cell surfaces can specifically activate the alternative C pathway (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Ehrnst, 1977, 1978). This pathway is activated directly via C3 without the participation of C1, C4, and C2 (Götze and Müller-Eberhard, 1976). Other studies have revealed activation of the alternative C pathway by aggregated human IgE via its Fc portion (Ishizaka *et al.*, 1972a,b). In addition, aggregated human IgA myeloma proteins activate the alternative C pathway most efficiently (Spiegelberg and Götze, 1972; Götze and Müller-Eberhard, 1971; Boackle *et al.*, 1974). However, Burritt *et al.* (1977) have described activation of the classical C pathway by Fc fragments isolated by various procedures from IgA myeloma proteins; and B. D. Williams *et al.* (1976) have reported

activation of the classical C pathway by salivary anti-C3 antibodies of the IgA class after complexing with the C3 antigen. In contrast to the above studies, Colten and Bienenstock (1974) using anti-blood group A antibodies of the IgA class derived from human milk found no activation of either the classical or alternative C pathways. The pathways of C activation by various classes and subclasses of murine and guinea pig Ig have been reviewed by Spiegelberg (1974) and Götze and Müller-Eberhard (1976), and the C1q binding sites have been localized on the C_H2 domain not only for IgG from humans, but also from rabbits (Hill *et al.*, 1967; Allan and Isliker, 1974a,b; Ovary *et al.*, 1976), mice (Kehoe and Fougereau, 1969; Kehoe *et al.*, 1974), and probably guinea pigs (Tracey and Cebra, 1974). It should be noted that Ovary *et al.* (1976) and Allan and Isliker (1974a,b) presented evidence implying that regions in the C_H3 domain of IgG were also necessary for full anticomplementary activity.

Recently, Medicus (1978) showed that C3 consumption in serum caused by such classical C pathway activators as heat-aggregated human IgG (AHG) and ICs is strongly dependent on an intact alternative C pathway. No C3 is consumed in serum depleted of C4 (an essential component of the classical pathway) and factor D (an essential component of the alternative pathway), but 5–10% of C3 is consumed in C4-depleted or factor D-depleted serum and 30–50% in intact or fully reconstituted serum. In contrast, C3 consumption by particulate complexes, such as IgM antibody-sensitized sheep red blood cells, does not depend on the alternative C pathway. Radiolabeled factor B, properdin, C3, and C4 are taken up by AHG and ICs in a time-dependent manner. The maximum uptake per milligram of activator is: 0.1–1 μg of factor B, 0.2–0.4 μg of properdin, 5–25 μg of C3, and 1–2 μg of C4.

C appears to have dual effects on IC solubility. Thus, earlier studies showed increased precipitability of ICs after activation of the classical C pathway and fixation of C1q (Maurer and Talmage, 1953; Forster and Weigle, 1963). This phenomenon has been used to detect soluble ICs in biologic fluids (Agnello *et al.*, 1970). In contrast, partial solubilization of immune precipitates mediated by C present in fresh serum has been recently described by Nussenzweig and his associates (Miller and Nussenzweig, 1975; Czop and Nussenzweig, 1976; Takahashi *et al.*, 1976, 1977). The solubilization is mediated by the alternative C pathway, since factor B, factor D, properdin, C3, and Mg^{2+} must be present, and seems to result from intercalation of C3b into the immune precipitates and formation of a properdin and factor B-dependent C3 convertase. The classical C pathway in itself is incapable of inducing

solubilization of immune precipitates, possibly because the assembled classical C3 convertase ($C\overline{42}$), in contrast to the properdin- and factor B-dependent C3 convertase ($\overline{C3b, P, B}$), has very short half-life and therefore binds too few C3b molecules to disrupt the antigen–antibody lattice. Although the classical pathway cannot solubilize aggregates by itself, it greatly enhances the solubilizing activity of the alternative pathway (Takahashi *et al.*, 1978). According to this same study, nascent C5–9 seems to lack binding affinity for immune aggregates, unlike nascent C3b and C4b. The role of this phenomenon in immunobiology is not clear although participation of the alternative C pathway may be important in the resolution of ICs deposited in the kidney after experimental acute serum nephritis develops (Bartolotti and Peters, 1978).

The mechanisms by which antigen binding to antibody triggers C fixation remains undeciphered at the molecular level (Metzger, 1974). One hypothesis on this subject—that a conformation change within antibody combined with antigen accounts for the newly expressed effector function—has received little experimental support (Steiner and Lowey, 1966; Cathou *et al.*, 1968; Ashman *et al.*, 1971). Another suggestion is that the aggregation of IgG molecules, which takes place after antibody combines with polyvalent antigens, incites the effector function. Strong evidence for this hypothesis comes from the observation that polyvalent antigens can trigger C fixation, whereas monovalent ones cannot, and that nonspecific aggregation of Ig (from heat, chemical means, etc.) is as effective as antigen-involved aggregation (Müller-Eberhard, 1968; K. Ishizaka and Ishizaka, 1960; T. Ishizaka and Ishizaka, 1959). Recent evidence that conformational changes occur in Fab and Fc parts of the antibody molecule as a result of antigen binding came from measurements of circularly polarized fluorescence (Givol *et al.*, 1974; Schlessinger *et al.*, 1975; Jaton *et al.*, 1975). However, Jaton *et al.* (1976), who used ICs formed between rabbit antibody and oligosaccharides of increasing size, demonstrated that changes in the conformation of the Fc part of the antibody molecule were insufficient per se for triggering C fixation.

As a result of C activation by ICs, the following biologic activities are generated and mediated either by the IC-fixed C components or by activated C components and their fragments in fluid phase (Table II).

1. Immune adherence: ICs with fixed C components bind to the surfaces of C3b-C4b receptor-bearing cells, e.g., lymphocytes, neutrophils, macrophages, and primate erythrocytes (Gigli and Nelson, 1968; Cooper, 1969; Nussenzweig, 1974; Ross *et al.*, 1973; Ross and

TABLE II
BIOLOGIC ACTIVITIES GENERATED BY IC-INDUCED C ACTIVATION

Activities	Mediators
Immune adherence	C3b, C4b
Chemotaxis	C5a
Anaphylaxis	C3a, C5a
Direct and indirect cytotoxicity	C56789
Leukocytic response	C3e
Macrophage activation	Bb
IC solubilization	C3b
Release of cell-bound ICs	C3b

Polley, 1975; Theofilopoulos *et al.*, 1974a,b; Theofilopoulos, 1977). Binding of ICs to C3b-C4b immune adherence receptors may trigger phagocytosis, the release of biologically active substances from phagocytic cells and lymphocytes (Mackler *et al.*, 1974; Sandberg *et al.*, 1975; Wahl *et al.*, 1974; Koopman *et al.*, 1976), and lymphocyte activation (Hartmann and Bokisch, 1975), as well as cellular interactions that may play a role in immune responses (Dukor *et al.*, 1974; Pepys, 1974a, 1976; Dierich and Landen, 1977).

2. Chemotaxis: Leukocytes migrate provoked by C5a and possibly the $\overline{C567}$ complex (Hugli and Müller-Eberhard, 1978). Although it was once thought that C3a also exerts chemotactic activity (Ward *et al.*, 1965), recent experiments by Taylor *et al.* (1977) and Fernandez *et al.* (1978) have clearly refuted these earlier findings and suggested that the observed chemotactic activity of C3a and possibly $\overline{C567}$ was supplied by minute contaminating amounts of C5a.

3. Anaphylaxis: Anaphylatoxin activity occurs via the binding of C3a and C5a on mast cells, basophils, and possibly other cells followed by the release of vasoactive and spasmogenic amines (reviewed by Becker and Henson, 1973; Hugli and Müller-Eberhard, 1978).

4. Direct and indirect cytotoxicity: Cells can be lysed either directly by the assembly of the terminal attack sequence of C following C activation by cell-bound ICs, or indirectly in a bystander fashion after activation of C by ICs in fluid phase (Thompson and Lachmann, 1970).

5. Leukocytic response: Characteristically factors released during C activation induce release of leukocytes from bone marrow (Rother, 1972). Recently, Ghebrehiwet and Müller-Eberhard (1979) demonstrated both *in vivo* and *in vitro* that this activity resides in a small fragment of C3, designated as C3e. Others have observed *in vivo* neu-

trophilia after activation of the C system by endotoxin or ICs (McCall *et al.*, 1974; Andrews, 1910).

6. Activation of macrophages: Rapid spreading of murine peritoneal macrophages, an early parameter of the activated state, may be induced by agents that initiate activation of the alternative C pathway (Bianco *et al.*, 1976). This phenomenon, also evident with human macrophages, is mediated by the $\overline{Bb}$ fragment of factor B of the alternative pathway (Götze *et al.*, 1979) and may play a significant role in the accumulation of macrophages in sites of inflammation. Moreover, macrophages release lysosomal enzymes after interacting with soluble C3b (Schorlemmer *et al.*, 1976).

The participation of the C system in the immunopathology associated with IC-disease is unquestionable (Dixon, 1971; Cochrane and Koffler, 1973; Wilson and Dixon, 1976). However, as stated above, C may not always be necessary for the induction of certain lesions characteristic of IC disease.

B. Interaction of Immune Complexes with Cells

Particulate as well as soluble ICs, especially those with extensive lattice structures, can activate or inactivate a variety of cells by interacting with various surface receptors, such as Fc and C receptors and antigen receptors. Table III lists the Fc and C receptors of various human cell types.

1. Erythrocytes

In 1953 Nelson observed that *Treponema pallidum* incubated with corresponding antibody and C adheres to human erythrocytes. This phenomenon, designated as immune adherence, has been defined as the specific attachment of primate erythrocytes to ICs with fixed C3b (Gigli and Nelson, 1968). However, as discussed below, immune adherence (C3b) receptors are present on the surfaces of many other cell types. In addition to C3b-mediated immune adherence, Cooper (1969) has described its mediation by C4b; in fact, receptors for C3b and C4b on the red cell surface are the same (Ross *et al.*, 1973). Since there are no obvious biologic functions that are unique to primate erythrocytes by virtue of their having C3b-C4b receptors, this receptor may have no critical role. Although immune adherence of sensitized organisms to erythrocytes enhances their phagocytosis by neutrophils *in vivo* (Nelson, 1956; Robineaux and Pinet, 1960), erythrocytes bind to soluble ICs much less efficiently *in vivo* than *in vitro*, since in the former situation the ICs can be released continuously from the red cell

TABLE III

RECEPTORS FOR FC AND C ON HUMAN BLOOD CELLS

Cell type	Fc receptors				C receptors						
	IgG	IgM	IgA	IgE	C1q	C3a	C3b-C4b	C3bi	C3d	C5a	C5b
Erythrocytes	–	–	–	–	–	–	+	–	–	–	–
Platelets	+	–	–	–	+	–	–	–	–	–	–
Neutrophils	+	–	+	–	–	+	+	+	+[a]	+	?[b]
Macrophages	+	–	–	–	–	?	+	+	+[a]	?	–
Eosinophils	+	–	–	?	–	+	+	–	+	+	–
Basophils-mast cells	+	–	–	+	–	+	?	–	–	+	–
B lymphocytes	+	+	+	+	+	–	+	–	+	–	+[c]
T lymphocytes	+	+	+	+	–[d]	–	–[e]	–	–[e]	–	–

[a] Recent findings suggest that neutrophils and macrophages do not have C3d (CR_2) receptors.

[b] Not formally shown.

[c] Receptors for C5b are present on certain human lymphoblastoid (Raji) cells. The receptors for C5b have been found to be identical to C3b receptors (Landen and Dierich, 1979).

[d] It was originally suggested that both B and T cells have C1q receptors. However, recent studies indicate that C1q receptors are present only on B cells.

[e] C3 receptors have occasionally been described on T-type lymphoblasts.

surface by plasma factors such as C3b inactivator and β_1H that convert C3b and C4b to C3d and C4d (see Section III,B,3). It has been established that red cells do not have C3d or C4d receptors (Nussenzweig, 1974; Ross *et al.*, 1973).

2. *Platelets*

Human platelets bear receptors for the Fc portion of all subclasses of human IgG, but not of the other Ig isotypes (Henson and Spiegelberg, 1973; Pfueller and Lüscher, 1972; Israels *et al.*, 1973). Human platelets, in contrast to nonprimate platelets, are devoid of immune adherence (C3b-C4b) receptors (Müeller-Eckhardt and Lüscher, 1968; Henson and Cochrane, 1969; Henson, 1969, 1970; Becker and Henson, 1973). In fact, fixation of C by ICs *in vitro* inhibits their binding to platelets' Fc receptors (Henson and Spiegelberg, 1973; Pfueller and Lüscher, 1974). It has been claimed that human platelets have receptors for C1q (Suba and Csako, 1976; Cazenave *et al.*, 1976; Wautier *et al.*, 1976), and recent experiments by Wautier *et al.* (1977) have indicated that C1q bound to AHG promotes platelet aggregation. This is done not via a classical receptor, but by interaction of C1q with C1s produced by and present on the platelet surface. These authors suggest that C1q may be attached to the Fc fragment of antibody in the IC by its globular portion and to C1s on platelets by its collagen-like moiety.

Platelets interacting with ICs of appropriate size (>19 S) aggregate and release various constituents, such as serotonin, permeability factors, and nucleotides (Movat *et al.*, 1965; Penttinen *et al.*, 1969, 1971; Pfueller and Lüscher, 1972; Osler and Siraganian, 1972; Becker and Henson, 1973), that play an important role in the pathogenesis of IC disease as indicated above. Some experiments indicate that metabolic energy and possibly Ca^{2+} are required for IC-induced platelet aggregation (Mustard and Packham, 1970). Infusing ICs made *in vitro* into experimental animals has shown that thrombocytopenia occurs only when the infused ICs contain polyvalent antigens, not when they contain monovalent ones (Kekomaki *et al.*, 1977).

3. *Neutrophils*

Among the various known receptors on human neutrophils, the most important are those for the Fc portion of IgG and for the fragments of C3, C4, and C5. As shown by rosette techniques with sensitized red cells and by release of various cellular enzymes, neutrophils bind aggregates of all subclasses of human IgG, with IgG_1 and IgG_3 slightly more efficiently bound than IgG_2 or IgG_4 (Messner and Jelinek, 1970;

Spiegelberg, 1974; Lawrence *et al.*, 1975; Henson *et al.*, 1972; Henson, 1976b; Anwar and Kay, 1977a). These cells also seem to bind aggregated IgA myeloma proteins, but it is not known whether the IgA Fc receptors are the same as or different from the IgG Fc receptors (Lawrence *et al.*, 1975; Wilton, 1978). No other Ig isotype binds to these cells (Henson *et al.*, 1972; Lawrence *et al.*, 1975). The Fc portion of IgG, or more specifically the C_H3 domain, mediates the binding of IgG to neutrophils (MacLennan *et al.*, 1974; Messner and Jelinek, 1970). However, Spiegelberg (1975) using a myeloma half-molecule concluded that binding sites in both the C_H2 and C_H3 regions might be involved. It is not yet clear whether the binding of complexed or aggregated IgG to leukocytes results from cooperation among Fc receptors on a number of adjacent IgG molecules that exponentially increases adherence of the whole complex or from a configurational change in the Fc portion upon aggregation or fixation to antigen (Phillips-Quagliata *et al.*, 1969; Kazmierowski *et al.*, 1971; Henson, 1976b). The binding of ICs to neutrophils' Fc receptors is independent of Ca^{2+}, and the receptors are resistant to both trypsin and neuraminidase (Lay and Nussenzweig, 1968).

Apart from the Fc receptors, neutrophils also have receptors for fragments of C3, C4, and C5 (Nussenzweig, 1974; Theofilopoulos, 1977; Gigli and Nelson, 1968; Ross *et al.*, 1973, 1978; Eden *et al.*, 1973a; Rabellino *et al.*, 1978a,b; Anwar and Kay, 1977a). Antibody-sensitized sheep red blood cells mixed with fresh serum fix C4b and C3b that are rapidly cleaved by certain plasma regulator C proteins, namely, the C3b inactivator (C3bINA), or conglutinogen activating factor (Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1971; Cooper, 1975; Shiraishi and Stroud, 1975), and the β_1H, or accelerator of C3bINA (Whaley and Ruddy, 1976). Cleavage of C3b yields the respective c and d fragments, the d fragment remaining on the complex.

Contrary to previous accounts cited above, recent evidence indicated that C3bINA in itself cannot cleave C3b or C4b but requires the presence of β_1H (Pangburn *et al.*, 1977; Whaley and Thompson, 1978). Furthermore, Pangburn *et al.* (1977) found that, whereas C3bINA and β_1H combined can cleave C4b to C4c and C4d, an additional, unidentified, trypsinlike serum protein must be present to cleave C3b into C3c and C3d. Whether such interactions between particulate complexes and serum regulatory proteins also take place with soluble C-fixing ICs remains to be determined. C4b bound to ICs may be protected from the action of the inactivators via the interaction either with C2 or with the C4b-binding protein (Cooper, 1975; Ferreira *et al.*,

1977; Scharfstein *et al.*, 1979). IC-bound C3b may also circumvent inactivation by interacting with properdin and factor B of the alternative C pathway (Fearon and Austen, 1975; Schreiber *et al.*, 1975; Medicus *et al.*, 1976). At any rate, studies by many investigators (Ross *et al.*, 1973, 1978; Ross and Polley, 1975; Eden *et al.*, 1973a; Theofilopoulos *et al.*, 1974a,b; Theofilopoulos, 1977; Nussenzweig, 1974) have established the presence of two types of C receptors, designated by Ross as CR_1 and CR_2, on neutrophils and on other cells, such as macrophages, eosinophils, and lymphocytes (see below). Recently, Ross and Rabellino (1979) described a third type of C receptors (CR_3) that are specific for a site in C3bi that is between the c and d regions (C3e?) of the C3b. CR_1 receptors, or immune adherence receptors, fix soluble or IC-bound C3b or C4b. CR_2 receptors bind soluble or IC-bound C3d. If the IC-bound C3b is not cleaved by the regulatory proteins, then it may bind to cells' C3b receptors via the C3c portion and to cells' C3d receptors via the C3d portion. C4b binds to cells only via the C3c receptors (immune adherence), whereas the C4b cleavage products, C4c and C4d, are both believed to be unreactive with C receptors (Bokisch and Sobel, 1974). C3d binds to cells only via the CR_2 or C3d receptors.

It was originally believed (Eden *et al.*, 1973a; Ross *et al.*, 1973) that human neutrophils have only immune adherence receptors (C3b-C4b), but subsequent studies (Anwar and Kay, 1977a; Rabellino *et al.*, 1978b; Ross *et al.*, 1978) indicate that a subpopulation of these cells also has receptors for C3d and that expression of C3b-C4b or C3d receptors alone or in combination relates to various stages of maturation of the neutrophil. Thus, Ross *et al.* (1978) have described human neutrophils on which the following C receptor makeup defines potential stages of maturation of neutrophils: Ia-like antigens → C3d receptors → C3b-C4b receptors + C3d receptors → C3b-C4b receptors. Thus, C3d receptors are present only on immature neutrophils, which constitute approximately 20% of the peripheral blood neutrophils, whereas C3b-C4b immune adherence receptors are the only C receptors on mature neutrophils. In contrast to human neutrophils that lose their C3d receptors upon terminal maturation, murine neutrophils retain both the immune adherence (C3b-C4b) and the C3d receptors even at terminal maturation (Rabellino *et al.*, 1978b). The conclusion that neutrophils have receptors for C3d (CR_2) was challenged by Ross and Rabellino (1979). These investigators found that neutrophils actually lack receptors for C3d but do have receptors for C3b (CR_1) and for C3bi (CR_3). The receptors for C3bi are undetectable on mature neutrophils and this is due not to the loss of CR_3 but to the acquisition of

surface elastase that converts C3bi to C3d. The presence of C5b receptors on neutrophils is also possible, since opsonization of yeast particles for phagocytosis by neutrophils was defective when sera from mice deficient in C5 or serum from a patient with abnormal C5 were used for opsonization (Shin *et al.*, 1968; Nilsson *et al.*, 1974; Miller and Nilsson, 1974). However, such a receptor has not been formally shown. Furthermore, neutrophils have approximately 1 to 3 $\times$ 10^5 surface C5a binding sites per cell (Chenoweth and Hugli, 1978), and these cells respond chemotactically to C5a (Hugli and Müller-Eberhard, 1978). Evidently, the bulk of the C5a molecule is required for binding to neutrophils, but the COOH-terminal five residues of the molecule seem to play a critical role in governing receptor–ligand interactions and in eliciting the cellular response. C3a does not inhibit the binding of C5a to the receptor, but separate receptors for C3a do exist on neutrophils (Glovsky *et al.*, 1979).

A variety of neutrophil functions can be stimulated through the Fc, C3b-C4b, C3bi, C3a, and C5a receptors. These functions include phagocytosis and secretion of lysosomal enzymes that produce tissue injury, enhance glucose oxidation and intensify metabolism (Henson and Oades, 1975; Becker *et al.*, 1974; Goetzl and Austen, 1974), release prostaglandins (Zurier and Sayadoff, 1975), H_2O_2 (Root *et al.*, 1975), etc. Large ICs stimulate phagocytosis and neutrophil secretion better than the small ones (Henson, 1971a,b), and release of lysosomal enzymes follows the interaction of neutrophils either with phagocytosable soluble ICs or with nonphagocytosable surface-bound ICs (Henson, 1971a,b; Hawkins, 1971; Becker and Henson, 1973). However, surface-bound nonphagocytosable aggregates are more than 500 times as active in inducing secretion as aggregates in suspension (Henson, 1976b). This finding might indicate that ICs adhering *in vivo* to membranes, such as GMBs, synovial membranes, and blood vessel walls, might be especially potent in inducing release of injurious enzymes from neutrophils. The mechanism of secretion is different after neutrophils interact with phagocytosable ICs and with nonphagocytosable ICs (Henson, 1971b).

The contributions of IgG Fc and C receptors in phagocytosis of ICs are not yet clearly elucidated. Mantovani (1975) using murine neutrophils and Scribner and Fahrney (1976) using human neutrophils indicated that C receptors are primarily involved in the attachment phase of particulate complexes to cells, whereas participation of the IgG Fc receptor is necessary for inducing the mechanism of phagocytosis. However, the role of these receptors in phagocytosis of soluble ICs has not yet been explored.

4. *Macrophages*

The plasma membranes of human mononuclear phagocytes (macrophages) contain externally disposed receptors for the Fc portion of IgG_1 and IgG_3 subclasses, but not for IgG_2, IgG_4, or any other Ig isotypes (LoBuglio *et al.*, 1967; Huber and Fudenberg, 1968; Huber *et al.*, 1971; Hay *et al.*, 1972; Spiegelberg, 1974; Lawrence *et al.*, 1975; Dorrington, 1976). Experiments by Rhodes (1973) have suggested the presence of Fc receptors for monomeric (7 S), but not pentameric, IgM on guinea pig macrophages in addition to the IgG Fc receptors; Capron *et al.* (1975) and Joseph *et al.* (1977) found IgE Fc receptors in addition to IgG Fc receptors on rat macrophages. Human IgG_1 and IgG_3 bind to macrophages cytophilically in monomeric form. IgG_2 and IgG_4, as stated above, do not usually bind cytophilically to macrophages but may do so when aggregated (Spiegelberg, 1974). Similarly, Fc receptors on macrophages of mice bind monomeric IgG_{2a} and to a lesser extent IgG_{2b} but not IgG_1, and Fc receptors of guinea pigs bind IgG_2 but not IgG_1 (Berken and Benacerraf, 1966; Dorrington, 1976; Unkeless and Eisen, 1975; Dissanayake and Hay, 1975). It is of interest that a similar distribution of classical C-fixing activity exists among all the subclasses of IgG, although the two effector functions are mediated by different regions of Fc. Early studies showed that the effector site for macrophage cytophilic binding of IgG is within the Fc region (Liew, 1971; Arend and Mannik, 1972). Now the binding site for the Fc receptor has been localized to the C_H3 region (Yasmeen *et al.*, 1976; Okafor *et al.*, 1974) and more specifically to a decapeptide sequence in the C_H3 disulfide loop (Ciccimarra *et al.*, 1975).

Binding of IgG antibody-sensitized red cells with macrophages, in contrast to that of monomeric IgG, requires metabolically active macrophages, an intact cytostructure, and suitable environmental conditions, such as appropriate temperature and concentrations of cations (MG^{2+}, Ca^{2+}) (Passwell *et al.*, 1978). Some heterogeneity exists within a single population of monocytes in respect to their affinity for IgG, and there are some indications that receptor expression and affinity may increase with cell activation (Rhodes, 1975; Arend and Mannik, 1973; Zuckerman and Douglas, 1979). Moreover, it was originally suspected and later convincingly shown by binding assays and sensitivity to various enzymes that the receptors on murine macrophages for IgG_{2a} and for IgG_{2b} subclasses are different (Walker, 1976; Unkeless, 1977; Heusser *et al.*, 1977; Anderson and Grey, 1978; Diamond *et al.*, 1978). As yet, the existence of separate receptors for IgG_1 and IgG_3 on human macrophages has not been shown, but such separation could be functionally significant. For example, if human IgG_1 and IgG_3 did not

have separate receptors, the IgG_1, which is much more highly concentrated in serum (about 10-fold), would occupy the majority of receptors. In contrast, a separate receptor for IgG_3 would ensure that antibodies of this subclass have appropriate access to macrophage effector mechanisms. It should be emphasized that the affinity of the Fc receptors for ICs made with polyvalent antigens and of large lattice structure is much greater than for monomeric IgG (Phillips-Quagliata *et al.*, 1971; Arend and Mannik, 1973; Heusser *et al.*, 1977; Diamond *et al.*, 1978), and the possibility that there may be different receptors for antigen-associated polymeric IgG and monomeric IgG has been considered (Walker, 1976; Heusser *et al.*, 1977; Anderson and Grey, 1978). In general, it has been concluded that murine macrophages have two types of Fc receptors, namely FcR1 for monomeric or aggregated IgG_{2a} and FcR2 for IgG_{2b} aggregates or ICs. The FcR1 is trypsin-sensitive, whereas the FcR2 is trypsin-resistant (Unkeless, 1977; Heusser *et al.*, 1977; Walker, 1976; Unkeless and Eisen, 1975; Unkeless *et al.*, 1979). Moreover, it has been suggested that the two Fc receptors on murine macrophages may mediate different functions. For example, in one study the Fc receptors for IgG_{2a} mediated phagocytosis, whereas the Fc receptors for IgG_{2b} were responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) (Walker, 1977). The movement and capping of IgG Fc receptors on the surfaces of macrophages after interaction with particulate ICs *in vitro* have been described (Romans *et al.*, 1976; Thrasher *et al.*, 1975).

Apart from the IgG Fc receptors, macrophages have surface receptors for fragments of C3 and C4. The receptors for C3b on macrophages were demonstrated by Lay and Nussenzweig (1968), Huber *et al.* (1968), and Henson (1969). These authors observed that IgM antibody-sensitized red cells cannot bind to macrophages unless the red cells first interact with fresh serum containing C3. Subsequent experiments indicated that macrophages can bind, via their C3b receptors, sensitized red cells bearing C4b (Ross and Polley, 1975). Moreover, receptors for C3d are also present (Wellek *et al.*, 1975; Reynolds *et al.*, 1975; Rabellino and Metcalf, 1975). The loss of these C3d receptors on murine macrophages is associated with their terminal differentiation into activated cells (Rabellino *et al.*, 1978b; Griffin *et al.*, 1975). As in the case of neutrophils, recent findings by Ross and Rabellino (1979) indicate that macrophages actually lack C3d receptors, but they have C3b (CR_1) and C3bi (CR_3) receptors.

The role of the IgG Fc and C3 receptors in the attachment and phagocytosis of ICs by macrophages has been examined by various investigators. IgG Fc receptors mediate both binding and ingestion of

particulate complexes, whereas C3b and C3d receptors mediate binding, but not ingestion, of C3-coated antibody-sensitized particles (Mantovani *et al.*, 1972; Gordon and Cohn, 1973; Griffin *et al.*, 1974, 1975; Bianco *et al.*, 1975; Ehlenberger and Nussenzweig, 1977). In addition, IgG, C3b, and C3d bound to opsonized particles have a synergistic effect in inducing phagocytosis as shown by Ehlenberger and Nussenzweig (1977), who observed that erythrocytes opsonized with 500 molecules of C3b or C3d and 600 molecules of IgG were ingested in much greater amounts by macrophages than erythrocytes opsonized with 10,000 molecules of IgG without C3. However, Wellek *et al.* (1975) have shown phagocytosis of IgM-sensitized red cells bearing C3d by guinea pig macrophages, and Griffin *et al.* (1974) and Bianco *et al.* (1975) found that activated murine peritoneal macrophages induced by thioglycolate irritation of the peritoneal cavity could endocytose IgM sensitized-C3b-bearing red cells, but native nonactivated peritoneal macrophages could not, as cited above. Phagocytosis of opsonized particles via IgG Fc and C3 receptors seems to be a different process than that of nonopsonized particles such as zymosan (Michl *et al.*, 1976), since inhibition of phagocytosis of the former by 2-deoxyglucose does not inhibit phagocytosis of the latter. Recently, Van Snick and Masson (1978) and Kijlstra *et al.* (1979) demonstrated, first, that C markedly assists mouse and guinea pig peritoneal macrophages to ingest soluble ICs containing IgG antibodies and, second, that soluble heat-aggregated IgM is ingested in significant amounts in the presence of C. These findings indicate that the endocytosis of soluble ICs, in contrast to particulate ICs, does not necessarily involve IgG Fc receptors.

ICs phagocytosed by macrophages are degraded by lysosomal enzymes, although small amounts of antigen and antibody may remain undigested or partially digested (Cruchaud and Unanue, 1971; Cruchaud *et al.*, 1975; Steinman and Cohn, 1972). Once macrophages phagocytose ICs, the result is release of various hydrolytic enzymes that participate in inflammatory processes (Becker and Henson, 1973; Cardella *et al.*, 1974; Weissmann *et al.*, 1971). Moreover, interaction of preactivated macrophages (by endotoxin or ingestion of latex particles) with soluble ICs has been found to induce secretion of plasminogen activating factor *in vitro* (Gordon *et al.*, 1974). Additionally, as indicated above, interaction of macrophages with the $\overline{Bb}$ fragment of factor B results in their activation and immobilization (Götze *et al.*, 1979). The $\overline{Bb}$-dependent monocyte spreading appears to be mediated by C5 synthesized by these cells (Sundsmo and Götze, 1979). Furthermore, interaction with soluble C3b results in release of enzymes, which in

turn can cleave native C3, thus contributing to the chronicity of inflammation. C3a may likewise induce macrophages to release acid hydrolases, but this response is associated with a loss of cell viability (Schorlemmer and Allison, 1976). Synderman *et al.* (1971) also showed chemotaxis of guinea pig macrophages exposed to C5a. Finally, ICs attached to plastic surfaces can selectively paralyze macrophages' ability to phagocytose ICs, but not nonopsonized particles such as latex particles and yeast cell walls (Rabinovitch *et al.*, 1975).

5. *Eosinophils*

Human eosinophils have receptors for IgG Fc, C3b-C4b (immune adherence receptor), and C3d (Henson, 1969; Tai and Spry, 1976; S. Gupta *et al.*, 1976; Anwar and Kay, 1977a). In contrast, murine eosinophil colony cells grown *in vitro* were found to have only receptors for IgG Fc but not for C3 fragments (Rabellino and Metcalf, 1975). The IgG Fc and C3 receptors on human eosinophils appear to be of lower affinity or density than those on neutrophils, and this finding may explain the lower rate of ingestion of opsonized particles seen with the former over the latter (S. Gupta *et al.*, 1976; Anwar and Kay, 1977a). Immature eosinophils obtained from patients with eosinophilia seem to express fewer C3b surface receptors than mature normal eosinophils (Anwar and Kay, 1977a). A selective marked increase in the number or affinity of C3b receptors, but not IgG Fc receptors, expressed by human eosinophils occurred *in vitro* in the presence of chemotactic factors, such as peptides of eosinophilic chemotactic factor of anaphylaxis and histamine (Anwar and Kay, 1977b, 1978). Eosinophils are considered to be devoid of cytophilically bound IgE (Sullivan *et al.*, 1971; Ishizaka *et al.*, 1967; S. Gupta *et al.*, 1976). However, Hubscher (1975) reported IgE on the surface of 25–30% of human eosinophils. Human eosinophils have also been reported to have receptors for C3a and C5a (Glovsky *et al.*, 1979).

The role of the eosinophil in immediate-type hypersensitivity is thought to be that of a homeostatic cell affecting mediator release, mediator inactivation, and mediator replenishment by mast cells (Kay, 1976; Zeiger and Colten, 1977). Yet, eosinophils may function in helminth disease as a cytotoxic cell requiring the participation of IgG, analogous to lymphocyte-antibody-dependent cell-mediated cytotoxicity (Butterworth *et al.*, 1974, 1975). As will be discussed in Section V, circulating ICs present in the sera of patients with schistosomiasis may be responsible for the observed relative inability of eosinophils from such patients to kill the larvae *in vitro* (Butterworth *et al.*, 1977a,b).

6. *Basophils and Mast Cells*

Basophils and mast cells have IgE Fc receptors (Spiegelberg, 1974; Ishizaka, 1970, 1975; Becker and Henson, 1973) that are most fastidious, showing affinity for IgE only of the same or closely related species; hence this IgE is called homocytotropic antibody. These receptors are also the highest in affinity (10^8 to 10^9 M^{-1}) among the Fc cellular receptors. The receptors on basophils and mast cells for IgE and the receptors on macrophages for IgG are probably unique among blood cells in showing high affinity for monomeric Ig. Thus, *in vivo* these cell types carry Ig on their surfaces even in the absence of antigen. In contrast, IgG Fc receptor occupancy is likely to be low on neutrophils, platelets, and lymphocytes, because of the low intrinsic affinity, unless a polyvalent array of Fc regions is presented in the form of ICs. The localization of the binding site of IgE within Fc is not known, but more than one domain may be involved (Dorrington and Bennich, 1973).

Homocytotropic antibodies of the IgE class have been found in many species other than man, including rat, mouse, rabbit, and guinea pig (reviewed by Spiegelberg, 1974). Mast cells and basophils of rodents have receptors not only for homocytotropic IgE antibodies, but also for homocytotropic antibodies of the IgG class (Ovary *et al.*, 1963, 1965; Bach *et al.*, 1971; Zvaifler and Robinson, 1969; Becker and Henson, 1973; Spiegelberg, 1974). However, degranulation of mouse mast cells after interaction with IgG antibody and antigen is much less pronounced than that after interaction with IgE antibody and antigen (Barnett and Justus, 1975). A separate receptor for IgG Fc on human basophils was recently described (Ishizaka *et al.*, 1979).

Basophils and mast cells also bind C3a and C5a (Johnson *et al.*, 1975; ter Laan *et al.*, 1974; Petersson *et al.*, 1975; Glovsky *et al.*,1979; Hugli and Müller-Eberhard, 1978), and receptors for C3b were recently found on rat mast cells (Sher, 1976; Sher and McIntyre, 1977). After these cells interact with ICs or with C3a and C5a, degranulation occurs followed by the release of various biologically active substances, such as heparin, histamine, slow-reacting substance of anaphylaxis (SRS-A), and PAF. The role of PAF in the pathogenesis of IC-disease was discussed in Section I.

7. *Lymphocytes*

Lymphocytes from a number of species interact with ICs via receptors for antigen, receptors for the Fc portion of different Ig classes, and receptors for C components. A detailed coverage of the vast amount of literature describing the various surface receptors on lymphocytes is

beyond the scope of this review; therefore, only a summary and selected pertinent references to the topic under discussion will be given below.

Bone marrow-derived (B) cells, apart from their surface-bound Ig that serves as an antigen receptor, have low-affinity receptors for IgG Fc and high-affinity receptors for C3b-C4b, C3d, and C1q (Basten *et al.*, 1972; Dickler, 1974, 1976; Dickler and Kunkel, 1972; Alexander and Henkart, 1978; Lay and Nussenzweig, 1968; Ross *et al.*, 1973; Ross and Polley, 1975; Bokisch and Sobel, 1974; Dierich *et al.*, 1974; Sobel and Bokisch, 1975; Theofilopoulos *et al.*, 1974a,b; Theofilopoulos, 1977; Tenner and Cooper, 1979; Bianco *et al.*, 1970). These receptors are physically distinct entities, and ICs with fixed C appear to bind lymphocytes almost exclusively via the C receptors, presumably owing to steric hindrance of Fc sites on the antibody by the fixed C3 or C4 fragments (Theofilopoulos *et al.*, 1974b; Eden *et al.*, 1973b; Kammer and Shur, 1978). It has been claimed that a_1-antitrypsin serves as a C3 receptor molecule on membranes of lymphocytes and erythrocytes (Landen *et al.*, 1979). Release of ICs bound to cellular C receptors via activation of the alternative C pathway has been described (Miller *et al.*, 1973; Miller and Nussenzweig, 1974, 1975; Nussenzweig, 1974), and a role for this phenomenon in IC disease has been postulated (Miller *et al.*, 1975). Initially Forni and Pernis (1975), and more recently Abbas and Unanue (1975), have demonstrated a provocative association between surface Ig and the IgG Fc receptors of murine lymphocytes. When the surface Ig of freshly prepared splenocytes is capped with $F(ab')_2$ fragments of anti-Ig, the majority of Fc receptors co-cap selectively, indicating that ligand binding to surface Ig induces an alteration that allows a physical association between the otherwise separate surface Ig and Fc receptors. However, direct capping of IgG Fc receptors by ICs does not alter the distribution of surface Ig on the cells. Scribner *et al.* (1977) found that this associative interaction is lost after B cell activation and suggested that the loss represents a regulatory mechanism by which B cells escape Fc receptor-mediated regulation (see Section III,C). The physical association of Ia antigens on murine lymphocytes with IgG Fc receptors has also been shown (Dickler and Sachs, 1974), but recent studies indicate that these two structures are not identical (Dickler, 1976).

Apart from the IgG Fc and C receptors, recent reports contend that a subpopulation of B cells has receptors of low affinity for IgE Fc (Gonzalez-Molina and Spiegelberg, 1976, 1977), for IgM Fc (Ferrarini *et al.*, 1977; Pichler and Broder, 1978; Romagnani *et al.*, 1978) and for IgA Fc (S. Gupta *et al.*, 1979). There is no association between IgM Fc receptors and Ia-like antigens (Pichler and Knapp, 1978).

Thymus-derived (T) cells from humans and mice can also bind ICs via receptors for IgM Fc and receptors for IgG Fc (Moretta *et al.*, 1975; McConnell and Hurd, 1976; Ferrarini *et al.*, 1976; Gmeling-Meyling *et al.*, 1976; Romagnani *et al.*, 1978; Lamon *et al.*, 1975a,b; Basten *et al.*, 1975; Anderson and Grey, 1974; Stout and Herzenberg, 1975; Stout *et al.*, 1976; Santana, 1977). Moreover, receptors for IgA Fc on a subpopulation of murine (Strober *et al.*, 1978) and human (Lum *et al.*, 1979) T cells and receptors for IgE Fc (Yodoi *et al.*, 1979) have been described. Human IgM Fc receptor-bearing T cells, representing the majority of T cells (approximately 75%), are distinct from IgG Fc receptor-bearing cells (Moretta *et al.*, 1975, 1977). However, a recent report has indicated that the latter cells treated *in vitro* to remove ICs are converted to IgM Fc receptor-bearing cells (Pichler *et al.*, 1978). These authors suggested that IgG Fc and IgM Fc receptors are not markers for distinct T-cell subsets and that a subset of T cells expresses one or both of these classes of receptors at different functional stages. T cells with IgG Fc receptors are devoid of locomotion toward chemoattractants, whereas T cells with IgM Fc receptors are endowed with such locomotive activity (Parrott *et al.*, 1978). The presence of C1q receptors has also been claimed, but not definitely shown, on human T cells (Sobel and Bokisch, 1975), and there are occasional reports of C3b receptors on activated or malignant T cells (Shevach *et al.*, 1974; Barrett, 1978; Chiao *et al.*, 1974; Toben and Smith, 1977).

Unclassified human lymphocytes (K cells) that mediate ADCC *in vitro* are known to have high-affinity receptors for IgG Fc, especially for the IgG_1 and IgG_3 subclasses (Perlmann *et al.*, 1977; Dickler, 1976; Winfield *et al.*, 1977b; Cordier *et al.*, 1976; Spiegelberg *et al.*, 1976).

The binding of IgG to B, T, and K cells depends on the degree of aggregation (Heusser *et al.*, 1977) and on the subclass of IgG. Aggregated myeloma proteins of the IgG_1 and IgG_3 apparently bind more avidly than IgG_2 and IgG_4 on human lymphocytes, and IgG_1 and IgG_{2a} bind better than IgG_{2b} on murine lymphocytes (Lawrence *et al.*, 1975; Dickler, 1976; Dorrington, 1976; Wisløff *et al.*, 1974; Spiegelberg *et al.*, 1976). The receptors for the various subclasses of human IgG on activated murine T cells were found to be the same (Klein *et al.*, 1977). The binding site of IgG for lymphocytes appears to be on the C_H3 domain (Ramasamy *et al.*, 1975; Neauport-Sautes *et al.*, 1975), but a contributory role of the C_H2 domain has been suggested (Klein *et al.*, 1977). For the binding of IgG to K cells, Michaelsen *et al.* (1975) have provided evidence suggesting that either close association of the paired C_H2 domains or quaternary interactions between C_H2 and C_H3 are necessary. In addition, sites that trigger ADCC may be located in the C_H2 region, whereas sites in the C_H3 region seem to promote effi-

cient binding of the effector cells to the target cells (Spiegelberg *et al.*, 1976; Yasmeen *et al.*, 1976). The receptors for IgM Fc on T cells appear to have affinity for a structure located in the Fc portion of pentameric IgM (Ferrarini *et al.*, 1976), but monomeric 7 S IgM has also been claimed to bind to the IgM Fc receptors of T cells (Preud'homme *et al.*, 1977). IgM apparently binds to the T cell receptors by its C_H4 domain (Conradie and Bubb, 1977).

8. Other Cell Types

Many other cell types may interact with ICs via IgG Fc and C3 receptors inherently present on their surfaces or acquired secondarily. Recent studies have demonstrated IgG Fc receptors on a variety of tumor cells, other than those of lymphoid origin (Wood *et al.*, 1975; Tonder *et al.*, 1974; Braslawsky *et al.*, 1976a,b). In addition, cytomegalovirus- or herpes simplex virus-infected fibroblasts acquire IgG Fc receptors on their surfaces (Westmoreland *et al.*, 1976; Westmoreland and Watkins, 1974; Yasuda and Milgrom, 1968; Keller *et al.*, 1976; Rachman *et al.*, 1976; Watkins, 1964; Furukawa *et al.*, 1975; Nakamura *et al.*, 1978). It has been claimed that binding of Ig or Fc fragments to the Fc receptors on cells infected *in vitro* with herpes viruses inhibited viral replication (Costa *et al.*, 1977). ICs formed *in vitro* and aggregated γ-globulin from humans and rabbits, but not ICs with $F(ab')_2$ antibodies, can bind to the surfaces of isolated human and rabbit hepatocytes (Hopf *et al.*, 1976). Apart from IgG Fc receptors, the above authors also described receptors for C3 and IgA Fc on such cells (Hopf *et al.*, 1976, 1978). Also receptors for IgG Fc, possibly of importance in Ig transport from mother to fetus, have been found in fetal yolk sac membranes of rabbits (Hillman *et al.*, 1977; Wild and Dawson, 1977) and mice (Elson *et al.*, 1975) and in placental tissues of humans and mice (McNabb *et al.*, 1976; Elson *et al.*, 1975; Jenkinson *et al.*, 1976; Johnson *et al.*, 1973, 1976; Matre *et al.*, 1975; Matre and Haugen, 1978). Binding to the rabbit yolk sac membrane is mediated by the C_H2 domain of rabbit IgG (Tsay and Schlamowitz, 1978).

Human placental tissues have a single class of receptors with an intrinsic affinity of $4 \times 10^6 M^{-1}$ for the IgG_1. The order of affinity for IgG subclasses is $IgG_1 = IgG_3 > IgG_4 > IgG_2$; neither IgM nor IgA binds to these receptors. Whereas Fc isolates of IgG_1 bind as well as the intact molecule, fragments corresponding to the C_H2 or C_H3 domains are completely without activity. McNabb *et al.* (1976) interpreted these findings to mean that the binding site is formed from regions on both domains or that the site is present on one domain but active only in the presence of the second. Thus, the placental Fc receptor is unlike

those found on most cell surfaces where the binding is mediated by the C_H3 domain. The placental Fc receptors appear to be localized to the apical surfaces of trophoblasts and endothelial cells of the fetal stem vessels (Matre and Haugen, 1978; Johnson *et al.*, 1973, 1976). Human dendritic cells of the epidermis, referred to as Langerhans cells, have receptors for complexed IgG and for C3b (Stingl *et al.*, 1977). The physiologic role of these cells is still obscure. Finally, and possibly most important, receptors for ICs bearing C3b, but not C3d, exist on human glomeruli but not those of any other species studied so far (M. C. Gelfand *et al.*, 1975; J. A. Gelfand *et al.*, 1976; Sobel *et al.*, 1976; Moran *et al.*, 1977). Shin *et al.* (1977) used scanning electron microscopy to establish the precise location of these receptors within the renal corpuscle and determined that the receptors occupied visceral epithelial cells. The presence of binding sites for the Fc fragment of IgG in the renal interstitium of man and a variety of other species has also been reported (Gelfand *et al.*, 1979). The precise role of these receptors in the trapping and deposition of C3 and C-fixing ICs in some forms of immunologically mediated renal disease has not yet been defined. More recently, evidence for IgG Fc and C3b receptors in human choroid plexus has been obtained (Aathen *et al.*, 1979).

C. Effect of Immune Complexes on Immune Functions

ICs can modulate humoral and cellular immune responses both *in vivo* and *in vitro* by interacting with B and T cells having Fc, C, and/or antigen receptors. ICs may suppress or augment immune responses depending on the molar ratio of the antigen and antibody; the epitope density of the complex; steric and chemical conformation of antigens; and mass, class, and affinity of antibody. The possible effects of ICs on immune responses are summarized in Table IV.

Suppression of the antibody response and induction of tolerance *in vivo* and *in vitro* by antibody or ICs at antibody excess has been observed by many investigators (Smith, 1909; Uhr and Möller, 1968; Feldmann and Diener, 1970, 1971, 1972; Rowley *et al.*, 1973; Dive *et al.*, 1974; Cantor and Dumont, 1967; Weigle, 1975; Sinclair, 1969; Chan and Sinclair, 1971; Lees and Sinclair, 1973; Morgan and Tempelis, 1977; Andre *et al.*, 1975; Oppenheim, 1972). A prime example of this phenomenon in man is the therapeutic administration of anti-Rh antibodies, which prevent maternal sensitization by Rh isoantigens (Finn *et al.*, 1961; Freda *et al.*, 1966). At first it was suggested that antibody inhibits immune responses by masking or shielding antigenic determinants (peripheral block), thus making them inaccessi-

TABLE IV
POSSIBLE EFFECTS OF IMMUNE COMPLEXES ON IMMUNE RESPONSES

Humoral responses
A. Suppression
Through interaction with both Ag and Fc receptors on B cells (antigen-specific suppression)
Through interaction with Fc receptors on B cells (non-antigen-specific suppression)
Release of suppressor factor(s) from B cells
Effector cell blockade
Activation of suppressor T cells
Blockade of antigen receptors on T cells
Blockade of T and B cell interaction
Effect on macrophages
B. Enhancement
Increased DNA synthesis of B cells
Rapid localization of antigen in lymphoid follicles
Enhanced binding of antigen to antigen receptor-bearing cells
Enhanced processing of antigen by macrophages
Stimulation of helper T cells
Close approximation of collaborating cells via Fc and C3 receptors
Cellular responses
Inhibition or enhancement of antibody-dependent cell-mediated toxicity
Blockade of cell-mediated lymphocytic reactions
Blockage of delayed hypersensitivity
Altered traffic of lymphocytes

ble to antigen receptor-bearing lymphocytes (Uhr and Möller, 1968). However, this early concept that the antigen-related part of the antibody, the Fab portion, determines inhibitory activity cannot explain the observation, first made by Sinclair and associates (Sinclair, 1969; Chan and Sinclair, 1971; Lees and Sinclair, 1973, 1975; Sinclair *et al.*, 1968, 1970, 1974) and later by others (Tew *et al.*, 1976; Morgan and Tempelis, 1978; Wason and Fitch, 1973; Abrahams *et al.*, 1973; Kappler *et al.*, 1973; Stockinger and Lemmel, 1978), that an antigen-unrelated part of the antibody, the Fc portion, determined its inhibitory activity.

Several current explanations that separately or in concert may explain the antibody or IC-induced suppression of antibody production are as follows:

1. ICs could directly block antibody production at the B cell level through interaction with both antigen and Fc receptors of antigen reactive cells. The related data of Oberbarenscheidt and Kolsch (1978) and Stockinger and Lemmel (1978) certainly suggest that cross-linking

of antigen receptors (surface Ig) and Fc receptors by ICs is a direct blocking signal for B cells, without participation of T cells or macrophages. The *in vitro* experiments of Oberbarenscheidt and Kolsch (1978) indicated that the cross-linking blockade was antigen specific, did not require T cells, and was not overcome with lipopolysaccharide (LPS) and helper T cell replacing factor.

2. ICs might block immune responses nonspecifically by interacting with Fc receptors on B cells. The importance of IgG Fc receptors on B cells in immune responses was shown *in vitro* when LaVia and LaVia (1978) observed that aggregated IgG could nonspecifically inhibit immune responses of murine B lymphocytes to both T-dependent and T-independent antigens. These authors postulated that B lymphocytes are activated by antigen as follows: (*a*) interaction of antigen with B lymphocyte surface Ig; (*b*) redistribution of the surface Ig-antigen complexes; (*c*) interaction of these complexes with Fc receptors; (*d*) activation of B lymphocyte metabolic and differentiative pathways. In this connection, as indicated earlier, Forni and Pernis (1975) and Abbas and Unanue (1975) reported that, when complexes of surface Ig-anti-Ig are induced to cap, Fc receptors co-cap, and Ramasamy *et al.* (1974) suggested that Fc receptors may serve as "pro-receptors" for surface Ig molecules. Moreover, Sidman and Unanue (1976) found that anti-Ig antibodies bound both to surface Ig and to surface Fc receptors inhibit LPS-induced DNA synthesis. In the same context it should be noted that insoluble ICs inhibit stimulation of lymphocytes by LPS and concanavalin A (Con A), as evidenced by both mitogenesis and production of antibody-forming cells (Ryan *et al.*, 1975; Ryan and Henkart, 1976a). In contrast, soluble ICs apparently cannot inhibit LPS stimulation (Ryan *et al.*, 1975; Ramasamy, 1976). The possible mechanisms by which B-cell activation is inhibited by Fc receptor blockade were outlined by Ryan and Henkart (1976b). These include (*a*) blockage of the surface binding event(s) that triggers the cell to become activated; (*b*) inhibition or "freezing" of the movement of surface molecules necessary for activation; or (*c*) transmission of a central inhibitory signal affecting intracellular levels of cGMP and cAMP. Ryan and Henkart (1976b) have preliminary data indicating an increase in the intracellular level of cAMP after mouse spleen cells interact with immobilized ICs.

3. ICs could block immune responses in a non-antigen specific manner by interacting with IgG Fc receptor-bearing B cells, resulting in release of soluble immune response suppressor factors from these cells. This possibility is based on the recent studies of Masuda *et al.* (1978), who observed that responses to dinitrophenylated keyhole

limpet hemocyanin (DNP–KLH) are greatly enhanced in irradiated syngeneic recipients of Fc receptor-minus (FcR^-) B cells, but much diminished in those receiving Fc receptor-positive (FcR^+) B cells. Furthermore, FcR^+ B cells significantly suppress the plaque-forming response of FcR^- B cells. This suppression is mediated by a factor released from FcR^+ B cells first reacted with ICs but not secreted by FcR^- B cells or unseparated cells not preincubated with ICs. Data by these authors indicate that this suppressor factor, which is a molecule of 30,000–63,000 daltons, is absorbable with the corresponding anti-H-2 sera but not with anti-Ig antisera or AHG, suggesting that the factor is neither Ig nor the Fc receptor itself. The target cells for this suppressor factor are precursor B cells but not helper T cells (Miyama *et al.*, 1979).

4. ICs may inhibit antibody production by a mechanism similar to that termed by Schrader and Nossal (1974) "effector cell blockade." This term means inhibition of antibody secretion from terminally differentiated B cells after interaction with small amounts of antigen *in vivo* or *in vitro*. The phenomenon appears to involve a true reduction in secretion, not just temporary absorption of secreted antibody to cell-attached antigen. Nossal and Pike (1974) stated that, at least in preliminary experiments, ICs are powerful inducers of B cell effector cell blockade. Abbas and Klaus (1978) examined this concept further and found that anti-DNP antibody producing murine plasmacytoma cells (MOPC315) cultured with DNP–anti-DNP antiserum complexes specifically inhibit IgA antibody synthesis and secretion. The complexes are much more effective than the DNP antigen alone, although the Fc portion of the anti-DNP antibody does not appear to play an obligatory role in this phenomenon.

5. ICs may supress immune responses via activation of Fc receptor-bearing suppressor T cells or blockade of antigen receptor-bearing T cells. Thus, experiments by Moretta *et al.* (1977) indicate that the pokeweed mitogen (PWM)-induced differentiation of human B cells to plasma cells is suppressed by T cells that interact via their IgG Fc receptors with ICs. T cells with IgG Fc receptors that have interacted with ICs suppress the induction of polyclonal B cell differentiation by acting indirectly via their released soluble factors on helper T cells (Moretta *et al.*, 1979). The same authors showed that IgM Fc receptor-bearing T cells are helpers in the PWM-induced differentiation of B cells. However, in later experiments by Hayward *et al.* (1978), suppressor activity was not confined to the IgG Fc receptor-bearing T cell population but was also expressed by Con-A stimulated IgM Fc receptor-bearing T cells. A loss *in vitro* of Fc receptors for IgG from human T lymphocytes exposed to IgG ICs was

described by Cordier *et al.* (1977) and Moretta *et al.* (1978). These last authors suggested that this phenomenon may represent a mechanism to prevent an excess of suppression *in vivo* during normal immune responses. FcR^+ murine T cells with inherent suppressor activity in primary as well as secondary responses to both particulate and soluble antigens were also described (Yodoi *et al.*, 1978). Experiments by Gorczynski *et al.* (1974) similarly suggested that ICs mediate suppression through T cells by blocking the antigen receptors on these cells. Furthermore, Kontianen (1975) and Kontiainen and Mitchison (1975), working *in vitro* and *in vivo* with adoptive transfers, considered that suppression of antibody formation occurs via ICs locked onto the T-cell surface by virtue of the cells' antigen receptors. Moreover, interaction of human IgG FcR^+ T cells with insoluble ICs was shown to induce an impaired proliferative response to phytohenagglutinin (PHA), Con A, and PWM (Samarut *et al.*, 1979).

6. ICs may suppress via soluble factors released from T cells. Thus, Fridman and Goldstein (1974) and Gisler and Fridman (1975, 1976) found that alloantigen-activated T cells release a factor that combines with the Fc fragment of antigen complexed-IgG. This Ig binding factor (IBF) may be identical to the T-cell Fc receptor, since IBF binds IgG and inhibits C fixation by IgG, although as stated the binding site for Fc receptors on IgG molecules is different from the C1 binding site. IBF inhibits IgM and IgG responses to T-dependent and T-independent antigens *in vitro*, but this inhibitory effect is neutralized by aggregated IgG.

7. ICs may suppress immune responses by interfering with T- and B-cell interactions. Hoffmann *et al.* (1974) and Hoffmann and Kappler (1978) proposed that for cellular antigens, two mechanisms of antibody-mediated suppression of antibody production exist: one is operative at low antibody concentration, depends on the Fc portion of the antibody, and is reversed by factors that substitute for helper T-cell function; the other requires high concentrations of antibody and is not reversed by factors that replace T cells. The later mechanism may be based on the masking of antigenic determinants, which makes them inaccessible to responsive lymphocytes. The former mechanism provides the possibility that one antibody molecule, by reacting with one determinant of a large antigen and attaching its Fc portion to cellular Fc receptors, removes all other determinants of the same carrier from the circulation. Thus attached to cell surfaces, antigen would remain recognizable by T cells and B cells, but could no longer serve as a bridge of communication between them. This last concept can be applied only to complex antigens with many antigenic determinants.

8. Finally, ICs may inhibit antibody production via T cells and

macrophages. Taylor and Basten (1976) have suggested that ICs activate suppressor T cells, which in turn stimulate macrophages to release nonspecific factors capable of suppressing both T and B cells. In the recent experiments by Ptak *et al.* (1978) macrophages were an obligatory intermediate in the transfer of T cell-derived suppressor signals to other T cells. These authors also showed that suppressor factors specific for delayed-type hypersensitivity responses in mice could be adsorbed onto peritoneal exudate macrophages via cell surface structures. Since this binding could be blocked with aggregated γ-globulin, indications were that the structure had Fc receptor-like characteristics.

ICs can have opposite effects (augmentation vs. inhibition) on the immune response. Several investigators have reported that ICs, especially those in antigen excess, are more immunogenic than free antigen both for primary antibody responses and for secondary antibody responses *in vivo* and *in vitro* (Morrison and Terres, 1966; Terres and Wollis, 1961; Terres *et al.*, 1972; Uhr and Möller, 1968; Diener and Feldmann, 1972; Siegre and Kaeberle, 1962; Laissue *et al.*, 1971; Houston *et al.*, 1974; Dennert, 1971; Revoltella *et al.*, 1975). This enhanced responsiveness has been attributed to increased binding of complexed antigens to lymphocytes (Revoltella *et al.*, 1975), to the rapid localization of ICs in lymphoid follicles (White, 1975) and to improved "processing" by macrophages (Seeger and Oppenheimer, 1970). Romano *et al.* (1975) have further suggested that ICs bound to the surfaces of B cells may mediate helper T–B cell cooperation in the humoral immune response. Studies on the direct activation of normal, unsensitized lymphocytes by ICs have produced conflicting results. Bloch-Shtacher *et al.* (1968) and Möller (1969) reported that ICs can stimulate DNA synthesis in normal human peripheral lymphocytes. However, this finding was not reproduced with mouse cells (Möller and Coutinho, 1975; Ramasamy, 1976; Ryan *et al.*, 1975), and it was concluded that lymphocytes were not directly activated by the Fc receptors. Recently Berman and Weigle (1977) and Berman *et al.* (1979) described strong DNA synthesis, polyclonal antibody synthesis, and blast transformation in murine B lymphocytes after interaction of these cells with isolated Fc fragments of human IgG, IgM, IgA, and IgD, but not of IgE. In contrast, intact Ig and Fab fragments of the same classes had no effect. These authors found that sites in addition to the C_H3 domain are required for optimal cell activation. Thus, they postulated the enzymic cleavage of IgG produces a mitogenically active Fc fragment with reactive sites similar to those of the Fc portion of Ig in ICs or aggregated Ig. Macrophages have been found to be neces-

sary for Fc-mediated mitogenic stimulation of B cells (E. L. Morgan and W. O. Weigle, unpublished).

ICs also may enhance immune responses by interacting with C receptor-bearing cells. Dukor and Hartman (1973) hypothesized that binding of activated C3 to C receptors on B lymphocytes acts as a necessary second signal for antibody production. In their model, T-independent antigens and B-cell mitogens generate this second signal by their ability to activate C3 via the alternative pathway. In contrast, they suggested that T-dependent antigens cannot directly activate C3 but depend upon proteases released from activated T cells to cleave C3. Several experimental findings, however, argue against the above hypothesis. First, in many cases there is no direct correlation between C3 activation and T-independence (Pryjma *et al.*, 1974); second, B-cell mitogens and T-independent antigens seem to operate in the absence of C3 (Diamantstein and Blitstein-Willinger, 1975; Feldmann and Pepys, 1974; Janossy *et al.*, 1973; Pepys, 1974a; Pryjma and Humphrey, 1975; Waldmann and Lachmann, 1975); and third, C3 receptor-bearing cells are not obligatory for B cell-T cell cooperation in the 7 S response (Mason, 1976). On the other hand, Pepys (1974a) showed that C3 depletion results in depression of T-dependent antibody responses but leaves T-independent responses unimpaired. Pepys postulated that C3 may have a role in presentation of T cell-bound antigen–antibody complexes to B cells and macrophages bearing C3b receptors, thereby enhancing approximation of the different cell types involved in cooperation. The observation of spontaneously reversible, C3-dependent mixed adherence of macrophages, lymphocytes, and other cell types upon activation of the C system and generation of C3b (Arnaiz-Villena and Roitt, 1975; Pepys, 1974b) lends support to this second theory. More recent studies in support of this concept have been reviewed by Pepys (1976). C3b generated by C activation from ICs or C3b fixed to ICs may also stimulate DNA or RNA synthesis by B cells directly (Hartmann and Bokisch, 1975; Soderberg and Coons, 1978; Logue and Huang, 1977). However, others have been unable to demonstrate proliferation of lymphocytes by soluble C3b or ICs with fixed C3b (Sandberg *et al.*, 1975; Koopman *et al.*, 1976; Möller and Coutinho, 1975). Finally, binding of ICs to C receptor-bearing cells appears to be important for their transportation and localization in lymphoid follicles (Papamichail *et al.*, 1975; Klaus, 1979a).

In the context of antibody and IC-induced regulation of immune responses, the concept of idiotypes and anti-idiotypes should also be discussed briefly (for detailed review, see Eichmann, 1978). To sum-

marize, the immune system interacts with antigens in the environment via the variable domains of receptor molecules on lymphocytes. In addition to a binding site for antigen, each variable domain sequence carries a unique set of antigenic determinants, the combination of which is now termed idiotype and can be recognized by a particular set of anti-idiotypic antibodies. The concept that antibodies can be the target for recognition by immune cells was first demonstrated by showing that antigen-specific structures of antibodies can be antigenic in the same or different species (Kunkel *et al.*, 1963; Oudin and Michel, 1969). Recent work by several investigators (Sirisinha and Eisen, 1971; Yakulis *et al.*, 1972; Rodkey, 1974) shows that an animal can recognize its own Ig and mount an immune response that is specific for the antigen-specific portion of that Ig molecule (V region). Antibodies directed against the individual's own receptors are the essential ingredients in the network theories of immune regulation proposed by Jerne (1973, 1974, 1976) and extended by others (Köhler, 1975; Richter, 1975; Hoffmann, 1975; Raff, 1977). These investigators attempt to explain the behavior of the immune system in terms of the interactions of antigens, idiotypes, and anti-idiotypes. The idiotypes and anti-idiotypes may be either B or T cells or their antigen reactive products, antibodies. Logically, the anti-idiotypes themselves can also be recognized in turn by other receptors, anti-anti-idiotypes. Interactions between idiotypes and anti-idiotypes can facilitate or suppress associated immune reactivity, thereby acting as a dynamic network that regulates itself (Jerne, 1976). Administration of large amounts of anti-idiotypic antibody into mice before or shortly after immunization with antigen may result in a long-lasting unresponsiveness (Hart *et al.*, 1972; Nisonoff and Shyr-Te, 1976; Eichmann, 1974, 1975, 1978; Cosenza *et al.*, 1977; Bangasser *et al.*, 1975). Administration of such quantities of soluble anti-idiotype antibody may result in permanent dramatic alterations of the lymphocyte compartments, which become evident as (*a*) an amplification of suppressor T cells; (*b*) a shift in the ratio of idiotype-positive to idiotype-negative precursor B cells; (*c*) a shift in the number of idiotype-positive to idiotype-negative T helper cells; and (*d*) an increase in the responsiveness of B cells bearing anti-idiotypic receptors for the idiotype (Eichmann, 1978). Under certain circumstances, anti-idiotypic antibodies given alone in small quantities or in the form of idiotype–anti-idiotype complexes may stimulate rather than suppress (Eichmann, 1974, 1978; Trenkner and Riblet, 1975) immune responses. It is of particular interest to note that antibody, given as an antigen–antibody complex, acts as a very effective adjuvant for inducing immunologic memory for antibody produc-

tion (Klaus and Humphrey, 1977) and that antibody complexed with antigen enhances the production of anti-idiotypic antibodies against self-idiotypes ("site-directed" and "non-site-directed") (Klaus, 1978, 1979b). This last finding is compatible with network theories of immunoregulation and suggests that antibodies acquire marked autoimmunogenicity only when complexed with the eliciting antigen.

Apart from the effects of ICs on the humoral immune response, they may have effects on many other immunologic reactions, such as antibody-dependent cellular cytotoxicity (ADCC) and cell-mediated lympholytic reactions. ADCC has been advocated as a possible mechanism involved in allograft rejection, elimination of viral infections, tumor immunity, and autoimmune diseases. The mediator of ADCC is an Fc receptor-bearing nonimmune unclassified lymphocyte (K cell) and requires the presence of IgG anti-target cell antibodies (Perlmann *et al.*, 1977). In contrast to B cells' Fc receptors, the IgG Fc receptors on K cells are not associated with Ia molecules on the cell surface, and anti-Ia antibodies fail to inhibit ADCC (Schirrmacher *et al.*, 1975). Recently, ADCC involving IgM antibodies has also been shown for both murine (Lamon *et al.*, 1975a,b) and human lymphocytes (Perlmann *et al.*, 1977; Fuson and Lamon, 1977) primarily of the T type (Perlmann *et al.*, 1977). Depending on their molar ratio, ICs may inhibit or enhance ADCC. In antigen excess, ADCC is inhibited, whereas at antibody excess "arming" of lymphocytes and enhancement of ADCC occurs (MacLennan, 1972; Perlmann *et al.*, 1972; Greenberg and Shen, 1973; Sakselä *et al.*, 1975; Lustig and Bianco, 1976). Armed lymphocytes carrying ICs that have some free antibody valences may attack the cellular target for which the free antibody-combining sites have specificity.

ICs also interfere with cell-mediated cytolytic reactions. Thus, some sera of humans and animals with malignancies contain substances, termed blocking factors, that specifically inhibit cell-mediated cytolysis of tumor cells by sensitized lymphocytes (Hellström and Hellström, 1974). As discussed in detail in Section V,B, there is considerable evidence that these blocking factors in cancer sera often represent tumor antigen–antibody complexes (Sjögren *et al.*, 1971; Hellström and Hellström, 1974; Baldwin and Robins, 1975, 1976). Additionally, ICs made with tumor antigens and antibodies *in vitro* can specifically block the destruction of tumor cells by sensitized lymphocytes (Gorczynski *et al.*, 1975) and macrophages (Gershon *et al.*, 1974), and putative tumor antigen–antibody complexes eluted from peripheral mononuclear cells of tumor-bearing patients can inhibit stimulation of the patients' lymphocytes by tumor antigens (Hattler

and Soehnlen, 1974). Working with mice, Stout and Herzenberg (1975) and Stout *et al.* (1976) have shown that IgG FcR^+ T cells perform different functions from the FcR^- cells. Thus, FcR^- cells are not responsive to Con A in the absence of FcR^+ T cells, contain the precursors of cytotoxic effectors of cell-mediated lympholytic responses, and contain the helper T cells but not the amplifier cells. In contrast, the FcR^+ T cells are responsive to Con A (Con A-responsive cells are known to be predominantly suppressor T cells), contain the differentiated cytotoxic effector cells of cell-mediated lympholytic responses, and contain the amplifier T cells but not the helper T cells. How these FcR^+ T cells function after interaction with ICs has not yet been determined. ICs also have been postulated to inhibit delayed-type hypersensitivity reactions (Mackaness *et al.*, 1974a,b; Lagrange and Mackaness, 1975) possibly because the responsible cells migrate from the circulation to the spleen (Lagrange and Mackaness, 1978). Last, ICs have been observed to affect the traffic patterns of lymphocytes (Stutman, 1973).

IV. The Detection of Immune Complexes

Circulating soluble ICs in man and lower animals are responsible for, or associated with, a diverse array of diseases. These include autoimmune disease, neoplastic diseases, infectious diseases due to bacteria, viruses and parasites, and other unclassified disorders.

Both exogenous and endogenous antigens can trigger pathogenic immune responses, resulting in IC disease. The awareness of IC's important role in many diseases has stimulated development of techniques for demonstrating them in tissues and biological fluids.

The presence of ICs in pathologic tissue specimens can be inferred from several lines of evidence. Histologically, the patterns of injury may appear similar to those known to occur in experimental animals in which IC disease has been induced (Wilson and Dixon, 1976). Presumed IC deposits in tissues can be identified also by electron microscopy and by conventional histochemical techniques. Of the immunohistochemical techniques, the most widely used is immunofluorescence (Wilson and Dixon, 1976), which allows Ig, C components, and in some cases specific antigens, to be indentified. When these materials deposit in a granular, discrete pattern, in all likelihood the individual has an IC disorder. In addition, if enough of the diseased tissue is available, deposited ICs can be eluted from it by using low pH buffers (citrate buffer, HCl–glycine buffer) (Oldstone, 1975a; Koffler *et al.*, 1971; Woodroffe and Wilson, 1977; Bartolotti, 1977) or chaotropic agents (KI, KSCN) (Edgington, 1971; Woodroffe and Wil-

son, 1977), etc., after which the antibodies and sometimes the antigens can be recovered, quantitated, and identified (see Section VI).

Recently, interest has increased in developing immunologic techniques for demonstrating ICs directly in bodily fluids; therefore, we will present in detail the techniques currently used as well as the problems inherent in these assays.

Although historically the development of assays for soluble ICs started with physicochemical rather than immunologic techniques, the latter are more commonly used now. It is useful to divide all the available methods into two main groups: antigen-specific tests, that is, detection of a specific antigen complexed with antibody or, by far the larger and more readily applicable group, antigen-nonspecific tests (Table V).

TABLE V
ANTIGEN-NONSPECIFIC METHODS FOR DETECTING CIRCULATION IMMUNE COMPLEXES

1. Physical techniques
   - Analytical ultracentrifugation
   - Sucrose density gradient centrifugation
   - Gel filtration
   - Ultrafiltration
   - Electrophoresis
   - Polyethylene glycol (PEG) precipitation
   - Cryoprecipitation
2. Methods based on the biologic characteristics of immune complexes
   a. Complement techniques
      - Microcomplement consumption test
      - Assays based on the interaction of ICs with purified Clq (Clq precipitation in gels, Clq-PEG test, Clq deviation tests, Clq solid-phase radioimmunoassays)
      - Assays of breakdown products of C3 and C1
      - The C3 precipitation assay
      - The conglutinin radioimmunoassay
   b. Antiglobulin techniques
      - Rheumatoid factor tests
      - Other antiglobulin tests
   c. Cellular techniques
      - The platelet aggregation test
      - Inhibition of antibody-dependent cell-mediated toxicity
      - Intracytoplasmic staining of polymorphonuclear leukocytes
      - Release of enzymes from eosinophils and mast cells
      - The macrophage inhibition assay
      - Rosette inhibition tests
      - The Raji cell assay
      - The L1210 murine leukemia cell assay
      - The human erythrocyte assay
   d. Other methods
      - Binding to staphylococcal protein A

A. Antigen-Specific Methods

In the few instances of pathogenic immune responses in which the inciting antigen is known, detection of circulating ICs composed of the known antigen and corresponding antibody is rather easily accomplished. For example, ICs containing viruses as antigens can be shown in the sera of animals and humans, by immunoprecipitation with an antiserum to host γ-globulin or antiserum to host C, because the titers of virus or viral products in the supernatant decrease markedly (Notkins *et al.*, 1966; Oldstone and Dixon, 1969). However, when the antigenic excess is very large and a small proportion of the antigen is bound to antibody, such an immunoprecipitation may not obviously decrease titers of virus or viral proteins. In such instances preparatory procedures, such as sucrose density gradient fractionation or gel filtration, that separate the large quantities of free antigen from the small quantities of antibody-bound antigen should precede precipitation. Thereafter, the fractions should be analyzed by methods such as radioimmunoassays for the distribution of the known antigenic substance. Fractions containing antigenic material heavier than free non-Ig bound antigen should then be separated and the antigen tested for its association with host Ig and/or C. This kind of association becomes evident if the antigen is removed by immunoprecipitation with anti-Ig or anti-C3 or by absorption to Sepharose-immobilized anti-Ig, anti-C3, or staphyloccal protein A. We used such a procedure in demonstrating the presence of gp70 (retroviral envelope antigen)-anti gp70 complexes under conditions of large antigen excess in sera of mice with autoimmune syndromes (Izui *et al.*, 1979b). Viruses such as hepatitis virus and polyoma virus complexed with antibodies and C can also be identified by electron microscopic examination of pellets in ultracentrifuged sera from the infected host (Almeida and Waterson, 1969; Oldstone *et al.*, 1974). Moreover, microbial antigens such as pneumococcal antigen specifically complexed with Ig have been detected by counterimmunoelectrophoresis (Preheim *et al.*, 1978). Enhancement of anti-DNA levels in sera of patients with SLE after DNase treatment also denotes the presence of DNA–anti-DNA complexes (Harbeck *et al.*, 1973). Similarly, Jayarao *et al.* (1973), employing acid dissociation and removal of insulin with charcoal, demonstrated an increased titer of antibody to insulin in some diabetic patients, suggesting the presence of insulin–anti-insulin complexes in their sera. Antigens complexed with antibody may additionally be identified by using specific fluor-labeled or radiolabeled antisera after ICs bind to substrates such as cells with Fc and C receptors (Theofilopoulos *et al.*, 1976a, 1977; Long *et al.*, 1977).

These methods have found much less application thus far than the antigen nonspecific methods. It is unlikely, in view of the marked heterogeneity of antigens involved in circulating ICs, that antigen-specific assays will ever be applied on a large scale in clinical immunologic studies. However, for the study of a particular disease in which the antigen is known, these techniques have important uses.

B. Antigen-Nonspecific Methods

The antigens in the ICs of most spontaneous diseases of animals and humans are unknown and, therefore, in these instances antigen specific techniques are not of use. In these situations, one attempts to identify ICs by relying on the different physiochemical and biological properties of antigen-complexed Ig as opposed to free Ig. Inherent in all the methods to be discussed hereafter is the possibility that nonspecifically aggregated Ig may be detected by these assays and assumed to constitute ICs. These methods have varying specificity, sensitivity, complexity, clinical applicability and idiosyncrasies, factors that preclude the detection of all species of ICs. Moreover, as will be detailed below, materials other than ICs or aggregates may interfere with the results in a majority of these assays, leading to false positive or false negative results.

1. Methods Based on Physical Characteristics of Immune Complexes

These techniques are based on the increased molecular size, different solubility and charge of complexed, compared to free, antigens or antibodies. The methods include analytical and sucrose density gradient centrifugation, gel filtration, electrophoresis, polyethylene glycol (PEG) precipitation, ultrafiltration, and precipitation in the cold.

Analytical ultracentrifugation of serum has been employed to detect and identify the IgG-IgM rheumatoid factor (RF) and IgG-IgG RF complexes in patients with rheumatoid arthritis (Franklin *et al.*, 1957; Kunkel *et al.*, 1961), but it is too insensitive for use as a routine IC assay. Sucrose density gradient (Kunkel *et al.*, 1961; Hannestad, 1967; Theofilopoulos *et al.*, 1976a, 1977; Amlot *et al.*, 1976; Brostoff *et al.*, 1977; Gabriel and Agnello, 1977; Benveniste and Bruneau, 1979) and gel filtration (Soothill and Hendrickse, 1967) are important tools for identifying macromolecular IgG or C3, presumably in complex form. With these techniques, sera are fractionated together with radiolabeled molecular markers, after which the collected fractions are assessed by double immunodiffusion or immunoelectrophoresis for the position of IgG and C3.

Polyethylene glycol, an uncharged, water-soluble, linear polymer

that has been used to fractionate plasma proteins, has unusual properties that are useful in detecting ICs. This substance concentrated to 20% precipitates most native Ig and many other proteins. When the PEG concentration is decreased to 3.5–4%, the precipitation of monomeric Ig decreases significantly, without preventing precipitation of ICs formed *in vitro* or *in vivo* (Creighton *et al.*, 1973; Zubler and Lambert, 1976; Digeon *et al.*, 1977). This enhancement of the precipitation by polymers has been explained as a steric exclusion of the ICs from the domain of the polymer (Laurent, 1963). Although not clearly understood, this differential precipitation may not necessarily relate to the size of the ICs but instead to conformational changes, since large molecular weight proteins, such as α_2-macroglobulin and β-lipoproteins, are not precipitable by PEG (Digeon *et al.*, 1977). Low concentrations of PEG (3.5–4%) are now being used to assess the presence of ICs in sera of patients with various diseases, based on the assumption that greater amounts of IgG in complex form precipitate than monomeric IgG (Mohammed *et al.*, 1976; Digeon *et al.*, 1977). Obviously, such a technique cannot have much specificity, and results may be influenced by the abnormally high concentrations of IgG in sera of patients with autoimmune and other disorders. In certain instances, antigens and antibodies may be identified in the precipitates after acid dissociation (Digeon *et al.*, 1977; Carella *et al.*, 1977), and C fixation by the precipitates can be demonstrated (Harkiss and Brown, 1979). Identification of C components in the precipitate, although not conclusive, may further suggest the presence of ICs in sera (Digeon *et al.*, 1977), but verification requires showing by size fractionation procedures, such as sucrose density gradient, that any Ig, C1q, or C3 present is in a high molecular weight form.

Cryoglobulins, or cold-precipitable globulins, were first observed in patients with reticuloendothelial malignancies (Winthrobe and Buell, 1933). Subsequent studies of these proteins have focused on the physicochemical nature of cryoprecipitation and immunochemical properties that determine their unique solubility characteristics (Grey and Kohler, 1973). Some investigators consider cryoglobulins to be circulating ICs because of the demonstration that they may contain antigens, antibodies, or other immunoreactants that may relate to the pathogenesis of the disease they accompany (Barnett *et al.*, 1970). Although commonly found in patients with lymphoproliferative, autoimmune, and other disorders (Brouet *et al.*, 1974; Winfield *et al.*, 1975a; Levo *et al.*, 1977; Fakunle *et al.*, 1978; McPhaul, 1978; Weisman and Zvaifler, 1975), not all such cold-precipitable fractions constitute ICs; some contain other proteins (e.g., cryofibrinogen). Three

types of Ig cryoglobulins have been described (Brouet *et al.*, 1974). These are monoclonal nonantibody Ig (type I), monoclonal rheumatoid factors associated with polyclonal Ig (type II), and polyclonal antibodies (e.g., rheumatoid factor) associated with antigen (type III). Autoimmune diseases such as SLE, rheumatoid arthritis, and Sjögren's syndrome are mainly associated with type III cryoglobulins. Recently, Middaugh *et al.* (1978) used many criteria to study monoclonal cryoglobulins and showed that these proteins cannot be distinguished from the noncryoglobulin reference proteins analyzed in parallel; however, certain hydrodynamic and spectroscopic properties of the proteins indicated that cryoimmunoglobulins differ in tertiary structure relative to their cold-soluble counterparts. The lack of cryoprecipitability of cryoimmunoglobulin subunits or bivalent fragments and the failure of fragments to inhibit or enhance cryoprecipitability of the parent molecules in their experiments argue against classical antibody–antigen complex formation in this instance. By definition, type II and type III cryoglobulins represent ICs composed of monoclonal or polyclonal rheumatoid factors and polyclonal IgG. However, in other instances extensive collaborative study is obviously necessary to demonstrate the IC nature of cryoprecipitates in sera. Such analysis should involve demonstration of antigens, antibodies, and possibly C components by immunodiffusion or other immunochemical procedures, demonstration of a specific concentration of antigens and antibodies higher than that in the corresponding serum, and evidence of macromolecular Ig and possibly C1q or C3.

2. *Methods Based on the Biologic Characteristics of Immune Complexes*

Upon the complexing of antibody with antigen, changes occur that endow the antibody molecule with the ability to react with certain serum proteins and cellular receptors (see Section III).

a. Complement Techniques. A variety of techniques have been developed for detecting aggregated or antigen-complexed antibody by utilizing its reactivity with the C system. Of course, C-fixation assays do not detect ICs made with non-C fixing antibodies or small ICs.

i. *The microcomplement test.* In this test (Mowbray *et al.*, 1973; Shulman and Barker, 1969) presumptive evidence for the presence of ICs in serum is obtained by assessing the consumption of a standard amount of added C. Residual C activity is measured by the degree of lysis of IgM antibody-sensitized sheep red blood cells. This assay is sensitive and may detect complexes regardless of the pathway by which the complexed antibody initiates C activation. However, a dis-

advantage is that the test serum must be heated, which may create Ig aggregates, and the assay may give positive results with a variety of materials that can consume C or inhibit C-induced lysis of sensitized red cells, the end point of the test.

ii. *Assays based on the interaction of ICs with purified C1q.* As discussed in Section III,A, C1q molecules may bind weakly monomeric IgG_1, IgG_2, IgG_3, and IgM, and the binding is enhanced when these proteins aggregate because of heating or antigen (Augener *et al.*, 1971; Müller-Eberhard and Calcott, 1966; Müller-Eberhard and Kunkel, 1961). The C1q assays not only fail to detect ICs made with non-C activating antibodies (IgG_4) but also do not detect ICs made with antibodies that activate C preferentially via the alternative pathway (IgE, IgA). Furthermore, C1q interacts preferentially with ICs larger than 19 S (Gabriel and Agnello, 1977; Lurhuma *et al.*, 1976). The binding of C1q to ICs appears to be reversible; therefore, ICs containing C1q in serum can interact with exogenous C1q. C1q is purified from human serum by the method of Agnello *et al.* (1970) or by the more recent techniques of Volanakis and Stroud (1972) or of Yonemasu and Stroud (1971). Zubler and Lambert (1976) have published a detailed modified procedure for the isolation and radioiodination of C1q. C1q may be iodinated either by the lactoperoxidase technique of Heusser *et al.* (1973) or by the chloramine-T method of McConahey and Dixon (1966). Whatever the procedure, assays based on using C1q must be interpreted with the knowledge that various polyanions, DNA, endotoxin, C-reactive protein, heparin, and viruses may bind to the C1q, reducing its specificity as a molecular probe for ICs (Agnello *et al.*, 1970, 1971; Sobel *et al.*, 1975; Woodroffe *et al.*, 1977; Rossen *et al.*, 1978; Cooper and Morrison, 1978; Cooper *et al.*, 1976). Some of these problems have been overcome in recent variations of the C1q assay (Gabriel and Agnello, 1977; Zubler and Lambert, 1976; Zubler *et al.*, 1976b).

(a) The agarose C1q precipitation test. This test described by Agnello *et al.* (1970, 1971) is the basis for all C1q assays described since. In this test the interaction in agarose gels of C1q with AHG or IgG complexed with antigen, but not monomeric IgG, results in precipitin line but is not sensitive enough (sensitivity $> 100\ \mu g$ AHG/ml serum) for routine use. Further, this method requires heating of serum prior to testing, detects ICs bigger than 19 S, is time consuming, and gives positive reactions with substances other than ICs or aggregates, such as DNA and endotoxin (Agnello *et al.*, 1970, 1976). Reduction and alkylation of the serum sample and abolishment of C1q precipitating activity after such treatment may be of help in discriminating Ig from

non-Ig-induced C1q precipitation, since such treatment is known to destroy the C-fixing activities of Ig.

(b) The C1q-binding combined with PEG precipitation test. In this test, which was developed by Nydegger *et al.* (1974) and modified by Zubler and Lambert (1976) and Zubler *et al.* (1976b), radiolabeled C1q is separated from C1q bound to ICs by differential precipitation with PEG. In the modified assay, pathologic sera are treated with ethylene diaminotetraacetate (EDTA) instead of being heated to prevent the incorporation of radiolabeled C1q into the endogenous, high-molecular-weight C1qrs complex in serum (Zubler *et al.*, 1976b). However, Lurhuma *et al.* (1976) found such treatment with EDTA to be disadvantageous, since C1q released from C1 in the test serum can compete with the exogenous radiolabeled C1q added to the reaction mixture and thus decrease the sensitivity of the assay. The authors recommended avoidance of heating or addition of EDTA to the serum sample prior to testing in the C1q assays. The C1q–PEG method has the advantage over other C1q tests that no positivity results from DNA or endotoxin, presumably because complexes of these substances with C1q are not precipitable by the concentration (2.5%) of PEG employed (Nydegger *et al.*, 1974). However, free DNA in serum strongly inhibits binding of C1q to aggregated γ-globulin (Tappeiner *et al.*, 1977). The disadvantages of this assay seem to be its insensitivity (detects > 80–100 μg of AHG per milliliter of serum) and the false positive results obtained in the presence of heparin (Woodroffe *et al.*, 1977). Additionally, there is no assurance that labeled C1q binds all C-fixing ICs if, for example, the Ig sites are fully occupied with endogenous C1. Other factors influencing the results of this test have been described by Rossen *et al.* (1978). In a recent modification of this technique, no exongenous C1q is added to serum, but after precipitation with 2.5% PEG, the precipitate is examined by double immunodiffusion for C1q bound to ICs *in vivo* (Grangeot-Keros *et al.*, 1978).

(c) The C1q deviation tests. These tests evaluate inhibition of the binding of radiolabeled C1q to a particulate substrate, such as IgG antibody-sensitized sheep red blood cells (Sobel *et al.*, 1975), IgG-coated latex particles (Medof and Sukhupunyaraksa, 1975), or IgG-coated Sepharose particles (Gabriel and Agnello, 1977). All these methods are very sensitive variants of the C1q tests (5–10 μg of AHG per milliliter of serum). However, in many instances heating of serum is required prior to testing; the presence of RF may lead to falsely positive results; and positive results occur with DNA, endotoxin, and other C1q-binding non-IC materials. In the test described by Gabriel

and Agnello (1977), positive reactions due to interfering non-IC substances, such as DNA, can be avoided by increasing the test's ionic strength. Testing inhibition of the agglutination of IgG-coated latex particles by C1q has also been employed (Lurhuma *et al.*, 1976) as an alternative to testing the inhibited binding of radiolabeled C1q to such particles.

(d) The C1q solid-phase radioimmunoassays. These tests are based on the interaction of ICs or aggregates with C1q bound to polystyrene tubes. In these assays pathologic sera are incubated in tubes internally coated with C1q; thereafter, the amount of bound IgG is assessed with radiolabeled or enzyme-linked anti-IgG sera (Hay *et al.*, 1976; Ahlstedt *et al.*, 1976) or staphylococcal protein A instead of antiglobulin (Farrell *et al.*, 1975). Inhibition of radiolabeled AHG binding to C1q-coated tubes has also been used for the detection of ICs in sera (Svehag, 1975). These tests appear to be very sensitive (1–10 μg of AHG per milliliter) and have the distinct advantage over fluid-phase C1q tests in that they are Ig specific. Since an antiserum to IgG is used as a final reagent, one avoids false positive results from DNA, endotoxin or other interfering substances. Major disadvantages are that serum is usually heated and that RF interferes with the results. Heating can be avoided by adding EDTA to the sample prior to testing (Tung *et al.*, 1978).

iii. *Assays of breakdown products of C3 and C1q.* Measuring levels of C3 breakdown products, namely C3c and C3d, has been used as indirect evidence that C-fixing IC-like materials are present in serum (Perrin *et al.*, 1975; Nydegger *et al.*, 1977). Blood is collected in EDTA; the derived plasma is mixed with 22% PEG, incubated, and then centrifuged. Native C3, C3b, and C3c are precipitated under these conditions, whereas the very small molecular weight fragment C3d remains in the supernatant and can be quantitated by means of radial immunodiffusion. C3 breakdown products in serum can also be detected by counterimmunoelectrophoresis (Arroyave and Tan, 1976).

Activation of C1 in human sera is readily detected and quantitated in double and radial immunodiffusion studies with anti-C1q, anti-C1r, and anti-C1s sera (Ziccardi and Cooper, 1978a,b). The sensitivity of this last approach in detecting various classical C pathway activators, such as ICs, and its clinical applicability have not yet been determined. Again, the participation of nonimmune activators of C1 and C3 may render this assessment extremely nonspecific, but these tests certainly represent a helpful adjunct in the study of IC-associated diseases.

iv. *The C3 precipitation assay and the solid phase anti-C3 as-*

say. These assays appear to be useful for the detection of C-fixing ICs. In the former assay, C-fixing ICs are first precipitated out of serum or plasma by an anti-C3 antibody (Jacobs and Reichlin, 1979), whereas in the latter assay C-fixing complexes bind to Fab'2 anti-C3 fixed on plastic surfaces (A. B. Pereira and A. N. Theofilopoulos, unpublished). Quantitation of precipitated or bound ICs is achieved by measuring the Ig by a direct reaction with radiolabeled antihuman Ig. These assays have not yet been applied in clinical material.

v. *The conglutinin radioimmunoassay.* Conglutinin is an unusual protein (mw 750,000) that occurs naturally in the sera of some ruminants, including cattle (Lachmann, 1967; Lachmann and Coombs, 1965). Because of its strong affinity for IC-fixed C3 fragments, conglutinin has been used for many years in various serologic tests. Recent information regarding its molecular structure and specificity of binding confirms that bovine conglutinin is not an Ig and is therefore distinct from immunoconglutinins, which are found in essentially all species. The biologic role of conglutinin is not known. Conglutinin has the important property of producing strong agglutination of sheep red blood cells coated with IgM antibody and C. The binding of conglutinin to ICs is C and Ca^{2+} dependent and appears to be specific for the inactivated form C3bi of the C3b (Lachmann, 1967; Gitlin *et al.*, 1975; R. A. Eisenberg *et al.*, 1977). Conversion of IC-bound C3b to C3bi occurs very rapidly as shown by the binding of conglutinin to red cells sensitized by prior incubation for 1–2 minutes with fresh serum from any of various species including man, horse, guinea pig, and mouse (R. A. Eisenberg *et al.*, 1977; Linscott *et al.*, 1978). The IC-bound C3bi can be further fragmented, possibly within a matter of hours, to C3c and C3d, with the C3d remaining on the complex and the C3c released into the medium. IC-bound C3b is fragmented to C3c and C3d by the action of the serum enzyme C3b INA (conglutinogen activating factor), which acts together with β1H (accelerator of C3b INA) (Nillson and Müller-Eberhard, 1965; Whaley and Ruddy, 1976) and with another unidentified trypsinlike serum factor (Pangburn *et al.*, 1977). Linscott *et al.* (1978) proposed that once IC-bound C3b converts to C3d, which may occur within 60–400 minutes depending on the activity of C3b INA and β1H in the serum used as a source of C, the interaction of ICs with conglutinin is minimal. Conglutinin's interaction with C-fixing ICs can be inhibited or, more importantly, reversed by adding chelators of divalent cations such as EDTA and by adding sugars with acetamido group, e.g., *N*-acetyl-D-glucosamine (NADG) (Lachmann, 1967; Leon and Yokohari, 1964; R. A. Eisenberg *et al.*, 1977). Based on the affinity of conglutinin for IC-fixed fragments

of C3, R. A. Eisenberg *et al.* (1977) and, independently, Casali *et al.* (1977) developed a solid-phase radioimmunoassay to detect C-fixing ICs in sera. Conglutinin may be purified in moderate quantities from bovine serum (Lachmann, 1967; R. A. Eisenberg *et al.*, 1977; Theofilopoulos *et al.*, 1978a; Casali *et al.*, 1977), but one must screen sera from many cows by using the conglutinin agglutination test (Coombs *et al.*, 1961; Theofilopoulos *et al.*, 1978a) with IgM antibody and horse C-coated sheep red blood cells to obtain sera with relatively high titers. Individual conglutinin titers can vary from about 1 : 8 to 1 : 2096 (Lachmann, 1967; Theofilopoulos *et al.*, 1978a). The assay is performed by placing test sera in microtiter plates coated with conglutinin then quantitating the amount of bound IgG with a radiolabeled or enzyme-linked anti-IgG antibody (R. A. Eisenberg *et al.*, 1977; Theofilopoulos *et al.*, 1978a; Casali *et al.*, 1977). By using AHG as a standard, the sensitivity of this assay was found to be on the order of 5–10 μg/ml. The chief advantages of this assay are its specificity and relatively simple technology as well as the stability of conglutinin at 4°C (freezing and thawing of the isolated conglutinin are not recommended, since we have observed a related loss of activity). The disadvantages are preferential reactivity with large ICs and, owing to the conglutinin's specificity for probably short-lived intermediate fragments of C3b, detection of only a portion of the C-fixing ICs.

b. Antiglobulin Techniques. A number of helpful antigen-nonspecific assays for detecting ICs irrespective of their C-fixing activity employ antiglobulin reagents.

i. *Rheumatoid factor tests.* Human polyclonal RFs (pRF) are low-affinity IgM (and IgG) antibodies to monomeric IgG that, because of their multivalency, have a high affinity for aggregated IgG or IgG-containing complexes (Eisenberg, 1976). Thus, RF may be used similarly to C1q for the detection of IgG aggregates or ICs containing IgG. Monoclonal 19 S RF (mRF) shows a greater ability for precipitating ICs than does the pRF commonly found in sera of patients with rheumatoid arthritis (Winchester *et al.*, 1971). To a great extent, the successful use of these reagent in detecting ICs depends on the mRF selected. There is marked variation in the amount of monomeric IgG required to inhibit the reaction of different mRF with complexed IgG. The greater the mRF's affinity for the complexed IgG, the more sensitive the method is in the presence of large amounts of monomeric IgG. The original test involving precipitation in agarose gels, which was described by Winchester *et al.* (1971) was relatively insensitive (100 μg of AGH per milliliter). This sensitivity can be enhanced

greatly by incorporating RF into radioassays. In one type of competitive inhibition assay, ICs are quantitated by determining the inhibited binding of radiolabeled mRF to IgG-Sepharose (Gabriel and Agnello, 1977). In a sensitive radioimmunoassay with pRF, ICs or aggregates are quantitated by determining inhibition of the reaction of pRF with radiolabeled AHG. The pRF is precipitated with an anti-IgM serum, and the precipitated counts are assessed (Cowdery *et al.*, 1975). Finally, the ability of ICs or aggregates to inhibit agglutination of IgG-coated latex particles by RF is testable (Lurhuma *et al.*, 1976). Radioimmunoassays involving RFs are very sensitive (1–25 μg of AHG per milliliter); they detect IgG complexes as small as approximately 8 S (Gabriel and Agnello, 1977; Agnello *et al.*, 1976; Lurhuma *et al.*, 1976) and are independent of the C-fixing properties of the ICs. The disadvantages of these methods are that the results may be influenced by high concentrations of monomeric Ig; the presence of intrinsic RF in the sample may result in falsely negative results; the assays do not detect complexes other than those of IgG type (Lurhuma *et al.*, 1976), and the mRF to be used requires careful selection.

ii. *Other antiglobulin tests.* Levinsky and Soothill (1977) have detected IC-like material in sera by measuring their inhibition of agglutination of IgG-coated latex particles with a low-affinity rabbit IgM anti-human IgG reagent. According to these investigators, this reagent has a low affinity for monomeric IgG but a high affinity for aggregated or antigen-complexed IgG. The sera are first fractionated through an IgG cellulose column in order to remove intrinsic RF, which interferes with the results. The inhibition of agglutination is measured by counting residual unagglutinated latex particles in a Coulter counter.

As indicated above, in all assays for ICs false positive results may be derived from nonspecifically aggregated Ig. A new antiglobulin assay described by Kano *et al.* (1978) seems to overcome this major drawback. Their test involves IC-induced inhibition of the agglutination by a human anti-antibody of group 0, R_1r erythrocytes, which are sensitized with anti-Rh serum "Ripley." The anti-antibody is an IgM serum factor that is similar but not identical to RF. Anti-antibody specifically combines with the IgG-antibodies of homologous species when they undergo molecular transformation in reactions with their corresponding antigens (Milgrom *et al.*, 1956; Milgrom, 1962). The serologic activity of anti-antibodies that are directed against the $F(ab')_2$ portion of the antibody molecules in the complex cannot be neutralized by normal or heat-aggregated IgG (Milgrom *et al.*, 1956; Milgrom, 1962; Fudenberg *et al.*, 1964). The assay was applied with

varying degrees of positive results to testing sera of patients with SLE, rheumatoid arthritis, parasitic diseases, and malignancies (Kano *et al.*, 1978). Disadvantages of this assay are the necessity of screening very large numbers of sera to select one with titers of anti-antibody activity; RF interferes with the results, and the procedure is only semiquantitative.

c. Cellular Techniques. The cellular methods to detect ICs are based on the principles that aggregates or ICs interact with Fc and C receptors on the surfaces of various cell types and that cells' activities change after binding ICs. The major drawback of all cellular techniques is the possibility of false positive results caused by various anticellular antibodies commonly found in sera of patients with autoimmune and other disorders.

i. *The platelet aggregation test.* Platelets aggregate after their surface Fc receptors interact with IgG type ICs or aggregates, an activity that forms the basis for detecting ICs in sera (Myllylä, 1973; Penttinen *et al.*, 1971; Penttinen and Myllylä, 1970; Palosuo *et al.*, 1976). The platelets used in this assay must be freshly collected, since surface alterations produced by ICs or aggregates are dependent on the platelets' metabolic state. This method, although sensitive (5–10 μg of AHG per milliliter), is troubled by platelet preparations that lack uniformity, aggregation of platelets by materials other than ICs or aggregates (antiplatelet antibodies, myxoviruses, enzymes), inhibition of aggregation by RF, and the failure of ICs with fixed C to induce aggregation. Wager *et al.* (1973) claim that antiplatelet antibody-induced aggregation is not inhibited by IgM RF, whereas IC-induced aggregation is inhibitable, and suggest that this difference may be used to differentiate IC-induced from antiplatelet-induced aggregation.

ii. *Inhibition of antibody-dependent lymphocyte-mediated cytotoxicity.* ADCC resulting from lysis of IgG antibody-coated target cells by unsensitized lymphocytes has been advocated as a possible mechanism of tumor immunity, autoimmune diseases, viral infections, and allograft rejection. Lysis of antibody-coated target cells is induced by contact of the target cells with a subclass of lymphocytes bearing IgG and possibly IgM Fc receptors (Perlmann *et al.*, 1972, 1977; MacLennan, 1972). Inhibition of ADCC, as a consequence of soluble IgG- or IgM-type ICs in the serum competing with antibody-coated target cells for the appropriate Fc receptors on the effector lymphocytes, is a means of detecting ICs (Jewell and MacLennan, 1973; Hallberg, 1974; Fye *et al.*, 1977; Barkas *et al.*, 1976; Scheinberg and Cathcart, 1976; Roberts-Thompson *et al.*, 1976; Kammer and Shur, 1978). The assay seems to be sensitive (5–10 μg of AHG per milliliter),

but may be faulty when monomeric IgG in addition to antilymphocyte antibodies inhibits target cell lysis (Barkas *et al.*, 1976; Feldmann *et al.*, 1976). Furthermore, because certain antibody-coated target cell types are sensitive to C-dependent lysis, decomplementation by heating the pathologic sera before testing is required, although one may bypass this problem by adding EDTA. It should be noted also that ICs with fixed C bind to cells *in vitro* preferentially via the C3 receptors, not via the Fc receptor (Section III,B); therefore, such C-fixing complexes do not inhibit ADCC (Kammer and Schur, 1978).

iii. *Polymorphonuclear leukocytes.* Immunofluorescence has been used to detect cytoplasmic Ig in polymorphonuclear (PMN) leukocytes presumably caused by phagocytosis of ICs. Cytoplasmic IgG and IgM RF have been found in PMN cells from synovial tissues and fluids of patients with rheumatoid arthritis (Kinsella *et al.*, 1969; Vaughan *et al.*, 1968; Cats *et al.*, 1975). Ig has also been found in the cytoplasms of PMN cells of patients with Felty's syndrome (Hurd *et al.*, 1977) and patients with malignant melanoma (The *et al.*, 1978), as well as in Reed–Sternberg cells from patients with Hodgkin's disease (Kadin *et al.*, 1978). Steffelaar *et al.* (1976, 1977) detected ICs both in a direct test that demonstrates *in vivo* phagocytosed ICs and an indirect test in which normal PMN cells are incubated *in vitro* with pathologic sera and then checked by immunofluorescence for inclusions of Ig, C, and antigen. Since cellular destruction *in vitro* may allow release of antigens that then complex with free antibodies in serum, as in many other assays, blood must be collected so as to avoid cell breakage, and blood for the direct test must be mixed immediately with an inhibitor of phagocytosis (i.e., 2-deoxyglucose, etc.) to prevent artifactual activity.

Inhibition of *Escherichia coli* killing by PMN cells is also being used as a means to detect ICs or aggregates in serum (Lambert *et al.*, 1978). In this test, PMN cells are incubated with test sera, washed, mixed with *E. coli* and then compared to untreated PMN cells for their ability to phagocytose and kill *E. coli.*

iv. *Eosinophils and mast cells.* Eosinophils are used as IC reactants (Takenaka *et al.*, 1977) by calculating the amount and rate of peroxidase they release after interacting with ICs. Complexes formed with IgE and rabbit anti-IgE are particularly effective. This technique has not been applied to the study of clinical materials. Measuring histamine release from mast cells is a similar assay for detecting IC in sera (Baumal and Broder, 1968).

v. *The macrophage inhibition assay.* This method is based on the competitive inhibition of uptake of radiolabeled AHG by macrophages in the presence of pathologic sera containing ICs or aggre-

gates (Onyewotu *et al.*, 1974). Problems with this assay are the requirement for isolation of macrophages from guinea pigs, interference of RF with the results (Onyewotu *et al.*, 1975), and the fact that its clinical applicability has not been adequately tested.

vi. *Rosette inhibition tests.* Inhibition of rosette formation between lymphocytes bearing Fc and C receptors and erythrocytes sensitized with antibody or antibody and C is a means of detecting ICs (Morito *et al.*, 1976; Smith *et al.*, 1975). These assays have little specificity, since high concentrations of monomeric Ig, breakdown fragment of C3, and antilymphocyte antibodies may interfere with the results and produce false positivity.

vii. *The Raji cell assay.* As mentioned earlier, B lymphocytes and lymphoblastoid cells in continuous culture bind soluble ICs or aggregates by receptors for Fc and receptors for C (Dickler, 1976; Nussenzweig, 1974; Theofilopoulos *et al.*, 1974a,b; Theofilopoulos, 1977; Eden *et al.*, 1973a,b). Therefore, these cells can be used as *in vitro* detectors of ICs in biologic fluids. Human cultured lymphoblasts are better than peripheral B lymphocytes for this purpose in that they are relatively homogeneous and easily accessible. After examining the surface markers of several human B-type lymphoblastoid cell lines, we found the Raji cell line (derived from a Burkitt's lymphoma) most suitable for detecting ICs since these cells lack surface Ig, have few or low-affinity receptors for IgG Fc (therefore, bind little 7 S IgG), and have large numbers of high-affinity receptors for C1q, C3b, C3d, and other C components (therefore, bind large amounts of C-fixing ICs or aggregates) (Theofilopoulos *et al.*, 1974a,b,c; Sobel and Bokisch, 1975; Dierich and Landen, 1977). Theofilopoulos *et al.* (1974a) demonstrated that the majority of C-fixing ICs bind to Raji cells via receptors for C3b and C3d. However, Gupta *et al.* (1978a) recently attempted to show that soluble C-containing ICs, in contrast to particulate complexes, bind to Raji cells only via C1q receptors, not via C3b-C3d receptors. In our view, these experiments by no means indicate that C3 receptors are unimportant in binding soluble IC to Raji cells. Furthermore, Sauter and Nelson (1978) and Manthei and Strunk (1979) have confirmed that, although ICs may bind to the C1q receptors on Raji cells, as originally shown by Sobel and Bokisch (1975), the binding is enhanced 6-fold in the presence of fresh human serum containing C3. Several other investigators have shown the importance of C3 in mediating binding of soluble ICs to B-type lymphocytes (Eden *et al.*, 1973b; Nussenzweig, 1974; Papamichail *et al.*, 1975). Initially, Theofilopoulos *et al.* (1974c) used the C receptors on the Raji cells to develop a nonquantitative fluorescence assay, which was subse-

quently adapted to a quantitative radioimmunoassay (Theofilopoulos *et al.*, 1976a; Theofilopoulos and Dixon, 1976). Because Raji cells are devoid of membrane-bound Ig, the technique quantitates ICs bound to the cells in terms of the uptake of radioactive antibody by IgG in the complexes. The amounts of ICs in the test sera is extrapolated from comparison with a standard curve of radiolabeled antibody uptake obtained by incubating cells with various amounts of AHG mixed with normal human serum as a source of C.

Overestimation or underestimation of the actual amount of ICs present is possible, depending on the molecular size of the ICs in the pathologic sera that determines the ICs' ability to fix C and thus to bind to Raji cells' C receptors. The Raji cell method is sensitive (6–12 μg AHG/ml) and has been used successfully for detecting ICs in sera from humans and animals with a variety of disorders (see Section V). The technique is rather easy to perform, is reproducible, and requires little serum. Moreover, antigens related to the disease under study can sometimes be identified when one stains the surfaces of these cells with immunofluorescent antibodies or checks uptake of radiolabeled antibodies directed against the suspected antigens (Theofilopoulos *et al.*, 1976a, 1977). The major disadvantages of the Raji cell assay, and of all cellular techniques, are, first, false positive results owing to the presence of warm reactive noncomplexed IgG-type antibodies with auto- or allospecificity to cellular surface components; and, second, interaction of antibodies in serum with cytoplasmic or nuclear antigens liberated from dead cells. When the latter problem is suspected, the immunofluorescent Raji cell assay (Theofilopoulos *et al.*, 1974c) should be used because it detects Ig bound only to live cells. When 30 SLE sera were examined by both the Raji cell immunofluorescent test and the Raji cell radioimmunoassay, all but one of these sera were positive in both tests.

Possibly because of removal *in vivo*, warm reactive IgG antilymphocyte antibodies (in contrast to IgM cold-reactive antibodies), seem to be present in sera only in low amounts and/or affinity (Winchester *et al.*, 1974) and react poorly with Raji cells under the conditions of the test. This conclusion is based on the observations that (*a*) very little or no IgG from SLE sera binds at 37°C to Raji cells with blocked Fc and C receptors (Theofilopoulos *et al.*, 1976a); (*b*) $F(ab')_2$ fragments of IgG isolated from SLE sera bind very poorly, if at all, to Raji cells and only when used in a concentration similar to that in undiluted serum (the pathologic sera are tested at 1 : 4 dilution) (Theofilopoulos *et al.*, 1978a); (*c*) levels of ICs correlate poorly with cytotoxic titers of sera against Raji cells (Woodroffe *et al.*, 1977); (*d*) sera from

multiparous women, which often contain antilymphocyte antibodies, are uniformly negative in the Raji assay (Lambert *et al.*, 1978; Gleicher *et al.*, 1978; and (*e*) results obtained with the Raji cell assay on various human sera correlate frequently with results obtained by other assays (C1q, conglutinin) that are not influenced by antilymphocytic antibodies (Lambert *et al.*, 1978; R. A. Eisenberg *et al.*, 1977; Lawley *et al.*, 1979; Woodroffe *et al.*, 1977; Tung *et al.*, 1978). Possibly antilymphocyte antibodies even assume the form of ICs in serum (Day *et al.*, 1976). Considering the above findings, the bulk of IgG bound to Raji cells from pathologic sera is probably not derived from free antibody *in vivo*, yet, as in all the cellular techniques, IgG antilymphocyte antibody could cause some falsely positive results in some instances (R. C. Williams *et al.*, 1976).

viii. *The L1210 murine leukemia cell assay.* Another radioimmunoassay has been developed in which the L1210 murine leukemia cell (Poskitt and Poskitt, 1978) replaces the Raji cell as a substrate for detecting ICs. ICs bind to IgG Fc receptors, after which cell-bound IgG is quantitated with a radiolabeled anti-IgG serum. The authors found that Fc receptors on these cells bind 7 S IgG from various species poorly. A major disadvantage is that the assay may not actually detect C-fixing ICs, since fixation of C3 appears to interfere with Fc receptor binding (Eden *et al.*, 1973b; Theofilopoulos *et al.*, 1974b; Kammer and Schur, 1978). In addition, this cell line requires *in vivo* intraperitoneal passage for maintenance although *in vitro* cultures of this cell line have recently been established.

ix. *The human erythrocyte radioimmune assay.* Instead of using Raji cells, C-fixing ICs can also be detected using human red blood cells as a substrate (Tsuda *et al.*, 1979). This assay, although it overcomes the need for tissue culture facilities and handling, has several disadvantages: It detects only C3b- or C4b-bearing ICs but not C3d-bearing ICs; the red cells to be used should be carefully selected since there is a wide range of variation in the number of immune adherence receptors (C3b-C4b) present on the surfaces of red blood cells from different donors; only "O" group Rh-negative individuals can be used as donors; and false positive results can be obtained due to the presence of anti-erythrocyte antibodies so commonly found in many diseases.

d. Other Methods. Interaction of ICs with staphyloccal protein A. Protein A from the bacterium *Staphylococcus aureus* binds the Fc piece of human IgG_1, IgG_2, and IgG_4, as well as various murine IgG subclasses and possibly IgM (Forsgren and Sjöquist, 1966; Grov *et al.*, 1970; MacKenzie *et al.*, 1978). This property has been put to use in assays for ICs (Hallgren and Wide; 1976; McDougal *et al.*, 1979). Removal of monomeric IgG by gel filtration is a prerequisite in this tech-

nique, RF interferes with the results, and C-fixing ICs may not be detectable (Scharfstein *et al.*, 1979). *Nephelometric assays* have also been developed for the detection of ICs (Höffken *et al.*, 1979; Whitsed *et al.*, 1979; Roberts-Thomson and Bradley, 1979).

V. Application of Assays for Immune Complexes

Assays for ICs may be useful in establishing a diagnosis or prognosis and in monitoring therapy. There follows a summary of the various human disease states (see Table VI) that involve endogenous or exogenous antigens and to which these assays have been applied.

A. Immune Complex-Associated Diseases

1. Autoimmune Diseases

a. Rheumatoid Arthritis. Classical rheumatoid arthritis is a systemic disease characterized by erosive polyarthritis and associated with the presence of IgM RF and IgG RF in serum. The principal

TABLE VI
DISEASES ASSOCIATED WITH IMMUNE COMPLEXES

Autoimmune diseases
Rheumatoid arthritis, Felty's syndrome, systemic lupus erythematosus, Sjögren's syndrome, mixed connective tissue disease, periarteritis nodosa, systemic sclerosis
Glomerulonephritis
Exogenous and endogenous antigens
Neoplastic diseases
Solid and lymphoid tumors
Infectious diseases
Bacterial: Infective endocarditis, meningococcal infections, disseminated gonorrheal infection, recurrent infections in children, infected ventriculoarterial shunt, streptococcal infections, leprosy, syphilis
Viral: Dengue hemorrhagic fever, cytomegalovirus infections, viral hepatitis, infectious mononucleosis, SSPE (subacute sclerosing panencephalitis)
Parasitic: Malaria, trypanosomiasis, schistosomiasis, filariasis, toxoplasmosis
Other conditions
Dermatitis herpetiformis and celiac disease, ulcerative colitis and Crohn's disease, myocardial infarcts, idiopathic interstitial pneumonia, cystic fibrosis, sarcoidosis, multiple sclerosis, amyotrophic lateral sclerosis, myasthenia gravis, uveitis, otitis media, atopic diseases, arthritis associated with intestinal bypass procedure for morbid obesity, sickle-cell anemia, thrombotic thrombocytopenic purpura, primary biliary cirrhosis, kidney and bone marrow transplantation, pregnancy, preeclamptic and eclamptic syndrome, Lyme arthritis, steroid-responsive nephrotic syndrome, xanthomatosis, vasectomy, oral ulceration and Behçet's syndrome, pemphigus and bullous pemphigoid, IgA deficiency, thyroid disorders, ankylosing spondylitis, iatrogenic diseases

antigenic site for reactivity of most RFs appears to be the Fc portion of IgG, but specificities for other sites have also been described (Henney, 1969; Williams, 1964; Waller and Blaylock, 1966). RFs can be found in sera of patients with many other disorders, e.g., infectious endocarditis, sarcoidosis, leprosy (Carson *et al.*, 1978; Kunkel *et al.*, 1958; Williams and Kunkel, 1962). Recent *in vivo* and *in vitro* experiments have shown induction of IgM RF production by lymphocytes after interaction with antigens, and polyclonal B cell activators such as LPS (Dresser, 1978; Izui *et al.*, 1979a) and Epstein–Barr virus (Slaughter *et al.*, 1978).

Antiglobulins of the IgG, and IgM classes have been shown to form ICs in serum and joint fluid either by self-association (Pope *et al.*, 1974) or by reaction with native IgG and such ICs seem to be involved in the pathogenesis of rheumatoid arthritis. The synovial fluids of these patients frequently contain ICs of the IgG-IgM RF and of the IgG-IgG RF varieties (Hannestad, 1968; Winchester *et al.*, 1970, 1971; Zubler *et al.*, 1976a; Zvaifler, 1974; Hashimoto *et al.*, 1977; Luthra *et al.*, 1975; Agnello *et al.*, 1970). These ICs react strongly with C1q and IgM mRF in precipitation assays (Agnello *et al.*, 1970; Winchester *et al.*, 1969, 1970, 1971; Winchester, 1975; Zubler *et al.*, 1976a; Luthra *et al.*, 1975; Halla *et al.*, 1978a) and may be responsible for the intraarticular activation of the classical and alternative C systems known to occur in synovial fluids (Winchester *et al.*, 1969, 1970; Hedberg, 1963; Pekin and Zvaifler, 1964; Zvaifler, 1969, 1978; Ruddy *et al.*, 1971; Lambert *et al.*, 1975; Perrin *et al.*, 1975). Intermediate molecular weight complexes of IgG in rheumatoid synovial fluids were shown to have platelet-activating activity (Valone *et al.*, 1979). Synovial fluids may also contain cryoimmunoglobulins with RF and antinuclear antibody activities (Cracchiolo *et al.*, 1971; Marcus and Townes, 1971; Zvaifler, 1978). ICs are phagocytosed by neutrophils in the synovial fluids and other phagocytic cells in the synovial tissues, a process that is enhanced by IgM RF binding to IgG–IgG RF complexes (Kinsella *et al.*, 1969; Hurd *et al.*, 1970; Munthe and Natvig, 1971). It has been proposed (Cooke *et al.*, 1975; Jasin and Cooke, 1978) that such ICs sequestered in periarticular tissues may persist for a long time and may be responsible for the chronic inflammation. Parenthetically, receptors for IgG Fc, C3b, and C3d were found recently on the surfaces of cultured synovial cells from normal persons and rheumatoid arthritics (Theofilopoulos, 1978). Histochemical and other criteria suggested that the cultured cells bearing Fc and C3b receptors were synovial lining cells. Such cells after phagocytosis of ICs could release lysosomal enzymes that in turn may cleave fluid-phase C3, yielding

C3b and C3d. These cleavage units of C3 can then bind to lining cells via C receptors and stimulate the release of enzymes, etc., leading to chronic inflammation. This mechanisms resembles that described by Schorlemmer *et al.* (1976) to explain the role in inflammatory processes of macrophages activated by soluble C3b, and the subsequent cleavage of native C3 by lysosomal enzymes released from the activated macrophages. Presence of IC-like materials in pleural and pericardial effusions of rheumatic patients have also been suggested by the finding of selective depletion of C components and the presence of C1q-reactive materials (Natali and Ashton, 1978).

Several of the techniques for detecting circulating ICs described earlier in this review have been used to study ICs in sera of patients with rheumatoid arthritis. In the classical studies of Franklin *et al.* (1957), physicochemical factors such as size and density were used to separate from rheumatoid sera the 22 S sedimenting protein component that reacted in sheep cell agglutination and latex fixation tests and was composed of IgG–IgM RF complexes. In addition, Kunkel *et al.* (1961) and Schrohenloher (1966) determined by direct ultracentrifugation analysis that some rheumatoid sera contained globulin ICs varying from 9 to 17 S. These ICs were easily dissociated into 7 S units with acid buffers or urea and were shown to be IgG–IgG RF complexes. Cryoglobulins containing IgM RF and sometimes IgG RF have also been described in sera of some patients with rheumatoid arthritis, especially those with vasculitis (Weisman and Zvaifler, 1975). Varying degrees of positivity for ICs or like materials in sera of patients with rheumatoid arthritis are obtained with the C1q deviation tests (Gabriel and Agnello, 1977), the C1q-PEG test (Zubler *et al.*, 1976b), the mRF precipitation test (Winchester *et al.*, 1971), inhibition of AHG binding to mRF (Luthra *et al.*, 1975; Halla *et al.*, 1978a), inhibition of mRF binding to IgG-Sepharose (Gabriel and Agnello, 1977), the platelet aggregation test (Norberg, 1974), inhibition of ADCC (Roberts-Thompson *et al.*, 1976; Fink *et al.*, 1977; Diaz-Jouanen *et al.*, 1976; Scheinberg and Cathcart, 1976), the macrophage inhibition test (Onyewotu *et al.*, 1975), the histamine-release assay (Russell *et al.*, 1974), the inhibition of Fc rosette formation (Hashimoto *et al.*, 1977), the conglutinin assay (Casali *et al.*, 1977), and the staphyloccal protein A test (Hallgren and Wide, 1976) and combinations of the above tests (R. C. Gupta *et al.*, 1979; Halla *et al.*, 1979a). Under auspices of the World Health Organization (WHO) (Lambert *et al.*, 1978), 18 techniques were evaluated, 6 of which indicated significantly elevated incidences of positivity; 75 and 81% of sera were positive in the Raji and the C1q-PEG binding test, respectively. Zubler *et al.* (1976a) and

Hay *et al.* (1979) found that the serum C1q binding activity in patients with rheumatoid arthritis and extraarticular disease manifestations is significantly increased compared to that in patients with joint disease alone. Similarly, Luthra *et al.* (1975) stated that the presence of material detected with an mRF inhibition test correlates with severity of disease. Others have associated IgG RF with vasculitis (Theofilopoulos *et al.*, 1974d), and deposits of IgG and C in the sural nerves and rheumatoid nodules of patients with rheumatoid vasculitis have been reported (Conn *et al.*, 1976; Munthe and Natvig, 1971; Pernis *et al.*, 1963).

ICs in sera of patients with rheumatoid arthritis are predominantly of the intermediate IgG–IgG RF variety (Winchester *et al.*, 1971; Norberg, 1974; Gabriel and Agnello, 1977). This finding may explain the optimal reactivity of these complexes with mRF, their low reactivity with C1q (Winchester *et al.*, 1970; Agnello *et al.*, 1970; Gabriel and Agnello, 1977), and the comparatively little C activation in the patients' sera (Vaughan *et al.*, 1951; Glass and Schur, 1977). Yet another explanation of the latter may be an increased synthesis of C components associated with the inflammatory syndromes, masking increased catabolism. In fact, catabolism of radiolabeled IgG, C3, and factor B is heightened in turnover studies, and C3 breakdown products are present in the plasma of some rheumatoid patients (Weinstein *et al.*, 1972; Ruddy and Colten, 1974; Versey *et al.*, 1973; Lambert *et al.*, 1975; Krick *et al.*, 1978; Catalano *et al.*, 1977). Nydegger *et al.* (1977) correlated plasma C3d levels with circulating ICs, as measured by determination of C1q binding activity, and found that levels of C3d were higher in rheumatoid patients with extraarticular disease than in those with joint disease alone.

Collectively these studies indicate that rheumatoid arthritis is associated with circulating as well as articular ICs which appear to play an important role in the pathogenesis of the disease. Although the majority of these ICs seem to be IgG–IgG RF complexes or IgG–IgM RF complexes and combinations thereof, one cannot exclude the possibility that other antigen–antibody systems are responsible for the immunopathology of this disease. In fact, sera from approximately two-thirds of rheumatoid patients contain antibody reactive to nuclear antigen(s) in human B-lymphocyte cultures (Alspaugh and Tan, 1976). Evidence was presented that this nuclear antigen(s) is associated with Epstein–Barr virus-transformed lymphocytes and differs from previously described Epstein–Barr virus nuclear antigens (Alspaugh *et al.*, 1978). These findings although provocative do not necessarily imply an etiologic relationship between Epstein–Barr virus infection and rheumatoid arthritis.

b. Felty's Syndrome. Felty's syndrome consists of the triad of rheumatoid arthritis, leukopenia (neutropenia), and splenomegaly (Felty, 1924). Common clinical adjuncts are lymphadenopathy, anemia, thrombocytopenia, and vasculitis. Patients with this syndrome usually have high titers of RF and frequently are positive for antinuclear antibodies. Most of their sera examined by analytical centrifugation contain intermediate complexes, possibly IgG–IgG RF. The large inclusions with IgG, IgM, and C3 seen by immunofluorescence in normal neutrophils incubated with sera from patients with Felty's syndrome and the similar inclusions in the patients' own neutrophils (Andreis *et al.*, 1978; Hurd *et al.*, 1977; R. C. Gupta *et al.*, 1976) are further indications of presence of ICs in the sera of such patients. These investigators suggested that phagocytosis of circulating ICs by neutrophils *in vivo* may interfere with the function of these cells in combating infections and also render them susceptible to removal from the circulation, thus leading to the development of neutropenia. An additional mechanism for the neutropenia may be the presence of anticellular antibodies.

c. Systemic Lupus Erythematosus. SLE, the prototype of IC disorders in humans, is characterized by a variety of autoimmune responses and various manifestations of IC disease, such as glomerulonephritis, vasculitis, and arthritis. The etiologic agent(s) remains unknown, but notable signs are the hyperactivity of B cells in producing various autoantibodies against self-components, cellular and noncellular (reviewed by Glass and Schur, 1978), and possibly imbalances in T-cell function (Morimoto, 1978; Bresnihan and Jasin, 1977).

Various types of ICs may be formed in patients with SLE due to the presence in their sera of a spectrum of antibodies that react with native or altered autologous antigens. Among them are antibodies to nuclear components, to cell surface and cytoplasmic antigens of lymphocytes, red cells, platelets, PMN cells, and neuronal cells, and to IgG (RFs). Antibodies against nuclear material, the hallmark of the disease, are common, and they may be directed toward a variety of antigens, including DNA (Tan *et al.*, 1966; Koffler *et al.*, 1971; Robbins *et al.*, 1957), deoxyribonucleohistone (Holman and Kunkel, 1957), histone (Holman *et al.*, 1959), RNA (Schur and Monroe, 1969), nucleolar antigens (Pinnas *et al.*, 1973; Miyawaki and Ritchie, 1973), nuclear antigens expressed only on transformed cells (Miyachi *et al.*, 1978), and constituents of the soluble nuclear extract, among which the most prominent are the nuclear ribonucleoprotein antigen (RNP) (Mattioli and Reichlin, 1971; Sharp *et al.*, 1972), the Sm antigen (Tan and Kunkel, 1966a) and the Ha antigen (Akizuki *et al.*, 1977a). Antibodies to

native DNA and Sm seem to be highly specific for SLE, and anti-DNA antibodies in high titers generally accompany active lupus nephritis (Koffler *et al.*, 1971; Tan *et al.*, 1966; Robitaille and Tan, 1973; Schur and Sandson, 1968). The antilymphocyte antibodies, which may be directed against T cells, or B cells or both, obtained from normal individuals or those with lupus are predominantly of the IgM cold-reactive variety, but those of the IgG class also exist (Winchester *et al.*, 1974; Winfield *et al.*, 1975b; Terasaki *et al.*, 1970; Mittal *et al.*, 1970; Stastny and Ziff, 1971; Bluestein and Zvaifler, 1976). Some of these lymphocytotoxic antibodies cross-react with brain cells and may play a role in the development of the central nervous system complication frequently seen in patients with SLE (Bluestein and Zvaifler, 1976; Bluestein, 1978). However, Winfield *et al.* (1978) found no correlation between levels of antilymphocyte antibodies and the presence or severity of central nervous system manifestations.

Investigation of some of the mechanisms of tissue injury suggests that SLE is at least in part an IC deposition disease. Deposits of DNA, Ig, and C found in the kidneys of SLE patients resemble those found in animals with experimentally induced nephritis (Koffler *et al.*, 1973; Andres *et al.*, 1971; Glass and Schur, 1978). Moreover, Ig and C are detected commonly at the dermal–epidermal areas of skin from patients with SLE (Gilliam *et al.*, 1974; Tan and Kunkel, 1966b; Pohle and Tuffanelli, 1968; Schroeter *et al.*, 1976). Similar Ig and C deposits have been observed in the small blood vessels of SLE patients with necrotizing vasculitis (Tan and Kunkel, 1966b), in the choroid plexi of some patients with cerebral involvement (Atkins *et al.*, 1972), and in the lung (Eagen *et al.*, 1979). The Ig eluted from nephritic kidneys of patients with SLE is highly enriched relative to serum levels of anti-native DNA and anti-single-stranded DNA antibodies (Koffler *et al.*, 1967, 1971, 1973, 1974; Krishman and Kaplan, 1967; Winfield *et al.*, 1977a; Agnello *et al.*, 1973). Antibodies to the retroviral antigen p30 and p30 antigen were claimed to be present in glomeruli of biopsied kidneys of a few such patients (Mellors and Mellors, 1978). Sera and kidney eluates of murine strains (NZB, NZB × NZW, MRL, BXSB) with spontaneous SLE-like syndromes contain, to varying degrees, antibodies similar to those found in humans with SLE, including anti-DNA, anti-RNA, anti-Sm (only in MRL mice), anti-thymocyte, RFs, and, in addition, anti-retroviral gp70 (Andrews *et al.*, 1978a; Eisenberg *et al.*, 1978, 1979; Yoshiki *et al.*, 1974; Shirai and Mellors, 1971; Shirai *et al.*, 1978; Izui *et al.*, 1979b).

Cryoglobulins, which in some instances may represent ICs, are frequently present in sera of SLE patients, and their quantity correlates

well with the severity of SLE, high levels of anti-DNA antibody, and reduced serum C levels (Christian *et al.*, 1963; Stastny and Ziff, 1969; Agnello *et al.*, 1971; Druet *et al.*, 1973; McPhaul, 1978). Cryoprecipitates contain RFs, anti-DNA, anti-RNP (Winfield *et al.*, 1975a; Zvaifler and Bluestein, 1976) and C components, especially C1q (Winfield *et al.*, 1975a; Agnello *et al.*, 1971). DNA is sometimes demonstrable in the cryoprecipitates (Lee and Rivero, 1964; Forsen and Barnett, 1968; Winfield *et al.*, 1975a; Davis *et al.*, 1978), but not always (Hanauer and Christian, 1967; Stastny and Ziff, 1969). Despite the specific concentration of antilymphocytic antibodies found in cryoglobulins of SLE sera (Winfield *et al.*, 1975b; Zvaifler and Bluestein, 1976), the search for lymphocyte cell-membrane antigens remains unsuccessful (Winfield *et al.*, 1975b; Zvaifler and Bluestein, 1976). Finally, although cryoglobulin levels correlate with disease activity, the titer of lymphocytotoxic antibody in the cryoglobulins does not correlate with that of anti-DNA antibody, with the clinical diagnosis of nephritis, or with the involvement of any other organ system (Zvaifler and Bluestein, 1976).

IC-like materials have been detected in sera of SLE patients by a variety of procedures. Agnello *et al.* (1970) analyzed hypocomplementemic SLE sera using gel systems and described C1q precipitating materials whose presence correlated with disease activity; after treatment they disappeared. When the same authors used C1q and mRF radioimmunoassays, the majority of SLE sera were positive for IC-like materials (Gabriel and Agnello, 1977). However, the C1q tests showed a higher incidence of positives and higher levels of positivity than the mRF assay. In some SLE sera studied by sucrose density gradient analysis, the C1q reactive materials were of two different sizes, one approximately 7 S and the other larger than 19 S (Agnello *et al.*, 1971; Gabriel and Agnello, 1977). The material with high molecular weight reacts with both C1q and mRF whereas the low molecular weight material reacts only with C1q. The high molecular weight substances do not seem to be DNA–anti-DNA complexes (Agnello *et al.*, 1970). The exact nature of both these materials is still unknown, but their presence is associated with hypocomplementemia, active disease, and a high degree of cryoprecipitation (Agnello *et al.*, 1971, 1973). The hypothesis has been advanced that interaction of IgM RF with circulating complexes and C1q might potentiate their deposition and result in tissue injury (Agnello *et al.*, 1971; Winfield *et al.*, 1975a). Evidence for the possible significance of the RFs in the nephritic process was obtained by Agnello and co-workers using serologic and renal biopsy studies (Agnello *et al.*, 1971) on a group of SLE patients followed

serially. Some of these patients had relatively large amounts of cryoprecipitates that contained RFs. These patients had significant proteinuria and on renal biopsy showed heavy deposits of IgM in a granular pattern in their glomeruli. In some of the biopsies anti-γ-globulins were detected by staining with fluorescein-labeled AHG. Thus, cryoglobulinemia in this situation appears to be an *in vitro* manifestation of circulating ICs, which *in vivo* are deposited in the glomeruli. More direct evidence for this was obtained by demonstrating that the same anti-γ-globulins were in the isolated cryoprecipitins and glomerular deposits. This was accomplished by staining the renal deposits with fluorescein-labeled idiotypic antiserum to the isolated anti-γ-globulins (Agnello *et al.*, 1971).

Nydegger *et al.* (1974) and Zubler *et al.* (1976b) used the C1q-PEG method and found significantly increased C1q binding values in sera of more than 60% of patients with SLE; particularly high were the values in patients with active disease. Similarly, Theofilopoulos *et al.* (1976a) using the Raji assay, Cano *et al.* (1977b) using the C1q deviation test, R. A. Eisenberg *et al.* (1977) and Casali *et al.* (1977) using the conglutinin technique, Morito *et al.* (1976) using an Fc rosette inhibition test, Scheinberg and Cathcart (1976) using an ADCC inhibition test, Digeon *et al.* (1977) using PEG precipitation, Levinsky *et al.* (1977) and Kano *et al.* (1978) using antiglobulin tests, Losito and Lorusso (1979) using immunofluorescence of PMN cells, and Davis *et al.* (1977) using combinations of the above tests, all found varying degrees of positivity and not only a rather direct correlation between levels of ICs or like materials and clinical activity, but also an inverse correlation with hemolytic C levels. Moreover, large amounts of C3 breakdown products were found in sera of SLE patients with glomerulonephritis (Perrin *et al.*, 1975), and serum C levels were decreased in most patients with SLE at some time during their illness, especially when the disease was active (reviewed by Glass and Schur, 1978). A WHO study has confirmed the presence of IC-like materials in SLE sera as six different methods for detecting ICs clearly discriminated between normal controls and a group of patients with SLE (Lambert *et al.*, 1978). Results from the Raji cell test, C1q-PEG test, C1q solid-phase test, and platelet-aggregation test, all correlated significantly in the WHO study. None of the tests based on interaction of ICs with RFs, polyclonal or monoclonal, showed a significant number of abnormal values in SLE, and this confirmed previous observations (Winchester *et al.*, 1971; Gabriel and Agnello, 1977). The concurrence among tests in the WHO study, the positive results obtained by various investigators using almost every technique currently available,

and the demonstration that the positive materials in many instances are high molecular weight IgGs, all suggest the true presence of ICs. Interestingly, IC-like materials were recently reported in sera of patients receiving procainamide (Becker *et al.*, 1979).

In the management of SLE, IC determinations are found useful by many investigators (Levinsky *et al.*, 1977; Pussell *et al.*, 1978; Davis *et al.*, 1977), since ICs disappear with remission and sometimes elevated IC levels are the first indication of recurring disease. According to these investigators, testing patients with SLE for DNA binding is not always a reliable indicator of disease activity, although high values can be obtained. Davis *et al.* (1977) suggested that measurement of DNA-binding, C levels, and IC levels together may provide better means of following these patients. Moreover, plasmapheresis has been advocated as an effective method for treating IC disease (Lockwood *et al.*, 1977; Verrier-Jones *et al.*, 1976), and IC levels fall immediately after this treatment in patients with SLE (Verrier-Jones *et al.*, 1976, 1978).

While glomerular elution studies suggest a role for DNA–anti-DNA complexes in the pathogenesis of SLE nephritis (Koffler *et al.*, 1973, 1974), direct evidence of circulating DNA–anti-DNA complexes has been difficult to obtain. In 1973 Harbeck *et al.* tested SLE sera and observed that the majority of samples had significantly increased titers of anti-DNA antibodies after DNase digestion, which indicated to the authors that DNA had bound *in vivo* to the anti-DNA in the sera. However, with few exceptions (Bruneau *et al.*, 1977), other experiments were not confirmatory (Feltkamp, 1975; Hughes, 1975), including those of Izui *et al.* (1977), who used a variety of procedures. Izui *et al.* (1977) concluded that the presence of large amounts of DNA–anti-DNA ICs in circulating blood is unlikely and that most of the ICs detected in SLE sera involve other antigen–antibody systems. The possibility that DNA–anti-DNA complexes form in serum and are rapidly phagocytozed or deposited was not, however, excluded. Rapid removal and localization of the DNA–anti-DNA complexes in glomeruli may be facilitated by the demonstrated affinity of DNA for collagen or collagen-contiaining structures such as the GBM (Izui *et al.*, 1976). Emlen and Mannik (1978) showed that the murine liver is the major organ for removal of circulating single-stranded DNA ($\simeq$90%), whereas the kidney takes up only 2–5%, confirming prior observations (Tsumita and Iwanaga, 1963; Chused *et al.*, 1972; Dorsch *et al.*, 1975). The clearance mechanisms of DNA from the circulation become saturated when large amounts of DNA are injected into mice (Emlen and Mannik, 1978), thus large molecular weight DNA can continue to circulate and is available for combination with antibodies

to form ICs for local deposition in tissues. Later studies (Bruneau and Benveniste, 1979) indicate that the majority of SLE plasmas do contain DNA–anti-DNA ICs, the antigen being of low molecular weight.

In the aforementioned murine strains (NZB, NZB × NZW, BXSB, MRL/1) with autoimmune SLE-like syndromes, circulating ICs detectable by the Raji cell assay increase quantitatively as the disease progresses (Dixon *et al.*, 1978; Andrews *et al.*, 1978a). Sera from some of these mice, expeically the MRL/1 strain, also have large amounts of cryoglobulins, the removal of which does not significantly reduce the levels of ICs. Regarding the nature of these circulating ICs, Izui *et al.* (1979b) have shown that the sera of these autoimmune-prone mice contain a heavy form of retroviral envelope glycoprotein gp70 with sedimentation rates from 9 S to 18 S in sucrose density gradients. This heavy gp70 appears with the onset of disease and persists throughout its course. By contrast, strains that do not develop SLE sometimes have large concentrations of gp70 in their sera, but not this heavy form. The fact that the heavy gp70 is selectively absorbed with anti-mouse IgG antibodies or with *Staphylococcus aureus* protein A and that the heavy gp70 is not present in sera of cyclophosphamide-immunosuppressed mice strongly suggests that the heavy form of gp70 is gp70–anti-gp70 complexes. The importance of these gp70–anti-gp70 complexes in the pathogenesis of glomerulonephritis seen in these mice remains to be determined. Whether the gp70 that evokes the immune response in question is derived from xenotropic or ecotropic retroviruses or recombinations thereof is not yet known. MRL/1 mice have, in addition to gp70 ICs, intermediate IgG–IgG RF complexes (Eisenberg *et al.*, 1979).

d. Other Autoimmune Diseases

i. *Sjogren's syndrome.* This syndrome, also known as sicca syndrome, is a chronic inflammatory, autoimmune disease that has a broad clinical and histopathologic spectrum ranging from lymphocytic infiltration of lacrimal, salivary, and other exocrine glands to a widespread lymphoproliferative process and lymphoreticular malignancy (Bloch *et al.*, 1965). The disorder can occur alone or in conjunction with another autoimmune disease, such as rheumatoid arthritis or SLE. Patients with Sjögren's syndrome have a high incidence of non-organ-specific autoantibodies, such as RF, as well as antibody to an extractable nuclear antigen termed SS-B (Alspaugh and Tan, 1975; Alspaugh *et al.*, 1976) or Ha (Akizuki *et al.*, 1977a,b) and are known to develop vasculitis, myositis, neuritis, glomerulonephritis, and tubulointerstitial nephritis (Bloch *et al.*, 1965; Kaltreider and Talal, 1969; Moutsopoulos *et al.*, 1978; Winer *et al.*, 1977). In examining sera of patients with

Sjögren's syndrome for ICs, Theofilopoulos *et al.* (1976a) used the Raji assay and Lawley *et al.* (1979a) used both the Raji and the C1q-PEG assays. In the former study, 6 of 10 patients with Sjögren's syndrome accompanied by cryoglobulinemia and vasculitis had circulating ICs. The latter study involved a large number of patients about 85% of whose sera were positive in both assays with an excellent concordance of results. Since the C1q-binding material could not be destroyed with 2-mercaptoethanol and since the activity was removed with protein A, it was concluded that these ICs were distinct from 19 S IgM RF found in these patients.

ii. *Mixed connective tissue disease syndrome.* MCTD may represent a variant of SLE (Reichlin, 1976), but the patient's unusual serologic features, high titers of antibody to RNP, and rarity of antibody to double-stranded DNA suggest that MCTD is a distinct clinical syndrome (Sharp *et al.*, 1976). In preliminary studies sera of patients with MCTD were examined for circulating ICs by the Raji cell assay, the mRF radioimmunoassay, and the C1q-PEG assay (Halla *et al.*, 1978b, 1979b; Cunningham *et al.*, 1978). Approximately 80–90% of the sera were positive by at least one of the tests, and 65% were positive by two or more methods. Changes in levels of ICs seemed to parallel clinical activity in the majority of patients, although the ICs' nature was not determined. Parker and Marion (1977) found all 8 patients studied with MCTD to be positive in the Raji cell assay and concluded that the absence of nephritis among such patients, despite the presence of circulating ICs, indicated a fundamental difference in the pathologic potential of ICs involving DNA and those involving RNP. However, kidneys of such patients do have IC deposits according to a report of Bennett and Spargo (1977), and renal involvement in MCTD may be more prevalent than previously indicated.

iii. *Periarteritis nodosa.* This is a disease, frequently of unknown etiology, characterized by widespread necrotizing vasculitis involving small-to-medium-sized arteries. Gocke *et al.* (1970, 1971) and Trepo and Thivolet (1970) reported the association of the hepatitis B antigen and periarteritis nodosa, but the pathogenic significance of that association remains unclear. Immunofluorescence studies of tissue from patients with periarteritis showed hepatitis B antigen, IgG, and C3 deposited in blood vessel walls along the elastic membrane (Gocke *et al.*, 1970, 1971). The majority of these patients also had hepatitis B antigen–antibody complexes in their sera when tested by various procedures (Gocke *et al.*, 1970; Trepo *et al.*, 1974). Nowoslawski *et al.* (1971) injected sera containing ICs from patients with hepatitis into the skin of rabbits, producing an Arthus-like reaction indicative of the

sera's phlogogenicity. However, Prince and Trepo (1971) could not correlate such ICs with the presence or absence of polyarteritis nodosa. These last authors suggest that, although the pathogenesis of polyarteritis nodosa probably involves immune mechanisms, these are not mediated by pathogenic ICs composed of hepatitis B antigen and antibody, but rather by cellular immune reactivity. In another study, Fye *et al.* (1977) found that ADCC of patients with hepatitis and polyarteritis nodosa decreased during periods of disease activity and was almost normal during remission. Furthermore, the patients' sera could block ADCC in normal lymphocytes, and the blocking ability correlated with the concentration of ICs as determined by the Raji cell assay.

iv. *Systemic sclerosis (scleroderma).* Systemic sclerosis is a connective tissue disease with three possible mechanisms—vascular alterations, abnormal collagen metabolism, and/or immunologic processes (Winkelmann, 1971). Approximately half of the patients with systemic sclerosis studied by Husson *et al.* (1976) had serum cryoglobulins that contained IgM and IgG or IgA and sometimes C3. RF activity was present in sera and cryoprecipitates of these patients. Moreover, Cunningham *et al.* (1978) and Pisko *et al.* (1979) found that approximately 40% of patients with systemic sclerosis were positive in the Raji cell assay, but there was no correlation between IC levels and severity of disease.

2. *Glomerulonephritis*

The immunologic renal injury produced by the formation and deposition of ICs has been detailed by Wilson and Dixon (1976). Kidney tissue can be injured by antibodies in two ways. The first and less common pathway occurs when antibodies specific for renal structural components, mainly GBM, bind to their respective tissue-fixed antigens. The second, and by far the commonest, pathway (70–80% of glomerulonephritides) of immunologic glomerular damage occurs when antibodies complexed with circulating antigens deposit in the vessels and filtering structures of the kidney. Exogenous antigens, such as drugs and infectious agents (bacteria, parasites, and viruses), as well as endogenous antigens, such as DNA, thyroglobulin, tubular antigens and tumor antigens, may participate in nephrotoxic IC formation (Wilson and Dixon, 1976). Glomerulonephritis due to circulating ICs may be a component of systemic disease such as SLE.

Anti-basement membrane antibodies accumulate smoothly all along the basement membrane and can be shown by immunofluorescence in a characteristic linear pattern, while ICs that deposit irregularly from

the circulation are visible in a broken, granular pattern. Ig, C components, and sometimes the inciting antigen can be identified by immunofluorescence with these granular deposits. Methods are also available to elute ICs from the glomerular tissues (Woodroffe and Wilson, 1977; Bartolotti, 1977), after which the specificities of the deposited antibodies can be identified.

A new dimension in the identification and management of patients with IC glomerulonephritis is possible in view of the techniques described above. Some of these patients have depressed serum C levels (Gotoff *et al.*, 1969), C3 breakdown products in the circulation (Perrin *et al.*, 1975) cryoglobulins (Adam *et al.*, 1973; McIntosh *et al.*, 1975) or RF (Rossen *et al.*, 1976), any of which suggest the presence of ICs. With both C1q and Raji cell assays, most patients who have IC-induced glomerulonephritis that complicates systemic disease are positive, and virtually all those with active SLE show circulating IC-like materials (Woodroffe *et al.*, 1977; Tung *et al.*, 1978). Persons with acute primary glomerulonephritis, such as poststreptococcal glomerulonephritis, are relatively infrequently positive, and those with chronic forms such as IC-induced membranous glomerulonephritis rarely have detectable circulating ICs (Woodroffe *et al.*, 1977; Digeon *et al.*, 1977; Ooi *et al.*, 1977a,b; Tung *et al.*, 1978). The infrequent detection of circulating ICs in patients with primary glomerulonephritis was confirmed in the WHO collaborative study that evaluated techniques for IC detection (Lambert *et al.*, 1978). This low incidence of positive results may indicate that the glomerulonephritides of these patients results from recurrent, brief, and not easily detected bouts of IC deposition. Other possibilities are differences in size and composition of ICs in systemic diseases from those in primary glomerulonephritis.

B. Neoplastic Diseases

Tumor cells expressing new antigens on their surface membranes (Haughton and Nash, 1969; Ferrone and Pellegrino, 1978) become foreign to the host and elicit both humoral and cell-mediated immune responses (I. Hellström and Hellström, 1969; Hellström *et al.*, 1971a; K. E. Hellström and Hellström, 1969; Oettgen and Hellström, 1974; Morton, 1971; Berke and Amos, 1973; Mizelewski, 1973; Cerottini and Brunner, 1974; Ferrone and Pellegrino, 1978). These responses should produce a condition detrimental to the malignant cells and hence conducive to continued survival of the host, but under certain circumstances, they do not. For example, interaction of tumor-specific antibodies with neoantigens on the tumor cells' surfaces may mask,

modulate, or remove these antigens, thus preventing their recognition by effector cells carrying specific antigen receptors. Moreover, free tumor antigens can combine with specific antibodies in the circulation to generate ICs that deposit in tissues or interact with effector lymphocytes, thereby interfering with the host's defense mechanisms.

Two types of tumor antigen–antibody ICs can be envisaged in patients with malignant disease. The first type, as just mentioned, results when antitumor antibodies interact with cell surface-associated tumor antigens. ICs formed on the tumor cells' surfaces may undergo endocytosis or be released into the cells' environment. The second variety of ICs occurs when circulating tumor antigens interact with antitumor antibodies. Malignant disease, analogous to chronic viral and autoimmune diseases, may be characterized by continuous production and release of tumor antigens into the circulation (Currie and Basham, 1972; Thomson, 1975; Thomson *et al.*, 1973; Vaage, 1974). Persistent antigenemia along with any degree of antibody production should cause circulating tumor antigen–antibody ICs to form continuously. Although one would expect to find tumor-antigen–antibody ICs at one time or another in the circulations of patients with malignant disease, typical manifestations of IC disease, such as glomerulonephritis or vasculitis, are infrequently found, possibly because these individuals' ICs are not of the appropriate size and composition. However, occasionally, glomerulonephritis does develop and may present as nephrotic syndrome (Glassock and Bennet, 1976; Kaplan *et al.*, 1976). Nephrotic syndrome or glomerulonephritis has now been observed in association with melanoma (Weksler, 1974), carcinoma of the lung (Lee *et al.*, 1966; Loughridge and Lewis, 1971; da Costa *et al.*, 1974), breast (Loughridge and Lewis, 1971), stomach (Cantrell, 1969; Weintroub *et al.*, 1975), and colon (Lee *et al.*, 1966; Costanze *et al.*, 1973; Couser *et al.*, 1974), Burkitt's lymphoma (Oldstone *et al.*, 1975), leukemia, lymphosarcoma, and Hodgkin's disease (Lyman *et al.*, 1973; Plager and Stutzman, 1971; Lee *et al.*, 1966; Ghosh and Muercke, 1970; Froom *et al.*, 1972; Sutherland *et al.*, 1974; Gault *et al.*, 1973; Lokich *et al.*, 1973), and Waldenström's macroglobulinemia (Lin *et al.*, 1973; Martello *et al.*, 1975). Several reports that proteinuria disappears after surgical removal of the primary tumor strongly suggest that the relationship between neoplastic and renal diseases is not fortuitous (Rizzuto *et al.*, 1965; Lumeng and Moran, 1966; Cantrell, 1969; Lee *et al.*, 1966; Hopper, 1974; Plager and Stutzman, 1971). In more than half of these cases, immunofluorescence and electron microscopy have demonstrated "lumpy" IC deposits in the kidney. In two patients, one with colon carcinoma and the other with malignant melanoma, both asso-

ciated with nephrotic syndrome, carcinoembryonic antigen and melanoma antigens, respectively, could be detected in the renal glomeruli in addition to IgG and C (Costanza *et al.*, 1973; Weksler, 1974). Examination of renal glomeruli from two patients with African Burkitt's lymphoma revealed host IgG and C3 (Oldstone *et al.*, 1975). Elution and subsequent immunologic assays indicated that the IgG in the glomeruli of these patients contained antibodies to viral capsid and early antigens, but not antibodies to C-fixing nuclear antigens or membrane antigens of Epstein–Barr virus. Furthermore, IgG eluted from kidneys of a patient with squamous cell carcinoma of the lung reacted specifically with the patient's tumor cells (Lewis *et al.*, 1971). These studies provide direct evidence that renal disease in some patients with cancer may be mediated by ICs composed of tumor antigens and antitumor antibodies. Severe IC disease in cancer patients may be rare because death from the neoplasm intervenes, but inapparent IC disease may be more frequent than is generally realized; for example, IgG and C deposits are found in kidney biopsies of patients with lymphoma but without clinical or histologic evidence of glomerulonephritis (Sutherland *et al.*, 1974). It has been proposed that any patient over 40 years of age with nephrotic syndrome should be checked thoroughly for malignant disease (Lee *et al.*, 1966; Glassock and Bennett, 1976; Hopper, 1974; Fichman and Bethune, 1974).

Glomerulonephritis due to the deposition of ICs has also been described in mice and rats with virus-associated leukemia, lymphoma, and sarcoma (Oldstone, 1975a; Oldstone *et al.*, 1972a,b, 1976a; Dixon *et al.*, 1974; Yoshiki *et al.*, 1974; Pascal *et al.*, 1973; Hirsch *et al.*, 1969; Jenette and Feldman, 1977). AKR mice, which spontaneously develop leukemia due to Gross murine leukemia virus (MuLV), have a high incidence (90%) of IC glomerulonephritis (Oldstone *et al.*, 1976a) with viral antigens, host Ig, and C detectable as glomerular deposits. Antibodies recovered from these IC deposits have specificities for the major classes of MuLV structural components gp70, gp45, and p30 (Oldstone *et al.*, 1976a). Circulating ICs and IC deposits in kidneys also appear in mice with melanoma (Poskitt *et al.*, 1974) and neuroblastoma (Oldstone, 1975b). The deposited ICs in these mice consist of tumor antigens and specific antibodies.

Assuming that the theory of immune surveillance is correct, the mechanisms by which cancer cells escape surveillance and produce tumors in persons with no identifiable immunologic deficiencies must be explained. One escape mechanism thought to function in oncogenesis involves "blocking factors" in sera of individuals with tumors. This concept was originally proposed and developed by the

Hellströms (K. E. Hellström and Hellström, 1970) following the development of *in vitro* assays of lymphocyte cytotoxicity for target tumor cells. Blocking factors may mask tumor antigens on malignant cells, preventing antigen recognition by effector cells, or they may react directly with effector cells, resulting in interference or alterations of these cells' functions (Hellström and Hellström, 1974; Baldwin and Robins, 1975, 1976). Factors that block *in vitro* cytotoxicity may be soluble tumor antigen–antibody complexes (Hellström and Hellström, 1974; Baldwin and Robins, 1975, 1976) or soluble free tumor antigens (Baldwin *et al.*, 1973b; Zoller *et al.*, 1976; Plata and Levy, 1974; Shellam and Knight, 1974; Vaage, 1974). Evidence to support the conclusion that tumor antigen–antibody ICs exert a blocking effect *in vitro* has been reviewed by Baldwin and Robins (1976) and can be summarized as follows:

1. Blocking factors obtained from serum, after elution from surgical specimens of tumor cells or from the patient's own lymphocytes, can be dissociated into low- and high-molecular-weight components with subsequent loss in blocking activity (Sjögren *et al.*, 1971, 1972; Hattler and Soehnlen, 1974).
2. Blocking factors have been generated *in vitro* by combining tumor-specific antibodies obtained from rats with hepatoma D23 and tumor-specific antigens prepared from hepatoma D23 membrane fractions (Baldwin *et al.*, 1972), or by combining sera from patients in remission from neuroblastoma with neuroblastoma membrane antigens (Jose and Seshadri, 1974).
3. Finally, blocking activity in the sera of patients with malignant disease can be abrogated (unblocked) by adding to the sera an excess of tumor-specific antibodies (I. Hellström and Hellström, 1970; Bansal and Sjögren, 1971, 1972, 1973; Robins and Baldwin, 1974; Hellström *et al.*, 1971c). These unblocking studies can be interpreted in terms of antibody-induced changes of IC size in tumor-bearing sera. Numerous studies of tumor-bearing humans and animals have indicated that the presence of blocking factors in serum correlates with the size of the tumor and indicates a poor prognosis (I. Hellström and Hellström, 1969; Hellström *et al.*, 1971b; K. E. Hellström and Hellström, 1974; Baldwin *et al.*, 1973a, 1974; Hayami *et al.*, 1974; Baldwin and Robins, 1975; Grosser and Thomson, 1976).

The awareness of the possible importance of ICs in cancer has stimulated the clinical application of IC detection. Therefore, Theofilopoulos *et al.* (1976a, 1977) used the Raji cell assay to test patients with melanoma, colon carcinoma, osteogenic sarcoma,

breast carcinoma, and esophageal carcinoma and found a high degree of positivity. Patients with active disease had higher levels of ICs than those with no clinical evidence of disease, particularly patients with malignant melanoma. Moreover, patients who were considered cured after surgery had lower incidences and levels of positivity than those considered not cured. Immunization of melanoma patients with BCG and tumor cell vaccine increased their levels of ICs. In follow-up studies of a few patients with melanoma, positivity in the Raji assay sometimes correlated with tumor burden, or concentrations of ICs rose before tumor recurrence. The ICs in selected cancer sera examined by sucrose density fractionation were of intermediate size; this provides an explanation for the infrequency of overt IC disease in cancer patients. Tumor antigens and IgG, presumably in the form of ICs at antigen excess, were identified with immunofluorescence and radiolabeled antibody techniques on the surfaces of Raji cells incubated in selected IC-containing sera from patients with melanoma, osteogenic sarcoma, and colonic carcinoma (Theofilopoulos *et al.*, 1977). The presence of carcinoembryonic antigen (CEA)–antibody complexes in sera of patients with gastrointestinal cancers was recently documented (Kapsopoulou-Dominos and Anderer, 1979). The presence of ICs in melanoma sera was again suggested when The *et al.* (1978) found IgG and C3 inclusions in neutrophils obtained from the blood of melanoma patients with advanced disease. Others used the Raji cell assay and obtained evidence of ICs in sera of patients with head and neck cancers (Veltri *et al.*, 1978) and with osteosarcoma (Tsang *et al.*, 1979). Brandeis *et al.* (1979), who used the Raji cell assay for assessing the prognostic significance of IC measurements in children with neuroblastoma, found that levels of ICs increased significantly as disease advanced. When patients at the most advanced stage were subdivided into "before," "during," and "after" treatment groups, there was a significant decrease in IC levels as treatment progressed. IC levels were significantly higher in patients who were deceased than in those who remained alive. Long *et al.* (1977) also used the Raji assay and obtained evidence of ICs in sera of patients with Hodgkin's disease. These authors described specific removal of IC reactivity after absorbing the sera with cultured Hodgkin's tumor cells. Amlot *et al.* (1976, 1978) also observed ICs or like materials in sera of patients with Hodgkin's disease tested by the macromolecular C3 technique and the C1q-PEG assay. More than 90% of symptomatic patients were positive in these studies compared with only 30% of asymptomatic patients, and there was a good correlation between positive results for ICs and histologic types with poor prognosis.

C1q and monoclonal RF radioimmunoassays have yielded positive

results in sera from patients with a variety of malignancies. Thus, Jerry *et al.* (1977) and Samayoa *et al.* (1977) using the C1q deviation test and the mRF test found IC-like materials in melanoma patients, and the reactive material was of heavy (>19 S) and intermediate (7 S to 19 S) varieties. Serial determinations revealed cyclic variations in the levels of reactivity and fluctuations in response to therapy. Some of these patients whose kidney biopsy specimens were available had deposits of Ig and C. Others (Hoffken *et al.*, 1977) using the C1q-PEG assay measured ICs in patients with breast cancer and observed that after mastectomy patients identified with clinicopathologic tests as having a good prognosis had almost normal plasma levels of ICs. By contrast, patients with detectable dissemination on diagnosis or those who died within 22 months after mastectomy had significantly raised plasma levels. Teshima *et al.* (1977), Rossen *et al.* (1977), and Heier *et al.* (1977) using the C1q deviation and the C1q-PEG test charted positive results in approximately 50% or more of patients with various malignancies. The reactive material had sedimentation rates between 10 S and 30 S, contained IgG and dissociated under acid conditions. In general, these studies described a prevalent association between the presence of serum ICs and extent of ongoing disease.

Recurrence or progression of tumor growth occurred more frequently in lung cancer patients with high C1q-binding activity (Rossen *et al.*, 1977). Carpentier *et al.* (1977) and Casali *et al.* (1977), using the C1q-PEG assay, the conglutinin radioimmunoassay, and the Raji cell assay found IC-like material in patients with leukemia, especially those with acute or chronic myeloid leukemia. The results obtained with these three tests in both studies had a highly significant correlation. Carpentier *et al.* (1977) found that the C1q-binding material sedimented at 14–28 S on sucrose density gradients, it contained IgG, and it was found most commonly during the blastic stage of leukemia. The median survival times of the patients without detectable ICs during the acute stage was more than 18 months in acute myeloid and acute lymphatic leukemia and more than 8.5 months in blastic crises of chronic myeloid leukemia. The corresponding median survival times in patients with these three forms of the disease and with circulating ICs at this stage were only 64, 135, and 90 days, respectively. Remission took place in 75% of patients with no detectable circulating ICs, but in only 32% of those with detectable ICs. Further, antilymphocyte antibodies and lymphocyte antigens were identified in the ICs of a patient with chronic lymphocytic leukemia (Day *et al.*, 1976).

Finally, in a large percentage of patients with African Burkitt's lymphoma both the Raji cell assay (Theofilopoulos *et al.*, 1976a; Oldstone

et al., 1975) and the C-consumption test (Heimer and Klein, 1976; Mukojima *et al.*, 1973) yielded positive results. The material with anticomplementary activity sedimented between 10 S and 19 S and contained IgG. This material was retained by Con A–Sepharose columns and eluted by α-methyl-D-manoside, suggesting that the complexed antigens might be glycoproteins (Heimer and Klein, 1978). The presence of IC-like materials in Burkitt's lymphoma sera was confirmed by various assays in the WHO-sponsored study (Lambert *et al.*, 1978).

Although tumor antigen–antibody ICs seem to play an important role in the immunopathology and course of neoplastic diseases, the way in which they act is not yet fully characterized. In conditions of antibody excess, ICs may mask, modulate, or cause shedding of tumor-associated antigens so that effector lymphocytes no longer recognize their antigenicity (Hellström and Hellström, 1974; Ting and Herberman, 1975; Baldwin and Robins, 1975). Conversely, tumor antigen–antibody ICs may inhibit humoral and cellular antitumor immune responses by the various mechanisms described earlier in this review (Section III,C). Further studies should aim at assessing the relationships between ICs, antitumor antibodies, and blocking factors and at studying the relationship of circulating ICs to cellular immune functions in patients with cancer.

C. Infectious Diseases

1. Microbial Diseases

a. Infective Endocarditis. Infective endocarditis is a major infectious disease of humans (Lerner and Weinstein, 1966; Kaye, 1973). Many of its clinical and histologic features, once assumed to be a consequence of tissue invasion and injury by the causative microorganism, are now considered to be immunologically mediated (Gutman *et al.*, 1972; Levy and Hong, 1973; Williams, 1971). Moreover, the characteristic tissue lesions in endocarditis are consistent with deposition of ICs (Gutman *et al.*, 1972; Levy and Hong, 1973; Boulton-Jones *et al.*, 1974). Recently, patients with infective endocarditis had a >90% incidence of circulating ICs as measured by Raji and conglutinin assays (Bayer *et al.*, 1967). Circulating IC levels correlated directly with duration of illness, extravalvular manifestations, and hypocomplementemia. Patients with right-sided endocarditis had significantly higher levels of ICs than patients with left-sided involvement. In general, levels fell to zero with successful antimicrobial or surgical therapy. This drop was concurrent with disappearance of ex-

travalvular signs, termination of septicemia, and rise in serum C levels. These findings support the concept that ICs may be important in the pathogenesis of infective endocarditis.

Distinguishing between infective endocarditis and noncardiac septicemias is of utmost prognostic as well as therapeutic importance. Bayer *et al.* (1979a) used IC-measurement by the Raji assay for distinguishing these two disorders; 98% of patients with infective endocarditis versus 44% of septic patients without endocarditis developed detectable circulating ICs. The IC levels in the infective endocarditis group were significantly higher, that is, 15/35 in this group had IC levels >75 μg of AHG eq per milliliter as opposed to only 1/36 with noninfective endocarditis. Similarly, none of the patients without infective endocarditis had IC levels >100 μg/ml, in contrast to 10/35 with infective endocarditis. Thus, in patients without the classic clinical syndrome of endocarditis, measurements of serial IC levels in serum may be of conjunctive, diagnostic importance. If circulating ICs are undetectable, endocarditis is an unlikely diagnosis; alternatively, levels above 75–100 μg/ml would support a valvular rather than nonvalvular septic focus.

An important problem in management of infective endocarditis is establishing laboratory procedures that are sensitive and reliable indicators of therapeutic efficacy. Because circulating C-fixing ICs have been detected in most humans with infective endocarditis, Bayer *et al.* (1979b) studied the natural history and concentrations of serum ICs in response to penicillin therapy given to rabbits with experimental *Streptococcus viridans* endocarditis. In the successfully treated animals, IC levels fell sharply during the first week of therapy while in either control animals or treated animals with refractory endocarditis, IC levels remained at preinduction levels, even though serum bactericidal titers remained >1 : 32 in these latter treated rabbits. These findings suggest that serial IC measurements during antimicrobial therapy for infective endocarditis may aid in monitoring therapeutic efficacy.

While RF of both the IgM (Williams and Kunkel, 1962; Gutman *et al.*, 1972) and IgG (Carson *et al.*, 1978) varieties are frequently found in infective endocarditis, their occurrence and course do not necessarily parallel those of ICs. Thus, Carson *et al.* (1978) found that the absolute amounts of ICs did not correlate with the amount of IgG RF or IgM RF even though the appearance of ICs and RF was temporarily related. The peak level of ICs occurred prior to that of IgM RF or IgG RF in 62% of these patients. In only 25% did either IgM RF or IgG RF levels peak before those of circulating ICs, while in 13% they peaked

simultaneously. The mean interval between the zenith of IC levels and RF was 9 days. In most patients, IgG RF and IgM RF levels peaked simultaneously and decreased to normal levels with antibiotic treatment, although not so rapidly as did levels of circulating ICs. These results suggest that (*a*) IgG RF, like IgM RF, is not found exclusively in rheumatoid arthritics; (*b*) both RFs are part of the polyclonal antibody response to elevated levels of circulating ICs; and (*c*) the continued production of IgG RF and IgM RF in endocarditis may require a stimulus by other ICs that do not contain RF.

b. Meningococcal Infections. Allergic complications such as arthritis, vasculitis, and skin lesions occur in meningococcal disease, possibly due either to a Shwartzman reaction or to the deposition of ICs containing meningococcal antigens. Thus, Greenwood *et al.* (1973, 1976) postulated the presence of ICs in sera of patients with acute meningococcemia from evidence of IC deposition in the tissues examined by immunofluorescence, low levels of circulating C3, and activation of both the classical and alternative C pathways. Subsequently, others (Davis *et al.*, 1976; Larson *et al.*, 1977) using a variety of techniques demonstrated IC-like materials in sera and synovial fluids of a few patients with meningococcal disease complicated by arthritis. However, attempts by these authors to demonstrate meningococcal antigens in such complexes were unsuccessful.

c. Disseminated Gonorrheal Infection. Many clinical features of disseminated gonorrheal infections resemble manifestations of acute IC disease, including vasculitis, transient arthritis, and cutaneous lesions. It is not clear whether most of the peripheral clinical manifestations are mediated immunologically or bacterially. Walker *et al.* (1978) using the Raji cell assay and the C1q-solid-phase assay detected IC-like materials in the majority of sera from patients with disseminated gonorrheal infections, but in very few patients with local gonococcal infections. The putative ICs were 19 S or larger and associated with C activation. However, others (Ludivico and Myers, 1979), using a monoclonal RF test, did not obtain evidence of ICs in sera of patients with gonococcal arthritis–dermatitis syndrome.

d. Immune Complexes in Children with Recurrent Infections. Recurrent upper respiratory tract infection is common in children, and Delire and Masson (1977) using the latex agglutination–inhibition test described the presence of circulating ICs in these patients. Administration of a human IgG preparation containing antibodies against antigens present in the sera of these children appeared to have therapeutic effects, presumably by altering the composition of ICs and promoting their removal.

e. Infected Ventriculoatrial Shunts. Chronic bacteremia is not uncommon in infected ventriculoatrial shunts surgically implanted for relief of hydrocephalus. The infection usually is due to coagulase-negative *Staphylococcus* and is associated with hepatosplenomegaly, thrombocytopenia, neutropenia, hypergammaglobulinemia, RF, cryoglobulinemia, C depletion, IC formation, and glomerulonephritis (Black *et al.*, 1965; Stickler *et al.*, 1968; Kaufman and McIntosh, 1971; Dobrin *et al.*, 1975; Harkiss *et al.*, 1979).

f. Streptococcal Infections. The renal injury following infection with nephritogenic streptococcus is generally considered to have an IC pathogenesis (Zabriskie *et al.*, 1973). This subject has been reviewed in detail previously (Wilson and Dixon, 1976), and the studies related to the incidence of IC in sera of patients with poststreptococcal glomerulonephritis are discussed in Section V,A2). IC-like materials were found by both the Raji cell assay and the solid-phase C1q assay in patients with acute poststreptococcal sequelae, acute rheumatic fever, and acute poststreptococcal glomerulonephritis (van de Rijn *et al.*, 1978). Since the incidence and molecular size of the ICs in these two poststreptococcal sequelae were identical, it was concluded by the investigators that other factors, such as the nature of the antigen in the complex, are more important in the pathogenesis of these complications than the actual presence or the absolute levels of ICs.

g. Leprosy. There are basically two clinical forms of the disease, lepromatous and tuberculoid, characterized by different levels and types of immune responses. Humoral immunity against mycobacteria predominates over cellular immunity in lepromatous leprosy, and the reverse occurs in tuberculoid leprosy (Ridley and Jopling, 1966). Thus, IC formation may accompany the former, and is less likely in the latter, form of the disease. Deposits of Ig and C3, and sometimes mycobacterial antigens were detected with immunofluorescence in skin areas with erythema nodosum leprosum (Wemambri *et al.*, 1969) and kidneys (Shwe, 1972) of patients with lepromatous leprosy. In addition, several investigators observed C1q precipitation and platelet-aggregating activity (Wager *et al.*, 1978) in sera from these patients (Moran *et al.*, 1972; Rojas-Espinoza *et al.*, 1972; Gelber *et al.*, 1972). More recently, Bjorvatn *et al.* (1976), using the C1q-binding assay, found almost equal incidences of positivity in patients with lepromatous and tuberculoid forms of the disease. The reactive material sedimented in a 10–25 S range, and suggestive evidence indicative of ICs that contained mycobacterial antigens was obtained. The same authors found increased levels of C3d in most of the plasmas from patients with erythema nodosum leprosum, but rarely in the plasmas of patients with uncomplicated lepromatous leprosy and

never in tuberculoid leprosy patients' plasma. However, C3d levels correlated poorly with C1q-binding activity in lepromatous patients, suggesting to these investigators that large quantities of C-activating ICs were generated extravascularly in this condition.

The presence of IC-like materials in patients with leprosy was also suggested in the WHO study (Lambert *et al.*, 1978) when seven methods for IC testing discriminated the leprosy patients as a whole from normal controls. Three techniques (conglutinin, platelet aggregation, neutrophil inhibition) showed a higher incidence of positives among sera from patients with lepromatous than with tuberculoid forms of the disease.

h. Syphilis. Glomerulonephritis is associated with both congenital (Hill *et al.*, 1972; Kaplan *et al.*, 1972) and secondary (Braunstein *et al.*, 1970) syphilis. Presumed IC deposits of Ig and C3 were seen by immunofluorescence and electron microscopy in some of these patients and antitreponemal antibody was eluted from glomerular deposits of a patient with acquired syphilis (Gamble and Reardan, 1975). The glomerulonephritis can be resolved with penicillin therapy, which results in eradication of the antigenic source. The presence of circulating ICs in patients with syphilis has not been examined.

2. *Viral Diseases*

Viral infections are frequently associated with the development of ICs and IC disease. The virus is a self-replicating agent that provides a supply of macromolecular antigens both in the form of virions and virus-associated molecules and, in most instances, elicits a host immune response. The interaction of viruses with antibody and C and the development of IC disease in animals (mouse, mink, horse, pig) due to infections with various RNA and DNA viruses have been reviewed in detail by Oldstone (1975a). Below are three examples of human viral diseases associated with ICs.

a. Dengue Hemorrhagic Fever. The dengue viruses, a subgroup of group B arboviruses (flaviviruses) are presently divided into four distinct antigenic types with pronounced serologic cross-reactivities (Hammon *et al.*, 1960). Humans infected with dengue viruses manifest dengue fever symptoms or the more severe syndrome, dengue hemorrhagic fever (DHF), sometimes accompanied by shock. DHF has been associated with the advent of a second heterologous virus infection, and this association suggests that immunopathologic processes figure importantly in this more severe form of the disease (Halstead *et al.*, 1970). In fact, DHF and shock may relate to activation of the C system by both classical and alternative pathways (Bokisch *et*

al., 1973). Intravascular coagulation is also observed in these patients, as manifested by thrombocytopenia, reduction in coagulation factors, especially factor VIII, and the presence in plasma of fibrinogen and fibrinogen degradation products. The mechanisms leading to activation of these effector systems in DHF are poorly understood, although circulating ICs are considered the most important common denominator. Accordingly, IC-like materials were found in sera of patients with DHF by both the Raji cell assay (Theofilopoulos *et al.*, 1976a; Ruangjirachuporn *et al.*, 1979) and the C1q deviation test (Sobel *et al.*, 1975; Ruangjirachuporn *et al.*, 1979) and association of the degree of positivity in the latter test and severity of the hemorrhagic syndrome was reported. In addition, presumed IC deposits are occasionally demonstrated in tissues and on the surfaces of a small proportion of circulating lymphocytes from these patients (Boonpucknavig *et al.*, 1976). Macrophages are the major cell type in which dengue viruses replicate (Theofilopoulos *et al.*, 1976b; Halstead *et al.*, 1977). It was found *in vitro* that ICs formed by IgG nonneutralizing homotypic and heterotypic antibodies to Dengue-2 virus were more efficient in infecting macrophages than the virus alone and that the enhanced infectivity was dependent on the Fc portion of the antibody (Halstead and O'Rourke, 1977a,b). This finding may explain the severity of the disease in infants with circulating maternal antidengue antibodies. Dengue viruses replicate *in vitro* not only in macrophages but also in B type lymphoblastoid cells and possibly antigen-stimulated peripheral B cells (Theofilopoulos *et al.*, 1976b; Halstead *et al.*, 1977). These viruses also replicate in human endothelial cells *in vitro* (Andrews *et al.*, 1978b), suggesting an additional way of accounting for the vascular pathology and the resultant hemorrhagic shock syndrome.

b. Cytomegalovirus Infections. When natally acquired, cytomegaloviruses produce a chronic infection lasting several years. Persistent viral replication occurs in salivary glands, renal parenchyma, and possibly other sites (Stagno *et al.*, 1975). The elicited specific humoral immune responses are substantial and prolonged whether the infection is productive or latent. Stagno *et al.* (1977) using a microcomplement consupmtion test and the Raji cell assay, detected IC-like materials in sera of 35–45% of patients with congenital or natal cytomegalovirus infections. The frequency of reactivity in these assays was highest in children with severe intrauterine infection, and during the first year of life paralleled the patterns of viral excretion and humoral immune response. Circulating ICs seemed to be heavier (18–22 S) in sick, as opposed to asymptomatically infected, infants, in whom intermediate-size complexes (12–16 S) were found. In symp-

tomatic patients whose demise was due to severe congenital cytomegalovirus infection, granular deposits of Ig and C3 were detected along the GBM. Therefore, circulating ICs could contribute to this insidious, progressive disease either by interfering with host defense mechanisms or by direct tissue damage.

c. Viral Hepatitis. This disease results from infection with one of at least two different viruses (reviewed by Dmochowski, 1976). The first, type A hepatitis virus, induces infectious or epidemic hepatitis; and the second, type B hepatitis virus, induces serum hepatitis. It is now known that three antigenic systems are associated with viral hepatitis type B: hepatitis B surface antigen (HB_sAg or Australia antigen), hepatitis B core antigen (HB_cAg), and a third antigen, or complex of antigens, referred to collectively as *e* antigen.

The spectrum of responses that follow infection with hepatitis B virus, includes (*a*) asymptomatic infection, transient production of HB_sAg, and the development of protective titers of anti-HB_s antibody; (*b*) asymptomatic infection, persistent production of HB_sAg, minimal synthesis of anti-Hb_s antibody, and minimal or no cellular immune response to HB_sAg ("chronic carriers" of hepatitis B virus); (*c*) acute hepatitis, presence of HB_sAg and antibody to it detectable during the convalescent period (4–6 weeks after onset); (*d*) chronic hepatitis, which may develop from acute hepatitis or may have an insidious onset. HB_sAg is usually found in the patient's blood, and humoral and cellular immune responses to the virus are also detectable. Persons with any of these four responses usually develop antibody to the viral core antigen.

In terms of circulating ICs, Shulman and Barker (1969) were the first to describe a very high anticomplementary activity in sera from patients with acute serum hepatitis; the activity was reversed by the addition of excess HB_sAg or antibody. Millman *et al.* (1970) unmasked HB_sAg determinants after adding enzymes capable of digesting antibody to the centrifuged serum pellet. Almeida and Waterson (1969) examined pellets of centrifuged sera from hepatitis patients by electron microscopy and observed structures suggesting the presence of HB_sAg–antibody complexes. Others, using physicochemical techniques detected IgG antibody–HBe antigen ICs in sera of patients with hepatitis (Takekoshi *et al.*, 1979). Nydegger *et al.* (1974) and Thomas *et al.* (1978), using the C1q-binding test, detected ICs in patients with acute transient hepatitis and with chronic persistent hepatitis, but not in healthy carriers of HB_sAg. There was no correlation between the increased C1q-binding activity in the sera of patients with acute hepatitis and clinical status, but the C1q-binding activity

declined as the circulating HB_sAg disappeared. In persons with chronic active hepatitis, the degree of C1q-binding matched the severity of disease (Thomas *et al.*, 1978). Moreover, concentrations of various C components decreased during the acute phase of hepatitis (Kosmidis and Leader-Williams, 1972; Thompson *et al.*, 1973; Charlesworth *et al.*, 1977). Using the Raji cell assay, Theofilopoulos *et al.* (1976a) detected ICs in sera of more than half of patients with acute serum hepatitis with or without detectable HB_sAg in their sera. In contrast, only 13% of asymptomatic carriers with circulating HB_sAg were positive. In the majority of these patients, the amount of ICs diminished markedly as the elevated serum enzyme levels fell during convalescence. Upon sucrose density fractionation, sera positive for ICs had IgG, C3, and HB_sAg migrating in lower portions of the gradient, whereas sera negative for ICs did not, suggesting that these components comprised the complexes detected. When Raji cells were then incubated with the contents of this lower portion of the gradient and were stained with fluorescein conjugated antisera to HB_sAg, clear fluorescence positivity indicated the presence of this antigen on the cells' surfaces, presumably as part of ICs. Carella *et al.* (1977), using PEG precipitation, also detected HB_sAg and specific antibody in the precipitates obtained from sera of patients with acute and chronic hepatitis. Similar ICs have been detected in sera and cryoprecipitates of hepatitis patients by various other procedures (Lurhuma *et al.*, 1976; Digeon *et al.*, 1977; Prince and Trepo, 1971; McIntosh *et al.*, 1976; Myllylä, 1973; Tiku *et al.*, 1979; Madalinski and Bragiel, 1979; Pernice *et al.*, 1979).

Persons who develop anti-HB_s antibody, probably by virtue of responding to the group-reactive *a* determinant, appear to be immune to rechallenge with hepatitis B virus (Krugman and Giles, 1973). However, some carriers of HB_sAg may develop secondary infections from another subtype of hepatitis virus (Sasaki *et al.*, 1976; Koziol *et al.*, 1976; LeBouvier *et al.*, 1976). In such instances, apart from the ICs that may form between the antigens of the new subtype and their corresponding antibodies, if antibodies also arise against the group-reactive determinant *a*, then large amounts of ICs in antigen excess may develop due to the interaction of the anti-a antibody with the excess of circulating HB_sAg derived from the first infection (LeBouvier *et al.*, 1976).

Several reports have confirmed an association between HB_sAg and polyarteritis (Gocke *et al.*, 1970, 1971; Trepo and Thivolet, 1970; Baker *et al.*, 1972; Kohler, 1973; Duffy *et al.*, 1976), and ICs have been detected in sera as well as tissues of these patients (Gocke *et al.*, 1970, 1971; Trepo *et al.*, 1974; Prince and Trepo, 1971; Nowoslawski *et al.*,

1972). Moreover, ICs might be associated with vasculitis and arthralgia syndrome seen as a prodrome in patients with acute viral hepatitis (Alper *et al.*, 1971, 1972; Onion *et al.*, 1971; Gocke *et al.*, 1971). Wands *et al.* (1975) demonstrated the presence of C-fixing HB_sAg and antibody ICs in cryoprecipitates derived from sera of such patients. Similar ICs are probably involved in some cases of essential mixed cryoglobulinemia, since Levo *et al.* (1977) recently reported finding HB_sAg or antibody in the majority of cryoprecipitates from patients with this syndrome.

Several histologic forms of glomerulonephritis also accompany serum hepatitis and are typified by granular deposits of Ig and C, as well as electron-dense deposits along the GBM (Brzosko *et al.*, 1974; Randall *et al.*, 1971; Eknoyan *et al.*, 1972). Ig deposits noted in tubules (Brzosko *et al.*, 1974; Randall *et al.*, 1971) suggest additional extraglomerular IC deposition consistent with the systemic nature of this IC disease (Wilson and Dixon, 1976). Moreover, HB_sAg or HBe are identified occasionally in the glomerular IC deposits examined by immunofluorescence and electron microscopy (Brzosko *et al.*, 1974; Nowoslawski *et al.*, 1972; Combes *et al.*, 1971; Köhler *et al.*, 1974; Knieser *et al.*, 1974; Eknoyan *et al.*, 1972; Knecht and Chisari, 1978; Takekoshi *et al.*, 1979). All these results strongly suggest that the extrahepatic manifestations of hepatitis B infection are mediated by HB_sAg-antibody ICs.

d. Other Viral Infections. Many other viral infections are associated with glomerular changes, and it seems likely that viral antigens may contribute to nephritogenic IC formation in such diseases as mumps (Hughes *et al.*, 1966), varicella (Minkowitz *et al.*, 1968), infectious mononucleosis (Tennant, 1968; Woodruff *et al.*, 1973; Wallace *et al.*, 1974), and Coxsackie B infections (Burch *et al.*, 1969). IC-like materials are detected frequently in sera of patients with infectious mononucleosis by both the C1q-binding and the Raji cell assay, especially at the acute phase of the disease (Charlesworth *et al.*, 1978).

3. *Parasitic Diseases*

The study of ICs is an important step toward improving serology and understanding the mechanisms of persistent infection and immunopathology of chronic parasitic diseases in which large amounts of parasitic antigen, corresponding antibody, and ICs may be present.

Circulating ICs seem to be associated with the immunopathology of malaria, since low C levels and circulating IC-like materials have been observed in association with *Plasmodium falciparum* infections (Houba and Williams, 1972). Granular deposits of Ig, C, and malaria

antigens accumulate in glomeruli of some humans with transient glomerulonephritis due to *P. falciparum* or chronic glomerulonephritis and nephrotic syndromes due to *P. malariae* (Houba *et al.*, 1970, 1971, 1976; Houba and Lambert, 1974; Bhamarapravati *et al.*, 1973; Berger *et al.*, 1967; Allison *et al.*, 1969). Additionally, cryoglobulins, low C3 levels, anticomplementary activity, macromolecular C3, and soluble ICs have all been detected in sera of patients with chronic malaria associated with tropical splenomegaly syndrome (Soothill and Hendrickse, 1967; Ziegler, 1973; Fakunle *et al.*, 1978). Moreover, malarial IC and C components occupy the surfaces of red cells from some patients with malaria and may contribute to the associated anemia (Ssebabi *et al.*, 1975; Woodruff *et al.*, 1973).

American trypanosomiasis may also involve IC formation either in serum or locally in tissues where the parasite resides (Galvao-Castro *et al.*, 1978). Recent experiments have shown that both malarial antigens and trypanosomal antigens can act as polyclonal B cell stimulators (Wyler and Oppenbein, 1974; Greenwood and Vick, 1975; Kobayakawa *et al.*, 1979; Rosenberg, 1978), thus explaining the various autoantibodies found in these diseases.

The involvement of ICs in the renal injury associated with *Schistosoma mansoni* infection, particularly the hepatosplenic form of the disease, has long been suspected since schistosomal antigens, IgG and C granular deposits have been found frequently in glomeruli, particularly the mesangia, of infected humans and animals (Andrade *et al.*, 1971; Hoshino-Shimizu *et al.*, 1976; Falcao and Gould, 1975; Natali and Cioli, 1976). ICs composed of schistosomal antigens and IgG or antibodies were also described in sera of rats and mice with experimental infections (Santoro *et al.*, 1978; Digeon *et al.*, 1979). Additional recent evidence located ICs containing schistosomal antigens and antibodies in the sera of such patients (Phillips and Draper, 1975; Smith *et al.*, 1975; Madwar and Voller, 1975; Bout *et al.*, 1977; Lawley *et al.*, 1979b). The presence of IC-like materials in sera of these patients was also suggested by the WHO study cited above (Lambert *et al.*, 1978), in which positivity in various IC assays differed quantitatively between Kenyan patients with schistosomiasis and European controls. The possible interference of ICs with cellular defense mechanisms against *Schistosoma* was intimated recently when Butterworth *et al.* (1974, 1975, 1977a,b) preincubated eosinophils with either ICs or sera of patients with schistosomiasis and inhibited the cells' attachment to and killing of antibody-sensitized *Schistosoma*. Moreover, eosinophil-mediated antibody-dependent killing of *Schistosoma* was much less apparent when eosinophils of persons infected with *S. mansoni* were used over those obtained from control, uninfected individuals.

IC-like material was found in C1q-binding tests of patients with onchocerciasis (filariasis) (Ngu and Blackett, 1977). Increased positivity of sera from persons with filariasis over appropriate controls with a variety of cellular and C1q-based IC-assays was evident in the WHO study (Lambert *et al.*, 1978).

Congenital toxoplasmosis is sometimes associated with glomerulonephritis, and Ig, C, and *Toxoplasma gondii* antigens are identifiable in the glomerular deposits (Ginsburg *et al.*, 1974; Shahin *et al.*, 1974). However, circulating ICs have not been sought in persons with this condition. Many other studies in experimental animals indicate the significant role of ICs in the pathogenesis of all these parasitic diseases (reviewed in World Health Organization Report, 1977).

D. Other Conditions

Assays for the detection of ICs have been applied to the study of many other diseases. These include the diseases discussed below.

1. Dermatitis Herpetiformis and Celiac Disease

Mowbray *et al.* (1973), Mohammed *et al.* (1976), and Doe *et al.* (1973) described IC-like materials in almost all sera from patients with these two disorders. The material sedimented at 8–10 S after sucrose density fractionation. Originally, patients receiving normal diets seemed to have a higher incidence of positivity than those with gluten-free diets (Mowbray *et al.*, 1973; Doe *et al.*, 1973), suggesting that a dietary antigen was involved in the formation of ICs. However, this finding was not confirmed in later studies (Mohammed *et al.*, 1976), and the authors concluded that the varied types of ICs per se detected in these patients were not responsible for either the gut or the skin lesions of dermatitis herpetiformis. Using the Raji cell assay (A. N. Theofilopoulos, unpublished), we found significant quantities of ICs in only 2% of the sera from patients with dermatitis herpetiformis.

2. Ulcerative Colitis and Crohn's Disease

IC-like materials have been reported in both these conditions (Doe *et al.*, 1973; Nielsen *et al.*, 1978a,b; Jewell and MacLennan, 1973; Hodgson *et al.*, 1977). All the investigators found circulating ICs or like materials most commonly in the sera of patients with active disease and with extraintestinal manifestations, particularly acute arthritis, spondylitis, and liver disease.

3. Myocardial Infarcts

Circulating ICs may be associated with myocardial infarcts. ICs in such patients may be formed secondarily due to the escape of myocar-

dial antigens from the infarct or in some instances preexisting ICs might contribute to the onset of the infarct. Thus, Farrell *et al.* (1977) using a solid-phase C1q-binding assay found IC-like materials in more than 50% of patients with acute myocardial infarcts. Between 1 and 3 weeks after the onset, positive reactions appeared, but the test was negative 6 weeks after infarction. Preliminary observations by these authors indicated an association between IC levels and the size of the infarct (aspartate-aminotransferase levels). A high incidence of myocardial infarcts was observed in mice with autoimmune syndromes and large quantities of circulating ICs which apparently induced coronary vascular lesions (Andrews *et al.*, 1978a; Accinni and Dixon, 1979).

4. Idiopathic Interstitial Pneumonias

Several investigators have noted the presence of Ig and C deposits in the alveolar walls of such patients (Schwarz *et al.*, 1977; H. Eisenberg *et al.*, 1977). Dreisin *et al.* (1978) attempted to determine the role of type III mechanisms in idiopathic interestitial pneumonia by measuring IC-like materials in the patients' sera with the Raji cell assay. Circulating IC concentrations were elevated in all but 3 of 16 patients with cellular interstitial disease, but in none of 7 with diffuse fibrosis. Granular deposits of IgG, usually with C3, were present along alveolar walls of 94% of patients with elevated IC levels, but in only 11% of those with normal levels. A better radiographic and physiologic response to corticosteroid therapy was seen in treated patients with initially elevated IC levels than in those with normal values.

5. Cystic Fibrosis

Because cystic fibrosis patients suffer from chronic and recurrent bacterial infections, ICs formed between soluble bacterial antigens and antibacterial antibodies may contribute to the pulmonary tissue damage. In fact, presumed IC deposits have been described in various organs of such patients (McFarlane *et al.*, 1975). In addition, circulating C-fixing materials, presumably ICs, were observed in patients infected with *P. aeroginosa* (Schitz *et al.*, 1977).

6. Sarcoidosis

Sarcoidosis is a multisystem granulomatous disease of unknown etiology. Ig and C can deposit within the granulomas of the lymph nodes, lungs, and cutaneous tissues (Ghose *et al.*, 1974; Wanstrump and Elling, 1968; Quismorio *et al.*, 1977). Moreover, the platelet aggregation test yielded evidence of IC-like materials larger than 19 S in the sera of approximately 25% of patients with acute sarcoidosis (Hedfors and Norberg, 1974).

7. *Multiple Sclerosis and Other Neurologic Disorders*

Tachovsky *et al.* (1976) using the Raji cell assay judged approximately 50% of patients with multiple sclerosis, nonsymptomatic optic neuritis, and Guillain-Barré syndrome as positive. Measles virus, considered to be associated with multiple sclerosis, could not be identified in the Raji cell bound ICs. The frequency of ICs in sera did not correlate with the clinical status of patients with multiple sclerosis. Other studies have shown IgG and C deposits in the brain tissues of such patients (Tavolato, 1975) and occasionally in the kidneys (Whitaker *et al.*, 1971).

Oldstone *et al.* (1976b), using a C1q assay, recorded the presence of IC-like materials in sera of patients with amyotrophic lateral sclerosis. However, Tachovsky *et al.* (1976) did not obtain positive results using the Raji cell assay. Sera of patient with subacute sclerosing panencephalitis are among those with IC-like materials (Theofilopoulos *et al.*, 1976a). Such sera contained blocking factors removed by anti-C3 or RF in one study (Ahmed *et al.*, 1974), and IgG and C3 deposits were noted in the vessels of some of these patients (Whitaker and Engel, 1972). A few sera from patients with myasthenia gravis, caused by an autoimmune reaction to the nicotinic postsynaptic acetylcholine receptor, were uniformly negative when examined by the Raji assay (Tachovsky *et al.*, 1976), but deposits of IgG and C3 at the motor endplate have been observed (Engel *et al.*, 1977).

8. *Uveitis*

In experimental uveitis models, circulating ICs can cause immunologically mediated ocular tissue destruction (Howes and McKay, 1975; Levine and Ward, 1970). There is suggestive evidence that ICs may be present in the sera of patients with retinal vasculitis and heterochromic cyclitis (Dernouchamps *et al.*, 1977; Andrews *et al.*, 1977); however, neither the nature of these complex-like materials, nor their correlation with disease status, has yet been delineated.

9. *Chronic Serous Otitis Media*

Maxim *et al.* (1977) using the Raji cell assay obtained evidence of ICs in effusions of patients with chronic otitis media. The authors postulated that ICs formed locally may be responsible for the chronicity of the disease.

10. *Atopic Diseases*

Brostoff *et al.* (1977) examined patients with atopic eczema and hay fever and found macromolecular IgE, presumably complexed with antigen, in the majority of such sera. Similarly, using the Raji cell assay

we obtained evidence of ICs in most test sera of patients with dog dander sensitivity (A. N. Theofilopoulos, unpublished), and others (Cano *et al.*, 1977a; Stein *et al.*, 1978) observed high C1q binding activity in sera of patients with atopic allergy or allergic rhinitis. Allergic persons undergoing maintenance immunotherapy did not seem to have higher incidences or levels of C1q binding materials in their sera than allergic controls without immunotherapy (Stein *et al.*, 1978).

11. Arthritis Associated with Intestinal Bypass Procedure for Morbid Obesity

Arthritis and tenosynovitis can complicate intestinal-bypass procedures performed to treat obesity. Wands *et al.* (1976) found that sera from such patients contained cyroproteins composed of Ig and C and suggested that ICs may be important in the pathogensis of the arthritis. However, the Raji cell assay offered no evidence of circulating ICs in such patients (A. N. Theofilopoulos, unpublished).

12. Sickle-Cell Anemia

A variety of renal structural and functional abnormalities have been associated with sickle-cell anemia. Strauss *et al.* (1975) and Pardo *et al.* (1975) found not only dense deposits of Ig, C, and tubular antigens in the glomeruli of such patients, but also circulating cryoglobulins that contained renal tubular epithelial antigen and antibody. These investigators postulated that the immunogenic tubular antigens were released in sera after tubular damage secondary to oxygenation and hemodynamic alterations related to sickle-cell disease.

13. Thrombotic Thrombocytopenic Purpura (TTP)

The disease is of unknown etiology and is characterized by microangiopathic hemolytic anemia, thrombocytopenia, fever, neurologic syndromes, and renal abnormalities. Since TTP patients recover completely after intensive plasmapheresis, the syndrome could be caused by circulating ICs of unknown composition (Bukowski *et al.*, 1977). However, Neame and Hirsh (1978) found no circulating ICs when performing the Raji cell assay in multiple samples taken at the acute phase from two patients with typical TTP. In a few patients with infectious endocarditis presenting with TTP symptoms, IC-like materials were detected, and the complexes disappeared after antibiotic treatment (Bayer *et al.*, 1977). Others (Lurhuma *et al.*, 1977) using latex agglutination inhibition assays obtained evidence of IC-like materials in the majority of patients with idiopathic purpura. The inhibitory factor contained IgG and DNA. The investigators suggested that the

DNA was of viral origin because the sera contained various commonly found viral antigens.

14. Primary Biliary Cirrhosis

Wands *et al.* (1978) examined sera from patients with this disorder and concluded that their high content of cyroglobulins and high levels of positivity in the Raji cell assay could be ICs. These authors found that isolated cryoglobulins from such patients activated that alternative C pathway. The role of ICs in this disorder is not clear. Penicillamine treatment was shown to reduce IC levels and to have favorable influence on the course of the disease (Epstein *et al.*, 1979). Moreover, a defect in the clearance of sensitized erythrocytes by receptors for C3b on Kupffer cells was found that was attributed to the occupation of these receptors by C-fixing ICs or by C3b generated by C activation (Jones *et al.*, 1979).

15. Kidney and Bone Marrow Transplantation

Patients given renal allografts were studied by Palosuo *et al.* (1976), who found by platelet aggregation testing that 3 of 16 such patients had IC-like material in their sera and that one of these patients with serum specimens available before and after transplantation was positive only after transplantation. Granular glomerular IgG and C deposits were detected in all 3 patients. Others (Bakkaloglu *et al.*, 1977) found that all sera from patients with acute rejections inhibited Fc rosettes *in vitro*, possibly via ICs or alloantibodies. However, the predictive value of this test for rejection was uncertain. Similarly, Ooi *et al.* (1977c) observed high C1q binding activity in the sera of the majority of patients with acute rejection after kidney transplantation. Serial studies by these investigators correlated increased C1q-binding activity with rejection; binding activity returned to within normal levels with reversal of rejection after appropriate therapy. The positive material had a sedimentation rate of 15 S to 18 S and contained IgG. Ig and C deposited granularly in glomeruli and tubules of such patients.

Presumed ICs deposited in the dermoepidermal junction of humans with acute and especially with chronic cutaneous graft-versus-host disease after allogeneic bone marrow transplants (Tsoi *et al.*, 1978; Ullman *et al.*, 1976) have also been described, suggesting that the associated skin lesions are caused by humoral as well as cellular immune mechanisms.

16. Pregnancy

The fetuses of all outbred mammalian species possess an immunogenic set of paternal membrane antigens that pass through the

placenta and enter the maternal circulation. In the mother, this antigenic exposure induces subsets of specifically sensitized lymphocytes and IgG antibodies against paternal lymphocyte HLA antigens (Jenkins and Hancock, 1972). The sensitized lymphocytes are detectable during culturing when specific recognition is signaled either directly by lymphokine production or as maternal T cells specifically lyse paternal peripheral blood leukocytes. Yet, neither of these *in vitro* cellular reactions occur if maternal serum is present (Hellström *et al.*, 1969; Ceppelini *et al.*, 1971; Jenkins and Hancock, 1972; Youtananukorn and Matangkasombut, 1973; Revillard *et al.*, 1973; Kasakura, 1971; Gatti *et al.*, 1973). The responsible blocking factor in maternal serum may be an IgG antibody (Pence *et al.*, 1975; Rocklin *et al.*, 1976) interacting with HLA-D antigens of paternal B cells or an IC of paternal histocompatibility antigens and maternal antibody. Rocklin *et al.* (1976) presented data indicating the absence of blocking factors in sera of women who have had multiple spontaneous abortions. Such histocompatibility antigen–antibody ICs may combine with the specific antigen receptors on maternal T cells, thereby blocking their function, or interact with IgG Fc receptor-bearing suppressor maternal T cells, thereby implementing release of soluble factors that suppress cytotoxicity. In addition, Oldstone *et al.* (1977) and Hayward and Lydyard (1978) identified activated IgG Fc receptor-bearing suppressor T ce'ls in human cord blood that inhibited both mitosis and Ig production by lymphocytes from the mother. At any rate, Masson *et al.* (1977) tested sera of pregnant women for ICs by inhibiting rabbit RF-induced agglutination of IgG coated-latex particles. Most of these sera inhibited agglutination, and 3–4 weeks before delivery the inhibition titers dropped to levels observed in nonpregnant women. The inhibiting factor contained IgG and C3 and could be dissociated by acid buffers into smaller noninhibitory components. Similarly, Stirrat *et al.* (1978) using a C1q and an antiglobulin test detected IC-like materials in sera of pregnant women. The conclusion was that sera of pregnant women contain ICs that may be the blocking factors responsible for the immune tolerance of the mother toward the fetus.

The possibility has also been proposed that ICs composed of maternal antibody and fetal antigens at appropriate ratios may be responsible for the preeclamptic and eclamptic syndrome in pregnant women (Kirkwood, 1975; Scott *et al.*, 1978) on the basis of the above-mentioned work by Masson *et al.* (1977) and Stirrat *et al.* (1978) and the description by others (Petrucco *et al.*, 1974) of Ig and C deposits in renal biopsies of women with preeclampsia. However, the studies of Knox *et al.* (1978) with the C1q and the Raji cell assay failed to demon-

strate any appreciable quantities of IC-like materials in sera of preeclamptic women. Moreover, in accord with the latter study, Gleicher *et al.* (1978), using the Raji assay, and D'Amelio *et al.* (1979), using the PEG assay, obtained negative results with sera of pregnant women taken throughout the gestation period, as well as sera of women with preeclampsia. The three latter studies differ from the work of Masson *et al.* (1977) and Stirrat *et al.* (1978), possibly either because of the idiosyncrasies and specificities of the methods employed or owing to the presence of interfering factors. It should be noted that Rocklin *et al.* (1976) and Pence *et al.* (1975) have shown that the blocking factors present in pregnant women's sera contain 7 S IgG.

17. Lyme Arthritis

This newly described epidemic disease occurs in regions of Connecticut and areas bordering Long Island Sound. The disease is characterized by attacks of asymmetric large-joint arthritis (without RF), skin lesions (erythema chronicum migrans), and sometimes meningoencephalitis, peripheral neuropathy, or cardiac conduction abnormalities (Steere *et al.*, 1977a). Thus far, efforts to identify a causative agent have been unsuccessful, but epidemiologic studies have indicated transmission by a tick vector (Steere *et al.*, 1978). Cryoglobulins occur in some of the patients, particularly those with active arthritis (Steere *et al.*, 1977b). More recently, the same group of investigators (Hardin *et al.*, 1979), using C1q-binding and C1q-solid-phase assays as well as the Raji cell assay, found 19 S IC-like material that contained IgG in sera of such patients. The reactive material was most prominent in the sickest patients, but was rarely seen during remissions; the concentrations fluctuated in parallel with changes in disease activity. The nature of the antigen in the ICs was not identified.

18. Steroid-Responsive Nephrotic Syndrome

This childhood syndrome, associated with atopy, is characterized by proteinuria and the patients respond favorably to corticosteroids. Histologically, the biopsied renal sections show minimal changes, and immunofluorescence study of these specimens is negative for Ig or C3 deposition (Mallick, 1977). Nevertheless, Levinsky *et al.* (1978), when measuring inhibition of the agglutination of IgG-coated latex particles by rabbit IgM antihuman IgG, located intermediate-size IgG-containing materials that did not fix C1q in the sera of such children, especially those in relapse. These authors raised the possibility that the material detected induced proteinuria by activating a lymphokine system.

19. Xanthomatosis

Antilipoprotein autoantibodies found sometimes in humans are considered to play a role in lipid metabolism resulting either in hyperlipidemia, if the antibody covers sites attacked by lipases, or in hypolipidemia, if the antibody enhances processing and degradation of the lipids (Beaumont, 1969; Riesen and Noseda, 1975). ICs and cryoglobulins observed in sera of a few patients with xanthomatosis were composed of IgG autoantibody and lipoproteins (Kodama, 1977).

20. Vasectomy

After vasectomy or vasoligation, humans and experimental animals can develop (reviewed by Hellema and Rümke, 1978) various antibodies including sperm agglutinating and sperm-immobilizing antibodies, autoantibodies against human protamine expressed on sperm heads and antibodies directed toward the acrosome and the tail (Samuel *et al.*, 1975; Tung, 1975). Most of these antibodies appear in the serum approximately 2–3 months after the operation, and the titers progress upward thereafter (Tung, 1975; Gupta *et al.*, 1975; Hellema and Rümke, 1978). The long-term immunologic consequences of vasectomy, if any, have not yet been established. Lesions in vasectomized guinea pigs and rabbits resembling experimental autoimmune orchitis as well as glomerulonephritis may occasionally occur (Bigazzi *et al.*, 1976).

21. Recurrent Oral Ulceration and Behçet's Syndrome

Uncomplicated recurrent oral ulceration is a common disease, but Behçet's syndrome, which is characterized by such ulceration plus uveitis, arthritis, erythema nodosum, and other multifocal tissue involvements, is rare. Antibodies directed against oral and other mucosal epithelial cells are frequently found in these patients. Macromolecular C3 has been detected in the sera of these patients (Williams and Lehner, 1977). The presence of IC-like materials, especially in patients with Behçet's syndrome, was noted in various tests (Levinsky and Lehner, 1978; Gupta *et al.*, 1978b). These authors found IC-like materials more frequently in patients with Behçet's syndrome who have neuroocular and arthritic manifestations than in those who have mucocutaneous lesions only, and there was a close association between levels of ICs and clinical activity. A curious finding was the significant correlation between IC levels and the presence of presumed damaged cellular membranes observed by electron microscopy in pellets of ultracentrifuged sera from patients with

Behçet's syndrome (Lehner *et al.*, 1978). It was concluded that cells are lysed in a bystander fashion after C activation by the ICs.

22. *Pemphigus and Bullous Pemphigoid*

Autoantibodies against epidermal and mucosa antigens have been implicated in the pathogenesis of these vesiculobullous skin diseases, and Ig as well as C components were identified in the characteristic skin lesions (Jordon *et al.*, 1973; van Joost *et al.*, 1972). Moreover, Jordon and McDuffie (1967) found anticomplementary activity in high molecular weight fractions of pemphigus and bullous pemphigoid blister fluids, and Tappeiner *et al.* (1977) estimated that 40% of the patients with pemphigus and 20% of those with bullous pemphigoid had elevated Clq-binding activity in their sera as a correlate to active disease.

23. *IgA Deficiency*

Selective IgA deficiency is among the most common of immunologic disorders, afflicting as many as one of every 700 individuals (Koistinen, 1975). The majority of these patients are apparently quite healthy, but a few complain of sinopulmonary infections, arthritis, allergies, and gastrointestinal disturbances (Amman and Hong, 1970). Various autoantibodies have been observed in such patients, and a frequent finding (50–75%) is the presence of precipitating antibodies to cow milk (Buckley and Dees, 1969). Recently, Cunningham-Rundles *et al.* (1978) observed that the majority of IgA-deficient patients with anti-milk antibodies, but not those without, also have ICs, as determined by the Raji cell assay. ICs are present in both healthy and symptomatic IgA-deficient patients, but the levels seem to be higher in those with arthritic and vascular symptoms. Moreover, IgA-deficient persons receiving milk in their diet develop high levels of ICs some time after ingesting milk. Milk antigens are present in the sera of such individuals, but not in sera of normal persons who receive the same amount of milk. Therefore, in humans with IgA deficiency, cow's milk protein may enter the circulation, become complexed to preexisting antibody, and circulate as an IC. The relevance of this observation in terms of the diseases to which IgA-deficient patients are prone remains to be determined. Circulating ICs in IgA deficiency have also been detected by the C1q solid phase assay (Kwitko *et al.*, 1979).

24. *Thyroid Disorders*

Using an ADCC inhibition assay and Clq assays, evidence has been obtained for the presence of IC-like materials in sera of patients with

Hashimoto thyroiditis and primary hypothyroidism (Barkas *et al.*, 1976; Brohee *et al.*, 1979). Electron-dense deposits in the follicular basement membrane of the thyroid gland of patient with Hashimoto thyroiditis have also been observed (Kalderon *et al.*, 1973). Moreover, in a few cases of glomerulonephritis associated with autoimmune thyroiditis, IgG, C3, and thyroglobulin glomerular deposits have been observed (O'Regan *et al.*, 1976; Ploth *et al.*, 1978).

25. *Ankylosing Spondylitis*

The pathogenesis of this disease remains unknown, but immunologic mechanisms have been implicated. An association between ankylosing spondylitis (AS) and HLA B27 histocompatibility antigen has been established (Brewerton *et al.*, 1973). Two-thirds of patients with AS were found to contain IC-like materials using an inhibition of ADCC assay, but there was a poor correlation between the materials detected and disease activity (Corrigall *et al.*, 1978). In a preliminary study using the Raji cell assay, we detected IC-like materials in the majority of patients with AS and those with Reiter's syndrome (J. T. Rosenbaum, A. N. Theofilopoulos, and H. O. McDevitt, unpublished). Moreover, breakdown products of C3 and C4 have been detected in AS sera (Sturrock *et al.*, 1974). However, others using the Clq-PEG assay did not obtain evidence of IC-like materials in sera of patients with AS (Zubler and Lambert, 1977).

26. *Pulmonary Eosinophilic Granuloma*

Sera from six patients with pulmonary eosinophilic granuloma have also been analyzed for ICs by the Raji cell assay (King *et al.*, 1979). Levels of ICs were elevated in five subjects. All but one of the subjects had an active cellular histology. Immunofluorescent studies revealed granular deposits of IgG and C3 in alveolar walls and blood vessels. These findings show that circulating ICs are present in cases with cellular disease and suggest that their formation and/or deposition may contribute to the pathogenesis of pulmonary eosinophilic granuloma.

27. *Iatrogenic Diseases*

A large number of foreign antigens in the form of foreign sera used as a passive antibody (Monaco *et al.*, 1967; Iwasaki *et al.*, 1967), vaccines (Peeler *et al.*, 1965; Bishop *et al.*, 1966; Joekes *et al.*, 1973), and drugs such as penicillamine (Jaffe *et al.*, 1968; Hayslett *et al.*, 1968), sulfa compounds (Owens *et al.*, 1974), trimethadione (Bar-Khayim *et al.*, 1973), mercury (Mandema *et al.*, 1963; Seedat *et al.*, 1974; Kibukamusoke *et al.*, 1974), gold (Silverberg *et al.*, 1970; Vaamonde

and Hunt, 1970; Katz and Little, 1973; Tönroth and Skrifvars, 1974), and halothane (Williams *et al.*, 1977) can lead to the development of glomerulonephritis with electron microscopic and/or immunofluorescent evidence of IC deposition. ICs formed in these conditions may contain antibodies against the drug or drug-associated haptenic conjugates or possibly endogenous antigens released through toxic effects of the drug (Wilson and Dixon, 1976). Moreover, ICs composed of bovine proteins as antigens and IgG antibodies of maternal origin have been found in the sera of neonates receiving cow's milk (Delire *et al.*, 1978).

VI. Isolation of Immune Complexes

As noted above, several antigen-nonspecific tests detect materials that are tentatively designated ICs in sera and tissues of patients with various diseases. However, definite proof that these materials are indeed ICs exists for a few diseases, such as rheumatoid arthritis, hepatitis, and possibly SLE. Therefore, techniques are needed for the isolation, dissociation, and identification of the materials that result in positive reactions in the IC assays. Such techniques may lead not only to demonstrating that the materials detected are indeed ICs, but also to identifying antigens involved in pathogenic immune responses.

With an array of elution procedures, ICs (antigens and specific antibodies) have now been recovered from various tissues, predominantly kidneys, quantitated, and characterized for reactants (Oldstone *et al.*, 1975, 1976a; Koffler *et al.*, 1971; Poskitt *et al.*, 1974; Lambert and Dixon, 1968; Costanza *et al.*, 1973; Weksler, 1974; Lewis *et al.*, 1971). The techniques used for eluting ICs from kidneys have been examined comprehensively by Woodroffe and Wilson (1977) and by Bartolotti (1977). Both groups agree that elution is most efficient when one uses buffers with pH values less than 4.0 or greater than 10.0 or substitutes chaotropic agents (KI, KBr, KSCN), which preserve the antibody activity best. It should be noted that a very little ($\simeq 10\%$) of the protein in such eluates represents ICs, but this percentage can be improved by neutralizing the eluate and then precipitating the complexes out of the impurities with ammonium sulfate (Woodroffe and Wilson, 1977). The eluted antibody's specificity for an antigen is demonstrable by double immunodiffusion, by radioimmunoassays and by absorption to cells or other insoluble matrices carrying the antigen to which the eluted antibody may be directed. Elution techniques, although of great value, have several limitations: (*a*) selective elution of the least avid antibodies; (*b*) modification or denaturation of eluted antibody or antigen that may not be capable of immunologic reaction (Woodroffe and Wilson, 1977; Bartolotti, 1977); (*c*) reassociation of

eluted antigen and antibody upon neutralization of the low pH eluate or after removal of the chaotropic agent by dialysis. In fact, Woodroffe and Wilson (1977) found that up to 75% of the eluted antibody from the kidneys of rabbits with serum sickness is lost to further study, because, unless eluted antigen is separated from antibody, the two recombine at the restoration of physiologic conditions. Partition can be achieved by sucrose density fractionation of the dissociated eluate, by immunoelectrophoretic techniques, or by enzymic digestion of the antigen, i.e., pepsin digestion of viral antigens such as gp70 (A. N. Theofilopoulos, unpublished) or DNAse digestion of DNA.

Because tissues are not always available and because ICs in serum do not always deposit in tissues, efforts are under way to isolate ICs directly from pathologic sera. Originally, physicochemical procedures were used to isolate circulating ICs. Thus, ICs were separated from sera by cold precipitation or differential PEG precipitation (Digeon *et al.*, 1977; Carella *et al.*, 1977; Winfield *et al.*, 1975a; Lee and Rivero, 1964). Although antibodies and C components are found in some precipitates, specific antigens usually are not, casting some doubt as to the IC nature of the isolated materials. It should be noted, however, that few attempts were made to dissociate antibodies from other materials in the precipitates; therefore, antigens may have been masked by excess antibody. Chenais *et al.* (1977) combined gel filtration and affinity chromatography in their attempts to isolate ICs from sera. They obtained a globulin-enriched fraction by precipitation with ammonium sulfate or PEG, then subjected this material to gel filtration to separate monomeric IgG from macromolecular IgG. Thereafter, the macromolecular IgG was passed over an affinity column coupled with protein A to remove the IgG-containing material from other contaminants. The bound material was subsequently eluted with low pH buffers. Sera from an SLE patient and from a rheumatoid patient so treated yielded, respectively, DNA–anti-DNA plus RNA–anti-RNA complexes and IgG RF complexes. A similar approach was employed by Tucker *et al.* (1978), who separated high molecular weight materials from free IgG by Sephadex G-150 filtration at neutral pH, then allowed the heavy molecular weight material from this step to react with staphylococcal protein A bound to Sepharose. Subsequently, the bound material was eluted with low pH buffer and further fractionated at low pH on a Sephadex G-150 column. The materials recovered in the various fractions were then pooled, dialyzed, and analyzed by double immunodiffusion and sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) electrophoresis. Application of these methods was useful for the isolation of gp70–anti-gp70 ICs from sera of rats bearing Gross virus-induced lymphomas. As for the use of staphylococcal pro-

tein A for the isolation of ICs, it should be noted that C-fixing ICs may not be reactive with this reagent (Scharfstein *et al.*, 1979). Heimer and Klein (1978) have used affinity chromatography of sera on Con A–Sepharose columns for isolating ICs containing glycoproteins as antigens. The putative ICs bound to Con A–Sepharose were eluted with α-methyl-D-mannoside. This approach, although of some usefulness, is not practical as an initial step in IC-isolation from sera, since many glycoproteins, such as C3, present plentifully in most sera compete with the binding sites on Con A, and antigens with few lectin-binding mannose residues may not bind to the lectin because of their coverage by the antibody molecules in the IC. At any rate, these investigators used the above procedure and found that sera from patients with Epstein–Barr virus-related cancer (Burkitt's lymphoma, nasopharyngeal carcinoma) contained materials, possibly ICs, with strong affinity for Con A, thus suggesting the glycoprotein nature of the antigens involved. The same approach was used by Izui *et al.* (1979b) to obtain an enriched fraction of gp70–anti-gp70 complexes from sera of autoimmune mice.

In one of several efforts to isolate ICs from sera, Svehag and Burger (1976) used affinity-purified Clq covalently coupled to agarose or absorbed to IgG-agarose resin with sera containing preformed virus-antibody ICs or rheumatoid arthritic sera. When these sera had passed through the columns, Clq-bound ICs were eluted with 1,4-diaminobutan at mild basic conditions. Three of five samples from rheumatoid arthritics treated as above were found to contain both IgG–IgM RF and IgG–IgG RF complexes, but two had only IgG–IgG RF complexes.

Raji cells were used (Theofilopoulos *et al.*, 1978b) to isolate C-fixing ICs from sera. ICs bound to the cells were radiolabeled while attached to the cell surface with lactoperoxidase. The labeled ICs were then eluted from the cells with isotonic citrate buffer at pH 3.2 or recovered by immunoprecipitation of cell lysates. The constituents of the complexes were then isolated by dissociating sucrose density gradient centrifugation or by SDS-PAGE. A variety of ICs formed *in vitro* were successfully isolated from serum with this approach. As little as 25–100 μg of antigen complexed with antibody per milliliter of serum would produce distinct peaks of radioactivity in the gels. This technique was then applied to the isolation of ICs from sera of rabbits with bovine serum albumin (BSA)-induced chronic serum sickness and rats with Moloney virus-induced sarcomas, both models of human diseases for which these procedures should be useful. Eluates obtained from Raji cells incubated with the rabbit sera showed radioactivity in gels corresponding to markers for IgG and BSA. Sera of rats to be trans-

planted with tumors were negative for ICs, and no peaks of radioactivity were observed upon SDS-PAGE analysis of the Raji cell citrate buffer eluates. However, analysis of the eluates derived from cells incubated with IC-positive serum samples obtained from the same animals 5–40 days after inoculation with tumor cells showed three major peaks of radioactivity corresponding to IgG, gp70, and p30 markers. The material comigrating with gp70 and p30 and then eluted from the gels was, indeed, envelope and core antigen of retroviruses which immunoprecipitated with specific goat antisera to gp70 and p30. Furthermore, the gp70 and p30 were associated with IgG antibody on Raji cell surfaces, since no peaks of radioactivity were seen after neutralizing and precipitating the eluate with ammonium sulfate prior to electrophoresis. From these conclusive results, the ICs examined were composed of antibody and gp70 plus p30 antigens. This technique has been applied to a small number of IC-containing sera from patients with SLE and cancer with no definite results as yet. The principles and techniques oultined above for isolating ICs using Raji cells could be adapted to other substances that bind ICs, e.g., conglutinin. ICs bound to insolubilized conglutinin are recovered after elution with EDTA and NADG and other buffers (R. A. Eisenberg *et al.*, 1977; Casali and Lambert, 1979).

In certain viral diseases, such as dengue fever, which is known to be associated with circulating ICs, the percentage of IgG-positive PMN cells and lymphocytes, presumably carrying ICs, is increased (F. J. Dixon, unpublished). High percentages of Ig-positive lymphocytes are also found in patients with autoimmune diseases (Winchester *et al.*, 1974). In these diseases Ig on lymphocytes may represent either antilymphocyte antibody or the antibody part of blood-borne IC. Moreover, evidence accumulated from studies of cancer patients suggests that tumor antigen–antibody ICs are absorbed *in vivo* on leukocytes (Bansal *et al.*, 1976, Hattler and Soehnlen, 1974). The techniques described above for recovery and isolation of ICs bound to Raji cells from serum, then, seem to be applicable to isolating ICs absorbed *in vivo* on a patient's own leukocytes. To test this possibility in rabbits with chronic serum sickness caused by injecting BSA, we obtained peripheral leukocytes by Ficoll–Hypaque centrifugation immediately before and 10 minutes after daily injections of the antigen. The leukocytes were washed and labeled with ^{125}I by the lactoperoxidase technique; then elutes obtained as above were subjected to SDS-PAGE analysis. ^{125}I peaks comigrating with the ^{131}I markers of IgG and BSA were observed only in eluates from leukocytes of postinjection samples. Eluates derived from peripheral leukocytes of a normal control rabbit

injected with similar amounts of BSA had no such peaks. This technique was applied to the study of peripheral blood cells of patients with Felty's syndrome, and peaks corresponding to IgM and IgG were observed. The IgM peak was found to represent RF. In this context, Jaquemin *et al.* (1978) purified IgG from fresh human blood cells of patients with chronic myelogenous leukemia and chronic lymphatic leukemia in blastic crises and found antibodies to reverse transcriptase from feline leukemia virus and to a lesser extent antibodies to reverse transcriptase from horizontally transmitted primate retroviruses.

Although Raji cells and other materials have been successful in isolating ICs, whether such an approach will yield enough ICs and antigens from pathologic sera for subsequent detailed studies is questionable. Therefore, we are developing techniques to produce antisera in animals against small quantities of antigen present in isolated ICs (Theofilopoulos *et al.*, 1978b). After repeated challenges with whole Raji cells to which ICs from human sera were bound or with eluates from such cells, rabbits tolerant to human IgG made antibody against antigens in the ICs, although reactivity against cellular and serum components was also elicited. Monospecific antisera were produced against the antigens in ICs removed from serum with Raji cells by immunizing IgG-tolerant rabbits with the alum-precipitated antigen eluted from polyacrylamide gels.

We should stress that, although these techniques may have some success when the identity of an antigen–antibody system is suspected, the identification of unknown antigens, even if they are isolated, would be extremely difficult because many antigen–antibody systems may coexist in an individual with a given disease. Any putative antigenic material isolated from serum should be further analyzed by (*a*) enzymic digestion and other chemical procedures to determine its composition; (*b*) absorption on insolubilized lectins to determine whether it is a glycoprotein; (*c*) interaction of the antigen with a battery of antisera whose specificities are known; (*d*) inhibition of certain antigen–antibody reactions with the antigenic material; and (*e*) specific stimulation with the antigen of lymphocytes or delayed hypersensitivity reactions from individuals suffering from the same disease as the individual from which ICs were isolated.

VII. Conclusions

An individual can, and usually does, make an immune response to a large number of exogenous and a smaller number of endogenous antigens. Depending upon the availability of antigen, the antibodies so

produced form ICs, which for the most part serve the purpose of aiding the host in eliminating potential pathogens.

Apart from eliminating the antigen, antibody production and IC formation appear to exert important regulatory influences on humoral and cellular immune responses by interacting with Fc, C, and/or antigen receptor-bearing lymphoid cells. Depending on the molar ratio of antigen to antibody, the epitope density of the complex, and the class and affinity of antibody, ICs may enhance or suppress immune functions, but the exact mechanisms of such effects are not well understood.

Because the complexes are handled harmlessly in large part by the reticuloendothelial system, only when ICs deposit in vascular structures, with subsequent activation of inflammatory mediator pathways such as the C system, does an "IC disease" emerge. What determines the fate and phlogogenic potential of circulating ICs is not completely understood, but it is recognized that important factors include the nature of the antibody and antigen, the molar ratio and production rate of the two reactants, and the state of the phagocytic system.

Since IC formation is a common event in life, why should some individuals develop IC disease while other individuals do not? Genetic factors may play an important role in this respect. For example, the amount and affinity of antibody formed against an antigen vary from one animal strain to another, perhaps because of their differing genetic backgrounds. In humans, HLA haplotype has been associated with the magnitude of responsiveness to certain antigens (Sasazuki *et al.*, 1978; de Vries *et al.*, 1977; Christiansen *et al.*, 1978). However, no direct relationship between the HLA system or an immune response gene and IC disease has been established as yet in man. The class and subclass of the antibody in an IC must also be considered in explaining that complex's phlogogenic capacity. Each class and subclass is distinctive in its capacity to activate C and to attach to cells with Fc and C receptors. Never have well-defined IC composed of the various Ig classes and subclasses been compared to establish the unique properties of each in IC diseases of intact animals. The presence of autoantibodies directed against phagocytic cells may also influence the fate and phlogogenic potential of ICs. Such autoantibodies directed against subpopulations of mononuclear cells and other cells circulate continuously in the blood of most patients with autoimmune disorders (Winchester *et al.*, 1974; Winfield *et al.*, 1975b; Pruzanski *et al.*, 1978). It would not be surprising if such autoantibodies have among other specificities anti-IgG Fc and anti-C receptor antibodies. Such antibodies would block the attachment of ICs to phagocytic cells and

promote persistence of ICs in the circulation. In fact, autoantibodies with antireceptor activities are found in some patients with autoimmune disorders (Pruzanski *et al.*, 1978). Moreover, preliminary experiments in which Hamburger *et al.* (1979) and Frank *et al.* (1979) followed the clearance of IgG antibody-sensitized red cells show that patients with Sjögren's syndrome and SLE clear these ICs more slowly than normal controls, possibly because IgG Fc or C receptor function is abnormal or the receptors are occupied by antimononuclear cell autoantibodies, ICs or C3b generated after C activation. Similarly, reduced phagocytic activity of monocytes from patients with SLE has been found in *in vitro* studies (Kavai *et al.*, 1979). The rate of IC formation in relation to the turnover of cellular IgG Fc receptors also may be of importance. Several investigators (Cordier *et al.*, 1977; Moretta *et al.*, 1978; Mingari *et al.*, 1978) have shown that after particulate or soluble ICs bind to IgG Fc receptors on human mononuclear cells, a process of dissociation occurs *in vitro*, and that cells from which ICs are dissociated become incapable of binding IgG-type ICs again or acting as effectors in ADCC. Furthermore, the IgG Fc receptor is not detectable at the cell surface even after prolonged incubation, suggesting that the IgG Fc receptor is rapidly shed and very slowly resynthesized after modulation. Thus, if an analogous phenomenon is operative *in vivo*, abundant continuously produced ICs will not find enough IgG Fc receptor-bearing cells to bind and will remain in the circulation. A similar mechanism could operate through C receptors. Thus, human mononuclear cells preferentially form rosettes with antibody-sensitized erythrocytes containing C3 of the F phenotype over that of the S phenotype (Avrilommi, 1974). Again, the possibility exists that subjects having C3S may be more prone to the development of IC disease because of insufficient binding and removal of C-fixing ICs by phagocytic cells. In the same context, adequate function of the C system would be important. ICs of certain composition may also initiate the production of anti-idiotypic antibodies (Klaus, 1978; Eichmann, 1978), which may contribute to IC disease by forming ICs with the relevant idiotype and so on. The result would be a variety of persistent idiotype–anti-idiotype ICs, independent of the release or retention of the antigen involved in the original stimulation. Finally, the nature of the antigen involved may be important, since certain substances such as DNA and lectins have been found to have affinity for basement membranes (Izui *et al.*, 1976; Golbus and Wilson, 1979).

Because ICs have effects on immune responses and are involved in the pathogenesis of many human diseases, detection techniques are

evolving rapidly. From the foregoing descriptions, it is clear that no universal and absolutely specific reagent is yet available for this purpose. Therefore, the search must continue for a specific, sensitive, and easily reproducible test capable of detecting ICs of all Ig classes, both C-fixing and non-C-fixing, and of all sizes. The degree of correlation among the results of various tests for circulating ICs in a given group of patients is not high, as indicated in the WHO comparative study (Lambert *et al.*, 1978). Because of the different specificities and idiosyncracies of the assays, patholgic sera should be analyzed with more than one assay, preferably by a combination of the solid-phase Clq assay or Clq-PEG assay, a mRF radioimmunoassay, and the Raji cell assay. Furthermore, adequate standards must be established before any of these methods can reliably be applied to the quantitation of ICs. This task will be extremely difficult, since ICs made *in vitro* or aggregates used for standardization cannot exactly mimic the composition of ICs present in sera of different individuals. Recently, the International Society of Immunologists together with the WHO initiated a study to establish standard reference preparations of AHG and of ICs formed *in vitro* for use by investigators in the field. When implemented, such standards will be of obvious benefit, particularly in expressing uniformly the activity found in pathologic sera by various IC assays. Despite all the limitations, any positive result from tests that detect ICs in fluids presently provide evidence that a given patient's disease may be associated with ICs. Then further analysis of the pathologic fluids, preferably by more than one of these techniques, would be appropriate before the conclusion that ICs are involved is finalized. It should be emphasized that the detection and demonstration of ICs in biological fluids does not necessarily indicate that the pathology of the disease under study results from these complexes. A further necessary requirement is the demonstration of ICs in the affected tissues.

Despite the aforementioned deficiencies of existing techniques, and apart from the importance of IC measurement in understanding the pathogenesis and immunopathology of diseases, IC determination, as indicated above, can be valuable in forecasting prognosis and monitoring various therapeutic modalities in patients with infectious and autoimmune diseases as well as cancer. It is hoped that routine availability of these assays in many institutions and further application with long-term observations of defined groups of patients will establish the usefulness of IC-measurement in clinical medicine.

An important by-product of IC measurement is the use of the detecting substances to concentrate and isolate ICs so that the antigens involved can be identified. Preliminary accounts of this use seem en-

couraging, but because multiple IC systems are involved in many diseases, the tasks of isolating ICs from human sera and identifying antigens will be extremely difficult. However, purified antigens, even though imcompletely identified, could be used diagnostically to assess the presence of specific antibodies in sera and the presence of cellular immunity manifested by induction of cutaneous delayed-type hypersensitivity or lymphocyte stimulation. Purified antigen also might be used therapeutically to enhance a weak immune response. Antisera raised against the isolated antigens may be useful in developing reproducible and sensitive techniques for detecting the corresponding antigens either in serum or in tissues of patients. In addition, the availability of such antisera will allow the use of immunochemical techniques, such as affinity chromatography, to purify large quantities of the respective antigens.

Since ICs seem to be active in many diseases, it would be desirable to develop therapies to attenuate their deleterious effects, as outlined by a World Health Organization expert committee (1977). The steps proposed in this study include (*a*) elimination of microbial antigens with appropriate chemotherapy and endogenous antigens with anti-inflammatory drugs; (*b*) alteration of the antibody quantity or quality by immunosuppressive or immunostimulatory agents and possible alteration of the antibody structure so as to avoid activation of C; (*c*) removal of ICs by plasmapheresis with simultaneous attempts to eliminate the antigen source; and (*d*) use of drugs to block the discharge of injurious constituents by effector cells (mast cells, neutrophils) and introduction of inhibitors to neutralize injurious products released by these cells. Continued study of the characteristics and handling of ICs should provide information necessary for the treatment of IC-associated diseases and perhaps their prevention.

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ADVANCES IN IMMUNOLOGY, VOL. 28

The Human Ia System[1]

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I. Introduction

The human major histocompatibility complex controls the expression of two distinct systems of highly polymorphic membrane-associated glycoproteins. The first type (type I) comprises the HLA-A, -B, and -C segregant series. They consist of molecules containing a 45,000 dalton heavy chain and a lighter chain, that is, β_2-microglobulin, and are widely distributed on most mature nucleated cells.

The second type (type II) consists of the human "B cell alloantigens" that are the equivalents of the murine Ia antigens. For simplicity this human Ia-like molecule will be referred to as "Ia" where the context is unambiguous. The dominant characteristics of the human Ia antigens are the sharply restricted distribution on different cell types and variations in amount during the chronology of a particular cell lineage. The molecules consist of two chains with apparent molecular weights entirely different from the type I molecules and lack β_2-microglobulin.

This review will examine the Ia antigens from two principal perspectives: first, as membrane components related to differentiation; and, second, as part of a genetically intricate system of molecules bearing alloantigens that have relevance to susceptibility to certain diseases. It is already likely that studies on the Ia antigens will uncover the relatedness of events in the regulation of the immune response to other systems of controlled cellular proliferation and specialization. Although the importance of Ia alloantigens as a tool for probing genetic factors in a number of common diseases is now evident, it is possible that analyses of the mechanisms of these diseases will result in novel insights into the function of the histocompatibility complex and its polymorphisms.

II. Historical Overview

The recognition of the human equivalent of the Ia system rested on a succession of developments in several distinct lines of investigation.

A. Early Observations

A series of earlier reports in several areas of investigation are, in retrospect, attributable to the presence of Ia alloantigens. These in-

clude studies on tumor-associated antigens, of anomalous HLA typing results given by lymphoid lines and certain leukemias, and serologic inhibition of mixed lymphocyte culture reactions.

1. Tumor-Associated Antigens

The presence of dominant antigens characterizing cells from the majority of patients with acute and chronic leukemias and certain other lymphoid malignancies, demonstrable by immunization of rabbits or other species (Mann *et al.*, 1971; Bentwich *et al.*, 1972; Billing and Terasaki, 1974; see Mohanakumar and Raney, 1978), have now been shown to consist principally of Ia antigens. The presumed tumor-associated antigens recognized by these sera were at the time not evident upon normal B cells because available methodologies did not recognize the small number of normal B lymphocytes that were positive.

2. Anomalous HLA Typing

Unexpected cytotoxic typing reactions with well characterized HLA allosera were encountered during attempts to HLA-type lymphoid lines or lymphocytes from patients with chronic lymphatic leukemia (Bernocco *et al.*, 1969; Herberman, 1969; Dick and Steel, 1971; MacIntosh *et al.*, 1971; Pegrum *et al.*, 1971; Walford *et al.*, 1971; Dick *et al.*, 1972; Ferrone *et al.*, 1972; Jeannet *et al.*, 1972; Moore and Woods, 1972). These reactions were initially interpreted in part as examples of leukemia-associated antigens. It was then recognized that these "extra" reactions consisted of non-HLA antigens (Dick, *et al.*, 1973; Pious *et al.*, 1974) that constituted a distinct genetically determined system (Gosset *et al.*, 1975).

3. MLC Inhibition

Alloantisera were found to inhibit specifically the allogeneic MLC. The nature of the inhibitory material was demonstrated to be antibodies that were not removed effectively by platelet absorptions, suggesting that other than conventional HLA antibodies were involved (Ceppellini *et al.*, 1971; Ceppellini, 1971; Revillard *et al.*, 1972). Inhibition specific for the stimulating cell that did not involve classic HLA antigens was obtained using the alloserum donor as the responder in the MLC, and an HLA identical subject as the stimulator (van Leeuwen *et al.*, 1973). The sera were devoid of cytotoxic activity when tested on peripheral blood cells; however, in immunofluorescence experiments staining of up to 25% of the mononuclear cells was observed, indicating that an alloantigen was being detected.

B. Recognition of B Cell Alloantigens

The resolution of the explanation of these phenomena had to await the development of methods for the analysis of the phenotype of peripheral blood lymphocytes in terms of T and B cells, along with techniques for their isolation. This permitted the recognition that the vast majority of chronic lymphatic leukemias involved selective B lymphocyte proliferation and that nearly all lymphoblastoid cell lines were in the B cell series. These advances in lymphocyte biology provided the basis for a series of reports describing the B cell alloantigen system (Jones *et al.*, 1975; Mann *et al.*, 1975a; van Rood *et al.*, 1975; Wernet *et al.*, 1975; Winchester *et al.*, 1975a; van Leeuwen *et al.*, 1975).

At the same time, in the course of isolating classic HLA antigens from B-type lymphoid lines after papain digestion, an antiserum was prepared against these components and, after absorption with T cells, precipitated a 35,000 and a 27,000 dalton glycoprotein from labeled B cell membranes (Cresswell and Geier, 1975).

C. Relationship to Murine Ia

It was now evident that these B cell antigens were homologous to the murine Ia system (see Shreffler and David, 1975). The homologies to the murine system included: the selective cellular distributions (Shreffler *et al.*, 1974; Delovitch and McDevitt, 1975; Cullen *et al.*, 1976; Sachs and Cone, 1973), two-chain structure with α and β chains of comparable size (Cullen *et al.*, 1976; Cullen and Schwartz, 1976; Silver *et al.*, 1977), and inhibition of MLC by anti-Ia sera (Meo *et al.*, 1975).

The significance of this association stemmed from the fact that the genes for the Ia molecules were initially defined through their regulation of specific immune responsiveness and involved aspects of cellular interaction in the immune response (reviewed in Benacerraf and McDevitt, 1972; Klein, 1975; Shreffler and David, 1975; Katz, 1977). A considerable body of evidence implicates the Ia molecules as the entities responsible for these *I* region regulatory functions. These include specific collaboration among T and B cells in the immune response (Katz *et al.*, 1974; Pierce *et al.*, 1976; Kappler and Marrack, 1976), suppressive control of the immune response (Murphy *et al.*, 1976; Tada *et al.*, 1976; Pierres *et al.*, 1978), and antigen-induced antibody synthesis and T cell proliferation (Schwartz *et al.*, 1976a, 1978; Frelinger *et al.*, 1975; Niederhuber and Frelinger, 1976).

The availability of inbred strains permitted the division of the *I* region into *I-A*, *-B*, *-J*, *-E*, and *-C* subregions (Shreffler and David, 1975;

Shreffler *et al.*, 1976). A noteworthy feature of the E/C molecules is the evidence for control by two closely linked *I* region genes, one in the *I-A* region and the other in the *I-E/C* region (Jones *et al.*, 1978). This appears to provide the molecular basis for the phenomenon of complementation in which antibody synthesis or T cell proliferation to certain antigens requires the complementary presence of two IR genes mapping in the *I-A* and *I-E/C* region (Dorf *et al.*, 1975; Schwartz *et al.*, 1976b).

D. Consolidation and Evolution

The crystalization of the central findings in the human Ia field by 1975 was followed by a rapid evolution of investigation along multiple but interrelated lines. The study of the tumor-associated Ia antigens principally contributed to the view of the Ia antigens as differentiation antigens and focused attention on the role of the Ia system outside the immune system. The study of Ia alloantigens has become one of the central points of focus of the histocompatibility field. The infusion of direction from the murine studies has resulted in confirmation of a parallel role of human Ia genes in control of the immune response. Last, the availability of new Ia alloantigens has brought out many new disease associations not apparent from the study of the classical HLA antigens. This last endeavor, apart from its obvious applicability to medical problems, promises new insights into the function of the MHC.

E. Basic Reagents and Applications

The two types of reagents used for the serologic identification of Ia are human alloantisera or heteroantisera. The human Ia alloantisera are derived from individuals who have been alloimmunized during pregnancy or in the course of organ transplantation or transfusion. Considerable selection and processing is necessary to be assured that a reagent serum is specific for Ia alloantigens.

The heteroantisera are of two varieties: those obtained by immunization of rabbits or comparable species with preparations containing Ia (Cresswell and Geier, 1975; Billing *et al.*, 1976; Humphreys *et al.*, 1976; Snary *et al.*, 1976; Winchester *et al.*, 1976) and the newer hybridoma reagents. Monoclonal hybridoma reagents reacting with common Ia antigens have been readily obtained in a number of laboratories. The origin and application of the heteroantisera in general relate to a degree to the area of tumor-associated antigens. Particularly in the area of leukemia-associated antigens, the use of the heteroantisera served to demonstrate that these were not leukemia specific, but rather

antigens that characterized the particular stage of differentiation characteristic of the leukemia (Janossy *et al.*, 1977; Winchester *et al.*, 1977). Most heteroanti-Ia sera have not detected any allospecific determinants and appear to react with framework antigens common to all Ia molecules. However, several investigators have obtained selected rabbit antisera raised against isolated Ia preparations that have some allospecificity when tested in cytotoxic assays (Welsh and Turner, 1976; Solheim *et al.*, 1978; Ferrone *et al.*, 1973a). Heteroantisera in general have had an important part in development of the field. However, it is already clear that the single specificity of each of the new hybridoma reagents, either to common or variant parts of the Ia molecule, makes these valuable reagents that will have wide application.

III. B Lymphocytes

A. Introduction

The relationship of the special characteristics of the B lymphocyte to the expression of Ia antigens is the subject of this section. The properties of the Ia antigens illustrate the dominant theme in studies on lymphocyte surface markers that the recognition of the receptors and cell surface molecules that characterize the cell are membrane specializations serving distinct functions at particular stages of differentiation. These membrane components are expressed in patterns of acquisition and subsequent loss that delineate broad stages in this differentiation process. The presence in peripheral blood of lymphocytes in several stages of differentiation in each of the pathways leading to functionally distinct subsets of cells clearly accounts for some of the considerable heterogeneity of lymphocytes in terms of surface markers.

B. Peripheral Blood Lymphocytes

1. General Status of Surface Markers

The occurrence of surface Ig is the classic marker of a B lymphocyte, while rosette formation with sheep erythrocytes is the most widely used primary characteristic of human T lymphocytes. Approximately 90% of lymphocytes in blood can be accounted for by cells with either one or the other of these markers. The remaining lymphocytes are termed the third or null-cell population and are defined by the absence of both surface Ig and the E rosette receptor (Froland and Natvig, 1973; McDermott *et al.*, 1975). The division of lymphocytes into these

three categories becomes less well delineated when the other major receptors are considered, including those reacting with complement components or Ig Fc region. The term "null" cell was particularly appropriate during the early stages of analysis of lymphocyte surface markers. At present caution must be exercised in its use because various laboratories employ it in different ways and it no longer necessarily implies a cell without markers, but rather a cell not readily classified as either B or T.

In the case of complement receptors (CR), all studies are in agreement that most lymphocytes with CR also express surface Ig and receptors for Fc (Moller, 1974; Ross *et al.*, 1973); however, CR^+ lymphocytes were identified that lacked sIg or formed rosettes with sheep erythrocytes (E) (Mendes *et al.*, 1974). Similarly, the IgG Fc receptor has been found on B and T, and lymphocytes of the third population (see Dickler, 1977; Winchester *et al.*, 1979). Since it was recognized that mIg is no longer detectable on the B lymphocyte surface after stimulation and during transformations into plasma cells (Fu *et al.*, 1974a; Pernis *et al.*, 1974; Preud'homme, 1977), the availability of the Ia system offered an alternative marker with which to analyze lymphocyte classification.

Accordingly, two major questions remained that could be approached by determination of the presence of Ia antigens on the cell surface: Are there B lymphocytes in peripheral blood without surface Ig? Is the third population an entity in itself or is it composed of mixtures of B and T cells with atypical marker profiles along with cells not in the lymphocyte lineage but superficially resembling lymphocytes?

2. *Relationship of Ia Antigens to Ig and E Rosette Markers*

Separation of lymphocytes according to the presence or the absence of the E receptor results in two populations with sharply contrasting percentages of Ia-bearing cells as shown in Table I. The results in a series of normal individuals show that an average of approximately 70% of the E rosette negative (E^-) population have readily demonstrable Ia antigens, while only 2% of the E^+ population have this marker. It is evident that the E^- population should be termed "B cell enriched," not simply "B cell," in view of the considerable number of undefined "third population" cells that are found in it. Within the E^- population nearly all cells with membrane Ig have readily detectable Ia antigens, and this and related observations led to the conclusion that Ia was an important characteristic of the B lymphocyte (Cresswell and Geier, 1975; Schlossman *et al.*, 1976; Winchester *et al.*, 1975a, 1976;

TABLE I

ENRICHMENT OF Ia-BEARING LYMPHOCYTES IN THE FRACTION OF LYMPHOCYTES THAT DO NOT FORM E ROSETTES: PARALLEL DETECTION OF Ia BY HETERO- AND ALLOANTI-Ia SERA (% POSITIVE CELLS)[a,b]

	E-rosette (−) fraction				E-rosette (+) fraction			
Normal subject	Hetero-anti-Ia	Alloanti-Ia serum[c]	Membrane Ig	E rosettes	Hetero-anti-Ia	Alloanti-Ia serum	Membrane Ig	E rosettes
1	47.9	43.5	27.6	2.5	1.8	0.9	1.0	87
2	60.1	55.0	23.0	7.3	3.6	0.3	0.6	94
3	81.8	60.4	53.6	6.0	5.9	5.0	2.3	90.5

[a] From Winchester *et al.* (1976).

[b] Hetero-Ia antibodies were used in direct immunofluorescence as $F(ab')_2$ fragments. Whole alloanti-Ia sera were used in indirect immunofluorescence and developed with $F(ab')_2$ fragments of antibodies specific for Ig_6. Monocytes were excluded from analyses.

[c] Only the highest positive staining given by the panel of five alloantisera with the nonrosetting fraction of each individual is given. The data obtained by the same alloantiserum is also given for the rosetting fraction.

Nelson *et al.*, 1977). Furthermore, these findings supported the reciprocal inference that a lymphocyte bearing Ia molecules is likely to be a B cell, an inference that has proved to be inexact in disease states where stimulated T cells express Ia antigens.

Among the E^+ or "T cell" population, a generally very small but variable percentage of cells contains sIg, in part comprising B cells with apparent specific affinity for determinants on sheep erythrocytes. Although these lymphocytes account for a portion of the Ia-bearing cells in this E^+ population, it was evident from the earliest experiments that a fraction of E^+ cells expressed the Ia marker without the presence of membrane Ig (Fu *et al.*, 1978). This topic will be considered further in Section VI.

In the case of the E^- population containing the B cells, in each individual the percentage of Ia-bearing cells exceeded the percentage of Ig-bearing cells by approximately 20% (Winchester *et al.*, 1976; Chess *et al.*, 1976; Humphreys *et al.*, 1976). This divergence was documented in double-label experiments. Thus, in a representative series of young adults, 12% of peripheral blood lymphocytes had sIg while an additional 4% had Ia markers without Ig (Winchester *et al.*, 1976). Since these latter cells were also E^-, they had been primarily considered to be in the "null" population (Chess *et al.*, 1976; Humphreys *et al.*, 1976). However, important clues concerning the relationship of these Ia^+Ig^- cells to the B lineage were provided by the observation that in patients with Bruton's X-linked agammaglobulinemia, a specific disorder of the B lymphocyte, the Ia^+Ig^+ as well as the Ia^+Ig^- cells were absent (Hoffman *et al.*, 1977). These results fit those which indicated that some cells in the null population matured *in vitro* to plasma cells (Chess *et al.*, 1976).

3. *Analysis of Complement-Receptor Lymphocytes*

In normal individuals an average of 17% of lymphocytes has complement receptors (CR). The two major complement receptors, CR_1 and CR_2, are respectively demonstrated by rosettes containing either C4b or C3b for CR_1, and C3d for CR_2. All lymphocytes with CR have CR_1, and slightly over half also have CR_2 (Ross *et al.*, 1978b).

Essentially all the lymphocytes with both CR_1 and CR_2 have membrane Ig and Ia markers; these account for nearly 10% of blood lymphocytes. An additional 2% of lymphocytes have Ig and Ia markers but express only CR_1 (Humphreys *et al.*, 1976; Ross *et al.*, 1978b). One possibility is that the sequence of differentiation is $Ig^+Ia^+CR_1^+ \rightarrow Ig^+Ia^+CR_1^+CR_2^+$. In addition to the $Ig^+Ia^-CR_1^+CR_2^-$ population, an equal number of $CR_1^+CR_2^-$ lack Ia or Ig; some of these

TABLE II
RELATIONSHIP OF Ia ANTIGENS TO mIg AND COMPLEMENT RECEPTOR MARKERS ON PERIPHERAL BLOOD LYMPHOCYTES (PERCENT)[a]

Ia (+) Ig (+) CR_1 (+)[b]	11.2
Ia (+) CR_1 (+)[b]	2.4
Ia (+)[b]	1.6
CR_1 (+)	2.7

[a] After Ross *et al.* (1978b).
[b] IgG Fc receptor (+), E rosette (−).

are E^+. This divergence between CR and Ia also exists in the reciprocal direction with an average of 1.6% of blood lymphocytes containing Ia antigens but lacking demonstrable complement receptors (Ross *et al.*, 1978b). The relationships are summarized in Table II where the data for CR_2 have been omitted.

4. *IgG Fc Receptors*

Fc receptors are found on virtually all B cells, a minor population of T cells, and the majority of null or third population cells (Dickler, 1977; Winchester *et al.*, 1979). The reactivity of the Fc receptors on the different lymphocyte populations varies widely with the particular type of test systems used for their demonstration (Arbeit *et al.*, 1977; Winfield *et al.*, 1977; Winchester *et al.*, 1979). Analyses of the lymphocyte population according to the presence or the absence of the Ia marker demonstrated that certain immune complex systems preferentially reacted with Ia^- cells regardless of the presence or absence of the E rosette receptor, whereas others primarily reacted with Ia^+ cells (Winchester *et al.*, 1979). The finding lent further support to the inclusion of the Ig^-Ig^+ lymphocyte within the B cell series and conversely provided evidence for the relationship of other third-population lymphocytes to the T cell series. Thus, in an average normal person, the 10–15% of lymphocytes lacking mIg or E rosette receptors are approximately equally divided into an Ia^+Fc^+ group, some of which contain CR_1, an Ia^-Fc^+ group, of which the Fc receptors are of the type present on T cells, and a few $Ia^-Fc^-CR^-$ cells of an undefined nature.

5. *Nonlymphoid Cells in the "Third Population"*

Metamyelocytes and other early forms of the granulocyte lineage have densities such that they will be isolated in the mononuclear cell layer (Winchester *et al.*, 1977; Ross *et al.*, 1978a). Definite identification of myeloid cells in these preparations has been made using antimyeloid sera (Niaudet *et al.*, 1979) and by morphologic and enzymic

criteria (Hoffman *et al.*, 1979). Particularly in certain patients with a variety of chronic diseases, the percentage of these early granulocyte forms sometimes exceeds the number of B lymphocytes (Hoffman *et al.*, 1979). The predominant cell closely resembles the myelocyte. It is slightly larger than a lymphocyte, as observed by phase microscopy, and lacks Ia, Ig, complement, and E rosette receptors. The Fc receptor is strong and binds avidly to various detector systems including anti-Rh-sensitized human erthrocytes.

C. Lymphoproliferative Diseases

The study of the various malignancies involving the lymphoid system has played an important role in orienting the subsequent direction of investigation, since the proliferating cells frequently represent in effect a highly purified population, fixed at a particular stage in a differentiation pathway. Furthermore, the possibility always exists that an analysis of the surface phenotype of the malignant cell will have predictive value for the clinical behavior of the malignancy as well as indicating which therapeutic agents are more likely to result in control. Within this context the Ia antigens have had several applications in defining surface marker profiles.

1. Lymphatic Leukemias

a. Chronic Lymphatic Leukemia. The chronic lymphatic leukemia (CLL) cells, by providing an abundant supply of essentially purified B cells, played an important role in illuminating the early studies on B cell alloantigens (Section II,B) (Gosset *et al.*, 1975; Lawler *et al.*, 1975; Legrand and Dausset, 1975; Winchester *et al.*, 1975a). Pooling of results from a number of laboratories in the VIIth Histocompatibility Workshop demonstrated that the frequency of alloantigens among all patients is within the expected range of alloantigen specificities in the normal population when corrected for racial composition (Lawler and Jones, 1978).

The vast majority of patients with CLL have malignant cells that generally resemble peripheral blood B cells, containing Ia antigens, Ig, and receptors for complement and Fc regions of IgG. The intensity of Ia antigen staining varies from case to case but is similar in general to that of peripheral blood B cells (Halper *et al.*, 1979). There is a complete parallel between the number of cells reacting with the heteroanti-Ia serum and those reacting with the alloantiserum of appropriate specificity (Winchester *et al.*, 1976). In approximately one-fifth of patients, the Ig staining is extremely weak or not detectable; however, the Ia marker remains bright by fluorescent staining. Uncommonly,

CLL consists of cells with normal morphology or Sezary cell morphology and have the phenotype of E rosette $^{+}Ia^{-}$. Both hetero- and alloanti-Ia reagents do not react with more than a few percent of cells from such T cell leukemias. Very rare unclassified CLL are characterized by cells that express Ia and form E rosettes (Fu *et al.*, 1978).

b. Acute Lymphatic Leukemias (ALL). The lymphocyte population present in patients with ALL represents a rapidly turning over, morphologically immature, cell that is lymphoid in appearance. Patients with ALL have proliferations of either of two principal types of lymphocytes that are morphologically indistinguishable. The surface phenotype of the most common variety of leukemia is characterized by Ia antigens and the absence of all other markers found on mature lymphocytes (Fu *et al.*, 1975b; Winchester *et al.*, 1976; Greaves *et al.*, 1977). The second principal variety of leukemic lymphocyte usually lacks detectable Ia antigens but contains receptors for sheep erythrocytes. Only an occasional case of ALL is characterized by lymphocytes that have Ia antigens on their surface and small quantities of intracellular or surface Ig. The true null-cell leukemia lacking E rosette and Ia is quite rare. The Ia on common ALL cells is contained on a two-chain molecule (27,000 and 35,000 daltons) resembling that present on B cells (Billing *et al.*, 1977).

It is generally thought that the $Ia^{+}E^{-}$ acute lymphatic leukemic cell represents either a progenitor or a very early stage within the B cell series, although T cell lineage remains a possibility (Fu *et al.*, 1975b; Janossy *et al.*, 1976a; Greaves *et al.*, 1977). Evidence for the relationship of the Ia^{+} cell of the common type of ALL to the lymphoid cell found in certain cases of chronic myelogenous leukemia has been presented (Janossy *et al.*, 1976b). The blast characteristics of the cell of common ALL as well as the absence of other receptors distinguish it from the $Ia^{+}Ig^{-}$ lymphocyte of normal peripheral blood.

2. *Multiple Myeloma*

The occurrence of these malignancies of the terminal stages of the B cell lineage allows the study of Ia expression at this phase of differentiation. The bone marrow of patients with myeloma of different Ig classes was stained for the presence of surface Ia and intracellular Ig. The typical plasma cells in the marrow preparations with plentiful cytoplasm staining brightly for Ig are uniformly negative for Ia (Schlossman *et al.*, 1976; Halper *et al.*, 1978).

Plasmacytoid lymphocytes representing an earlier stage in the B-cell series have been studied in the blood spleen and bone marrow of patients with Waldenström's macroglobulinemia. Here the percent-

age of plasmacytoid lymphocytes containing intracellular Ig along with surface Ia varied between 7 and 32%. In normal tonsils the small percentage of plasma cells resembles the plasmacytoid lymphocytes in terms of a variable pattern of Ia expression (Halper *et al.*, 1978).

The conclusion from these findings is that the Ia antigens represent differentiation antigens that are lost as the lymphocytes enter their terminal plasma cell phase.

D. Mitogen Stimulation of Normal B Lymphocytes

In contrast to plasma cells that result from endogenous differentiation either in normal or malignant sequences, the newly differentiated plasma cells induced by stimulation with pokeweed mitogen have surface membranes with abundant Ia. This is true whether the cells originate from tonsil or from peripheral blood (Halper *et al.*, 1978). Such stimulation is the basis for one type of assay for Ia antigens in which peripheral blood lymphocytes are cultured with pokeweed mitogen for 4–5 days. At this point in culture, the B cells have lost the major proportion of Ig and Fc receptors, while the amount of Ia antigen is increased. This results in a low background when indirect immunofluorescence is used as the assay (Winchester *et al.*, 1975b). In these stimulated plasma cells, in contrast to those mentioned above, the membrane reflected by Ia expression appears to be induced into a more primitive state than that usually found. The longer times of stimulation result in the appearance of Ia on T cells, as is discussed in Section VI.

IV. Chemistry of the Human Ia Antigens

Considerable progress has been made in the isolation of the human Ia antigens, and much of this work preceded that in the mouse (Springer *et al.*, 1976; Cresswell and Geier, 1975). They were first noted as by-products of the isolation of the classical HLA antigens. Two components were noted in papain-treated supernatants from B cell lymphoid lines of 23,000 and 30,000 daltons that reacted with specific alloantisera. In later work similar but larger components were noted for detergent extracts of B cell lymphoid-line cell membranes (Springer *et al.*, 1976; Snary *et al.*, 1976; Klareskog *et al.*, 1977a), and the original protein of 65,000 daltons and its component chains were isolated. The latter were used to produce specific heteroantisera as described above. The two chains were found to be tightly linked by noncovalent bands, and a boiling procedure was usually employed to separate them. Figure 1 illustrates the bands of 28,000 and 37,000

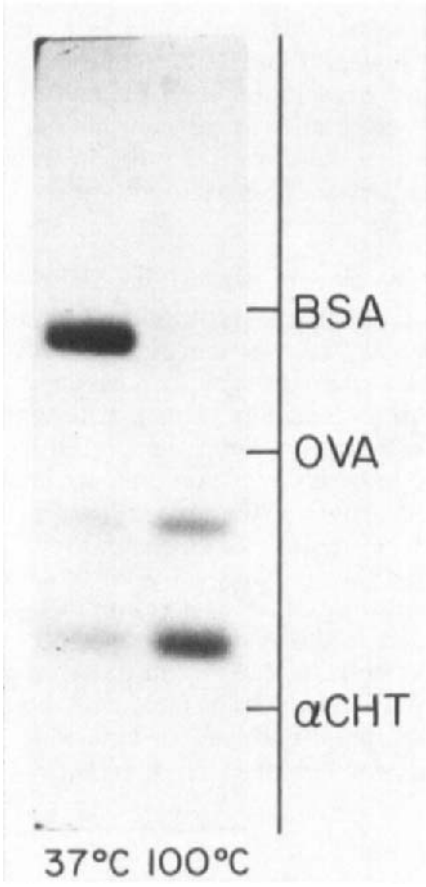

FIG. 1. Sodium dodecyl sulfate gel electrophoresis of a 65,000 dalton glycoprotein containing the Ia alloantigenic activity. At 100°C, treatment without reducing agents results in dissociation into 37,000 and 28,000 dalton chains (Wang, 1979). BSA, bovine serum albumin; OVA, ovalbumin; αCHT, α-chymotrypsine.

daltons obtained from the parent molecule by this procedure from experiments carried out in our laboratory (Wang, 1979; Winchester *et al.*, 1976).

In certain early studies preliminary evidence was obtained for the localization of the alloantigen variability in the human on the heavy chain (Barnstable *et al.*, 1976). This work was done with somatic cell hybrids and indicated that the light chain was not coded for by a locus in the HLA complex. In addition, Klareskog *et al.* (1978) found extensive structural variation in the heavy, but not the light, chain. More recently mobility differences have been observed in the light chains

from different DR alloantigens while the heavy chains have remained constant (Cook *et al.*, 1979; Kaufman *et al.*, 1979). This suggested primary variability in the light chains. This goes along with recent evidence in the mouse of considerable variability of similar light chains by peptide analysis (Cook *et al.*, 1979; McMillan *et al.*, 1979). However, this question in the mouse remains unsettled because some variation has been observed in the heavy chains (Cook *et al.*, 1979). In addition, suggestive evidence has been obtained that the heavy chain is encoded by the *E/C* region and the light chain by the *A* region of the Ia system (McMillan *et al.*, 1979). Preliminary results with peptide maps of the human heavy and light chains also have given evidence for the light chain as the primary variable constituent of the Ia molecule (Silver *et al.*, 1979).

Recently two-dimensional maps of the mouse Ia molecules brought down by specific antisera have given considerable further information regarding the molecular species determined by the different genetic loci of the Ia system (Jones *et al.*, 1979). Three polypeptide chains are observed by this technique, and one polypeptide is invariant in all haplotypes studied. The relationship between these peptides and the two described by others remains to be determined. The invariant peptide is not observed in surface labeling, but only in internal labeling experiments. The initial results with the same system in the human show a remarkable similarity in the band distribution to that observed in the murine system (Fig. 2) (Charron and McDevitt, 1979). The major polymorphism is in the region under the invarient component "I" and is indicated by an arrow. This is the region where muring $I\text{-}A_\beta$ gene products are found.

Certain evidence (Silver *et al.*, 1979) has suggested that the human DR system most closely resembles the E/C system of the mouse. Studies from our laboratory with the new Ia antisera that appear to recognize antigens controlled by loci other than that involved in the DR system indicate that a similar bimolecular complex giving chains of 28,000 and 37,000 daltons is involved, but the exact characteristics relative to the different murine systems have not been defined.

V. Cross-Reactions between Murine and Human Ia Antigens

Cross-reactions between H-2 and HLA-linked antigens have been observed by a number of observers (Colombani *et al.*, 1977b). These reactions were initially found in cytotoxicity experiments utilizing either mouse lymphocytes with human anti-HLA antisera or human lymphocytes with mouse anti-H-2 antisera. Some of these reactions

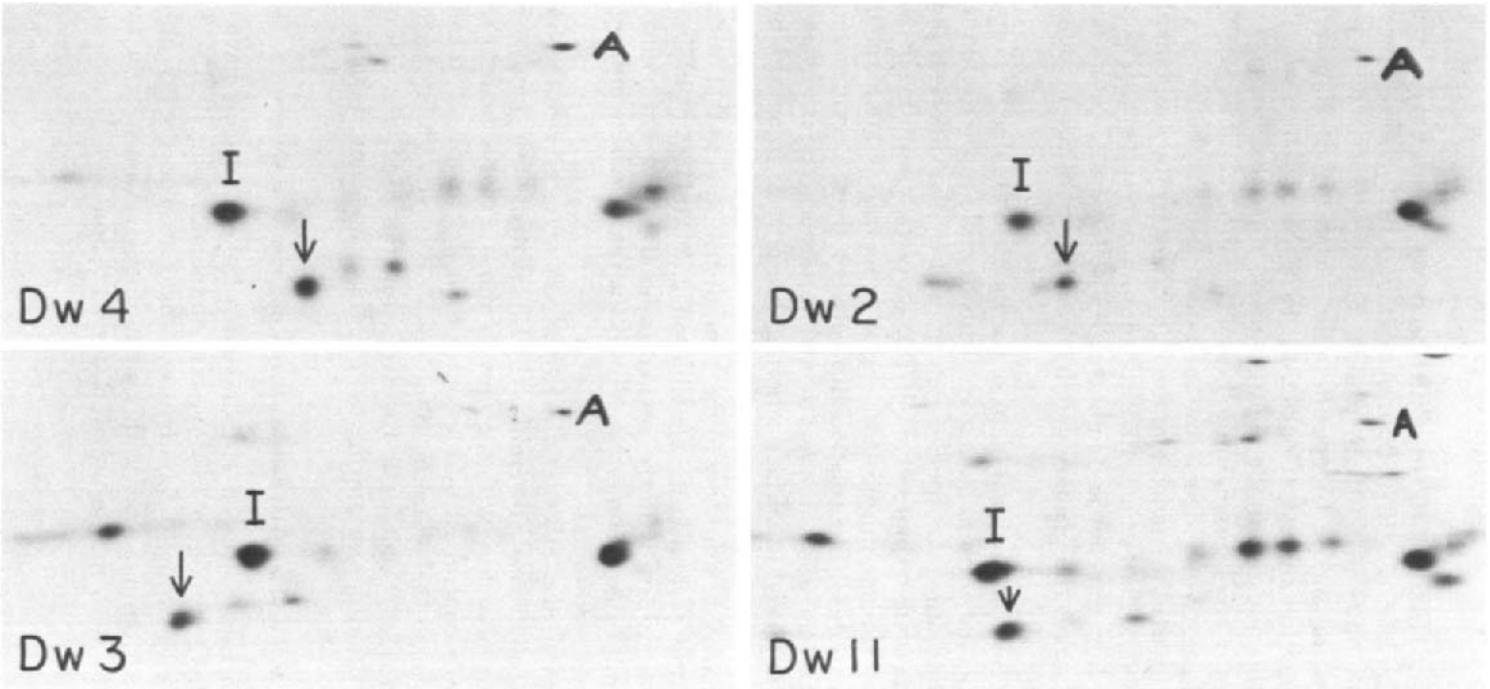

FIG. 2. Two-dimensional polyacrylamide gel. Different patterns given by Ia antigens precipitated from 4 B-cell lymphoblastoid lines derived from individuals who are homozygous for HLA-Dw4, 3, 2, or 11. The major area of polymorphism associated with each of the Ia alloantigen specificities is beneath the invariant spot (I), MW 31,000. Actin (A) is a reference component, MW 44,000. The portion of the gel autoradiograph above Actin has not been reproduced. The lymphoblastoid lines were internally labeled with [^{35}S]methionine and a monoclonal anti-human Ia reagent used for solid phase immunoprecipitation. The pH gradient electrophoresis was run at nonequilibrium conditions. From Charron McDevitt (1979).

were later found to be due at least in part to contamination with components of the Ia system, and mouse anti-Ia sera showed strong reactivity with human lymphocytes (Ivanyi *et al.*, 1977). Immunoprecipitation experiments also showed this cross-reactivity very clearly; a heteroantiserum to human Ia precipitated the two-chain components of the mouse system, and mouse alloantisera precipitated similar human Ia antigens (Kvist *et al.*, 1978).

Recently these studies have been extended considerably, and significant information on the human system is becoming available through these mouse Ia relationships (Delovitch and Falk, 1979). Most of this work has involved the use of mouse alloantisera and human B lymphocytes. Alloantisera with anti-I-E/C specificity clearly react more strongly with human lymphocytes than do anti-I-A. However, in the human these cross-reactive antigens ordinarily do not show polymorphism; in one instance some evidence of individual variability has been obtained (Lunney *et al.*, 1979). In all instances the murine antisera precipitate the characteristic 35,000 and 28,000 dalton molecules from the human cells. These studies suggest further that the human antigens relate closely to the mouse E/C group. This cross-reactivity approach should aid considerably in resolving the important problem in the human of the different Ia genetic loci.

VI. Ia Antigens on T Cells

A. Normal Occurrence and Increase on Stimulated T Cells

Recently a large accumulation of evidence has been obtained for the production of membrane Ia antigens by human T cells. Resting T cells show low staining with fluorescent anti-Ia reagents. However, a small but definite number of cells, ranging between 1 and 4%, can be identified in normal peripheral blood; these cells are Ia^+ and form E rosettes with sheep red cells (Fu *et al.*, 1978). However, after stimulation with mitogens, up to 75% of the T cells show this property (Ko *et al.*, 1979a,b). Antigen stimulation (Ko *et al.*, 1979b) and stimulation in mixed lymphocyte culture (MLC) reactions (Evans *et al.*, 1978; Suciu-Foca *et al.*, 1978a; Ko *et al.*, 1979b) also produce blast cells, a high percentage of which are Ia positive. Figure 3 illustrates the effect of pokeweed mitogen stimulation of highly purified T cells; Ia positive cells in large numbers appear at approximately day 7. In contrast, labeled thymidine uptake occurs considerably sooner. This delayed effect is observed with all mitogens studied including

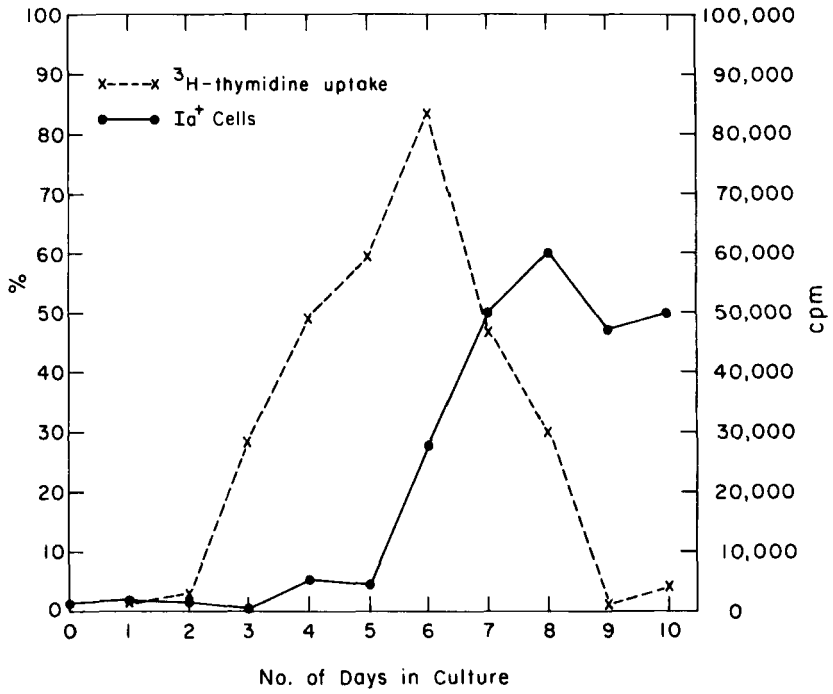

FIG. 3. Divergence in time between the appearance of blast transformation, determined by ^{3}H incorporation, and Ia antigen expression, determined by immunofluorescence, on highly purified T cells that have been stimulated with pokeweed mitogen (Ko *et al.*, 1979b).

phytohemagglutinin (PHA) and concanavalin A (Con A). Determination of blastoid cells indicates the same delayed appearance of the Ia determinants on the blastoid cells. However, not all such cells become positive.

Figure 4 shows the curve for the development of Ia positivity following primary and secondary MLC reactions. The rapid increase in Ia-positive cells in the secondary response is evident. In this experiment the secondary response is indicated by Ia determination with heteroantiserum as well as alloantiserum specific for the responder allotype. This experiment as well as other similar work (Ko *et al.*, 1979b; DeWolf *et al.*, 1979) both for lectin stimulation and MLC reactions indicates that the Ia on the responder cell is of the responder allotype. Recent experiments in our laboratory indicate that in the MLC reaction the stimulator allotype can be found on the responder cells for a very transitory period only. Studies with internal labeling of the Ia antigens have indicated that the blast cells of the MLC reaction

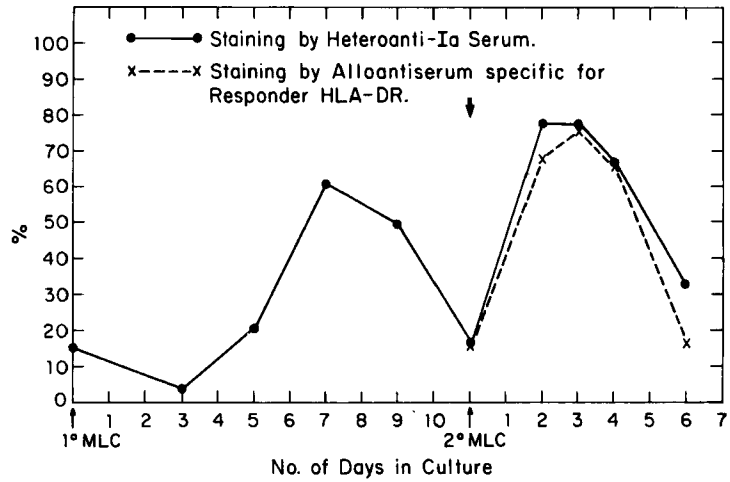

FIG. 4. Development of Ia antigens during primary and secondary MLC reactions determined by immunofluorescence. The rate of increase in the secondary reaction is greater than in the primary. Staining of the secondary MLC by an appropriate responder DR alloantiserum in indirect fluorescence (×---×) gave results parallel to those of the heteroanti-Ia reagent (●——●) (Ko *et al.*, 1979b).

synthesized the Ia antigens that were observed by fluorescence (Evans *et al.*, 1978; Wang, 1979), again indicating that the dominant proportion of these antigens are derived from the responding cells. It has been possible to type individuals for their DRw antigens by typing their stimulated T cells as well as their B cells (Suciu-Foca *et al.*, 1978a; Ko *et al.*, 1979b; DeWolf *et al.*, 1979). Evidence for new antigens on the T cells has been reported in one study (DeWolf *et al.*, 1979); however, this was rarely observed in our studies, where only occasional antisera showed small numbers of cells that appeared spuriously positive (Ko *et al.*, 1979b).

B. Detection of Stimulatory Cell Alloantigens on MLC Responding Cells

Studies in the mouse have demonstrated uptake of H2 and Ia antigens from the stimulating cell on the membrane of responder cells in MLC systems (Nagy *et al.*, 1976a,b; Elliot *et al.*, 1977; Geib and Klein, 1979). Similar experiments in the human system have been carried out in our laboratory (Yu *et al.*, 1979c). T lymphocytes from subjects of known Ia phenotypes were cultured with irradiated non T cells from subjects of different phenotypes. With the use of specific alloantisera, Ia antigens from the stimulatory cells could be detected on the

responder cells 3–4 days after initiation of the culture. The stimulatory antigens became much diminished 2–3 days later, at the height of blastogenesis and had disappeared by the time of maximum appearance of responder cell Ia antigens. Hence, after the antigens have been transferred to the responder cells, additional events followed, such as endocytosis or detachment, to account for these observations. The transfer of the Ia antigens from one cell to another was also found between T lymphocytes (Yu *et al.*, 1979c). This was demonstrated in experiments similar to those described above, with the exception that the cells that were mixed together were samples of mitogen-stimulated T lymphocytes which expressed different Ia phenotypes.

C. Occurrence in Disease

Leukemic T cells that are Ia positive have been observed in a number of different leukemias (Fu *et al.*, 1978). In addition, high levels of Ia positive nonmalignant T cells have been observed in a number of malignancies (Fu *et al.*, 1978). Also in a number of other conditions, especially infectious mononucleosis (Johnsen *et al.*, 1978) and during human transplantation rejection reactions (Reinherz *et al.*, 1979), high levels of Ia-positive circulating T cells have been observed. Recently it has become apparent that elevated levels of Ia^+ T cells may be found in a wide variety of conditions (Yu *et al.*, 1979b). An increase of such cells to as high as 30% of the T cells has been observed in certain patients with infectious diseases as well as rheumatoid arthritis. Simple booster shots of tetanus toxoid have been found to produce T cells that are Ia positive. It might be supposed that the Ia positivity represents a reflection of the blastogenesis that is known to occur in these situations (Yu *et al.*, 1979b). However, the number of blast cells observed under these conditions is much lower than the Ia positive cells, and many of the positive cells appear to represent small lymphocytes. These cells closely resemble the small number of Ia^+ T cells in the normal individual and the fluorescent staining is less intense than that for T blasts and B cells. The determination of Ia-positive T cells in disease may well prove to be a valuable procedure for evaluating the degree of T cell activation that is occurring *in vivo*.

D. Functional Effects

The small percentage of normal T cells that express the Ia antigens (1–4%) appears to represent small lymphocytes and not a few blast cells in normal peripheral blood. An occasional normal has been found to have as high a level as 10% positivity among the T cells. This population also shows weaker Ia staining by fluorescent reagents than B cells

and stimulated T cells. However, elimination of the Ia-positive T cells with anti-Ia antisera and complement has a number of marked effects. The residual population of T cells after such treatment shows a considerably lower number of Ia-positive cells that develop after pokeweed mitogen stimulation (Ko *et al.*, 1979b). In addition, such treatment abolishes the ability of these T cells to generate allogeneic helper activity (Chiorazzi *et al.*, 1979). Thus, it appears that this small subpopulation of T cells, or an additional population with Ia on its surface but undetectable by fluorescence, holds an important functional role.

VII. Ia Markers during Non-Lymphoid Cell Differentiation

A. Bone Marrow-Derived Cells

1. Introduction

Hematopoiesis is conventionally divided into three principal phases according to the biologic role of the constituent cells (see Lajtha, 1970; Price and McCullough, 1978). Various *in vitro* assays have provided insight into the characterization of these phases and factors involved in their control. Compared to the large number of cells in bone marrow, the number of precursor cells is extremely small, and these assays rely on the property of clonal expansion for the recognition and quantitation of these cells. The first phase consists of stem cells that are self-renewing and pluripotent in the sense that they are capable of giving rise to multiple lineages through selective induction. It has recently been possible to develop a continuous marrow culture assay for these pluripotent (CMC) cells (Moore *et al.*, 1979).

The second phase consists of progenitor cells committed to particular lineages. The progenitor cells have extensive but not unlimited proliferative potential and are selectively responsive to the various hematopoietic factors. The major series that give rise to megakaryocytes, erythrocytes, and granulocytes/monocytes are represented by separate progenitor cells that derive from the stem cell. It is in this developmental compartment that the principal regulation of the hematopoietic system occurs.

Within the progenitor cells committed to myeloid differentiation (CFU-C), there is considerable heterogeneity. The CFU-C that give rise to neutrophil granulocyte/macrophage colonies are distinct from eosinophil CFU-C (G. R. Johnson *et al.*, 1977). Furthermore, within

the neutrophil/macrophage CFU-C, three levels of maturity are recognized: the least mature pre-CFU-C, the progeny of which are the CFU-C determined by 14-day cultures, and in turn give rise to the more differentiated CFU-C determined by 7-day culture assays. The pre-CFU-C are distinguished by extremely low DNA synthetic rate indicating that they are in a noncycling state (Jacobsen *et al.*, 1979; Moore *et al.*, 1979). Early and late forms of the erythroid progenitor cells are also distinguishable (BFU-e, and CFU-e) (Iscove *et al.*, 1974; Iscove and Sieber, 1975).

The third phase of hematopoiesis comprises over 99% of the marrow elements and includes immature and fully functional members of the erythroid and myeloid series. Very little effective control over the ultimate number and composition of cells is possible at this level, because proliferation is limited to at most a few cell divisions (Price and McCullough, 1978).

2. *Expression of Ia Antigens on Stem and Progenitor Cells*

Ia antigens are demonstrable on the hematopoietic stem and progenitor cells in a pattern that indicates their appearance on cells derived from precursors that lack demonstrable Ia. The pluripotent stem cell in continuous marrow culture (CMC) lacks Ia antigens as illustrated by its resistance to elimination by anti-Ia heteroserum and complement (Moore *et al.*, 1979). In contrast, the earliest progenitor cell committed to myeloid differentiation is characterized by an extreme sensitivity to anti-Ia antibodies detectable at dilutions of 1:80,000 (Moore *et al.*, 1979). The subsequent stages of neutrophil/macrophage progenitor cell differentiation, the 14- and 7-day CFU-C, express Ia antigens (Cline and Billing, 1977; Winchester *et al.*, 1977; Janossy *et al.*, 1978). There is evidence of heterogeneity in the amount of Ia expressed on the CFU-C as is inferred from the gradual slope of the curve depicting elimination of CFU-C as a function of anti-Ia serum concentration, as well as in reciprocal experiments in which cell sorting on the basis of Ia expression was performed prior to the CFU-C assay (Janossy *et al.*, 1978). Further evidence for heterogeneity is provided by a decrease in susceptibility to lysis by comparable titers of anti-Ia serum in the day 7 CFU-C compared to the day 14 CFU-C (Moore *et al.*, 1979). Ia antigens are similarly demonstrable on erythropoietic colonies, with the more immature BFU-e exhibiting greater susceptibility to elimination (Winchester *et al.*, 1978a).

The results obtained with Ia heteroantisera in the progenitor cell assays have been confirmed by monoclonal and alloanti-Ia reagents (Winchester and Broxmeyer, 1979).

As will be discussed subsequently, the expression of Ia antigens in the third phase of hematopoiesis is limited to only the earliest immature cells. This, coupled with the absence of detectable Ia antigens on stem cells, provides a pattern of circumscribed Ia antigen expression that reaches a maximum in the progenitor cell and could explain the variation in quantity of Ia antigens on the progenitor population. This pattern of appearance and subsequent disappearance of a major cell surface glycoprotein in the course of a limited number of cell divisions is repeated for Ia antigens in other circumstances, for example, stimulated T cells (see Section VII). Here, the maximal representation of Ia occurs at the stage of hematopoiesis where maximal control over differentiative and proliferative events is exercised. This suggests that a particular cell/cell interaction mediated through the Ia molecules on the progenitor cell membrane occurs at this time, possibly related to the control of proliferation (Winchester *et al.*, 1977; Janossy *et al.*, 1977).

3. *Normal Bone Marrow Morphology Identification of Ia-Bearing Cells*

Unseparated bone marrow contains an average of 3% of cells that react with hetero- or appropriate alloanti-Ia reagents. When lymphocytes and monocytes are excluded, as, for example, by a double-marker format using the simultaneous determination of complement receptors, the percentage of residual cells bearing Ia falls below 1% (Ross *et al.*, 1978a), or below 3% if surface Ig is used as the second label (Janossy *et al.*, 1977).

The characteristics of the cells bearing Ia antigens can be studied by using low-density fractions isolated from the marrow (Winchester *et al.*, 1977; Ross *et al.*, 1978a). At 1.05 gm/ml the Ia-bearing cells are enriched over 20-fold and comprise an average of 44% of the myeloid cells (Table III) in fractions containing an appreciable enrichment of myeloblasts and promyelocytes. Phase microscopy in parallel with Wright–Giemsa staining permitted identification of the cells in the low-density fractions with Ia antigens as primarily myeloblasts with occasional promyelocytes. Here also, monocytes were excluded. Occasional cells identified as pronormoblasts were also found to express Ia antigens.

In higher-density fractions the number of Ia antigen-bearing cells rapidly declined. The preponderance of positive cells in all fractions were myeloblasts. Approximately one-third of the promyelocytes bore Ia antigens, and characteristically this staining was considerably weaker than that of the Ia antigens on the myeloblasts.

TABLE III

RELATIONSHIP OF Ia EXPRESSION AND GRANULOCYTE MATURATION IN NORMAL BONE MARROW SEPARATED BY DENSITY GRADIENT CENTRIFUGATION[a]

Density layer	Proportion of total cells	Percent of cells in each fraction with myeloid morphology positive for Ia antigen	Cells with myeloid morphology in fractions (Wright–Giemsa)[b]				
			Blast.	Pro.	Myel.-mta.	Band	PMN
1.05*	4	44	43	26	30	0	0
1.06*	17	31	44	33	21	2	0
1.07*	10	5	1	13	69	10	7
1.08	15	3	0	1	47	39	13
1.09	19	1	0	0	28	63	32
1.105	26	0	0	0	9	38	53
1.12	5	0	2	0	3	17	77
1.12*	4	1	6	0	10	16	68
Unseparated*	100	2	2	2	30	35	35

[a] After Ross *et al.* (1978a).

[b] Blast., myeloblast; Pro., promyelocyte; Myel.-mta., myelocyte or metamyelocyte; Band, band form; PMN, polymorphonuclear neutrophil.

This density separation method also permitted characterization of the other surface receptors of the differentiating granulocytes. Strong IgG Fc receptors appeared at the level of the promyelocyte, and the number of positive cells increased through the myelocyte stage. The CR_2 receptor appeared at the level of the myelocyte. Therefore, in contrast to the B lymphocyte and monocyte, where Ia antigens are present on functional cells, in the granulocyte series, the various receptors appear after Ia antigens are no longer detectable.

4. Megakaryocytes

With refinements in methods for the isolation of megakaryocytes from human bone marrow, the presence of Ia antigens on from 7 to 23% of cells was demonstrated using heteroanti-Ia reagents (Rabellino *et al.*, 1979). Nearly all megakaryocytes had Fc receptors, but complement receptors were not detectable. Since the absence of Ia antigens on normal platelets is well documented, the sequence of megakaryocyte differentiation represents another example of the loss of Ia with progression through differentiation. Presumably megakaryocytes that lack Ia are at a more advanced state of differentiation, however, the alternative explanation of two pathways cannot be excluded.

5. Monocytes and Related Cells

Monocytes were recognized to bear Ia determinants in the initial studies of peripheral blood (Jones *et al.*, 1975; Winchester *et al.*, 1975a). The average intensity of monocyte staining by heteroanti-Ia reagents exceeds that of B lymphocytes, although there is considerable variation among some monocytes with occasional cells appearing to lack detectable Ia (Winchester *et al.*, 1976). The explanation for this variation is not known, and the possibilities have been considered that it represents either distinct subsets or different stages in maturation.

The Langerhans cell, a dendritic-like cell present just above the basal layer in the epidermis, is characterized by the presence of IgG Fc and complement receptors (Stingl *et al.*, 1977), and of Ia (Rowden *et al.*, 1977; Klareskog *et al.*, 1977a; Stingl *et al.*, 1978). Available evidence places this cell within the monocyte-macrophage series. In guinea pigs the Langerhans cell can replace the immunologic functions of peritoneal macrophage cells (Stingl *et al.*, 1978). Recently the dendritic cells isolated from mouse spleens have been shown to be Ia^+ (Steinman *et al.*, 1979).

Evidence for a special relationship between Ia and the monocytoid cells producing granulocyte colony-stimulating factor has been pre-

TABLE IV

REPRESENTATIVE SEPARATION OF CML PERIPHERAL BLOOD LEUKOCYTES INTO FRACTIONS OF DIFFERING DENSITY: PARALLEL ENRICHMENT OF EARLY FORMS AND CELLS EXPRESSING Ia ANTIGENS[a]

Density (gm/ml)	Percent of total cells	Wright's stain cell morphology (%)						Percent of cells in each fraction with Ia antigen
		Myelo-blast	Promy-elocyte	Myelo-cyte	Meta	Band	PMN[b]	
1.06	0.26	15	32	24	11	5	0	15.1
1.07	25.0	0	2	30	59	8	1	0.2
1.08	10.8	0	0	15	61	19	5	0.16
1.09	16.6	0	0	3	10	23	64	0
1.105	45.5	0	0	0	7	14	79	0
Unseparated	100	0	0	3	15	13	70	0.18

[a] From Winchester *et al.* (1977).

[b] PMN, polymorphonuclear neutrophil.

sented (Broxmeyer, 1979). The subpopulation of these cells that respond to the addition of lactoferrin by cessation of colony-stimulating factor production are eliminated by anti-Ia and complement, leaving a residual non-Ia population that lacks the property of the lactoferrin-induced "off" response. Furthermore, the inhibitory effect of lactoferrin can be blocked by pretreatment of the monocyte preparations with high dilutions of anti-Ia sera, suggesting that the lactoferrin receptor could be in proximity to the Ia molecules (Broxmeyer, 1979).

6. *Leukemic Granulocytes*

The observation that Ia antigens are found on granulocyte progenitors in the blood of patients with acute myelogenous leukemia was the first evidence relating the Ia antigens to the granulocyte series (Billing *et al.*, 1976; Schlossman *et al.*, 1976). The Ia molecules were demonstrated by rabbit antisera and by human allosera with the appropriate specificities (Billing *et al.*, 1976; Schlossman *et al.*, 1976; Winchester *et al.*, 1976; Janossy *et al.*, 1977). Since polymorphonuclear leukocytes were known to lack Ia, one possibility that was considered was that the expression of Ia was an event related to leukemic transformation (Schlossman *et al.*, 1976).

It soon became apparent that the analogous stages of normal granulocytopoiesis had a similar pattern of Ia expression, and what appeared as a candidate for a tumor-associated antigen became a differentiation determinant (Winchester *et al.*, 1977; Janossy *et al.*, 1977). This finding has prompted a critical reexamination of previous work with heteroantisera raised against various human leukemias. It appears that many sera with presumed specificity for leukemic cells contain antibodies to Ia (Mohanakumar and Raney, 1978). However, irrespective of the status of the Ia determinants, their occurrence on certain leukemic cells could have consequences for the direction that an immune response to the malignancy takes.

The isolation of relatively pure preparations of the Ia-bearing leukemic myeloid cells from the blood of patients with either acute or chronic myelogenous leukemia led to the recognition that these cells were chiefly myeloblasts with small numbers of promyelocytes. This was accomplished by multistep density gradients (Winchester *et al.*, 1977) or fluorescence-activated cell sorting (Janossy *et al.*, 1977). In the case of the blood from patients with chronic myelogenous leukemia, the small number of circulating myeloblasts and promyelocytes are readily identified after enrichment in low-density fractions (Table IV and Fig. 5).

The use of leukemic cells also made possible the isolation of large

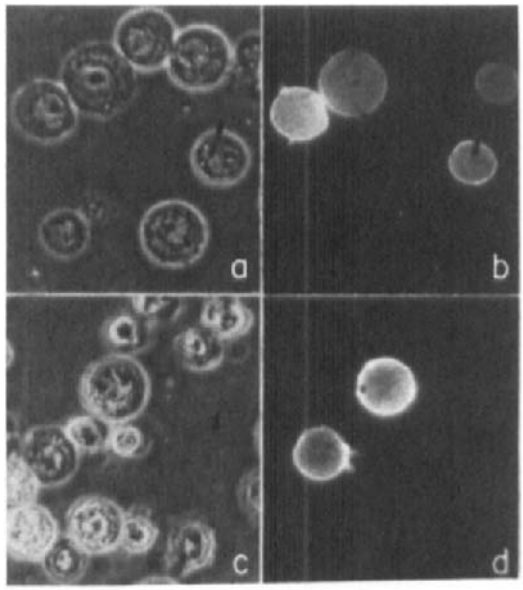

FIG. 5. Leukemic cells isolated in low-density fractions from a patient with chronic myelogenous leukemia. The unseparated cells contained 0.5% myeloblasts and 0.4% cells with Ia antigens. (a) Phase micrograph of < 1.06 gm/ml density fraction illustrating the enrichment of myeloblasts. (b) Fluorescence micrograph of the preceding field with positive staining by a fluorescent Ia antiserum on some cells. (c) Phase micrograph of 1.07 gm/ml density fraction illustrating a larger proportion of cells with cytoplasmic granules than was found in the lower density fraction. (d) Fluorescent micrograph of the preceding field illustrating only one cell with the appearance of a promyelocyte that bears Ia antigens. A myeloblast is also stained whereas several more mature cells are negative. [From (Winchester *et al.* (1977).]

quantities of myeloblasts in 1.06 gm/ml fractions. Upon the radiolabeling of the cells and subsequent immunoprecipitation with anti-Ia sera, the molecular profile characteristic of Ia molecules on B cells was obtained (Fig. 6). The yield of radiolabeled Ia antigens per myeloblast was about one-fourth the amount recoverable from a B cell lymphoblastoid line, a value compatible with the relatively weaker staining of myeloblasts. Absorption experiments performed using preparations containing over 99% myeloblasts demonstrated that all hetero-Ia determinants were fully shared by myeloblasts and B cells.

B. NONHEMATOPOIETIC CELLS

The study of various cultured tumor lines affords a relatively simple method of examining a variety of proliferating cell types at different

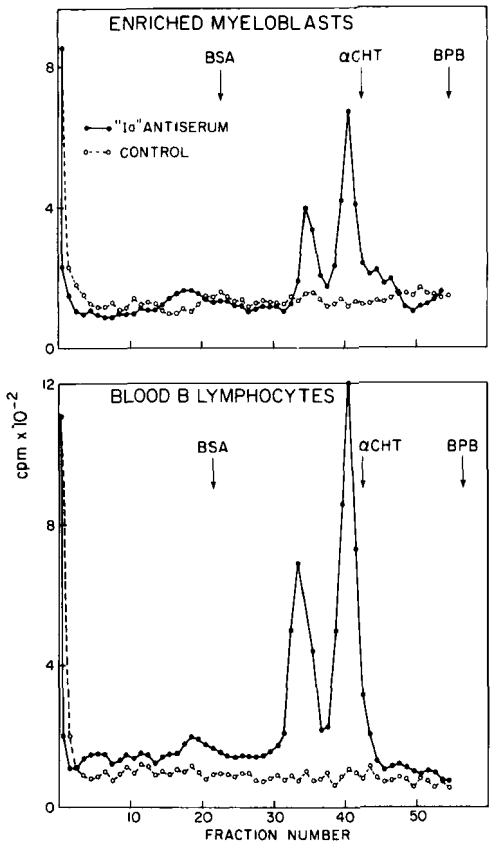

FIG. 6. Similar immunoprecipitation profiles of radiolabeled myeloblasts and B lymphocytes upon sodium dodecyl sulfate polyacrylamide gel electrophoresis with an Ia antiserum. *Upper:* A myeloblast preparation from a patient with acute myelogenous leukemia. *Lower:* Peripheral blood B lymphocytes depleted of adherent cells. 28,000 and 37,000 dalton molecules are detected in both samples. ●, Ia antiserum; O, control preimmune serum; BSA, bovine serum albumin; αCHT, α-chymotrypsinogen; BPB, bromophenol blue dye front. [From (Winchester *et al.* (1977).]

stages of differentiation for the expression of Ia antigens. A series of representative tumors shown in Table V emphasizes that Ia antigens are only infrequently found on most malignant cell lines. Both heteroantisera and monoclonal reagents demonstrated Ia antigens on Hep 2, a line derived from a laryngeal epidermoid carcinoma, one of two neuroblastoma lines, and one of two glioma lines (Winchester *et al.*, 1978). In addition, an epithelial cell line derived from an endometrial carcinoma is Ia^+ (Carrell *et al.*, 1979).

TABLE V
CONTRASTING EXPRESSION OF β_2-MICROGLOBULIN AND Ia ON DIFFERENT CULTURED CELL LINES[a]

Culture designation	Tissue type	Intensity and % of cells expression	
		$\beta_2\mu$	Ia
Hep 2	Epidermoid carcinoma	(2–4$^+$) 100%	(1–2+) 100%
U 20S	Sarcoma	(tr–1$^+$) 100%	0
Nemeth	Renal carcinoma	(1$^+$) 100%	0
J82	Astrocytoma	(1–2$^+$) 100%	0
Viola	Renal carcinoma	(4$^+$) 100%	0
T24	Bladder carcinoma	(3$^+$) 100%	0
Lawson	Lung carcinoma	(2$^+$) 100%	0
ME 180	Cervical carcinoma	(3$^+$) 100%	0
WI 38	Fetal lung	(2$^+$) 100%	0
Gm 43	Normal fibroblast	(3$^+$) 100%	0
ALAB	Breast cancer	± tr 100%	0
SK-Lu-1	Undifferentiated	1± (100)	0
SK-Ov-3	Ovarian cancer	2± (100)	0

[a] From Winchester *et al.* (1978b).

In contrast to the limited expression of Ia on the above tumor lines, lines derived from most patients with malignant melanoma have Ia detectable on the cell surface (Winchester *et al.*, 1978b; Wilson *et al.*, 1979). Of ten malignant melanoma lines (Table VI), eight had amounts of Ia varying from intermediate to the high levels evident in Fig. 7. Two lines had very low numbers of positive cells. Direct chemical quantitation using boro[^{3}H]hydride labeling demonstrated that up to 16.7% of the membrane glycoproteins were composed of Ia molecules in line SK-Mel-13 containing strongly staining cells. ^{125}I-surface radiolabeling and immunoprecipitation confirmed the presence of the antigens on the 37,000 and 28,000 dalton biomolecular complex characteristic of Ia.

The unanticipated intense expression of the Ia molecules on a lineage derived from the neural crest, embryologically quite distinct from the lymphohematopoietic system, raises again the question of the role of Ia molecules in a context unrealted to the immune system. Since freshly obtained melanoma tumors also bear Ia, their expression is not solely a result of culture conditions. By analogy with acute myelogenous leukemia, it is probable that this tumor-associated antigen reflects the surface phenotype of a stage of differentiation of the normal melanocyte, but the possibility cannot be excluded that the Ia

TABLE VI
VARIABLE EXPRESSION OF Ia DETERMINANTS ON MELANOMA CELL LINES, LACK OF RELATION TO β_2-MICROGLOBULIN[a]

Cell line designation	β_2-Microglobulin direct immunofluorescence		Ia direct immunofluorescence		Ia chemical isolation
	Relative intensity	Positive cells (%)	Relative intensity	Positive cells (%)	Percent of total glycoprotein
SK-Mel-13	2–4	100	3–4	100	7.8
SK-Mel-37	3–4	100	3–4	100	16.7
SK-Mel-42	tr–1	100	2–4	100	3.7
SK-Mel-29	2–4	100	tr–3	100	<2
SK-Mel-33	1–3	100	tr–3	100	<1
SK-Mel-41	tr–1	100	tr–3	100	N.D.
SK-Mel-28	2–4	100	tr–3	35	<1
MeWO	2–3	100	tr–1	24	<2
SK-Mel-19	2–4	100	tr–1	3	<2
SK-Mel-27	1–2	100	tr–1	2	<1

[a] From Winchester *et al.* (1978b).

antigens result from alterations in the genome that are a consequence of malignancy. The variation in quantity of Ia from tumor to tumor could represent tumors arising in melanocytes at different stages of maturation. The Ia antigens might, therefore, serve as markers of a more or less primitive state of the tumor and be associated with differences in clinical behavior and therapeutic response. The presence of Ia molecules on a tumor cell surface could also have significant implications for the state of antitumor immunity that is evoked, because of the possibility that Ia molecules could enhance the immune response through their capacity to induce lymphocyte proliferation. These and other speculations emphasize the potential for new directions in studies of the melanomas that result from the identification of their expression of Ia antigens.

VIII. Delineation of Ia Alloantigen Specificities

Present developments continue to reflect the unifying perception of early 1975 that allosera with the property of inhibiting the MLC define an alloantigen system selectively expressed on the minor population of B lymphocytes, and that these lymphocytes when expanded as B cell lines or in chronic lymphatic leukemia retain the same alloantigens

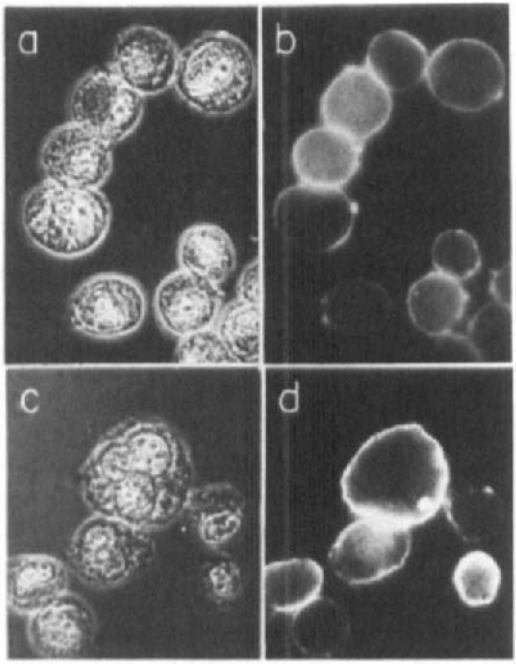

FIG. 7. Presence of Ia antigens on the surface of melanoma cell lines as demonstrated by heteroanti-Ia sera in direct immunofluorescence tests. (a and c) Phase micrographs of SK-Mel-13 and SK-Mel-37 cells, respectively. (b and d) Identical field under illumination for fluorescence microscopy. [From (Winchester *et al.* (1978b).]

(Jones *et al.*, 1975; Mann *et al.*, 1975a; van Rood *et al.*, 1975; Walford *et al.*, 1975; Wernet *et al.*, 1975; Winchester *et al.*, 1975a,b). The principal themes of the intervening investigations have centered on (*a*) the relationship of the Ia alloantigen specificities to determinants recognized in the MLC and (*b*) the utility of B lymphoid lines as reference standards and reagents in delineating these specificities.

A. METHODOLOGY

The technical problems inherent in typing an individual positive or negative for a B cell alloantigen are very considerable and have not yet been fully mastered for routine purposes. A detailed discussion of these problems has been reviewed (Ferrone *et al.*, 1978b). A series of factors relate to the biology of the B lymphocyte: (*a*) B cells comprise less than 15% of peripheral blood lymphocytes; (*b*) the expression of Ia antigens, even when determined by heteroantisera, varies in quantity

from cell to cell; (*c*) the profile of receptors on the surface of the B lymphocyte and its sensitivity to substances in certain rabbit sera interfere with the assay, usually by producing false positive reactions; and (*d*) other cells, particularly monocytes, are sources of major interference.

B cells are isolated either through negative selection, as with depletion of T cells by E rosette formations (Bentwich *et al.*, 1973), or positive selection, such as by physical adherence of B cells on nylon fibers (Lowry *et al.*, 1979; Handwerger and Schwartz, 1974) or using immunologic methods. These include insolubilized immune complexes to bind cells with Fc receptors (Mann *et al.*, 1975a,b), insolubilized Fab'_2 (Johnson *et al.*, 1977), or a rosette procedure using monoclonal anti-Ia antibody (Stocker *et al.*, 1979).

The object of the isolation is in most techniques to perform conventional cytotoxic assays but, because of dilution by unwanted Ia negative cells, some problems remain. In the case of isolation by depletion of T cells, the resulting "B" cell preparation contains approximately 30% of Ia negative lymphocytes because of the presence of the heterogeneous third-cell population (Winchester *et al.*, 1976). Also, in certain disease states, the yield of true B cells is very much lower owing to the presence of myeloid precursors that greatly increase the apparent "third-cell" population. Positive selection also has certain intrinsic limitations in that, for example, in the case of Fc receptor selection, the presence of these receptors on some T and third population cells also diminishes the number of Ia-bearing cells. At present it would appear that selection by anti-Ig reagents offers the most satisfactory approach from a theoretical viewpoint. For each of the methods, operationally satisfactory results can be obtained, but sometimes considerable circumspection is necessary when typing other than normal subjects.

A second approach initially reported by van Rood identifies the B lymphocyte in the freshly isolated mononuclear layer by virtue of the presence of Ig (van Rood *et al.*, 1976). The Ig is capped by an antiimmunoglobulin reagent tagged with fluorescein, and the presence of an Ia alloantigen is recognized in a cytotoxic reaction using ethidium bromide, which fluoresces in the red-orange range. The advantages of this method are that B lymphocytes need not be isolated and that the presence of contaminant antibodies with specificities, such as for HLA-A, B, or C, will be readily recognized through killing of the T cells. However, since Ig is readily capped and the caps are subsequently ejected by the cell, considerable skill is required in achieving the proper conditions for the assay.

A third approach involves short-term cultivation of peripheral blood mononuclear cells with a mitogen, pokeweed (Winchester *et al.*, 1975b) or phytohemagglutinin (Colombani *et al.*, 1977a). The resulting B cells with a variable addition of Ia-bearing T cells are examined by cytotoxicity or immunofluorescence. In the author's laboratory the procedure involves incubation with the alloantiserum, washing, and incubation with a mixture of rhodamine-tagged antihuman IgG and fluorescein-tagged heteroanti-Ia. The coincidence of both fluorochromes indicates a positive reaction. The disadvantage of a culture step makes this method suited for more specialized applications where the inherent precision is required.

The high level of isolated T cells that express Ia after cultivation with pokeweed mitogen (Ko *et al.*, 1979b) suggests that this could be a valuable alternative approach, with obvious applications in instances of B cell deficiency.

Monocyte contamination (which is involved in essentially all procedures, especially when nonnormal subjects are used) presents a major problem because the majority of monocytes have abundant Ia alloantigens (Winchester *et al.*, 1975a, 1976; Jones *et al.*, 1975; Albrechtsen *et al.*, 1977a). However, although the Ia alloantigen specificities parallel those on the B cells, they are frequently not killed under conditions that usually result in satisfactory B cell cytotoxic typing (Cicciarelli *et al.*, 1978; Drew *et al.*, 1978). This variable, cytotoxicity-negative absorption-positive phenomenon has the effect of diminishing the percentage of maximum cells that can be killed in a way that is difficult to control. In addition, the presence on monocytes of differentiation alloantigens unrelated to the MHC (Claas *et al.*, 1979) further emphasizes the need for their identification and exclusion from typing systems.

B. Complexity of Ia Alloantigens

The objectives of studies on the Ia alloantigens fall into four categories: (*a*) to refine the specificity of the reagent alloantisera in order to establish a relation to discrete single gene products; (*b*) to characterize the molecular basis of the discrete alloantigen specificities; (*c*) to define the genetic organization of the structural genes; and (*d*) to relate the structural genes to other genes mapping in the same region. It is becoming evident that the combination of newer serologic, genetic, and chemical studies is enlarging the concept of the human Ia alloantigens into a system at least as complex as that of the murine I region.

TABLE VII[a]
DRw GENE FREQUENCIES IN DIFFERENT POPULATIONS (%) AS DETERMINED IN THE VII INTERNATIONAL WORKSHOP ON HISTOCOMPATIBILITY TESTING

Allele	European Caucasoids	North American Caucasoids	American Blacks	African Blacks	Japanese	American Indians
DRw1	6.2	5.2	7.3	—[b]	4.5	—
DRw2	11.2	13.9	13.8	8.7	16.5	8.4
DRw3	8.9	11.8	12.4	11.7	—	9.1
DRw4	7.8	16.5	7.2	3.5	14.4	21.5
DRw5	15.1	11.9	15.4	7.4	5.4	6.0
DRw6	8.6	11.5	19.1	9.9	6.7	5.9
DRw7	15.6	12.4	12.0	6.6	—	3.7
WIA8	5.6	4.2	7.5	7.2	7.2	12.9
Blank	21.1	12.6	5.3	45.0	45.3	32.5
N	334	273	110	77	164	69

[a] From "Histocompatibility Testing 1977" (1978) (W. F. Bodmer, J. R. Batchelor, J. G. Bodmer, H. Festenstein, and, P. J. Morris, eds.). Munksgaard, Copenhagen.
[b] Alleles indicated—are not present in this population.

1. *Relationship of Ia Specificities to MLC Determinants*

Definition of DR Specificities. As an outgrowth of the earlier use of Ia allosera in MLC inhibition experiments (Ceppellini *et al.*, 1971; van Leeuwen *et al.*, 1973), the first phase of characterizing reagent specificities emphasized those sera that had a clear relationship to the HLA-D determinants (van Rood *et al.*, 1975; Winchester *et al.*, 1975b; Legrand and Dausset, 1975; Terasaki *et al.*, 1975; Walford *et al.*, 1976). The general correlation between the specificities of certain Ia alloantisera and HLA-D determinants was confirmed (Albrechtsen *et al.*, 1977b; Park *et al.*, 1977; van Rood *et al.*, 1977; Walford *et al.*, 1977) and formed one of the central contributions of the VIIth International Workshop. Both cluster analysis (Bodmer *et al.*, 1978b; Bodmer, 1978) and segregation of MLC and Ia specificities on the same haplotype in family studies (Lamm *et al.*, 1978) provided further support. The term "DR" was proposed to designate the relationship of the Ia specificities to the *HLA-D* locus alleles defined by MLC testing.

In the VII Workshop eight DR specificities were delineated. Of these, if any two are excluded from analysis, the frequencies of the remaining six fit well with a segregant series of six alleles at a single locus in Hardy–Weinberg equilibrium (Pickbourne *et al.*, 1978). Gene frequencies of the DR types calculated from these assumptions are shown in Table VII. From the available data it was not considered

necessary to postulate the existence of a second locus. The anomalous specificities that suggested the presence of new specificities appeared to be attributable to multispecific reagents.

The DR types were assigned on the basis of similar reactivities present in multiple typing sera. However, there was variability in the precision of the reagents defining different DR types. For example, in the VII Workshop, the sera defining the specificities DRw7, 3, 2, and 1 were characterized by the uniformity of their reactions as reflected in high intraclass correlation coefficients, whereas the assignment of DRw4, 5, 6, and 8 were based on reactions of typing sera with less closely related specificities (Bodmer *et al.*, 1978b).

The hierarchical relationships among these specificities suggested two principal DR families: one containing DRw2 and the closely associated DRw6 specificity as well as the more distinct DRw1 and DRw3 specificities, and the second containing DR7, the related DRw4 and 5 specificities and DRw8 (Bodmer *et al.*, 1978b, Bodmer, 1978). It was envisioned that additional refinements would become apparent, such as splitting of alleles into subtypes (Richiardi *et al.*, 1978; Ward *et al.*, 1979).

Subsequent developments have moved rapidly and the construct of Ia specificities as being completely attributed to an allelic set of DR specificities is now seen as an oversimplification, but a useful one that gave order and direction to a field in its early phases. From the extensive differences in mobility of the Ia antigens that are revealed in two-dimensional polyacrylamide gels (Fig. 2), it is apparent that these allelic variants have multiple charge differences and differ from one another by a number of amino acids. At this level alone it is probable that numerous epitopes comprise the alloantigen profile of a DR specificity with the implicit likelihood of considerable variability among individuals sharing the same primary specificity. It is possible that the reports of multiple related HLA-Dw specificities such as HLA-Dw7, -Dw11, and -13a being present on a single haplotype in association with DRw7 (Fuller *et al.*, 1978) could be a reflection of this level of variability. Of more fundamental importance is the prevailing direction of investigation that provides evidence for multiple genetic loci responsible for the Ia antigen system as discussed below.

The precise relationship of the determinants responsible for stimulation in the MLC to the DR specificities still remains an open question. The fact that no instance of a recombination between *HLA-D* alleles and the associated DRw specificities was encountered in the VII Workshop emphasized the similarity, if not identity, of the two determinants. However, even in the early studies that related the Ia

specificities to MLC types, it was evident that individuals reacted with Ia alloantisera yet did not have the anticipated HLA-Dw specificity (Winchester *et al.*, 1975b). This divergence is considerably more pronounced in non-Caucasian populations. For example, among the Navajo there is concordance between MLC typing for HLA-Dw7 and the presence of DRw7. However, individuals typed for DRw2, 3, or 4 were not found to have the anticipated HLA-Dw specificity (Troup *et al.*, 1978). Among Japanese and American Blacks the association between HLA-Dw specificities and DRw typing is largely different from that of the Caucasian population (Saito *et al.*, 1977; Sasazuki *et al.*, 1977; Ward *et al.*, 1979). For example, the *HLA-D* homozygous typing cell DHO expresses DRw2 but is a completely distinct MLC specificity from Caucasian HLA-Dw2 tying cells (Reinsmoen *et al.*, 1978; Tsuji *et al.*, 1978). In the reciprocal direction occasional individuals have been identified who inherit an HLA-Dw specificity inappropriate for the DRw specificity present on the same haplotype, such as Dw7 and DRw2 (Suciu-Fuca *et al.*, 1978c). In part the intricacy of these studies relates to the requirements of a typing response in the MLC; however, alternative techniques such as primed lymphocyte testing (PLT) emphasize the separate contributions of D and DR to the PLT specificities. The present findings of this actively investigated area do not favor the unitary view of an identity between Ia specificities and MLC determinants but point to a more complex and still incompletely delineated relationship. The reports of multiple HLA-D specificities on a single haplotype lend a further dimension to the problems of analysis (see Suciu-Foca *et al.*, 1978c; Stastny, 1978; Fuller *et al.*, 1978).

2. *B Cell Lines as Reference Standards for Alloantigen Specificities*

Lymphoblastoid lines of the B-cell variety served an important function in the initial definition of the Ia system (Bodmer *et al.*, 1975; Jones *et al.*, 1975; Winchester *et al.*, 1975) and in the definition of certain Ia alloantigen specificities (Bodmer *et al.*, 1977b; Naeim *et al.*, 1977a; Gibofsky *et al.*, 1978a; Hansen *et al.*, 1979). Apart from their obvious value as reference standards, they can be used in absorption procedures to eliminate undesired reagent specificities and have a clear potential for use in studies of the chemical basis of alloantigen specificity. It is amply documented that there exists a close parallel between the Ia alloantigens of B cells and autologous B-cell lymphoblastoid lines (Bodmer *et al.*, 1977; Dick *et al.*, 1977; Gladstone *et al.*, 1977; Strong *et al.*, 1978; Festeustein and Oliver, 1978; Hansen *et al.*, 1979). Problems that existed with cytotoxic assays on cell lines have

TABLE VIII[a]

Ia ALLOANTIGEN TYPING OF LYMPHOID CELL LINES DERIVED FROM HLA-D HOMOZYGOUS DONORS

Donor I.D.[c]	HLA-D	HLA-A,B haplotypes	Reactions with Ia alloantisera[b]																																	
			DRw1				DRw2				DRw3				DRw4 Ia 4 × 10			DRw4 Ia 4 × 7 × 10			DRw5				DRw6 3 + 6			DRw6 2 + 6			DRw7				Ia 883
			128	152	120	42	13	17	18	16	24	26	35	133	129	106	37	57	38	115	155	126	44	49	75	111	157	46	179	144	51	69	64	52	883
RMAY-1	w1	2,12/2,w35	3	3	4	—	—	—	—	2	—	—	—	—	4	—	3	—	—	—	3	—	—	2	—	—	—	—	—	—	—	—	—	—	—
MWOE-1	w1	3,w35,10,w18	4	3	3	2	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	2	—	4	—	—	—	—	—
CAH-4 (7W541)	w1	2,w35,2,w35	3	2	4	2	—	—	—	2	—	—	—	—	—	—	—	—	—	—	2	—	—	2	—	—	—	2	3	4	—	—	—	2	—
CMG-1	w2	3,7/3,7	—	—	—	—	3	4	4	4	—	—	—	—	—	—	—	—	—	—	—	—	3	4	—	—	—	3	4	3	—	—	—	—	—
AV-3	w2	2,7/2,7	—	—	2	2	—	4	3	4	—	—	—	—	—	—	2	—	2	—	—	—	—	3	3	—	2	2	4	4	2	—	—	—	—
FA-5 (7W546)	w2	9,18/2,18	—	—	—	—	4	4	4	4	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	2	—	—	—	—	—
RMcG-1	w3	1,8/1,8	—	—	—	—	—	—	—	—	3	4	4	2	—	—	—	—	—	2	—	—	—	—	3	3	—	—	—	—	—	—	—	—	—
SOM-1 (6W3002)	w3	1,8/1,8	—	—	—	—	—	—	—	—	2	—	4	—	2	—	—	—	—	—	—	—	—	—	3	3	3	2	—	—	—	—	—	2	—
HAL-1 (6W1008)	w3	1,8/1,8	—	—	2	—	—	—	—	—	4	4	4	3	—	—	—	—	—	3	—	—	—	—	3	4	—	4	—	—	—	—	—	2	—
HA-1 (7W547)	w4	2,15/2,15	—	—	—	2	—	—	2	—	2	—	—	—	4	4	4	4	4	4	2	2	2	4	—	2	—	3	—	3	2	—	2	—	—
ER-1 (6W3003)	w4	2,12/2,12	—	—	—	—	—	—	—	—	—	—	—	—	3	4	3	4	4	3	3	—	—	4	—	—	—	2	—	—	3	—	—	—	—
CK-1	w4	2,12/2,12	—	—	—	—	—	—	—	—	—	—	—	—	3	4	4	2	4	4	—	—	—	4	—	2	—	3	—	*	—	—	—	—	—
FS-2 (7W548)	w10	26,w38/26,w38	—	—	—	—	—	—	—	—	—	—	—	—	2	3	2	2	4	4	—	—	—	4	—	—	—	4	—	—	—	—	—	—	—
EM-3 (7W543)	w10	26,w38/26,w38	*	—	—	—	—	—	—	—	—	—	*	—	—	3	—	3	3	*	—	—	—	3	—	—	—	—	—	—	—	2	—	—	—
KH-4 (7W544)	Unknown	w24,5/w31,5	—	*	—	—	—	—	—	—	—	—	—	—	2	4	3	4	3	4	—	—	—	3	—	—	—	—	—	—	—	—	—	—	4

[a] This table was derived in major part from Hansen *et al.* (1979).

[b] Antisera of the *Histocompatibility* Workshop, Oxford, 1977. Serum I.D. numbers refer to 7th workshop. Serum 883 is a local reagent. Cytotoxicity score: "—" = negative, "2" = weakly positive (26–50% killing), "3" = positive (51–75% killing), and "4" = definitely positive (76–100% killing).

[c] I.D. of HLA-D homozygous donors. The letters identify the donor and the number identifies the individual line. I.D. numbers in parentheses refer to the 6th (6W) and 7th (7W) *Histocompatibility* Workshops.

been essentially surmounted either by alteration in technique (Naeim *et al.*, 1977b; Ferrone *et al.*, 1978b) or by initiating multiple cell lines from the same individual and selecting those with optimal properties (Hansen *et al.*, 1979).

Table VIII contains a summary of the reaction profiles of cell lines derived from individuals homozygous at the HLA-D locus, defined in mixed lymphocyte culture reactions.

The major proportion of the sera selected react in a simple pattern directly correlated with particular HLA-Dw determinants. The sera designated DRw1, 2, 3, and 7 illustrate this relationship. A second category of sera has a somewhat more intricate association with the reference cell lines in that they react with two or more HLA-Dw specificities. The sera designated Ia 4 × 10 (DRw4, Workshop) or Ia 4 × 7 × 10 (DRw4 × 7, Workshop) are clearly of this type. Absorption experiments (Gibofsky *et al.*, 1978a; Antonelli *et al.*, 1978; Hansen *et al.*, 1979) demonstrate that a single antibody specificity is present in sera with Ia 4 × 10 or Ia 4 × 7 × 10 specificities. A third pattern of reaction is given by serum 883 that reacts only with the cell line KH. KH is derived from an HLA-D homozygous Red Lake American Indian (7W544). Although KH bears the alloantigens of the DRw4 group (Ia 4 × 10/4 × 7 × 10), the MLC specificity present on KH is not associated with HLA-Dw4, w7, or w10 (Dupont *et al.*, 1978; Festenstein *et al.*, 1978) and remains undefined. Lymphoblastoid lines also express Ia alloantigens that are unrelated to DR specificities, including the Merrit group 5 that is coded for by a second locus (Naeim *et al.*, 1978b).

3. *Evidence for Multiple Loci Determining Ia Alloantigens*

a. Serologic Evidence. Despite the simplifying concept of DR as the major genetic locus for Ia alloantigens, a variety of evidence points to the conclusion that considerable genetic complexity underlies these alloantigens. Early studies provided evidence that certain Ia alloantisera gave reactions that could not be satisfactorily related to MLC specificities (Mann *et al.*, 1975b; Winchester *et al.*, 1975b; Legrand and Dausset, 1977; Park *et al.*, 1977; van Rood *et al.*, 1977; Walford *et al.*, 1977). These were interpreted as indicating the presence of additional linked loci in the Ia antigen system.

b. Chemical Evidence. In view of the difficulties inherent in the use of human alloantisera in serologic analyses, chemical approaches were exploited to refine the analysis of the molecular basis of the alloantigen specificities.

It became apparent that Ia alloantigens of a particular DR specificity account for only a fraction of the Ia molecules present on the cell

membrane since a considerably greater yield of radiolabeled Ia is precipitated by heteroanti-Ia sera than with DR alloantisera (Hoffman *et al.*, 1977).

Detailed examination of the sera reacting with the Daudi lymphoblastoid B cell line revealed the presence of two alloantigen specificities on different Ia molecules under the control of two linked genes (Tosi *et al.*, 1978). Through selective immunoprecipitation of labeled Ia molecules, these investigators defined a distinct allospecificity, dc1, that was frequently present in sera with DRw6 specificity. Evidence was presented that the two Ia molecular species bearing either DRw6 or dc1 differed in susceptibility to proteolysis.

Similarly, for the heterozygous B-cell line JY that expresses DRw4 and DRw6, removal of all Ia molecules bearing DRw4 or DRw6 alloantigens left Ia molecules that were detected both by heteroanti-Ia reagents, as well as by the alloantiserum Ia 172 (Mann *et al.*, 1979). In family studies the alloantigen detected by Ia 172 was determined by the same haplotype containing DRw6.

Furthermore, the results of amino acid sequence (see Sections IV and V) of the human Ia antigen suggest a homology to the murine *I-E/C* α chain. However, the two-dimenstional gel analysis of human Ia antigens points to a major polymorphism in the same region as the murine *I-A* β chains (Charron and McDevitt, 1979).

c. Disease Associations as a Means to Define Novel Ia Alloantigens. The screening of populations of patients with selected diseases using large panels of Ia alloantisera in an attempt to identify alloantigens associated with susceptibility to the disease affords a method of identifying novel Ia specificities (Moutsopoulos *et al.*, 1978; Reinertsen *et al.*, 1978; Gibofsky *et al.*, 1978a,b, Garovoy *et al.*, 1978; Patarroyo *et al.*, 1979). In this way a postulated common genetic factor in the disease is used in the same way that, for example, a particular MLC type was used to establish the DR associations. The approach is to initially establish an association in the disease population based on relative risk and then characterize the genetic control of the new specificity in normal individuals through population and family studies (Mann *et al.*, 1979; Laurence *et al.*, 1979).

d. Multiple Ia Alloantigen Expression. The identification of individuals with multiple Ia alloantigens provides further evidence for the existence of additional loci (Mann *et al.*, 1976; van Rood *et al.*, 1976; Park *et al.*, 1978; Fuller *et al.*, 1978; Naeim *et al.*, 1978b; Laurence *et al.*, 1979; Mann *et al.*, 1979). It is important to exclude the presence of contaminant second specificities in the reagent sera, since such reactions can account for some heterozygous individuals who type for

TABLE IX

TYPING FOR MULTIPLE Ia ALLOANTIGEN SPECIFICITIES IN FOUR SIBLINGS

		Alloserum identification number and designated specificity												
		DRw2			DRw3				Ia 4 × 10		Ia 4 × 7 × 10		DRw5	883
Individual	HLA type	1146	057	1239	1134	2134	932	277	191	924	1379	1479	1995	883
SK	A1,B8 /A31,B35	+	+	+	+	+	−	−	+	+	+	+	−	+
FH	A2,B14/A31,B35	+	+	+	+	+	+	+	+	+	+	+	−	+
JH	A2,B14/A1,B35[a]	+	+	+	+	+	+	+	+	+	+	+	−	+
LE	A1,B8 /A1,B39	−	−	−	+	+	−	−	−	−	−	−	+	+
Inferred assignment of above alloantigen specificities to HLA haplotypes														
	A1,B8	−	−	−	+	+	−	−	−	−	−	−	−	[b]
	A2,B14	−	−	−	+	+	+	+	−	−	−	−	−	
	A31,B35	+	+	+	−	−	−	−	+	+	+	+	−	
	A1,B35[a]	+	+	+	−	−	−	−	+	+	+	+	−	
	A1,B39	−	−	−	−	−	−	−	−	−	−	−	+	

[a] A–B recombinant.

[b] The 883 antigen is not assignable.

TABLE X
INDEPENDENCE OF MOLECULES BEARING DIFFERENT Ia ALLOANTIGEN SPECIFICITIES IN A B-CELL LYMPHOBLASTOID LINE DERIVED FROM SK, AN INDIVIDUAL WITH FOUR Ia ALLOANTIGENS[a]

Capping antiserum	Second antiserum	Cells with coredistribution of the two sera (%)
883 (Ia 883)	883 (Ia 883)	>95
1146 (DRw2)	1558 (DRw2)	>95
932 (DRw3)	1134 (DRw3)	>95
191 (Ia 4 × 10)	1379 (Ia 4 × 7 × 10)	>95
1146 (DRw2)	883 (Ia 883)	<5
932 (DRw3)	883 (Ia 883)	<5
191 (Ia 4 × 10)	883 (Ia 883)	<5
1146 (DRw2)	932 (DRw3)	<5
191 (Ia 4 × 10)	932 (DRw3)	<5
1146 (DRw2)	191 (Ia 4 × 10)	<5

[a] After Laurence *et al.* (1979).

three Ia specificities using antisera not characterized in absorption tests (Bodmer *et al.*, 1978b). The use of sequential immunoprecipitation (Tosi *et al.*, 1978; Mann *et al.*, 1979; Wang *et al.*, 1979) or coredistribution experiments (Laurence *et al.*, 1979) permits establishing evidence that the specificities reside on separate molecules and thus indicates multiple loci coding for Ia alloantigens.

Table IX contains a limited study of 4 siblings who express multiple Ia alloantigen specificities. Three of the individuals react with Ia alloantisera specific for DRw2, DRw3, and Ia 4 × 10, and Ia 4 × 7 × 10. The inferred assignment of the Ia alloantigen specificities is shown in the lower portion of the table. The *A-B* recombinant haplotype A1, B35 and the A31, B35 haplotype both appear to code for the specificities DRw2 and Ia 4 × 10/Ia 4 × 7 × 10, suggesting that both these specificities are under the control of closely linked loci on the *HLA-B* side of the recombination.

More extensive analyses are required to confirm this interpretation, however, cocapping experiments illustrated in Table X are consistent with the expression of multiple Ia alloantigen specificities. While Ia 4 × 10 and Ia 4 × 7 × 10 coredistributed and presumably reside on the same entity, the remaining specificities appeared to be present on distinct molecules. In experiments not illustrated in Table X, all of the Ia alloantigens coredistributed with heteroanti-Ia serum and were not redistributed with anti-β_2 microglobulin.

An interpretation of the specificities contained in the allosera designated Ia 4 × 10 and Ia 4 × 7 × 10 by virtue of their reaction pattern in Table VIII is that they contain a mixture of two specificities. One is directed to DRw4 and the second directed to an allele of a second Ia locus that is in linkage dysequilibrium with DRw4. In the case of the family illustrated in Table IX, DRw2 replaced DRw4; however, the allele of the second Ia locus was present in association with HLA-Bw35, giving rise to the multiple alloantigen typing. DuB 15 is a specificity that may be relevant to these findings since it is present in certain Ia 4 × 7 × 10 sera as a second specificity and defines an antigen mapping to the left of HLA-B. However, DuB 15 has not been identified as a second specificity determined by a haplotype with a defined DR allele (Johnson *et al.*, 1978).

The specificity Ia 883 present in the members of this family could not be assigned to a haplotype, but exists as a third Ia alloantigen. Immunoprecipitation experiments using the lymphoblastoid cell line KH reveal that the molecules bearing Ia 883 resemble the typical Ia molecule having the 37,000–28,000 dalton chain structure. In sequential immunoprecipitation experiments the Ia 883 molecules are separable from those bearing Ia 4 × 10/Ia 4 × 7 × 10. Cocapping experiments performed on KH demonstrate that Ia 883 coredistributes with heteroanti-Ia serum but not anti-β_2 microglobulin (Wang *et al.*, 1979; Laurence *et al.*, 1979). Taken as a group these preliminary findings provide support for the presence of at least one locus in addition to that responsible for the DR alloantigens and located in close proximity to it.

Further evidence exists for Ia loci distinct from that responsible for the DR antigens but mapping to the left of HLA-B (Park *et al.*, 1978). Two alleles, TE 21 and TE 22, have been delineated that map here and give rise to triplet antigen expression when they are found on the same haplotypes that contain DR specificities.

e. Ia Alloantigens Mapping between HLA-A and B. Evidence accumulated from a number of family studies provides evidence for the existence of another locus for Ia alloantigens that maps on the HLA-*A* side of the HLA-*B* locus, presumably between the *A* and *B* loci (Mann *et al.*, 1975b, 1976; Park *et al.*, 1977; van Rood *et al.*, 1977; Walford *et al.*, 1977; Abelson and Mann, 1978).

4. Models of Human MHC

In view of available evidence, three models of the human MHC warrant consideration. Because of the evident analogy of human and murine Ia antigens, for purposes of simplicity the region in the human

MHC that controls the synthesis of the Ia antigen bearing molecules will be designated "I."

a. Unitary I Region with One Locus (DR) Determining the Synthesis of the Ia Molecules. This model involving a single I region with one locus for DR alleles is the tentative conclusion of the VII Histocompatibility Workshop and suffices to account for many but not all of the present observations.

b. Tandem Type 1/Type 2 Duplication–Simple I Region. This model postulates a series of interrupted I regions, each associated with one of the major HLA-A, B, and possibly C regions. Each I region would be located on the centromeric side of the type 1 locus. Presumably, each of the interrupted I regions would be the site of one segregant series of Ia molecules. The DR series would be coded for by the I region in relation to HLA-B. This is the minimum model necessitated by the findings of Section 3e.

c. Complex I Region. The central feature of this model is that multiple linked loci determine a number of different Ia segregant series within a single I region. Two alternative forms of this model involve either one I region on the centromeric side of HLA-B, or multiple I regions separated by loci for Type 1 molecules as in the tandem model.

Taken together, the available findings favor the third model proposed above: The *I* region containing *HLA-D* resembles that of the mouse in that it contains multiple Ia loci. The evidence supporting the presence of loci determining Ia antigens that map between *HLA-A* and *-B* suggest the presence of an additional *I* region, so that it appears that two *I* regions exist in tandem in relation to the HLA-*B* and the HLA-*A* loci.

IX. Disease Association

A. Introduction

The availability of the polymorphic Ia antigen system provides a new dimension to the approach of defining the relationship of disease susceptibility to determinants of the MHC. In nearly all instances of diseases in which an MHC association has been found, if a pattern of inheritance of the disease had been previously recognized, it was non-Mendelian, and in some instances the diseases were considered to be nonfamilial. It has been emphasized that this is the pattern evident when multiple factors, both genetic and environmental, are involved in the development of disease (Svejgaard *et al.*, 1975).

The category of diseases that are the subject of this section has its

primary association with Ia alloantigens. In some instances, where the relevant Ia alloantigen is in strong linkage disequilibrium with HLA-*A* or -*B* locus alleles, the association has been anticipated by results of classic HLA typing. In other diseases the first evidence of association with elements of the MHC came from MLC typing, while new disease associations are beginning to be reported with Ia alloantigens where no prior measure of histocompatibility has been informative, such as in rheumatic fever (Patarroyo *et al.*, 1979). The weight of evidence, however, supports the increasing importance of the Ia antigens as playing a central role in these relationships.

In view of the evident genetic intricacies of the Ia antigen system, the approach has been utilized of examining selected patient groups with large panels of Ia alloantisera in order to identify Ia alloantigens that have a significant relationship to disease susceptibility or resistance.

Data defining disease associations with Ia alloantigens are derived from either of two sources: population studies in which a patient group and a normal control group are compared, and studies of families in which multiple members are affected. Each source provides different insight into the nature of the genetic events (Svejgaard *et al.*, 1975).

B. Measurement of Degree of Association

Several approaches have been developed to estimate the intensity of the association between disease occurrence and the presence of a particular alloantigen. The classic estimate is that of the relative risk (Woolf, 1955). This ratio is readily calculated as cross products from the 2×2 table used to calculate χ^2.

	Antigen	
	+	−
Patient	a	b
Control	c	d

relative risk RR = (ad)/(bc). When the ratio of antigen-positive to antigen-negative patients is the same as in the controls, the relative risk is 1. Positive whole numbers reflect a positive association of disease with the antigen whereas fractional numbers signify a negative association. The relative risk, however, lacks any intuitive probabilistic significance, and the more conservative estimate of the relative frequency of antigen in patients compared to controls has been advocated as a better statistic (Curie-Cohen, 1977). Relative frequency is:

$$[a/(a + b)]/[c/(c + d)] = (ad + ac)/(bc + ac)$$

A disadvantage of both methods is that negative associations are represented as fractional numbers. A logarithmic transformation has been used to equalize the scale of positive and negative associations (Svejgaard *et al.*, 1974).

C. Multiple Sclerosis

1. Role of Haplotype

Among patients with multiple sclerosis, there is a progressive increase in the relative risk of developing the disease with each successive allele in the Northern European haplotype A3-B7-Dw2-DRw2 (Jersild *et al.*, 1975; Terasaki *et al.*, 1976; Compston *et al.*, 1978; Svejgaard and Ryder, 1977). The disease association, however, is not with the entire haplotype, but is maximal with the Ia alloantigen (Thomson and Bodmer, 1979). For alloantisera in the cluster detecting DRw2, the percentage of positive patients varied from 47 to 84%, controls from 15 to 33%, and the relative risk from 4.3 to 10.4. This clearly establishes, for this population, an association of disease susceptibility with a gene mapping near the locus for DRw2.

One characteristic of the data from Caucasian patients is the variation in percentage of positive patients and relative risk given by different alloantisera in the DRw2 cluster (Terasaki *et al.*, 1976; Batchelor, 1977). A particular Ia alloantiserum used in two laboratories reacted with a very high proportion of patients (Winchester *et al.*, 1975c; Wernet, 1976). These findings raise the possibility that an Ia alloantigen allele in linkage with *DRw2* is the primary factor in the disease association. Family studies underway as a part of the VIIIth Histocompatibility Workshop should establish the locus of the susceptibility gene.

At a deeper level, associations obtained with a single serum raise a perplexing issue. On the one hand, the single serum could define an extremely important specificity not apparent in the more conventional typing sera. With fully reliable serology the segregation of this alloantigen should be easily characterized. Alternatively, when technical factors intrude on the reliability of typing, clusters of correlated sera provide a more secure but potentially less interesting result.

2. Ethnic Factors in Disease Association

The importance of ethnic factors in determining the nature of the disease association with particular alleles is emphasized by the com-

plete separation from DRw2 when populations other than Northern European Caucasians with multiple sclerosis are examined. This is evident in Arabs (Kurdi *et al.*, 1977; Compston *et al.*, 1978), Spanish (Ercilla *et al.*, 1978), Italians, Askenazi Jews, and Japanese (Naito *et al.*, 1978a; Batchelor and Morris, 1978). In each group, with the exception of the Spanish, a clear association with a D/DR alloantigen was evident. In the Japanese population multiple sclerosis is associated with the presence of DRw6, an Ia alloantigen that is in the cross-reactive group containing DRw2 (Bodmer, 1978; Antonelli *et al.*, 1978). Two sera identify a significant percentage of Japanese patients with multiple sclerosis, one relating to DRw2, relative risk 5.4, and the second to DRw3, relative risk 6.6 (Naito *et al.*, 1978a). However, among the Italians, Arabs, and Askenazi Jews with multiple sclerosis, the dominant DR specificity was DR4, with approximately half of the Askenazi Jew bearing DRw6 (Batchelor and Morris, 1978; Compston *et al.*, 1978).

Among American blacks, D/DRw2 was not encountered among 34 control individuals but was identified in 11/31 patients with multiple sclerosis (Dupont *et al.*, 1976). The low frequency in the control population of DR2 is not explained, since it has been reported to be present in American and African blacks at 13.8 and 8.7% (Pickbourne *et al.*, 1978), but not in association with Dw2 (Ward *et al.*, 1979).

In a reciprocal sense, the inhabitants of the Shetland and Orkney Islands who have an extremely high prevalence of multiple sclerosis as well as a high frequency of DRw2 (40%) do not have a significant association between the presence of DRw2 and multiple sclerosis, with the relative risk only 1.2 (Poskanzer *et al.*, 1979).

These studies raise several intriguing questions. The first is whether the genes predisposing to multiple sclerosis are the same in each of these populations. The Caucasian–Japanese relationship of DRw2–DRw6 suggests that this could be the case, and implies a common origin for the two DR specificities in association with a single disease susceptibility trait. Alternatively, the association with DRw4 evident in some Askenazi Jews and Italians favors a distinct susceptibility determinant because of the lack of relationship between DRw4 and DRw2. Despite the low frequency of the disease in the highly outbred North American Caucasian population, it should be possible to examine instances of individuals whose genotype contains haplotypes drawn from families with different ethnically associated determinants in order to determine if enhanced susceptibility or resistance is conferred by the simultaneous presence of two disease-associated deter-

minants. By the same consideration, the possibility exists that instances of DRw2 negative multiple sclerosis in the Caucasian population reflect an alternative susceptibility haplotype present because of earlier racial mixture.

D. Insulin-Dependent (Juvenile) Diabetes Mellitus

Taken as a group in all populations, insulin-dependent diabetes mellitus is distinguished from the maturity-onset type by the association of the former with alleles of the MHC. In the Caucasian population there are two haplotypes associated with the disease, B8-Dw3-DRw3 and B15-Cw3-Dw4-DRw4. In 293 Caucasian patients drawn from Europe and North America, the frequencies of B8, DRw3, B15, and DRw4 (4 × 7) were 32, 27, 20, and 39 with the respective normal frequences 16, 17, 10, and 15, with relative risks 2.4, 3.8, 2.1, and 3.5 (Svejgaard *et al.*, 1978; Batchelor and Morris, 1978). The primary association is clearly closer to the DR specificities than to the other HLA markers including Dw specificities (Svjegaard *et al.*, 1978; Cudworth and Festenstein, 1978; Suciu-Foca *et al.*, 1978b). In some studies considerably high frequencies for DRw3 were obtained—56%—and for DRw4 × 7—74–80%, with controls—27–42% (Garovoy *et al.*, 1978a); while in a study from Austria, only DRw3 was found to be elevated (Mayr *et al.*, 1978). In contrast, in a series from England, 73% of the patients had DRw4 and 53% DRw1, both highly significant associations, while the DRw3 was increased, but not to levels that were significant (Pickbourne *et al.*, 1978). The finding in this series of a family in which disease susceptibility was inherited with the haplotype containing DRw1 emphasizes the diversity of the association with MHC determinants. The possibilities exist that ethnic subsets of the Caucasian population are characterized by different DR associations of the factors involved in susceptibility and that there may be two forms of the disease.

A second feature of the patient population is a highly significant decrease in the frequency of Dw2/DRw2 that cannot be accounted for simply by the increase in frequency of DRw3 and DRw4 (Ilonen *et al.*, 1978; Svejgaard *et al.*, 1978; Batchelor and Morris, 1978). This is considered as a primary negative association rather than as a compensatory secondary alteration. This indicates that the development of insulin-dependent diabetes mellitus depends on the interaction of a resistance trait associated with DRw2 and susceptibility factors associated with DRw3 and 4.

Two central questions that are not yet conclusively answered are:

whether the susceptibility factor has a dominant or recessive mode of inheritance; and whether the two DR types are associated with the same or different susceptibility genes. On the basis of family studies, a "monogenic" recessive mode of inheritance has been proposed (Rubinstein *et al.*, 1977; Thomson and Bodmer, 1979). The penetrance of the recessive gene was calculated at 50%. In recombinant families the susceptibility gene on the DRw3 or DRw4 haplotype maps closest to the D/DR locus (Rubinstein and Suciu-Foca, 1978; Suciu-Foca *et al.*, 1978b). An alternative hypothesis is that two distinct dominant susceptibility effects are present (Svejgaard *et al.*, 1975, 1978), one associated with DRw3 and one with DRw4. The heterozygote DRw3/DRw4 would have an enhanced risk of developing diabetes because of the presence of both susceptibility factors.

A recent finding of considerable interest is that an Ia alloserum 7w-172 reacts with 60% of patients versus 8% of controls, relative risk 28 (Garovoy *et al.*, 1978b). This serum is in the specificity cluster merrit 5 that is considered to be under the control of a second locus distinct from but closely linked to *DR* (Walford *et al.*, 1975; Naeim *et al.*, 1978b). This raises the question of complementation between two linked Ia determinants as an important factor in determining susceptibility analogous to IR gene complementation. Since 75–90% of the patients in the series of Garovoy *et al.* (1978b) were either DRw3 or DRw4, this suggests that the gene product recognized by 7w-172 is found in association with both of the DR susceptibility markers.

Among the Japanese with insulin-dependent diabetes mellitus, there is a strong association with B22 and DRw4 × 7: patients, 38 and 65%, controls, 15 and 35, respectively; relative risk, 6.4 for DRw4 (Batchelor and Morrison, 1978). One DRw3-associated typing serum reacted with 47% of patients and 12% of controls. In this respect the finding that 15.8% of Japanese patients type for HLA DW3 whereas no normal subject among 67 controls had this specificity is of great interest (Sasazuki *et al.*, 1978b). It appears that the Caucasian and Japanese populations, although separated by a considerable genetic distance (Pickbourne *et al.*, 1978), share a genetic susceptibility for diabetes mellitus that is associated with the same or closely related DR specificities.

E. Autoimmune Diseases Associated with DRw3

A group of diseases characterized by an excess incidence in females and a varying spectrum of autoimmune features shares an association

in Caucasians with DRw3 and, by extension, an association with elements of the haplotype A1-B8-Dw3-DRw3. These same diseases in the Japanese population also appear to have a relationship to MHC alleles, primarily B12, B5, and Bw35 and DRw4, DRw4 × 7 (YT) or DRw2 (HO) (Sasazuki *et al.*, 1978a; Amos *et al.*, 1978). The diseases in this group, originally recognized by the common occurrence of HLA-B8 (Svejgaard *et al.*, 1975), are chronic active hepatitis, Addison's disease, myasthenia gravis, thyrotoxicosis, Sjögren's syndrome, and systemic lupus erythematosus.

1. Chronic Active Hepatitis

In the group of female Caucasian patients with chronic active hepatitis (periportal piecemeal necrosis) but lacking HB_s antigenemia, DRw3 is present in 76% of Australians (Mackay and Tait, 1978) and 68% of North Americans with progressive disease (Opelz *et al.*, 1977), the lower figure of 49% (Batchelor and Morris, 1978) having been revised upward by improved methodologies (Mackay and Tait, 1978). Evaluation of the patients for antinuclear and antismooth muscle antibodies revealed them to be associated also with the presence of DRw3 (Mackay and Tait, 1978; Batchelor and Morris, 1978). Of certain diagnostic interest was the finding that the frequency of DRw3 alloantigens in this patient group differentiated them from both those with primary biliary cirrhosis or HB_s antigen-associated chronic hepatitis (chronic active hepatitis B) (Mackay and Tait, 1978; Mazzilli *et al.*, 1978).

Among the Japanese the HLA-B antigen associated with chronic active hepatitis is B5 (Hoshino *et al.*, 1977). DR data are not yet available. The Japanese B5 haplotype contains DRw2 ($\Delta = 42.9$) (Pickbourne *et al.*, 1978); in view of the findings in Graves' disease, the possibility exists that among Japanese this category of autoimmune diseases is associated with DRw2.

2. Myasthenia Gravis

In myasthenia gravis the sex ratio is approximately 3 females to 1 male, and the frequency of HLA-B8 in the disease is 52% in Caucasian

females and 26% in males. The sex ratio of DRw3 frequency parallels the B8 data (Batchelor and Morris, 1978). Here, as in other diseases, the B8-DRw3 association serves to discriminate between two subsets of myasthenia gravis in that the group of patients with thymoma appears to lack the MHC allele association. Among North American Caucasians the occurrence of antireceptor antibodies was associated with the presence of DRw3 (Naeim *et al.*, 1978b). This relationship was not encountered in Scandinavia (Smith *et al.*, 1978).

Among Japanese with myasthenia gravis, the disease association is primarily with DRw4 as detected by the 4 × 7 reagents, with 70% of patients and 25% of controls expressing this alloantigen. In contrast to the Caucasian patients, there was no definite sex preponderance of DRw4 (Batchelor and Morris, 1978).

3. *Graves' Disease*

Among Caucasian patients with Graves' disease, the relative risks of HLA-B8 and Dw3 are 2.4 and 5.2, respectively. DRw3 was found in 68% of patients and 28% of controls, whereas 50% were HLA-B8 positive; relative risks 5.5 and 3.0, respectively (Farid *et al.*, 1979). Among Japanese patients with Graves' disease, the primary association is with a specificity defined by the MLC typing cell HO (Sasazuki *et al.*, 1978a). HO bears the DRw2 specificity but does not type for Dw2 (Sasazuki *et al.*, 1978b; Reinsmoen *et al.*, 1978).

Although, in Caucasian patients, a significant association with DRw2 was not apparent, a family with multiple cases of Graves' disease was encountered in which susceptibility was associated with a haplotype bearing DRw2 (Farid *et al.*, 1979).

4. *Addison's Disease*

Caucasian patients with idiopathic (autoimmune) Addison's disease have a significantly increased frequency of HLA-A1 and B8 accounting for 69–80% of individuals (Thomsen *et al.*, 1975; Irvine, 1978). The association of DRw3 with the presence of adrenocortical antibodies in patients with Addison's disease has been reported by Svejgaard (cited in Irvine, 1978).

5. *Sjögren's Syndrome*

Sicca syndrome is subdivided into two forms: primary sicca syndrome or Sjögren's syndrome and secondary sicca syndrome. The latter is primarily associated with rheumatoid arthritis or graft-vs-host disease. These two forms of sicca syndrome are clearly distinguished by their profiles of Ia alloantigens.

Sjögren's syndrome occupies a place in the DRw3-associated diseases that is midway between the preceding conditions, where one organ appears to be the principal target, and systemic lupus erythematosus, where the disease manifestations are protean. The association was originally detected with B8 and Dw3 (Gershwin *et al.*, 1977; Chused *et al.*, 1977; Ivanyi *et al.*, 1975; Hinzova *et al.*, 1977), and the relative risks are, respectively, 3.96 and 19.8.

In a study of 24 patients, 19 of whom typed for HLA-Dw3, all patients reacted with two Ia allosera designated 172 and AGS (Moutsopoulos *et al.*, 1978). In a control population the sera reacted with 37 and 24% of individuals, but their reactivities were not correlated, providing evidence that they detected different antigens. The specificity of serum 172 is intricate and, in the VIIth Histocompatibility Workshop, was considered to detect DRw3, 5, and 6 (Bodmer *et al.*, 1978). However, recent data demonstrate that the Ia specificity is the product of a locus distinct from, but closely linked to, that responsible for the DR specificities (Mann *et al.*, 1979). The frequency of DRw3 in patients with secondary sicca syndrome was only 9% while the frequency of Ia 172 was 82% (Moutsopoulos *et al.*, 1979). This finding of two Ia alloantigens that are coordinately involved in determining disease susceptibility suggests the occurrence of a mechanism analogous to IR gene complementation (Dorf *et al.*, 1975) and has important consequences in calculations of relative risk.

6. *Systemic Lupus Erythematosus*

For a number of years, it has been recognized that among patients with systemic lupus erythematosus there is a small but significant increase in the frequency of HLA-B8 (Grumet *et al.*, 1971; Kissmeyer-Nielsen *et al.*, 1975; Goldberg *et al.*, 1976). Analyses of the Ia alloantigens of patients in two clinics reveal the frequency of DRw3 to be 54 and 46% with controls of 20 and 22% (Reinertsen *et al.*, 1978; Gibofsky *et al.*, 1978a,b). The increase in DRw3 is of interest because it is not obtained with all allosera that have the DRw3 specificity (see Table XI). In addition, both studies demonstrated an increase in the frequency of DRw2 among patients—50 and 57%—compared to controls of 25 and 26%. Approximately three-fourths of all patients are

TABLE XI

CONTRASTING REACTIVITIES OF DIFFERENT DISEASE POPULATIONS WITH Ia ANTIGEN ALLOANTISERA[a]

Designated specificity	Reagent alloantiserum number	Normal caucasian control (%)	Caucasian patient groups: Rheumatoid arthritis $n = 41$ %	RR[b]	χ^2	Systemic lupus erythematosus $n = 20$ %	RR	χ^2	Idiopathic thrombocytopenic purpura $n = 20$ %	RR	χ^2
DRw1	1818	20	5	0.21	3.5[b]	50	4.07	5.9	10	0.45	0.5
	Bak.	25	5	0.15	6.1	50	2.94	3.4	11	0.35	1.2
DRw2	1558	19	12	0.57	0.6	50	4.07	5.9	68	8.82	14.7
	57	30	10	0.26	4.8	50	2.38	2.1	61	3.74	4.9
	1146	19	15	0.70	0.2	45	3.33	4.0	63	6.98	11.7
DRw3	2134[c]	28	20	0.62	0.6	25	0.85	0	30	1.10	0
	1033	20	15	0.7	0.1	55	4.9	6.0	—[d]	—	—
DRw4 (Ia 4 × 10)	191	17	71	11.88	30.2	20	1.23	0	10	0.55	1.6
	924	18	75	13.39	32.3	10	0.49	0.3	10	0.50	0.3
(Ia 4 × 7 × 10)	1283	22	73	9.06	24.5	20	0.87	0	11	0.40	0.7
	1379	20	68	8.77	24.1	10	0.46	0.5	5	0.21	1.5
DRw5	1995	7	5	0.68	0	15	1.47	0	0	0	0.4

[a] RR = relative risk = (patients +/patients −)(controls +/controls −); χ^2 calculated using the Yates correction.

[b] Significant negative associations are indicated by dashed enclosures and positive associations by solid enclosures.

[c] Serum 2134 detects an antigen also present on some individuals who are positive for DRw5.

[d] "—" indicates not tested.

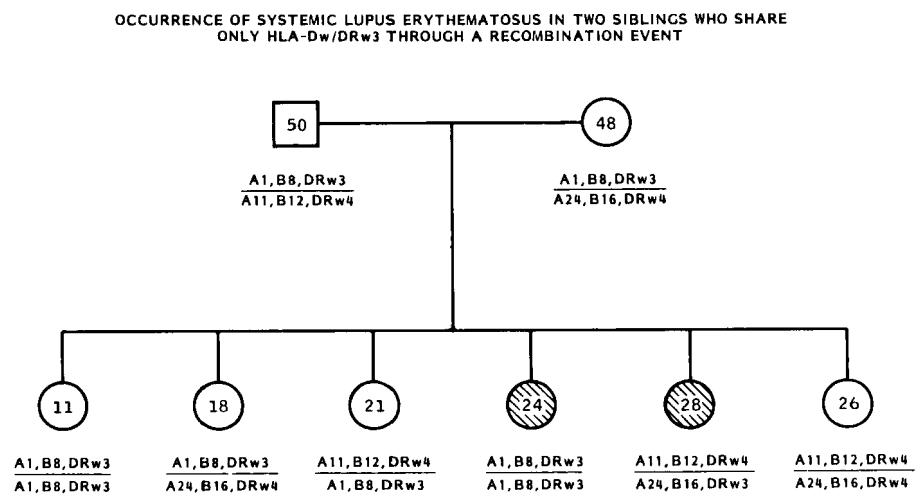

FIG. 8. A portion of a family in which two siblings aged 24 and 28 have systemic lupus erythematosus. They are disparate for HLA-*A*, B, and *C* alleles. Both share HLA-Dw3 and DRw3 that resulted from a maternal recombination between HLA-*B* and HLA-*D*/DR.

either DRw3 or DRw2 or both. In family studies systemic lupus erythematosus has been associated with the inheritance of both A1-B8 in 8 instances, and A2-B7 in 5 instances (Dawkins *et al.*, 1978).

The increment in frequency of a DR alloantigen over that of the B locus alloantigen provides evidence that the principal disease association maps near the DR locus. A B/D-DR recombinant family in which two siblings have systemic lupus erythematosus adds additional support to this interpretation. As shown in Fig. 8, the propositus is homozygous for the haplotype 1-8-DRw3. Her sister differs in HLA-A and -B type does not type for the DRw4 alloantigens anticipated on the basis of the haplotype, instead typing only for DRw3. Confirmation that this finding is the result of a maternal B/D recombination event was obtained through mixed lymphocyte culture results.

The fact that two diseases, idiopathic thrombocytopenic purpura and Sjögren's syndrome, that are considered to be closely related to systemic lupus erythematosus are primarily associated, respectively, with DRw2 or DRw3 suggests the possibility that a relationship of DR alloantigen to disease pattern or natural history might be found. A

tendency for more severe systemic lupus erythematosus to be associated with HLA-B8 (Goldberg *et al.*, 1976) or the A1-B8 haplotype (Dawkins *et al.*, 1978; Rigby *et al.*, 1978) has been reported. However, an alternative interpretation of the data on patients with idiopathic thrombocytopenic purpura is that the high frequency of DRw2 primarily reflects the ethnic composition of the patient population and that the DRw2-associated susceptibility gene originates in the Eastern Mediterranean. Support for this possibility is provided by the replacement of a specificity HO related to DRw2 for DRw3 in diseases such as thyrotoxicosis in the Japanese population (Sasazuki *et al.*, 1978a).

The association of a lupuslike disease with C2 deviciency (Agnello *et al.*, 1972) and the further association of C2 deficiency with the haplotype A10-Bw18-Dw2-DRw2 (Fu *et al.*, 1974b, 1975a) provides another example of the potential relevance of DRw2 to systemic lupus erythematosus.

The parallel between systemic lupus erythematosus to diabetes mellitus is an interesting one. Both diseases are associated with two DR alloantigens and both are characterized by one alloantigen specificity that occurs at a frequency low enough to suggest a resistance effect. In the case of systemic lupus erythematosus, this is DR4 and most probably the Ia 4 × 7 × 10 specificity. Indeed, an alternative interpretation of the role of the MHC in determining susceptibility to systemic lupus erythematosus is the less likely possibility that the prime genetic determinant is a disease-resistance effect associated with the presence of Ia 4 × 7 × 10.

The fact that DRw3 is associated with both diseases raises the question of whether this is accounted for by the operation of the same susceptibility factor. Against this possibility is the influence of sex on the expression of systemic lupus erythematosus and the contrasting association with DR2 and DR4 present in both diseases in terms of association with susceptibility or resistance.

Individual alloantisera discriminate more sharply between the systemic lupus erythematosus patient population and controls. One serum, No. 1066, reacted with 75% of patients and 34% of controls, relative risk 5.7. This serum contains at least two populations of antibodies with separate specificities that relate to determinants present on DRw2 or DRw3 reference cells, as determined in absorption experiments (Gibofsky *et al.*, 1978b). Another serum, Ia 715, with a specificity relating to DR1, 2, and 6, reacts with 75.6% of patients and 14.1%

of controls, relative risk 18.8 (Reinertsen *et al.*, 1978). Recent evidence indicates that this serum detects an alloantigen MC that is distinct from DR1. The elevated frequency of DR1 in patients with systemic lupus erythematosus (Table XI) could reflect the presence in the typing sera of a second specificity similar to Ia 172. These data emphasize the still incompletely resolved nature of the Ia system and the need for more comprehensive genetic studies in these diseases.

F. Rheumatoid Arthritis

Several early studies recognized that patients with rheumatoid arthritis were characterized by a high frequency of a particular Ia alloantigen (Ivaskova *et al.*, 1977; Panayi and Wooley, 1977; Winchester, 1977). One of the most significant contrasts in profiles of Ia alloantigens emerges when a group of Caucasian patients with rheumatoid arthritis is compared to patients with systemic lupus erythematosus (Table XI) (Gibofsky *et al.*, 1978a,b). This is particularly so because of certain similarities in pathogenic mechanism involving formation of autoantibodies that characterize both patient groups. Among patients with rheumatoid arthritis, the frequency of DR1, 2, 3, and 5 is proportionally decreased and the frequency of Ia 4 × 7 × 10 (DR4 × 7) and Ia 4 × 10 is increased to 68–75%, relative risk 8.8–13.4 (Table XI, Gibofsky *et al.*, 1978a). The frequency of DRw4 related specificities is 70%, relative risk 6.0 (Stastny, 1978a,b; Panayi *et al.*, 1978).

The higher relative risk and proportion of patients defined by the Ia 4 × 7 × 10 (DR4 × 7) alloantisera suggests that the primary association is with this determinant rather than with those more closely related to HLA-Dw4. This raises the possibility that the susceptibility determinants map at a site in the I region distinct from that associated with the usual DR specificity and closer to the locus of the gene determining the Ia 4 × 7 × 10 specificity. In the VIIth Histocompatibility Workshop, this same increase in relative risk was found comparing the DR4 × 7 and DR4 specificities. In the case of women, they were, respectively, 7.9 and 5.1; and for men, 6.7 and 3.1 (Batchelor and Morris, 1978).

With the primary association of rheumatoid arthritis with Ia 4 × 7 × 10, it would have been anticipated that there would not be a particular HLA-A or -B allele recognized because of the absence of strong linkage disequilibrium. The increase in Dw4, however, is unequivocal, ranging from 54% of patients, relative risk 6.1 (Stastny, 1978b); 36%, relative risk 3.0 (McMichael *et al.*, 1977); to 26%, relative risk 1.78 (Gibofsky *et al.*, 1978a). Dw10 is also significantly in-

creased to 13% from 6.9% in controls, relative risk 1.88 (Gibofsky *et al.*, 1978a). Presumably because of the linkage disequilibrium with Dw4, Cw3 has been identified in 34% of patients, relative risk 2.6 (McMichael *et al.*, 1977).

Among the Japanese the influence of ethnic factors on the nature of the MHC alleles associated with rheumatoid arthritis are apparent. Forty-eight percent of patients have Bw22, relative risk 2.8, as well as an absence of Bw12 in "malignant" rheumatoid arthritis (Toyodo *et al.*, 1977).

G. Autoimmune (Idiopathic) Thrombocytopenic Purpura

Among 20 Caucasian patients with idiopathic thrombocytopenic purpura, the frequency of DRw2 was 75%, relative risk 10.0. The borderline significant frequency of the DRw4 × 7 alloantigen (Ia 4 × 7 × 10) was 10% in the patient group compared to 30% in controls (Karpatkin *et al.*, 1979). This is of interest because it is a pattern resembling that of patients with systemic lupus erythematosus although a significant increase in DRw3 or DRw1 was not observed (Table XI). The predominance of Askenazi Jews in the patient population may play a role in the lack of DRw3, if the hypothesis that the DRw3-autoimmune associated gene is characteristic of Northern European Caucasoids. In a non-Askenazi Jew family in which multiple members had idiopathic thrombocytopenic purpura, the occurrence of disease was associated with the haplotype A1-B8-DRw3.

H. Lyme Arthritis

Lyme arthritis is a newly recognized illness characterized by brief but recurrent attacks of asymmetric large joint and oligo articular or migrating polyarthritis in both small and large joints. There are associated neurologic and cardiac abnormalities. The appearance of an antecedent skin lesion, erythema chronicum migrans, is the best clinical marker for the disease (Steere *et al.*, 1979). The geographic distribution of the disease along the coastal plains of New Jersey to Massachusetts parallels the distribution of a tick, *Ixodes scapularis*. There is strong evidence that Lyme arthritis is transmitted by this tick.

A subset of patients develops a chronic proliferative arthritis that resembles rheumatoid arthritis with the formation of pannus and cartilage erosion. However, of studies of 10 patients, 7 were clearly DRw2 and only 4 possessed the Ia 4 × 7 × 10 antigen associated with rheumatoid arthritis. These findings suggest that the pathogenic features of the disease are unrelated to rheumatoid arthritis. The frequency of DRw2 is elevated, uncorrected $p < 0.005$, and suggests the

possibility that the disease susceptibility is associated with DRw2 (Steere *et al.*, 1979).

I. Rheumatic Fever

Among patients with rheumatic fever, the frequency of a B cell alloantigen detected by a single serum, No. 883, was found to be 75% and 71% in two patient series, controls 16% and 17%, combined relative risk 12.9. The conventional DR specificities in the patients were not significantly different from those of the control group (Patarroyo *et al.*, 1979). The specificity of the alloantiserum designated Ia 883 was identified only on one reference homozygous typing cell, KH (see Section VIII). Among some patients and normal subjects, Ia 883 occurred as a third specificity in addition to two known DR allospecificities. Evidence was obtained that the Ia antigens recognized by serum 883 contained Ia determinants recognized by heteroanti-Ia reagents (Laurence *et al.*, 1979) and was present on typical Ia molecules (Wang *et al.*, 1979). This, in concert with the apparent segregation of the molecule within the MHC, suggests that it is the product of a gene within the MHC that is distinct from those controlling the DR specificities.

J. Juvenile Chronic Arthritis

Chronic arthritis with childhood onset comprises a number of distinct subsets primarily distinguished by a series of clinical findings (Schaller, 1977). Two types of juvenile chronic polyarthritis that appear to be interrelated are associated with the presence of HLA-B27 (Rachelefsky *et al.*, 1974). These are (*a*) polyarthritis with sacroiliitis and (*b*) the subset of Still's disease with pauciarticular onset affecting older male children and frequently involving hip joints (Schaller and Wedgewood, 1976; Schaller, 1977).

The distinction of juvenile chronic polyarthritis (previously termed juvenile rheumatoid arthritis) from adult rheumatoid arthritis was emphasized by the finding of a lower than normal frequency of HLA-Dw4 among the patient group (Stastny and Fink, 1979). MLC testing using an HLA-D homozygous patient as stimulator (TMo) revealed that 46% of patients with persistent pauciarticular arthritis bore the determinant TMo. TMo is related to HLA-Dw7/11 and DRw7 in a complex association (Stastny, 1978).

In contrast, a serum relating to DRw3 was found to react with 95% of patients from a different clinic (Gershwin *et al.*, 1977, 1978), whereas other sera with specificities relating to DRw3 were found to react at a

significantly reduced frequency. There are two interpretations of the divergent results between these laboratories. They may simply reflect different proportions of patients in attendance at the two clinics or, more interestingly, point to the presence of two Ia antigens that are together involved in determining susceptibility to juvenile chronic polyarthritis.

K. Disease Association Concepts

The conceptual significance of the various disease associations relates to the biologic function of the MHC in the individual as well as to the selective advantages of particular alleles in evolution. There are two dominant views regarding the origin of the polymorphisms of the entire cell surface glycoproteins of the MHC, in particular those of the Ia system. The first is that the present situation arose principally from the mixing of different populations that were essentially isolated for most of their history and the variations of which represent founder effects and other small-scale selective events (Dausset *et al.*, 1978). A second view emphasizes the adaptive value of these polymorphic genes and attributes selective pressure as being the dominant force in producing the present distribution of genes (Bodmer and Bodmer, 1978).

The various mechanisms of disease association have been the subject of several reviews (Sasazuki *et al.*, 1977; Dick, 1978; Svejgaard *et al.*, 1975; Dausset and Svejgaard, 1977). Among those diseases with primary susceptibility (or resistance) that map in the human *I* region analog, the fundamental question is whether this reflects the action or interaction of one or more IR genes or a linked trait not primarily a part of the histocompatibility system.

Second, if an Ia molecule is itself associated with disease, the question arises whether it is a mutant form distinct from other Ia molecules of the same allospecificity, or whether disease susceptibility is per se a reflection of the presence of the particular allele. Testing of this question will involve a comparison of families with multiple affected members in which the Ia antigen associated with susceptibility at the population level derives from either one or both parents. The ratio of disease in siblings with paternal or maternal haplotypes will provide the required estimate. The fact that insulin-dependent diabetes mellitus is exceedingly rare in any individual with DRw2 suggests that at least in this context the presence of any haplotype bearing DRw2 confers resistance.

Perhaps the most puzzling but potentially important finding that is

emerging from disease associations is the influence of ethnic factors on the allele with which a disease is associated. For example, in the Central and Northern European Caucasian, the series of autoimmune diseases associated with excess female incidence and DRw3 appears to become associated with DRw2 for the population of the Eastern Mediterranean and throughout the Orient. This implies either that the DRw2 determinants are different for the two populations or that a second gene closely linked with the relevant Ia antigen locus is responsible. In the case of these diseases, a likely candidate would be an enzyme that influences the level of estrogens (Lahita *et al.*, 1979). Other diseases, where the relationship is constant among different ethnic groups, favor a concept more related to the Ia alloantigen itself and possibly involving the mechanism of immune responsiveness.

The phenomenon of complementation is relevant to certain normal immune responses and is likely to provide important insights into disease susceptibility at two levels: (*a*) Complementation is the probable explanation for maintenance of the linkage disequilibrium that results in the major characteristic haplotypes and the presence of which permits many of the disease associations: (*b*) The occurrence of two independent Ia alloantigens on one haplotype associated with susceptibility to systemic lupus erythematosus or Sjögren's syndrome can be interpreted as the action of complementary genes resulting in disease.

X. Summary

Three types of antisera are now in use to detect Ia antigens: alloantisera, heteroantisera raised in rabbits or similar species, and monoclonal hybridoma reagents. These antisera all react with a similar 65,000 dalton glycoprotein component that contains the Ia alloantigens and consists of noncovalently bound chains of approximately 37,000 and 28,000 daltons. Available evidence, including antigenic cross-reaction, indicates that the human DR system most closely resembles the murine E/C system.

A striking property of the Ia antigens compared to the HLA-A, -B, and -C segregant series is the differential distribution of Ia on various cell populations. All peripheral blood B lymphocytes with surface Ig have Ia antigens. This is true also of the B cell leukemias and EB virus-transformed lymphoblastoid cell lines. Small percentages of non-T lymphocytes in normal blood with or without complement receptors have Ia antigens. The Ia antigens become undetectable as the B lymphocyte matures into a plasma cell. The pattern of expression of

Ia antigens in the B cell series, as well as in the monocyte series, is characterized by a relatively constant amount of Ia present prior to and during the function of these cells in the immune response. The common type of acute lymphatic leukemia also is Ia positive, but the cells involved have not been proved to be of B cell lineage.

In contrast, only a small percentage of resting T lymphocytes have Ia antigens on their surface. However, following the appropriate inductive event, abundant amounts of Ia antigens are found on T cells. The appearance of the Ia antigens is independent of a blastogenic response, but frequently associated with it. A remarkable feature of this Ia expression is the rapid appearance of Ia on a significant percentage of T lymphocytes in peripheral blood during a minor immune response to agents such as tetanus toxoid. The measurement of Ia antigens holds considerable promise as a method of measuring one aspect of T cell activation independent of blastogenesis. The immunologic significance of these events is a matter of considerable interest. The Ia alloantigens of the activated T cells closely resemble those present on B cells and no direct evidence has as yet been obtained for the existence of a special category of Ia-like antigens selectively present on human T cells.

A third pattern of Ia antigen expression is manifest on hematopoietic cells undergoing either normal or leukemic differentiation. The Ia antigens are found on progenitor cells of the erythroid and granulocyte series; but are not detectable on the pluripotential cell from which they are both derived. The Ia antigens are lost from the cell before reaching functional maturity. The pattern of appearance and disappearance resembles that of T lymphocytes, but in the case of the hematopoietic cells, the function of the Ia molecules is obscure and apparently not related to immune events. The possibility exists that the Ia molecules, through their presence on the cell surface, regulate cell-to-cell interactions that are involved in the events of induction and proliferation. In this sense the control of proliferation would be a feature common to events in the marrow as well as to events in the immune system. The presence of Ia antigens on melanoma and certain other tumors is probably a reflection of this same functional expression.

The molecular and genetic basis of the Ia alloantigen system has been the subject of a rapid expansion in knowledge. B cell lymphoblastoid lines derived from reference individuals who are homozygous for MHC determinants have played an important role in defining the alloantigens, particularly when they are used in immunoprecipitation or cocapping experiments. Evidence has been obtained from a variety of sources that suggests the existence of at least three Ia antigen loci

including that responsible for the defined DR alloantigens. Mapping of these loci from family data is proceeding rapidly, but remains incomplete. The non-DR Ia alloantigens have a polypeptide chain pattern that closely resembles the DR alloantigens.

A remarkable association of the Ia antigen polymorphisms with susceptibility as well as resistance to a variety of diseases has become very apparent. Diseases with little or no association with the classic HLA antigens often show strong Ia antigen system relationships. Included among these diseases are rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, and rheumatic fever. Other diseases, such as multiple sclerosis, diabetes mellitus, and a series of "autoimmune" diseases that were known to have an association with classic HLA antigens, have been demonstrated to have a closer relationship with Ia alloantigens.

It is apparent that the relationship of diseases to the Ia alloantigens is an intricate one likely to involve several distinct factors. In some diseases the association appears to be closely related to the particular Ia alloantigen itself, whereas in others, where the alloantigen associated with the disease changes in different ethnic groups, the possibility arises that factors linked to the Ia antigens are involved.

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Bacterial Endotoxins and Host Immune Responses

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I. Introduction

There has been a long-standing symbiotic relationship between the development of concepts fundamental to both immunology and microbiology. The very profound interrelationship between these two disciplines was underscored by the early recognition that the coexistence of infection and tumor often enhanced the survival of the host (Busch, 1866; Coley, 1898). The immunologic basis for this effect was not appreciated until independent research by microbiologists and biochemists succeeded in defining the biochemical nature of the bacterial products involved, and until immunologists succeeded in defining the cellular mechanisms of immune responses. The analysis of the structure of these immunologically active microbial products has resulted in the biochemical definition of many cell wall components including the extremely potent endotoxin complex. Similarly, a detailed analysis of the cellular basis of immune responses has demonstrated that a highly complex network of lymphoid cells must inter-

act and be self-regulated to produce functional immune responses (Cantor and Boyse, 1977). Therefore it might reasonably be expected that the interaction of this highly complex bacterial product with an equally complex multifunctional cellular network would result in such diverse reactions that they would defy rational analysis. The purpose of this review is to demonstrate that this prediction is not valid. To the contrary, defining the interaction of bacterial endotoxins with immunologically active cells has been one of the major factors contributing to current knowledge of the immune system and of its ability to function as our most potent defense against disease.

Bacterial endotoxins were first defined by their ability to produce fever (pyrogenicity). Thus the early history of endotoxin research characterized its pyrogenic nature. This topic has been admirably reviewed in a recent monograph by Professor Otto Westphal (Westphal *et al.*, 1977).

The demonstration of the potency of these bacterial products was established in the elegant studies reported by Sanarelli (1924) and by Shwartzman (1928). These investigators demonstrated that administration of two doses of a bacterial culture filtrate, a priming and a provocative dose, would elicit multiple pathologic sequelae that were primary to triggering of disseminated coagulation (reviewed in Landy and Braun, 1964; Morrison and Ulevitch, 1978). Since that time, multiple additional biologic activities resulting from direct and indirect toxic effects of endotoxins have been defined and characterized. These biologic activities have been the subjects of a number of recent reviews (Landy and Braun, 1964; Munoz, 1964; Nowotny, 1966, 1969; Weinbaum *et al.*, 1971; Kadis *et al.*, 1971; Kass and Wolff, 1973; Elin and Wolff, 1976; Schlessinger, 1977). The biochemical nature of bacterial endotoxins was first determined by Boivin and Mesrobeanu (1935). The importance of the lipopolysaccharide (LPS) component of endotoxins was first appreciated by Westphal, Lüderitz, and their co-workers, and much of the detailed knowledge concerning the structure of the lipid and polysaccharide has emerged from their laboratory and the laboratory of Professor Fritz Kaufmann.

A. Chemical Composition

Endotoxins are integral components of the outer membrane of gram-negative bacteria. They have not been found in gram-positive cell walls, mycobacterial cell walls, or fungal cell walls. Endotoxins are composed of protein, lipid, and polysaccharide. They were first termed endotoxins to distinguish them from exotoxins, the latter of which are actively secreted into the environment by both gram-

negative and gram-positive bacteria as well as a variety of other pathogens (Pfeiffer, 1892). A variety of extraction procedures have been developed to release and purify endotoxins from the cell wall (e.g. Boivin and Mesrobeanu, 1935; Westphal *et al.*, 1952; Leive, 1965; Galanos *et al.*, 1969; Morrison and Leive, 1975). By far the most widely used have been the trichloroacetic acid (TCA) extraction procedure of Boivin and Mesrobeanu (1935) and the hot aqueous phenol extraction procedure of Westphal *et al.* (1952). The former procedure results in the isolation of molecular complexes of endotoxin containing lipopolysaccharide (LPS) and protein; the latter procedure results in the purification of relatively protein-free preparations of LPS. LPS is composed of a lipid moiety, termed lipid A, and a polysaccharide moiety responsible for the serologic classification of the organism (reviewed in Lüderitz *et al.*, 1968, 1971; Galanos *et al.*, 1977). A detailed analysis of mutant *Salmonella* strains defective in polysaccharide synthesis (R mutants) has resulted in the elucidation of the structure and biosynthesis of this portion of the LPS molecule. The polysaccharide is composed of "core" sugars and specific "side chains" (O antigens).[1] The composition of the core is relatively constant in comparison to the O antigenic side chains (Lüderitz *et al.*, 1971). Similarly, the composition of the lipid A is remarkably constant among various species, and its structure has been defined in detail (Rietschel *et al.*, 1977). In contrast, the protein component of bacterial endotoxins has not been extensively biochemically characterized (Lüderitz *et al.*, 1968).

The terms "endotoxins" and "lipopolysaccharide" are often used interchangeably in the scientific literature. This is, in part, because many of the biologic properties characteristic of bacterial endotoxins can be reproduced by isolated purified lipopolysaccharides. It is, however, incorrect to assume that the more chemically precise lipopolysaccharide term is always preferable to the term endotoxin in defining immunologic responses to this bacterial product. Lipopolysaccharide, a protein-free endotoxin, is rarely, if ever, encount-

[1] The "O" property of gram-negative bacteria was originally used to describe the agglutination of select strains of *Proteus* sp. (Weil and Felix, 1917). These "O" species had the unique property of being transformed from "H" agglutinating strains by heat. *Proteus* species have the ability to swarm on agar with a morphology characteristic of condensation of breath (*Hauch*—"H") on cold glass. This term is now recognized to describe bacterial flagellar antigens responsible for motility. Upon heating, this characteristic was lost and such organisms were described as being without breath (*ohne Hauch*—"O"). This latter terminology is now recognized as defining the lipopolysaccharide (heat-stable) antigens.

ered in the environment. The advantages of using purified LPS or lipid A as molecular probes of lymphoid cell function are apparent. Nevertheless it is equally important to recognize that experiments with more gently extracted endotoxin complexes may, in some instances, more accurately reflect host responses to gram-negative bacteria. In a number of different biologic systems, profound differences have been observed between purified LPS activity and TCA- or butanol-extracted endotoxin activity (Kostka and Sterzl, 1962; Skidmore *et al.*, 1975a; Ulevitch and Cochrane, 1978; Curry *et al.*, 1979). In addition, potent biologic activity independent of and distinct from the biologic activity of lipid A has been found associated with isolated protein components of endotoxin (Sultzer and Goodman, 1976; Morrison *et al.*, 1976; Betz and Morrison, 1977; Morrison and Betz, 1977). In this review the term endotoxin will be used when the preparation has been incompletely specified or the results being discussed are not dependent on the detailed chemical characterization of the preparation. LPS will be reserved for those instances when the authors have made a specific effort to define their preparations or for those experiments in which the nature of the preparation could influence the interpretation of the results obtained.

Another important biochemical consideration in experiments employing endotoxins is that there are significant variations in the carbohydrate composition of purified lipopolysaccharides (Lüderitz *et al.*, 1968, 1971; Galanos *et al.*, 1977b). Although often overlooked as a potentially significant variable, it is important for investigators employing endotoxins to specifiy their characteristics, particularly with regard to polysaccharide composition and serotype. Evidence is accumulating that the polysaccharide is able to modulate the biologic activity of lipid A as well as have some activity of its own, and this may vary considerably between serotypes (Galanos *et al.*, 1971a; Nowotny, 1971; Morrison and Kline, 1977). This variable, in addition to that contributed by the protein component, should be considered in the interpretation of experimental data obtained using different endotoxin preparations.

During the last two decades, several standard endotoxin preparations with well characterized biologic and chemical properties have been prepared (Rudbach, 1971; Lüderitz *et al.*, 1978). The use of such standard reagents or equivalent highly purified well characterized commercially obtained reagents should do much to avoid problems with reproducibility among laboratories using endotoxins. This would be greatly facilitated by the specification of nucleic acid, lipid, carbohydrate, and protein content of such preparations.

B. Biologic Activities

The biologic manifestations of endotoxin interactions with *in vivo* and *in vitro* systems are protean. Thomas' (1974) suggestions that endotoxins are "read by our tissues as the very worst of bad news," and that, in response to these molecules "we are likely to turn on every defense at our disposal," elaborate beautifully the toxic aspect of these macromolecules. This was first defined by Pfeiffer (1892) and further elaborated by Tal and Goebel (1950), who suggested the presence in endotoxin of a "toxic principle." The proof that the potent activities of endotoxin are due to the presence of lipid A awaited the elegant studies of the Freiburg group (Lüderitz and Westphal, 1966; Westphal *et al.*, 1969; Gmeiner *et al.*, 1969). The characterization of rough hepatose-deficient (Re) mutants of *Salmonella* sp., which contained no polysaccharide in their endotoxin but retained virtually all the biologic activity of the parental strains (Kasai and Nowotny, 1967; Kim and Watson, 1967), provided considerable support for this conclusion. The cellular and molecular mechanisms by which endotoxins produce their toxicity have not been fully elucidated and remain a subject of intense study (see Kass and Wolff, 1973; Elin and Wolff, 1976; Morrison and Ulevitch, 1978).

Many of the cellular and molecular changes induced by endotoxins have, however, been characterized, and define an enormous spectrum of host responses. Indeed, the effects are so varied that Ivan Bennett has stated at the opening of the 1964 Rutgers Endotoxin symposium that "an investigator in almost any biological field is likely to obtain a positive result if he tries endotoxin in the experimental system he is using." For the purpose of this review, we will concentrate on the interactions that define host immune responses. We will therefore concern ouselves primarily with the interactions between endotoxin, macrophages, and lymphocytes. The interactions of endotoxins with other important host mediation systems have been the subject of a recent review article (Morrison and Ulevitch, 1978).

C. Immune Responses to Endotoxins

Nossal and Ada (1971) have stated that "evolution designed the humoral antibody system to help vertebrate species cope better with bacterial and viral pathogens." Although recent experimental evidence clearly indicates the important role that cellular immunity plays in defending against viral infections (Zinkernagel, 1979), there should still be no serious argument with the essence of this quotation. Bacteria

are extremely potent antigens. It has been found that a single injection of 10^{-15} gm of *Salmonella* O antigen is sufficient to immunize an animal (Nossal and Ada, 1971). Only several hundreds of molecules of LPS are sufficient to elicit a secondary response in an animal primed with LPS 14 days earlier (Rudbach, 1971). These data support the concept that bacterial antigens are among the most powerful antigens yet described and provide convincing experimental evidence for the suggestions of Nossal and Ada (1971) that "a perfect system for antigen handling would, on teleological grounds, be geared primarily toward small particulate microbes, present in relatively low total doses in the tissues, and toward the endo- and exotoxins produced by such microbes."

Immune responses to endotoxins may occur via the humoral system, resulting in the production of specific antibody. Such antibody could promote clearance of the endotoxin by the reticuloendothelial system or sensitize an endotoxin containing microbe to complement mediated lysis or phagocytosis. An antigen-specific cellular mechanism by which immunity to microbes is enhanced has been delineated as a result of the elegant studies of Mackaness (1964). Alternatively, an immune response may be mediated by cellular mechanisms, not involving antigen binding, but resulting in the production of activated cells that would aid in the phagocytosis and destruction of endotoxin containing microbes.

Another well studied aspect of the effect of endotoxins on the immune system has been their capacity to initiate nonspecifically the synthesis and secretion of antibody by immunocompetent lymphocytes in the absence of specific antigen. This phenomenon, termed polyclonal B cell activation (PBA)[2] by Möller and his colleagues, has become one of the hallmark features of endotoxin and LPS interactions with B lymphocytes. Information gained during the past decade from detailed analyses of the mechanisms of such interactions has been central to the formulation of current concepts of B cell activation and regulation. Antigen-dependent regulation of immune responses by endotoxins, (adjuvant activity, immunologic tolerance) were recognized as immunologic phenomena about 20 years before the specific

[2]The term, polyclonal B-cell activation (or activator) has gained popular acceptance in recent years as a generalized concept used to describe all interactions leading to nonspecific activation of B lymphocytes. As a number of distinct B-cell responses may be elicited by endotoxins, we have elected, for the purpose of this review to indicate the specific measurement used to assess B-cell activation rather than to refer to such responses as PBA.

recognition of endotoxins as activators of B lymphocytes. The research of the last 10 years has defined to a large extent the cellular mechanisms of, as well as the interrelationships between, these immunologic manifestations of bacterial endotoxins. The discovery and subsequent characterization of genetically unresponsive strains of mice has contributed significantly to these studies.

D. Limitations of this Review

In this review we will summarize the pertinent literature dealing with the interaction of bacterial endotoxins and purified lipopolysaccharides with macrophages and lymphocytes. Both *in vivo* and *in vitro* responses will be considered.

We will review recent research on the humoral immune response to lipopolysaccharide antigens. This will be done to introduce the complexity of these molecules on an immunologic basis and to provide a background for Section VII, in which biomedical applications of endotoxins will be discussed. However, the major focus of this review will be to summarize the potent effects that endotoxins manifest in the regulation of antibody responses to antigenic determinants unrelated to the endotoxin molecule.

In the field of cellular immunology, endotoxins have served primarily as probes of B lymphocyte activity in the mouse. While this area will be covered in some detail, it should be emphasized that we are not attempting to review concepts of the mechanism of B lymphocyte triggering. This topic has been the subject of numerous recent reviews and commentary to which the reader is directed (Coutinho and Möller, 1974, 1975; Cohn and Blomberg, 1975; Möller *et al.*, 1976a). Neither will an exhaustive attempt be made to compare the activities of endotoxins or lipopolysaccharides to other lymphocyte activators. We will, however, endeavor to describe in some detail the advances made in our understanding of macrophage and lymphocyte function through the use of these agents.

The biochemistry and serology of lipopolysaccharide antigens will not be discussed in this review. These important topics have been considered in some detail recently (Lüderitz *et al.*, 1966, 1968, 1971; Galanos *et al.*, 1977b) and may not be pertinent to much of the research to be discussed. Thus it is hoped that the reader will appreciate that much of the value of bacterial endotoxins to immunologists is based on the careful and elegant work of our biochemical colleagues.

Another significant area of endotoxin research that will not be covered in detail is the literature that has defined and characterized the mechanisms of endotoxic activity in the host. While it is recognized

that this represents some of the most pertinent research from a medical viewpoint, it could not be justly treated here. The reader is referred to recent reviews that address this topic (Elin and Wolff, 1976; Morrison and Ulevitch, 1978).

The final significant limitation that will be imposed in this review is that we will largely restrict ourselves to the literature since 1968. This will be done for two reasons. First, the literature is so vast that to consider in detail the earlier work would have made this task unwieldy. Second, there appeared in 1969 two excellent reviews on endotoxins in immunology to which the reader is directed for a discussion and references to the earlier literature (Neter, 1969; Nowotny, 1969). We will, of course, refer to selected earlier papers when it is deemed appropriate to the topic under discussion. Because of the widespread use of endotoxins as immunologic probes of lymphocyte function, there are undoubtedly relevant references pertinent to this review which, in spite of our efforts, have not been cited. We hope that these oversights have been infrequent.

II. Host Immune Responses to Endotoxins

Bacterial endotoxins, which are characteristically localized on the outer surface of gram-negative bacteria (Mergenhagen *et al.*, 1966; Shands, 1966; Dahlen *et al.*, 1978), represent major antigens in the host immune response to these infectious organisms. Consisting of polysaccharide, lipid, and protein, these antigens may elicit antibody with a number of diverse specificities. In spite of this potential antigenic diversity, however, the preponderance of evidence supports a primary immunodominant role for the specific polysaccharide component of the lipopolysaccharide portion of the endotoxin complex. First recognized as a specific soluble carbohydrate present in strains of smooth, but not of rough, typhoid bacilli (White, 1927), this immunogenic somatic antigen of the intact bacteria was firmly established by Landy and co-workers as being immunologically identical with purified, protein-free LPS (Webster *et al.*, 1955; Landy *et al.*, 1955). It is now generally recognized that the active immunization of man or experimental animals with either bacterial vaccines or purified endotoxins leads to the production of specific antibody directed mainly against the chemically diverse repeating oligosaccharide determinants of the LPS molecules (reviewed in Lüderitz *et al.*, 1966, 1968, 1971).

Because of this high degree of correlation between the antibody response to bacterial vaccines (formalin-treated or heat-killed bac-

teria) and to isolated endotoxins/LPS, it is attractive to consider these antigens as being immunologically interchangeable. There is, however, considerable evidence to indicate that host immune responses to intact bacteria are not always characteristic of the response to the isolated endotoxins. As the major emphasis of this review is on the immunologic properties of endotoxins, we will limit our discussion of bacterial vaccine-elicited immune responses to those studies that illustrate important similarities or differences in comparison with the isolated endotoxins.

As described above, the experiments of Landy and co-workers established that isolated endotoxins could serve as immunogens to elicit antibody responses specific for the somatic antigens of the intact organism. Additional comprehensive studies by these investigators (e.g., Landy *et al.*, 1965a,b; Sorkin and Landy, 1965) have contributed significant information on the characteristics of the endotoxin-elicited immune response. Of particular interest was the report by Landy and Baker (1966) demonstrating the extraordinary immunogenicity of these molecules. These authors provided data to suggest that injection of about 1000 molecules of purified endotoxin into rabbit popliteal lymph nodes was sufficient to elicit a primary immune response to the polysaccharide. Similarly Rudbach (1971) has established that as few as several hundreds of molecules of endotoxin, administered systemically, will prime mice for a detectable enhanced secondary response to endotoxin challenge. On the basis of these data, Rudbach (1971, 1976) has classified these molecules as "super antigens." In this section we will summarize recent experiments that have explored the molecular and cellular requirements that may be responsible for the potent immunogenicity of bacterial endotoxins.

A. Characteristics of the Response

1. Factors Affecting Immune Responses to the Polysaccharide

As indicated, endotoxins contain, in addition to the immunogenic polysaccharide component, both covalently bound lipid (lipid A) and noncovalent protein (lipid A-associated protein, LAP). Each of these components has the potential capacity to affect the immune response to the polysaccharide. In this respect, procedures have been utilized to modify selectively these components and subsequently analyze immune reactivity of the treated endotoxin. It is readily apparent that treatment of endotoxins to modify the polysaccharide, e.g., by periodate (Neter *et al.*, 1956) or succinate (McIntire *et al.*, 1967), abrogates the capacity to elicit specific antibody.

Attempts to modify selectively the lipid A component of endotoxins have been, at least in part, based upon the knowledge that toxicity of these molecules is a property of the lipid A region. Neter *et al.* (1956) reported that mild alkaline hydrolysis retained antigenicity of endotoxins but abrogated many of the toxic properties. Evidence that such endotoxins were immunogenic has been provided by a number of investigators (e.g., Britton, 1969a; von Eschen and Rudbach, 1976). Similar studies by Noll and Braude (1961), using $LiAlH_4$ to detoxify endotoxins indicated significant losses of ester-bound fatty acids without loss of immunogenicity. In addition, treatment of endotoxins with potassium methylate (Nowotny, 1968) led to detoxification without loss of antigenicity. Finally hydroxylaminolysis, which also selectively removed lipid A-bound fatty acids, was found to reduce only slightly antibody-neutralizing capacity (McIntire *et al.*, 1967). Thus, intact lipid A, which is necessary for the expression of toxic activities of endotoxins, does not appear to be required for immunogenicity of these molecules. Qualitative as well as quantitative differences, however, have been noted in the responses obtained. These will be discussed below.

Experiments utilizing a native protoplasmic polysaccharide (NPP) (Anacker *et al.*, 1964) have provided an additional means of establishing the lack of a requirement for lipid. This molecule may be isolated from the protoplasm of a number of gram-negative organisms (Anacker *et al.*, 1966) and is, with the exception of a complete absence of lipid A (Rudbach *et al.*, 1967), structurally and immunologically identical to the polysaccharide of the LPS portion of the endotoxin isolated from the same organism. The capacity of this lipid A-free polysaccharide to elicit a primary immune response in mice (von Eschen and Rudbach, 1974) would support the concept that lipid A is not necessary for immunogenicity of endotoxin (see, however, Section II,C). Of importance, however, is that NPP was not immunogenic in all species in that, although mice and rats were responsive, neither guinea pigs nor rabbits generated antibody to NPP.

Considerably less information is available on the role of LAP in modulating polysaccharide immunity. Leive *et al.* (1968) reported that EDTA-extracted endotoxins (containing 10% protein) were equal in antigen content to protein-free LPS prepared from the same organism; yet the former preparation was significantly more immunogenic in rabbits. More recently, Hepper *et al.* (1979) and D. C. Morrison and B. J. Curry (unpublished observations) have found little differences in primary immune responses of mice to protein-containing or protein-free endotoxins. However, Hepper *et al.* (1979) have established that

the presence of LAP is a highly significant factor in priming mice for a secondary challenge with endotoxin. The fact that the priming was due to protein was suggested by the abrogation of activity by proteolytic enzyme treatment or phenol extraction. The full spectrum of effects of LAP on endotoxin immunogenicity remain to be defined.

Several reports have demonstrated that pretreatment of mice with one bacterial serotype could enhance the secondary response to an immunologically unrelated serotype (Ahlstedt and Holmgren, 1975; Ahlstedt and Lindholm, 1977). There is much evidence to suggest that the increased immune responsiveness to the unrelated bacteria was induced by a protein component associated with the endotoxin. Thus in the intact organism, LAP may well serve as an additional immunogen.

As endotoxin complexes exist as high molecular weight aggregates, and are often particulate, several investigators questioned the effect of molecular weight on endotoxin immunogenicity. Early studies had shown that acid hydrolysis, which releases a low molecular weight hapten, resulted in loss of immunogenicity (Freeman, 1942). As an alternative approach, Tarmina *et al.* (1968) reported that deoxycholate-dissociated endotoxin of 20,000 molecular weight significantly reduced the plaque-forming cell response in rabbits. These results were not confirmed by Jackson (1969), who reported no loss of immunogenicity when such dissociated endotoxins were injected into mice. These latter results were also confirmed by Rudbach (1976). The differential relationship of these murine and rabbit responses to deaggregated endotoxins and to NPP remains as an interesting speculation. With regard to the effects of molecular weight on immunogenicity of endotoxins in rabbits, Neter *et al.* (1973) have reported marked loss of immunogenicity without concomitant loss of antigenicity in endotoxin or bacterial preparations heated to 100°C for 60 minutes. Immunogenicity could be restored by storage at 4°C or several cycles of freezing and thawing. These authors suggested a reversible aggregation/deaggregation mechanism as being responsible. It is noteworthy that a recent study has suggested conformational changes in gram-negative organisms as the result of freeze–thaw procedures (Kempler and Ray, 1978).

As will be discussed in detail in Section IV, covalent binding of haptens has also been used to modify endotoxin structure. Of interest, however, has been the recent demonstration that such haptenation, while not altering the antigenicity of the polysaccharide, can have marked effects on the *in vivo* immune response of some, but not all, strains of mice to the polysaccharide (Skelly *et al.*, 1979a). Reduced

responsiveness is independent of hapten or endotoxin but is limited to the primary immune response to the polysaccharide. The mechanism responsible for this reduced responsiveness has been shown to be under genetic control (Skelly *et al.*, 1979c) and controlled by both sex-linked and *H-2* region genes. (A contrasting lack of correlation with *H-2* region genes was earlier noted by Cerny *et al.* (1971) in an analysis of murine responses to *Vibrio* antigens.)

Genetic restriction in response to *Salmonella* LPS polysaccharides has been reported in an extensive series of studies by Di Pauli (1972, 1973, 1975, 1976, 1977). Selected mouse strains were shown to produce antibody to one LPS serotype which cross-reacted with other serotypes whereas antibody from other murine strains did not. Genetic analysis of backcross matings indicated that cross-reactivity was inherited as a single autosomal gene subject to allelic exclusion. Of importance is that this combining site marker was not linked to the heavy-chain allotype. Further, no correlation in the immune responses to LPS was observed in individual hyperimmunized mice when cross-reactivity patterns of IgM and IgG were compared.

More substantial differences in immune responsiveness to endotoxins are apparent in the C3H/HeJ (endotoxin unresponsive) mouse strain (see Section V) and the CBA/N (B lymphocyte maturational defect) mouse strain. The former mouse shows markedly reduced antibody titer and PFC responses to endotoxins (Watson and Riblet, 1974; Skidmore *et al.*, 1975a; Rudbach and Reed, 1977). This is not the result of an inability of C3H/HeJ lymphoid cells to recognize and respond to endotoxin (Section V). The CBA/N mouse is characterized by an altered immune response to a number of antigens that do not require T cells (see below and Section V). In addition, several murine strains, e.g., SJL/J (Smith, 1976) and NZB (Blankwater *et al.*, 1975), display a temporal decrease in immune responsiveness to endotoxin over a 1-year period in contrast to most strains, which are characterized by high responsiveness during their entire adult life (Smith, 1976).

2. *Antibody Responses to Lipid A*

The immunogenicity of the lipid A component of endotoxins has been the subject of a number of investigations. D. Watson and Kim (1963) suggested that intact LPS did not elicit antibody to lipid A; however, data were presented to indicate that the isolated lipid A was, itself, immunogenic. Support for these conclusions was provided by the extensive experiments of Galanos *et al.* (1971a), who postulated that antibody responses to lipid A were suppressed when immunization was carried out with purified LPS. Using heptose-deficient Re

mutants, some antibody responses to lipid A were elicited in rabbits; however, such responses were enhanced significantly when the Re bacteria were acid hydrolyzed to remove KDO and subsequently absorbed with free lipid A. The antibody to lipid A was shown to cross-react with lipid A isolated from a number of bacteria and to a variable extent with the intact LPS and bacteria, and to cause complement-dependent lysis of lipid A-coated liposomes (Kataoka *et al.*, 1971). Indirect evidence for cross-reactivity was suggested by Rietschel *et al.* (1973). Galanos *et al.* (1971a) further showed enhanced secondary responses to lipid A and the generation of both IgM and IgG antibody. Enhanced secondary antibody responses to lipid A were not, however, observed by Mita *et al.* (1977).

Several more recent investigations, however, have suggested that antibody to lipid A does not cross-react with the parent endotoxins or with the intact bacterium (Lugowski and Romanowska, 1974; Mattsby-Baltzer and Kaijser, 1979). These latter investigators also confirmed the lack of significant antibody responses to lipid A in rabbits immunized with a variety of smooth *Escherichia coli* serotypes, although, like Galanos *et al.* (1971a), they did observe such antibody in rabbits immunized with rough serotypes. It is of interest that Lugowski and Romanowska (1974) reported no loss of antigenicity of lipid A treated with hydroxylamine, suggesting that the amide-linked fatty acids (in particular 3-OH myristate), but not ester-linked fatty acids, were the immunodominant groups—a conclusion that supports the earlier suggestion of Lüderitz *et al.* (1973).

3. *Lymphoid Cells Responding to Endotoxins*

Most of the earlier studies on the generation of antibody in response to endotoxins assessed the production of serum antibody, which was detected by agglutination of bacteria or endotoxin-coated erythrocytes (Neter *et al.*, 1956). Additional modifications of this technique included agglutination of endotoxin-coated bentonite particles (Wolff *et al.*, 1963; Diena *et al.*, 1963). Immunodiffusion techniques (Holmgren, 1970a) have also been utilized. The use of the plaque-forming assay to assess antibody production by individual lymphoid cells, as adapted to antibody responses to endotoxins (Landy *et al.*, 1965a; Möller, 1965; Michael, 1966; Friedman, 1966) and modified to assess antibacterial antibody (Friedman *et al.*, 1969), has contributed significantly to the further delineation of immune responses to endotoxins.

As demonstrated by Landy *et al.* (1965b) in rabbits and Möller (1965) in mice, the injection of endotoxins results in the appearance in the spleen of lymphocytes synthesizing and secreting antibody (PFC)

to the polysaccharide component of the endotoxin. Detectable increases in PFC were noted as early as 2–3 days after injection, with peak responses at days 4 and 5. Although PFC responses decreased after the peak responses, the number of PFC in neither case returned to background levels. Secondary challenge with endotoxin led to a second rise in PFC; however, the ability to detect enhanced secondary responses was dependent upon the level of the initial response. The results of these and additional studies (Landy *et al.*, 1965a; Sorkin and Landy, 1965) also reported PFC to endotoxin in peripheral blood lymphocytes, lymph nodes, and thymus; in the thymus, however, the evidence suggested that the PFC were the result of infiltration by antibody-forming cells from other sites. A more detailed temporal analysis of splenic PFC formation indicated an initial drop in PFC following injection of endotoxin at 3 hours, a return to normal levels at 18 hours, and enhanced PFC at days 2–3 (Michael, 1966). Further, the kinetics of appearance of PFC was demonstrated to depend on the form of antigen injected, (e.g., bacterial vaccine vs. purified endotoxin) as well as the lymphoid organ examined (e.g., spleen vs. lymph node) (Field *et al.*, 1970).

The results of recent experiments by Benner and van Oudenaren (1976) have shown in detail the appearance of PFC following intravenous injection of endotoxin. The major primary response to low amounts of endotoxins was detected in spleen and later in the bone marrow; however, secondary responses with equivalent amounts led to detectable PFC in peripheral blood, lymph node, Peyer's patches, and thymus, as well as spleen and lymph node. Additional experiments by these authors (Benner and van Oudenaren, 1977), using splenectomized mice, have established that bone marrow cells generate *in situ* a PFC response to endotoxin independent of the spleen. These data also suggested that, following primary immunization, B lymphocyte memory cells migrate to the bone marrow and contribute to the response detected in that organ following secondary challenge with endotoxin.

The ability of the host to maintain enhanced PFC responses after endotoxin challenge [for periods as long as 1 year (Landy *et al.*, 1965b)] may well reflect the capacity of this antigen to be maintained in an immunogenic form in lymphoid tissues for extended periods. This was elegantly demonstrated by Britton *et al.* (1968), who immunized mice with endotoxin followed by lethal irradiation and adoptive transfer of nonimmune syngeneic lymphoid and bone marrow cells. The ability of the donor cells to respond with specific PFC to the endotoxin as long as 45 days after transfer attests to the long maintenance of antigen

in an immunogenic form in these animals. The use of radiolabeled endotoxins by Friedman (1971) and Rudbach (1976) has provided additional evidence for the presence of residual endotoxins in spleen as late as 3 weeks and 12 weeks, respectively, following endotoxin immunization. Given this unique capacity of endotoxins to be maintained in the host in an immunogenic form, the potent immunogenicity of endotoxins, and the relative ubiquitous nature of gram-negative organisms in association with mammalian species, it would not be surprising to find broad-spectrum antibody to these organisms in the sera of most individuals. Such "natural" antibody to endotoxins of both aerobic and anaerobic gram-negative organisms have been defined (Michael and Rosen, 1963; Hofstad, 1976).

The systemic administration of endotoxin leads to antibody production in a variety of lymphoid organs. Selective stimulation of antibody production in lymphoid organs can also be demonstrated following local administration of endotoxins. For example, the injection of low amounts of endotoxin into rabbit footpads leads to high levels of PFC in the stimulated popliteal lymph node and the spleen; however, markedly lower PFC are detected in the contralateral lymph node (Landy and Baker, 1966). Similarly, oral immunization of *E. coli* vaccines results in antibody secretion into saliva with little detectable antibody in serum (Ebersole and Molinar, 1978), and intranasal but not intramuscular immunization with *Pseudomonas* endotoxin generates antibody to endotoxin in bronchial secretions. As will be discussed in the following subsections, such selective immunization pathways also regulate the antibody isotypes synthesized and secreted in response to the endotoxin.

4. Classes of Antibody Responses to Endotoxins

In many of the ealier studies designed to examine the characteristics of the serum antibody generated by the host in response to endotoxin, administered either as bacterial vaccine or as the isolated molecule, considerable emphasis was placed upon the 19 S IgM antibody response. As reported by Landy *et al.* (1965b) the primary response of rabbits to isolated endotoxin was dominated by IgM antibody, with less than 1% of the response as IgG. Similarly, Möller (1965) reported that the murine primary response to soluble purified LPS was entirely 19 S antibody, even when the LPS was injected in complete Freunds' adjuvant. A later report by Britton and Möller (1968), however, suggested that 7 S IgG antibody responses could be elicited after repeated hyperimmunization. These experiments also reported cyclical appearances of serum antibody, which the authors attributed to feed-

back suppression of antibody synthesis by antibody. These results were essentially in agreement with the earlier studies of Pike and Schulze (1964), who indicated that, with a bacterial vaccine, 19 S antibody was predominantly synthesized even under conditions of repeated immunization. Essentially similar results were obtained by Field *et al.* (1970) using both isolated endotoxin and heated bacteria to immunize mice, and by Holmgren (1970b) using heated bacteria to immunize rabbits. In this latter study, secondary responses or responses to repeated injections lead to significant IgG antibody production. These data would be consistent with the conclusion that both IgM and IgG antibody may be elicited in response to endotoxin antigens depending upon the immunizing regimen, the form of the antigen, and the species of animal immunized. The following paragraphs will illustrate several points relevant to this conclusion.

The experiments of Barasoain *et al.* (1978) would indicate that the dose of endotoxin plays a relevant role in the type of antibody response elicited. In these experiments low doses of *Pseudomonas* endotoxin led to both IgM and IgG production although the temporal responses were different. High doses resulted only in the formation of IgM. This result appears somewhat surprising in light of the observations (Michael and Rosen, 1963; Hofstad, 1976) that most "natural" antibody to gram-negative organisms present in human serum is IgM. Nevertheless, as suggested by Dahlen *et al.* (1978), using two anaerobic bacteria to hyperimmunize rabbits, whereas both IgM and IgG responses to *Bacteroides oralis* could be detected, only an IgM response to *Fusobacterium* was elicited.

The experiments reported by Nowotny (1968) examined the antibody isotype elicited in rabbits to three distinct forms of a single endotoxin antigen including heated bacteria, purified endotoxin, and endotoxin detoxified by potassium methylate. The isolated endotoxin elicited almost exclusively IgM antibody, whereas repeated injections of the detoxified endotoxin led to primarily IgG antibody. In contrast, both types of antibody were produced in response to the heated bacteria. Similarly, Ahlstedt and Holmgren (1975) demonstrated that whole bacteria will elicit both IgM and IgG responses, but purified endotoxins produced only IgM. Of interest in these studies was the observation that endotoxin would prime for a secondary IgG response to challenge with whole bacteria but not for challenge with endotoxin. These authors correctly noted, however, that the use of whole bacteria for immunization and crude preparations of antigen for detection of antibody-forming cells makes difficult the exclusion of antibody responses to other bacterial antigens. These authors did, in

fact, subsequently provide experimental evidence for such responses (Ahlstedt and Lindholm, 1977).

An important role for lipid A-associated protein (LAP) in the secondary murine IgG responses to endotoxins has been suggested by the recent experiments of Hepper *et al.* (1979). These investigators noted markedly enhanced secondary IgG responses to endotoxins containing LAP. As Di Pauli (1977) has indicated an essential role for T lymphocytes in the murine IgG response to bacterial antigens, and as no antigen-binding T-cells have been detected for the purified LPS antigen (see Section II,C), this might suggest a role for LAP-specific T-helper cells in the IgG response to endotoxin antigens. Direct evidence for this concept will, however, await the results of future experiments.

Similar results using endotoxin to prime for secondary IgG responses were obtained by Friedman (1975) in murine responses to *Vibrio* antigens. The importance of the temporal relationship of the primary to the secondary dose of antigen with respect to IgG responses was indicated by the experiments of Ahlstedt *et al.* (1973). These authors indicated that a 10-day interval between antigen doses was not sufficient to allow an immediate secondary IgG response; however, 30 days was sufficient.

Analysis of the subclasses of IgG elicited in response to endotoxin antigens have defined distinct responses. Jackson and Walters (1972) reported that primary murine immune responses to intraperitoneal injection of endotoxin included an early IgM response followed by IgG_{2a} and IgG_{2b}, but not IgG_1 antibody. Secondary challenge led to synthesis of all three IgG subclasses. Similar results were obtained by Kateley *et al.* (1975), although these investigators did detect primary IgG_1 subclass antibody. Support for this latter conclusion was provided by the experiments of English *et al.* (1976) using an interesting experimental system in sheep. These investigators cannulated the afferent and efferent lymphatics, which were infused with endotoxin and [^{14}C]leucine. Analysis of the efferent lymph fluid indicated the early synthesis of IgM followed by both IgG_1 and IgG_2 antibody, which the authors reported to be the principal antibody secreted.

There is also good evidence to indicate that IgA antibody, but not IgE antibody, may be elicited in response to endotoxins and/or bacterial vaccines. With regard to the former antibody, while some reports have suggested IgA antibody in response to normal (e.g., intravenous, intraperitoneal) immunization protocols, most studies have utilized either oral or intranasal immunization and subsequent detection of antibody in secretory fluids. In this respect, antibody responses to

endotoxins of the IgA isotype have been detected in man (Rossen *et al.*, 1967), rabbits (Walters and Jackson, 1968; Eddie *et al.*, 1971; Reynolds *et al.*, 1974), and mice (Fubara and Freter, 1973; Ebersole and Molinari, 1978) as well as in pigs (Porter *et al.*, 1974). Neither isolated endotoxins nor bacterial vaccines have been demonstrated to elicit specific IgE antibody in mice (Danneman and Michael, 1976). Similarly, no IgE response was obtained when haptenated bacteria were used as immunogens (Shinohara and Tada, 1974).

B. Immunologic Tolerance to Endotoxins

Immunologic tolerance to bacterial endotoxins is characterized by the inability of antigen-specific B lymphocytes to respond to endotoxins by the synthesis and secretion of antibody directed against the endotoxin molecule. It is important to distinguish *immunologic* tolerance (unresponsiveness) to endotoxin from the more classically defined endotoxin tolerance. The latter form of tolerance is characterized by a decreased responsiveness of the host to the potential deleterious effects of endotoxins (see Section VII,B).

1. Neonatal Induction of Tolerance to Endotoxins

Experiments reported by Friedman (1966) established that injection of multiple low doses of a *Shigella* soluble antigen into neonatal mice resulted in the establishment of immunologic tolerance. This was demonstrated by an inability of such mice to respond to subsequent challenge with *Shigella* bacterial vaccines, as assessed either by serum titers of hemagglutinating antibody or hemolytic PFC. On the basis of these experiments it was concluded that lymphocytes capable of forming specific antibody in the tolerized mice were absent or were present only in low numbers. Evidence for specific participation of spleen cells in the tolerant state was shown by adoptive transfer of tolerance into irradiated recipients (Friedman, 1971). These studies further indicated that immunologic tolerance to *Shigella* antigens persisted for about 8–12 weeks.

Similar studies have also been described using a purified *E. coli* endotoxin (Allen and Friedman, 1970, 1971). In these experiments, injection of endotoxin into control adult mice led to the formation of hemolytic PFC as well as bacterial PFC (assessed by adherence of viable bacteria to splenic lymphoid cells of immunized mice). Induction of neonatal tolerance with the *E. coli* endotoxin led to a significant suppression of the hemolytic PFC upon subsequent challenge with an *E. coli* vaccine; however, the bacterial PFC response was essentially unaffected. These data suggested that minor antigens on the endotoxin

molecule also had the capacity to stimulate antibody formation and indicated the important finding that tolerance to major somatic endotoxin antigens may coexist with a concomitant immunity to other bacterial antigens present in the bacterial extracts used either for tolerance induction or antigen challenge.

2. *Adult Induction of Tolerance to Endotoxin*

The extensive experiments reported by Britton (1969a,b,c) defined and characterized the induction of immunologic tolerance to endotoxins in adult mice. Using a single injection of relatively high (10 mg) quantities of alkaline detoxified endotoxins, or multiple injections of lower doses, immunologic unresponsiveness to subsequent challenge with bacterial vaccines was established. However, unlike neonatal tolerance, adult tolerance to endotoxin was short-lived, with responsiveness (characterized by the presence of indirect as well as direct PFC) reestablished within 36 days. Tolerance to endotoxin was shown to be rapidly established. Injection of immunogenic doses of bacteria as early as 1 hour after a tolerant dose of endotoxin did not reverse tolerance. *In vitro* studies clearly demonstrated tolerance induction by incubation of spleen cells with endotoxin prior to adoptive transfer into irradiated mice. Optimal conditions required a 2-hour incubation at 37°C, although shorter times of incubation and 4°C were partially effective. Tolerance induction was reversed by treatment of spleen cells with trypsin prior to addition of endotoxin, suggesting a role for specific surface immunoglobulin.

An extension of these studies by Sjöberg and collaborators provided additional information about the endotoxin-tolerant state. Sjöberg and Möller (1970) reported that, although PFC to endotoxin in spleens of tolerant mice were markedly reduced, endotoxin binding cells (as assessed by rosette-forming cells, RFC) were significantly enhanced by comparison with unimmunized mice. The demonstration by Sjöberg (1971) that the RFC could be inhibited by free endotoxin or by rabbit anti-mouse Ig indicated that the binding was antigen specific. It was also reported (Möller *et al.*, 1971) that low zone tolerance to endotoxin was never established, which was suggested to correlate with the lack of demonstrable T cells having the capacity to bind endotoxin. An interesting set of experiments by Sjöberg (1972b) indicated that immunologic tolerance to endotoxin could be rapidly abrogated by adoptive transfer of tolerant spleen cells into irradiated recipients. Five days after transfer, immune responsiveness to bacterial vaccines was at normal levels. Equivalent regeneration of responsiveness could be established by *in vitro* culture of tolerant spleen cells for 20 hours prior to adoptive transfer into irradiated recipients. Sjöberg con-

cluded from these studies that the B cells were reversibly inactivated and that they had the capacity to be reactivated upon removal to a neutral environment.

C. Cellular Mechanisms of Immune Responses to Endotoxins

One of the most significant contributions of endotoxin immunobiology to current concepts of cellular immunology has developed from the demonstration that immune responses to endotoxin polysaccharide antigens do not require the participation of T lymphocytes. The classic experiments of Möller and Michael (1971) and Andersson and Blomgren (1971) independently established that adult thymectomized lethally irradiated mice reconstituted with bone marrow cells elicited perfectly normal immune responses to isolated endotoxins as well as heated bacteria. In contrast, immune responses to the T cell-dependent antigen, sheep erythrocytes, were markedly reduced. As an additional control against possible T cell function, Andersson and Blomgren (1971) utilized fetal liver cells to reconstitute irradiated recipients which, the authors reported, gave equivalent responses. These results established that endotoxins could indeed be classified as T-independent antigens.

The results of similar adoptive transfer experiments by Veit and Michael (1972a) provided data in support of these conclusions. Additional experiments by these investigators, and by Barth *et al.* (1973), using antibodies directed against murine T lymphocytes, also showed no adverse effects on antibody responses to endotoxin antigens under conditions where heterologous erythrocyte immune responses were almost completely abrogated. These data suggested that T cells neither enhanced nor suppressed the B cell response to endotoxin antigens. Similarly, *in vitro* treatment of spleen cells with anti-Thy-1 and complement prior to culture with either heterologous erythrocytes or endotoxin led to inhibition of the immune response to the former, but not the latter, antigen (Sjöberg, 1972a). In contrast to the *in vivo* data, Sjöberg in fact noted some enhancement in the PFC response to endotoxins. Another report by Veit and Michael (1972b) showed that adoptive transfer of spleen cells, previously treated *in vitro* with antigen and syngeneic serum, into irradiated recipients followed by antigen challenge led to reduced sheep erythrocyte immune responses, but no effects on the response to the bacterial antigen. As the syngeneic serum was shown to act selectively to inhibit T cell function, these experiments thus provided additional evidence for T cell independence of the response to endotoxin.

A series of experiments published by Reed and his co-workers, which demonstrated immune responses to endotoxins in congenitally athymic (Nu/nu) mice, was also instrumental in establishing the T-independence of the endotoxin immune response. As initially reported by Manning *et al.* (1972) immune responses to endotoxin, as measured by both splenic PFC and hemagglutination titers, were identical in both Nu/nu and heterozygous littermates. As the Nu/nu mice have no functional T cells, the T-independence of the response was clearly indicated. These results were also obtained when *in vitro* immune responses were examined (Aden and Reed, 1973). Further studies by these investigators (Reed *et al.*, 1973) demonstrated no differences between Nu/nu mice and normal littermates in secondary responses to endotoxins following an initial primary dose.

The experiments described above established that T cells were not essential for the induction of B cell antibody synthesis to endotoxin. To assess whether stimulation of T cells could mediate B cell responses to endotoxin, Poe and Michael (1975) employed several experimental systems to generate activated T cells. Mice were injected simultaneously with antigens and allogeneic spleen cells to initiate T cell activation (GVH responses) and stimulation of allogeneic effect factor (AEF). As assessed by PFC on day 6, AEF significantly enhanced the sheep erythrocyte PFC response, but had no detectable effect on the PFC response to endotoxin. Similarly, *in vitro* culture of mixed spleen cells to stimulate the mixed lymphocyte reaction also had no effect on the PFC response to bacterial antigen. This lack of demonstrable effects by activated T cells on immune responses to endotoxins further attests to the T-independence of this antigen.

As originally reported by Möller (1971), however, prior stimulation of GVH responses in mice significantly suppresses the subsequent capacity of host spleen cells to respond immunologically to *in vivo* stimulation with endotoxin or erythrocytes. Sjöberg (1972a) showed that GVH-stimulated spleen cells were equally incapable of responding to bacterial antigens *in vitro*. A potential role for adherent cells was suggested. It should be pointed out, however, that removal of adherent cells from normal or Nu/nu cultures of spleen cells has no effect on the PFC response to bacterial antigen. More recent experiments by Treiber and Lapp (1978) have provided data indicating that the reduced responsiveness to endotoxins of B lymphocytes from mice undergoing a GVH reaction can be overcome following multiple antigenic challenge with endotoxins. These data suggested that the suppression of B cell activity may be less stringent than T cell suppression in these mice.

D. Biochemical Mechanisms of T-Independent Immunogenicity

The demonstrated capacity of endotoxins to elicit antibody responses by an interaction that is limited only to B lymphocytes has had a profound impact on the development of concepts of B-cell triggering mechanisms. This has been in part the result of the comprehensive characterization of the antigen-independent interaction of endotoxins with B lymphocytes (see Section III). The potential interrelationship of these various immunologic activities was suggested by Chiller *et al.* (1973) on the basis of the close parallelism between the structure–function activities of various endotoxin preparations. The further development of this concept, with particular reference to the T-independence of the immune response, led to proposals of an obligate role for endotoxin-induced antigen-independent B-cell proliferation in the generation of T cell-independent antibody responses to endotoxin (reviewed in Coutinho and Möller, 1975).

As postulated, a requisite functional role of lipid A in the generation of antibody responses to endotoxins would be required. Several lines of evidence are not consistent with this hypothesis. First, native protoplasmic polysaccharide (NPP) which is antigenically identical to purified LPS but lacks detectable lipid A (and does not induce nonspecific murine B cell proliferation), will elicit T cell-independent antibody synthesis in mice (von Eschen and Rudbach, 1974; Rudbach, 1976). In addition, alkaline hydrolysis of LPS, which completely abrogates lipid A-mediated antigen-independent stimulation of B cells, inhibited antibody responses only slightly (von Eschen and Rudbach, 1976). This latter result confirmed the earlier results of Poe and Michael (1976a), who, in addition, used normal mouse serum to inhibit *in vitro* endotoxin-stimulated B cell proliferation. In contrast, *in vitro* antibody responses to the endotoxin were unaffected. Similar additional experiments by these investigators (Poe and Michael, 1976b) did, however, show that normal (or Nu/nu) mouse serum could inhibit endotoxin-elicited specific-antibody responses in cultures of Nu/nu spleen cells. The precise mechanisms responsible for these differences are currently not completely clarified. Finally, these authors have used the inhibitors of DNA synthesis, hydroxyurea and cytosine arabinoside, to demonstrate normal numbers of PFC to LPS under conditions where thymidine incorporation was inhibited by more than 95% (Poe *et al.*, 1978). Also described in this report were the results of elegant experiments that examined individual PFC to endotoxin for incorporated thymidine (using simultaneous plaque as-

says and radioautography). Only one-third of the PFC were shown to have synthesized DNA during the peak (24–48 hours) of the blastogenic B cell response. As $1/10^5$ B cells respond to the endotoxin antigen by PFC (Möller *et al.*, 1971) and whereas one-third of the B cells respond to endotoxin by proliferation (Andersson *et al.*, 1977a), these results would be entirely consistent with these two endotoxin-mediated events in the same B cell as being mediated entirely by chance.

The experiments summarized above support the concept that functional lipid A activity is not essential for immunogenicity of endotoxins in T cell-independent responses. Nevertheless, the data of von Eschen and Rudbach (1974, 1976) have provided convincing evidence that lipid A activity may be required in order to trigger a specific enhanced secondary response to endotoxins. These authors used various combinations of purified LPS (which does induce B cell proliferation) as well as NPP and alkaline-hydrolyzed LPS (which do not) as immunogens to elicit primary and secondary responses. Although all three molecules could prime for enhanced secondary responses to LPS, only LPS could elicit an enhanced secondary immune response. These investigators concluded that both the endotoxin antigen and a second (lipid A-mediated signal) play necessary but distinct roles in the generation of immune responses to the endotoxin.

Finally, the analysis of the immune response of the endotoxin-unresponsive C3H/HeJ mouse (see Section V) to endotoxins has provided additional data in support of this concept. Skidmore *et al.* (1975a) reported that both the C3H/HeJ and the histocompatible C3H/St endotoxin-responsive mouse strain made equivalent day 5 primary immune responses to two preparations of purified LPS. However, whereas the PFC response of the C3H/HeJ mouse rapidly waned, the C3H/St PFC response was maintained for up to 2 weeks. Similarly, Rudbach and Reed (1977) noted that the C3H/HeJ mouse could not generate enhanced secondary responses to endotoxins. As the C3H/HeJ mouse possesses a genetic defect that is manifested by an inability to recognize or respond to lipid A, these data would indicate at best a minor role for this moiety in the primary immune response to endotoxins, but a highly significant role in the secondary immune responses. In view of these potent immunostimulatory properties of lipid A, which are in part manifested by its effects on secondary responses, it might be anticipated that lipid A would play a major role in endotoxin immunobiology. Evidence in support of this concept will be summarized in the next section.

III. Antigen-Independent Effects of Endotoxins on Lymphocytes

The specific immune responses that can be elicited by bacterial endotoxins to antigenic determinants on the endotoxin molecules themselves have been summarized in the preceding section. The immune responses generated to the endotoxins have been documented to require an interaction only with specific B lymphocyte, not to require the participation of T lymphocytes. However, endotoxins have also been demonstrated to interact nonspecifically with lymphoid cells, and these interactions have been shown to have very profound effects on host immune reactivity.

The regulation of antibody synthesis and secretion by endotoxins is dependent upon the complex interplay of a number of variables, including the presence or absence of specific antigen and the participation of various lymphoid cell subpopulations. We have therefore elected to consider these variables separately. In Section III we will discuss the antigen-independent interaction of endotoxins with lymphocytes; and in Section IV, the antigen-dependent modulation of host immune responses by endotoxins. In Section VI we will consider the interaction of endotoxins with macrophages. The decision to consider macrophages separately is, in part, based on the wide variety of macrophage responses that can be elicited by endotoxins, only a few of which are directly relevant to host immune response regulation. Further, as will be established in this section, a vast majority of the effects of endotoxins on both B and T lymphocytes do not require the participation of macrophages. Therefore, except when such interactions bear directly on endotoxin–lymphocyte interactions, they will not be considered here.

An additional factor that will limit the scope of the research to be reviewed in these sections relates to the concept of using bacterial products (particularly endotoxins) and/or plant lectins (particularly concanavalin A and phytohemagglutinin) as biological tools (mitogens) to activate lymphocyte subpopulations. The generally accepted and widespread use of such tools would make a comprehensive comparative survey of the effects of endotoxins with these other lymphocyte activators virtually impossible. Therefore, except when particularly relevant to a given aspect of endotoxin–lymphocyte interactions, discussions of other activator–lymphocyte interactions will be limited. It is anticipated that for such information the interested reader will consult the appropriate references and/or reviews cited.

A. The Interaction with B Lymphocytes

The interaction of endotoxins with B lymphocytes *in the absence of antigen* leads to the initiation of several distinct and well-characterized morphological and biochemical alterations in these cells. These cellular responses have generally been categorized as leading to either proliferation or differentiation. Proliferative changes result in lymphocyte blast formation, initiation of RNA and DNA synthesis, and ultimately, cell division. Initiation of B cell differentiation is normally manifested by alterations in cell surface membrane markers, cytochemical morphology, and the initiation of synthesis and secretion of immunoglobulin. In the absence of antigen, the secreted immunoglobulin is representative of the complete repertoire of the set of variable regions of Ig that can be synthesized by those B cells responsive to endotoxin. Although B cell proliferation and maturation induced by endotoxins may not be mutually exclusive events in the lymphocyte cellular response, there is, as will be documented below, convincing evidence to suggest that these can occur as independent cellular responses. We will therefore, initially consider these separately in the following sections and then review the evidence indicating the independence of the induction of these B cell responses.

1. Antigen-Independent Proliferative Responses to Endotoxins

The first direct evidence for an endotoxin-elicited proliferative response of murine lymphocytes was provided by the *in vivo* experiments of Takano and Mizuno (1968) and Takano *et al.* (1968). These investigators examined the rate of incorporation of ^{32}P into the nucleic acids of spleen cells between 24 and 48 hours after the intraperitoneal injection of endotoxins. The results of these studies clearly indicated an increase in both DNA and RNA synthesis. Analysis of DNA/protein ratios suggested that lymphocyte cell division was taking place. Morphologic studies, coupled with ^{32}P kinetic analysis suggested two populations of lymphocytes with division times of about 20 hours (lymphoblasts) and 3 days (lymphocytes and reticular cells). The authors concluded from these studies the endotoxins primarily affected the former cells, by initiating RNA synthesis and maturation into differentiated cells, which subsequently had a less rapid division time.

A correlation of these *in vivo* studies on the proliferative response of murine spleen cells to endotoxin was provided by the experiments of Peavy *et al.* (1970). Using *in vitro* culture techniques, these authors established that splenic lymphocytes from five genetically unrelated strains of mice were all stimulated to blast formation and synthesis of DNA (as assessed by incorporation of [^{3}H]thymidine) following treat-

ment with endotoxin. These responses could be detected in as few as 24 hours after endotoxin stimulation, and as little as 0.5–1.0 μg of endotoxin per milliliter was required for significant responses. These authors reported, however, that stimulation was selective for murine splenocytes and no response was obtained with human peripheral blood lymphocytes under a variety of culture conditions. This latter conclusion must be modified by the results of more recent studies (see Section III,A,1,c).

Since these initial studies, considerable information has been gathered concerning both the endotoxin and the lymphocyte requirements for the initiation of proliferative responses.

a. Evidence for B Cell Response. Initial experiments aimed at probing the mechanisms of the splenocyte proliferative responses attempted to define the lymphocyte subpopulation responding to endotoxin. Using spleen cells from adult thymectomized, lethally irradiated, and bone marrow-reconstituted mice (AT × BM), Gery *et al.* (1972b) and, independently, Andersson *et al.* (1972a) demonstrated that such cells were fully capable of proliferative responses to endotoxin. In contrast, responses to a T cell activator were completely abrogated. Additional studies by the latter authors and by Piguet and Vassalli (1973) also indicated that treatment of normal spleen cells with anti-Thy-1.2 and complement had no effect on endotoxin-induced proliferation. Finally, the ability of endotoxin to induce proliferation in spleen cells from congenitally athymic (Nu/nu) mice precluded potential contributions of residual T lymphocytes in the observed response (Andersson *et al.*, 1972b). These combined observations, confirmed subsequently by numerous investigators, established that murine splenocyte proliferation in response to endotoxin was limited to the B lymphocyte subpopulation.

b. Species Variation in the Proliferative Response. The apparent specificity of the B lymphocyte proliferative response to endotoxin suggested that such responses might prove to be useful as a general means of defining B cell subpopulations. Results obtained by a number of investigators however, have indicated significant variability in the capacity of lymphocyte species to respond to endotoxin. As shown by Veit and Feldman (1976), rat lymphocytes have characteristic responses to endotoxins very much like those of mouse, with a selective proliferation of the B cell population. Zimmerman and Kern (1973) and Shek *et al.* (1974), in contrast, have reported minimal [^{3}H]thymidine uptake in rabbit spleen, lymph node, and peripheral blood lymphocytes stimulated by endotoxin. On the basis of treatment with antilymphocyte serum and complement, the latter authors sug-

gested that endotoxin was a B cell mitogen in rabbits. Other investigators, however, have been unable to obtain a proliferative response of rabbit spleen cells (Bona *et al.*, 1974b) or peripheral blood lymphocytes (Elfenbein *et al.*, 1973). These latter authors reported proliferative responses in B cells of guinea pigs lymph node, and spleen, whereas Ben-Efraim *et al.* (1975) suggested guinea pig B cells to be unresponsive to endotoxin. Undoubtedly much of this variability has been due to differences in culture conditions (see, e.g., Section III,A,1,c) as well as differences in endotoxins (see, e.g., Section III,A,3), both of which have been demonstrated to have profound influences on proliferative responses. Using adoptive transfer of embryonic bursa cells into B cell-depleted chickens followed by subsequent *in vitro* culture of peripheral blood and bursal sac lymphocytes, Weber (1973) provided convincing evidence for chicken B cell proliferative responses to endotoxin in the former, but not the latter, tissue. The author suggested that the failure of cells in the bursa to respond to endotoxin may be reflective of the immature differentiation state of these cells, an observation later confirmed in murine fetal liver lymphocytes. Large lymphocytes containing surface immunoglobulin have also been generated in cultures of newborn precolostral piglet spleen cells following stimulation with endotoxin (Jaroskova *et al.*, 1977).

Such blastogenic proliferative responses initiated by endotoxins have also been observed in a number of more esoteric experimental animals including axolotl (Collins *et al.*, 1975), hamster (Hart, 1978), and to a lesser extent newt splenic lymphocytes (Collins *et al.*, 1975), and lymph node, but not splenic, lymphocytes of the marmoset (Kateley *et al.*, 1977). In addition, spleen, peripheral blood, and anterior kidney lymphocytes of the rainbow trout were also stimulated in response to endotoxin (Etlinger *et al.*, 1976). These combined data suggest that the capacity of lymphoid cell subpopulations to initiate proliferative responses following stimulation with endotoxins may be a general property of such cells.

c. Proliferative Responses of Human Lymphocytes. As indicated above, Peavy *et al.* (1970) were unable to elicit proliferative responses in cultures of human lymphocytes in response to endotoxins over a wide range of endotoxin concentrations in either the presence or the absence of serum. These data essentially confirmed the earlier results of Oppenheim and Perry (1965), who obtained slight but detectable transformation of human peripheral blood lymphocytes in response to endotoxins only when the donor had measurable serum antibody titers. Similar experiments by Hedfors (1973) using peripheral blood

lymphocytes from patients with sarcoidosis and by Ivanyi and Lehner (1974) from patients with peridontitis also demonstrated low proliferative responses to endotoxins. Subsequent to these studies, a number of investigators examined the proliferative responses of a variety of human lymphoid tissues (e.g., peripheral blood, spleen, tonsil, bone marrow, lymph node) to various preparations of endotoxins (cf. Geha and Merler, 1974; Greaves *et al.*, 1974a; Ivanyi and Lehner 1974; Hsu, 1975; Schmidtke and Najarian, 1975; Fauci and Pratt, 1976; Turunen *et al.*, 1977, 1978; Schröder *et al.*, 1978). The combined results of these studies indicated responses ranging from negative to marginal, but statistically significant, positive responses depending upon the conditions of culture and the tissue source of lymphocytes. Of interest, Hsu (1975) reported that adult but not cord blood lymphocytes were stimulated to proliferate by endotoxin, whereas the opposite conclusion was reached by Turunen *et al.* (1977, 1978).

More recent experiments by Ringden and collaborators and by Miller *et al.* (1978) have established culture conditions under which substantial proliferative responses of human lymphocytes to endotoxin may be obtained. Ringden (1976) demonstrated that endotoxin will stimulate from 20- to 50-fold enhancement in thymidine incorporation in cultures of human spleen and abdominal lymph node lymphocytes. Substantially smaller responses were obtained with peripheral blood and bone marrow lymphocytes. Similar results using human spleen, tonsil, and peripheral blood lymphocytes were reported by Ringden and Möller (1975). Using defined culture systems with an apparent obligatory requirement for fresh frozen pooled human serum (Miller *et al.*, 1978) or 10% human type AB serum (Kunori *et al.*, 1978), substantial proliferative responses of peripheral blood lymphocytes have also been obtained, although the culture times required for optimal responses (5–9 days) are considerably longer than those required for optimal stimulation of murine lymphocytes. However, as with murine lymphocytes, the available evidence suggests a primary interaction between the endotoxin and the human B lymphocyte. In addition, several recent reports (Betz and Morrison, 1977; Goodman and Sultzer, 1978) have indicated that lipid A-associated proteins will also initiate proliferative responses in cultures of human lymphocytes.

2. *Antigen-Independent Differentiation Responses*

The ability of individual murine lymphocytes to respond to endotoxin by increased secretion of antibody with a specificity unrelated to the endotoxin was first demonstrated by the experiments of Ortiz-Ortiz and Jaroslow (1970). In a series of experiments designed to examine *in*

vitro the adjuvant effect of endotoxin on the immune response to sheep red blood cells (SRBC), these investigators showed that endotoxin, in the absence of antigen, elicited a statistically significant increase in the number of plaque-forming cells (PFC) to SRBC. Of importance is that both the kinetics and the magnitude of the antibody response elicited by endotoxin alone were strikingly different than those elicited in the presence of the SRBC antigen. The authors suggested that this response was probably not due to the presence of endotoxin antigenic determinants. They interpreted their results as most probably being due to "nonspecific" membrane permeability changes of the lymphocytes elicited by endotoxins.

In an extensive series of experiments, Rank *et al.* (1972) examined antibody responses to an impressive array of endotoxins from both smooth and rough strains of gram-negative bacteria, as well as several preparations of purified lipid A. As assessed by increased numbers of PFC to sheep erythrocytes in spleens of mice after injection of endotoxins, all preparations were found to be highly active. Equally significant responses were detected against erythrocytes from a number of species; however, no PFC were detected against mouse or rat erythrocytes (see Section III,A,2,b). The authors postulated from these studies that antibody to the common antigenic determinant of the endotoxin preparations, lipid A, cross-reacted with antigens present on the heterologous erythrocytes. Although this interpretation was later subject to modification, these data nevertheless established the potent capacity of endotoxins (and, importantly, lipid A) to elicit antibody with a broad spectrum of specificities.

The experiments of Andersson *et al.* (1972b) were designed to test directly the concept that endotoxins could initiate maturational responses in murine lymphocytes, manifested by increased synthesis and secretion of immunoglobulin. These authors clearly showed that, in addition to the proliferative changes induced by endotoxins, these molecules also increased the production of immunoglobulin, as assessed both by [^{3}H]leucine incorporation into 19 S protein and by an increase in the number of PFC against the unrelated antigens sheep and horse erythrocytes. These studies, performed with spleen cells from athymic Nu/nu mice, precluded an obligatory role for T cells in the antibody response. Of interest, these authors noted a strong similarity in the dose-response profiles of the proliferative [^{3}H]thymidine incorporation response and the maturational PFC response.

Although less extensively examined than the proliferative responses in species other than mice, increased nonspecific synthesis and secretion of antibody initiated by endotoxins has also been reported in

rabbit lymphocytes (Zimmerman and Kern, 1973), albacore tune lymphocytes (R. R. Skelly and D. C. Morrison, unpublished results), and human lymphocytes (Hoffmann *et al.*, 1978; Ringden and Möller, 1975; Fauci and Pratt, 1976; Ringden *et al.*, 1977; Kunori *et al.*, 1978). The vast majority of the studies performed to date, however, have focused upon murine lymphocyte responses to endotoxins, and we will concentrate primarily upon this system in the remainder of this section.

a. Nature of the Antigen-Independent Antibody Response. The experiments discussed above indicated the ability of endotoxin-activated murine B lymphocytes to secrete nonspecific antibody. Experiments in T cell-depleted cultures by Watson *et al.* (1973a), Andersson *et al.* (1973), and Coutinho *et al.* (1973a) demonstrated similar antibody responses to the haptenic determinants TNP, NIP, and NNP. Coutinho *et al.*, in fact, noted markedly stronger antihapten antibody responses when spleen cells from Nu/nu mice were compared with Nu/+ littermates. The endotoxin-elicited response was both qualitatively and quantitatively distinct from that induced in the presence of specific antigen and/or T-helper cells. *In vitro* the kinetics of the response were more rapid (Ortiz-Ortiz and Jaroslow, 1970; Watson *et al.*, 1973a). Further, the amount of antibody produced by endotoxin alone was significantly less than that generated in the presence of antigen (Sjöberg *et al.*, 1972; Saito and Nakano, 1975; Persson and Möller, 1975). These latter investigators, in addition, confirmed the earlier observations of Andersson *et al.* (1972c) indicating that the avidity of the antibody produced in response to endotoxin alone was lower than that produced in the presence of antigen.

Additional experiments by Andersson *et al.* (1972c) provided data to suggest that the subpopulation of B cells that normally respond to antigen are included within the population of B cells which respond to endotoxin in the absence of antigen. Using TNP bound to plastic dishes, these investigators selectively removed B cells which could bind TNP from Nu/nu spleen cell populations. When the bound cells were subsequently stimulated with either endotoxin or TNP-coupled SRBC a good anti-TNP antibody response was observed. The unbound lymphocytes did not respond to either endotoxin or TNP-SRBC with anti-TNP responses but were responsive to SRBC. Studies of a somewhat similar nature were carried out by Quintans and Lefkovits (1974). These authors, using limiting dilution analysis in microcultures of Nu/nu spleen cells, found that endotoxins were capable of activating a similar number of splenic precursor B cell independent of added antigen. They suggested that in the absence of antigen, the endotoxin-

initiated clonal expansion is limited, owing to "exhaustive maturation" (see, however, Section III,A,4,c).

b. The Requirement for Antigen. The experiments described above clearly document that endotoxin can initiate antigen-independent differentiation of murine B lymphocytes leading to antibody secretion. Yet not all investigators have been able to demonstrate such a response. For example, Watson *et al.* (1973b) indicated the absolute requirement for antigen in endotoxin-elicited B cell antibody responses and suggested that, although endotoxin alone could stimulate DNA synthesis and limited cell division, it was not sufficient to initiate the differentiation of precursor cells to antibody formation. Support for this concept was provided by Schrader's experiments (1974a) demonstrating an inability of endotoxin to initiate *in vitro* an antibody response to fowl γ-globulin (FγG) in cultures of Nu/nu spleen cells in the absence of antigen. He suggested that this represented a characteristic of the FγG B cells rather than an inability of the assay system to detect such cells, and that an additional population of B cells may exist that are responsive to the commonly assayed antigens (e.g., heterologous erythrocytes, DNP) as a result of prior exposure to cross-reactive antigens.

A similar conclusion was reached by Rosenberg and Cunningham (1975) on the basis of experiments designed to examine the role of antigen in the generation of fully mature splenic B lymphocytes. These authors adoptively transferred anti-Thy-1.2 and complement-treated spleen or bone marrow cells into irradiated recipients, either with or without antigen (SRBC). This was followed by subsequent culture of the recipient spleen cells with endotoxin *in vitro* in the absence of SRBC. The donor spleen cells injected with or without SRBC responded well to subsequent challenge with endotoxin assayed by PFC to the SRBC antigen. In contrast, donor bone marrow cells injected without SRBC required a considerable length of time in the spleen before they became responsive to endotoxin in comparison to bone marrow cells injected with SRBC antigen. The injection of endotoxin could not substitute for the SRBC requirement. These results strongly suggested that B cells required prior antigen exposure to become fully mature after stimulation by endotoxin.

Considerable support for these conclusions has been gained from the elegant experiments of Bretscher (1978) and of Kim and co-workers (1978; Kim, 1979). Bretscher examined the relationship between the intrinsic background response of spleen cells to six heterologous erythrocytes and bromelain-treated (Br) mouse RBC (which has been postulated to expose antigenic determinants similar to those generated during normal physiologic murine metabolism of aged MRBC) both

before and after endotoxin challenge. The results of these experiments indicated that (*a*) no PFC to rat RBC (RRBC) were obtained before or after endotoxin; and (*b*) the *ratio* of PFC to other RBC ± endotoxin was identical independent of the actual response obtained over a range of 300-fold in "background" PFC. [Similar results were actually obtained in an independent investigation several years earlier by Coutinho *et al.* (1973b) in response to six antigens, although this point was not noted at that time.] In addition, Bretscher demonstrated that administration of RRBC to mice initiated a "background" (or primary) response that was considerably enhanced by endotoxin. Finally, he demonstrated that the set of B cells stimulated by endotoxin showed considerable overlap in its capacity to produce antibody to both BrMRBC and SRBC (32%) whereas those B cells stimulated by SRBC immunization showed virtually no PFC for BrMRBC (<1%). These combined observations prompted Bretscher to hypothesize that both background and endotoxin-induced antibody responses are the result of ongoing antigen-dependent stimulation in normal mice.

A somewhat different approach was taken by Kim and collaborators, who nevertheless arrived at much the same conclusion. Using spleen cells from germfree colostrum-deprived piglets obtained by aseptic hysterectomy from specific pathogen-free Minnesota miniature swine 3 days prior to term, Kim was unable to demonstrate either background PFC or responsiveness to endotoxins. These lymphocytes were immunologically competent as judged by their excellent immune response to a number of antigens. These studies suggested to Kim that specific antigen is an obligatory requirement for induction of a true primary immune response.

Thus, these combined data would indicate that endotoxin alone is probably not sufficient to initiate a true B cell differentiation signal leading to synthesis and secretion of antibody. It is less clear, however, whether continued antigenic stimulation is required once B cells have seen antigen for the first time and subsequently differentiated into mature B cells.

3. *Endotoxin Requirements for B Cell Activation*

As indicated in Section I,C, bacterial endotoxins consist of mixtures of lipopolysaccharides (LPS) in some cases complexed noncovalently with protein (LAP). The LPS portion of endotoxins may be subdivided into a biologically active lipid A region and an antigenic O-polysaccharide region. The early experiments of Andersson *et al.* (1972a) described two observations to suggest that the antigenic determinants of the endotoxin–LPS were not responsible for its potent B

cell activity: (*a*) that the majority of B lymphocytes respond to endotoxin whereas only $1/10^5$ B cells respond to the LPS antigen; and (*b*) that mice made specifically tolerant to the LPS antigen were equally as responsive as untreated lymphocytes in response to endotoxin. Additional experiments by these and other investigators have clarified considerably the relationship of structure to function in preparations of bacterial endotoxins.

a. The Role of Lipid A. Because of the multiple biologic activities previously ascribed to the lipid A region of LPS, as well as the fact that it constituted the common biochemical component of a number of different endotoxin preparations, it was rapidly established by investigators in several laboratories that the *in vitro* initiation of B lymphocyte responses could also be attributed to lipid A (Andersson *et al.*, 1973; Chiller *et al.*, 1973; Peavy *et al.*, 1973; Rosenstreich *et al.*, 1973), a conclusion essentially reached earlier by Rank *et al.* (1972) (see Section III,A,2). These conclusions were based upon one or more of the following observations:

1. LPS isolated from various bacterial mutants that contained variable amounts of polysaccharide in relation to lipid A (in particular, LPS from the Re mutant of *S. minnesota* R595) were biologically active.
2. Acid hydrolysis of LPS to separate chemically the lipid A from the polysaccharide demonstrated activity in the lipid A, but not the polysaccharide, fraction.
3. Alkaline hydrolysis to modify the lipid A, but not the polysaccharide, abrogated activity.

Lymphocyte activities were variously assessed by DNA and protein synthesis using these different LPS preparations, as well as, in one report (Chiller *et al.*, 1973) adjuvant effects (see Section IV) and effects on immunologic tolerance (see Section IV,D). It was also reported in one of these studies (Rosenstreich *et al.*, 1973) that the inactive LPS preparations did not block the responses elicited by lipid A. It should be noted that these conclusions confirmed the earlier *in vivo* experiments of Nakano *et al.* (1971a), who demonstrated a low but significant antigen-independent PFC to SRBC in murine spleen cells 48 hours after one injection of Re595 endotoxin.

Several additional experimental protocols worthy of note have provided additional support for the major role of lipid A in B lymphocyte activation. Strong *et al.* (1974) and Knudsen *et al.* (1977) have demonstrated an 85–95% inhibition of endotoxin-initiated mitogenic responses with a rabbit antiserum to lipid A. As initially reported by

Jacobs and Morrison (1975a) and confirmed by Smith *et al.* (1976) and Axelrod and Shands (1977), the addition of the cationic antibiotic polymyxin B, which has been demonstrated to bind to the lipid A region of LPS (see Section III,A,5,e), to cultures of endotoxin-stimulated lymphocytes will inhibit both the proliferative response and the PFC response to unrelated antigens. Similar results have also been obtained by Smith and Hammarström (1976) using colistin methane sulfonite, which, the authors report, is less toxic than polymyxin B. However, the capacity of these molecules to inhibit specific antibody responses to endotoxins is currently unclear (see Section IV,C,1,a).

b. Chemical Modification of Endotoxins. Several chemical procedures have recently been described to modify endotoxins. These have resulted in a significant alteration in the ability of the endotoxins to activate B lymphocytes. The first involves treatment of endotoxins with mild alkali, a procedure reported to modify lipid A and abrogate endotoxin-initiated lymphocyte responses. A recent report by Goodman and Sultzer (1977), however, has indicated that, using carefully controlled conditions of alkaline hydrolysis, significant increases in proliferative responses (but not in PFC responses to TNP-SRBC) can be obtained. The results of similar, though less extensive, experiments have also been reported by Betz and Morrison (1977). It would thus appear that alkaline hydrolysis results in biphasic effects on some B-lymphocyte functions.

The experiments of McIntire *et al.* (1976) and Chédid *et al.* (1975, 1976) have examined the immunologic activities of endotoxins treated with reagents (sodium succinate, sodium phthalate) to substitute each hydroxyl group with a free carboxyl group. Although such procedures did not lead to a detectable decrease in lipid A-associated fatty acids, such derivatives had only 1/100 to 1/1000 the activity of the native endotoxin in initiating lymphocyte proliferative responses. Of interest, each of these modified endotoxins was equally active with respect to adjuvant properties (see Section IV).

As a further probe into the relationship of the chemical groups present on the lipid A to its ability to initiate a lymphocyte proliferative response, Lüderitz and co-workers (1978) have examined the activity of several modified lipid A preparations. These included: the complete lipid A, de-*O*-acylated lipid A, an acidic precursor of lipid A (isolated from a conditional lipid A-sensitive mutant lacking the nonhydroxylated fatty acids), and a de-*O*,*N*-acylated lipid A. Only the de-*O*,*N*-acylated lipid A lacked B lymphocyte activity, suggesting a functional role for the amide-linked fatty acids in the lipid A.

An interesting, if somewhat different approach, was taken by Rosenstreich *et al.* (1974) and Behling *et al.* (1976), who prepared synthetic glycolipids from D-glucosamine and long-chain hydrocarbon fatty acids in order to simulate the lipid A structure. These investigators found that, if appropriately inserted into liposomes, these glycolipids would function as B lymphocyte mitogens. Although some differences were noted depending upon the fatty acid used to substitute the amino sugar, striking differences were not obtained.

c. Lymphocyte Activity of Other Bacterial Products. In addition to the potent stimulatory activity of the lipid A region of endotoxin on B lymphocytes, a number of recent reports have provided evidence to support the concept that many other gram-negative bacterial cell wall products share this capacity to activate B lymphocytes. Although not all of these bacterial products are known to have a direct relationship with bacterial endotoxin, they manifest sufficient immunologic properties in common with endotoxins to warrant their inclusion in this section.

One of the major proteins of the outer cell wall of many gram-negative bacteria is a low molecular weight protein, often covalently associated with the murein layer of the cell wall. This protein—termed lipoprotein (LP)—consists of 57 amino acids and contains at its N-terminus two ester-linked fatty acids and one amide-linked fatty acid (Bessler *et al.*, 1977). As first demonstrated by Melchers *et al.* (1975a), this murein-free lipoprotein as well as muropeptides containing lipoprotein, but not the isolated murein, were potent immunostimulants of murine B lymphocytes as measured by both proliferative and nonspecific antibody responses assessed against TNP-SRBC. Of interest, these authors demonstrated that, like lipid A, activity of LP was extremely sensitive to alkaline hydrolysis. More recent experiments by Bessler *et al.* (1977) have demonstrated that Pronase generated peptide fragments of LP consisting only of 2–5 amino acids bound to diacylglyceryl-*N*-acylcysteinethioether are also potent B cell activators.

The lipid A-associated proteins (LAP) of the endotoxin complexes, have also been recently demonstrated to have B cell immunostimulatory properties distinct from those of the lipid A. The experiments of Skidmore *et al.* (1975a) first indicated that a component of endotoxins coisolated with the endotoxin, which had proliferative activity for B cells and whose activity could be altered by treatment of the endotoxins with aqueous phenol. Subsequently, Sultzer and Goodman (1976) and Morrison *et al.* (1976) isolated a protein-rich fraction in the phenol phase of such extracts which had properties distinct from those of lipid

A and had potent B cell activity, as assessed by proliferation and nonspecific synthesis of antibody. As with the other bacterial products, B cell activity was highly sensitive to alkaline hydrolysis (Betz and Morrison, 1977). The precise relationship of LAP to the murine-associated LP described by Melchers *et al.* (1975a) is unclear; however, the available evidence would indicate that, although LAP may contain LP as a minor constituent, its presence would not be nearly sufficient to account for the activity observed in LAP preparations (D. C. Morrison, unpublished observations).

Additional bacterial products that have recently been reported to have immunologic activity, as assessed by their capacity to initiate proliferation in cultures of murine B lymphocytes, include: purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Sultzer and Nilsson, 1972); a water-soluble mitogen (NWSM) extracted from *Nocardia* (Bona *et al.*, 1974a); a cell wall fraction from *Listeria* (Cohen *et al.*, 1975); an acidic polysaccharide produced by *Serratia piscatorum* (Matsumoto *et al.*, 1975); and an extract of *Actinomyces viscosus* (Engel *et al.*, 1977). It is, however, beyond the scope of this review to describe in any detail the immunologic properties of these bacterial products. Nevertheless, it does appear to be considerably more than coincidence that virtually all these bacterial products are associated with the external surfaces of bacteria and hence have maximal opportunity to interact with the external environment, and, in particular, the B lymphocyte.

4. Subpopulations of B Cells Responding to Endotoxins

In view of the multiple differentiation states of lymphocytes of both the B and T lineage and their capacity to manifest distinct morphologic and biochemical changes in response to antigen, a number of investigators have examined the potential for lymphocyte subpopulations to respond to endotoxins. As already indicated, lymphocyte responsiveness to endotoxins, as assessed by proliferative and maturational responses, were shown to be a property of the B lymphocyte. The results of additional studies have provided considerable support for the concept that different B lymphocyte subpopulations vary significantly in their responsiveness to endotoxin.

a. Relationship of Proliferation to Maturation. The early experiments of Takano and Mizuno (1968) and Takano *et al.* (1968) provided the first data to suggest that the initiation of DNA synthesis in murine lymphocytes following *in vivo* endotoxin stimulation was limited to a splenocyte subpopulation. Andersson *et al.* (1972b) demonstrated similar dose-response profiles in the *in vitro* initiation of 19 S protein

synthesis and [^{3}H]thymidine incorporation into DNA. Shohat *et al.* (1973) and Shands *et al.* (1973) published morphologic data in support of these proliferative and maturational lymphocyte responses to endotoxins, which, after stimulation, were shown at 24 hours to result in a significantly increased proportion of large lymphoblasts. (The latter study reported that 40–50% of the total cells were lymphoblasts.) A somewhat lower frequency of response to endotoxin was reported by Janossy *et al.* (1973), who, on the basis of radioautographic data, indicated that, even under optimal cultural conditions, only 20–30% of the B cells responded. Similar values were reported also by Hecht *et al.* (1976). The electron micrographic studies of Shohat *et al.* (1973) further showed that stimulation to lymphoblast formation was followed by the development of extensive endoplasmic reticulum and eventually the maturation of typical plasma cells. Significantly, a number of the endotoxin-elicited large lymphoblasts, as assessed by radioautography, were devoid of [^{3}H]thymidine, indicating that such cells had differentiated in the absence of DNA synthesis. Other cells, however, had clearly synthesized DNA and subsequently undergone blastogenesis.

The extensive and comprehensive studies of Melchers and Andersson (1974a,b) and Andersson and Melchers (1974) have provided considerable information on the biochemical and morphologic events following the interaction of B cells with endotoxins. These studies have been reviewed in detail by these investigators (Melchers and Andersson, 1973; Melchers *et al.*, 1975b) as well as by Janossy and Greaves (1975) and will be only briefly summarized here. Following endotoxin stimulation, the initiation of DNA synthesis can be first detected by incorporation of [^{3}H]thymidine at approximately 14–16 hours, which increases to a maximum at about 50 hours. Using high specific activity radiolabeled thymidine to "suicide" those lymphocytes synthesizing DNA, a critical period of 20–30 hours following stimulation was established, during which maturation to plaque-forming cells (assayed 36 hours later) could be substantially inhibited. These experiments indicated that most of the B cells emerging as PFC after endotoxin stimulation do so as a result of DNA synthesis and proliferation. In addition, these investigators demonstrated that hydroxyurea (which interferes with ribonucleoside diphosphate reductase activity), while completely inhibiting DNA synthesis of endotoxin-stimulated lymphocytes, led to no detectable decreases in synthesis or secretion of IgM as assessed at 48 hours. Andersson and Melchers (1974) also demonstrated that those cells stimulated to differentiate into immature plasmablasts in the absence of DNA synthesis were not precluded from subsequent proliferation in the absence of inhibitor.

These studies suggested two stages in the differentiation of B cells following endotoxin stimulation; the first occurs in the absence of DNA synthesis and proliferation and leads to the immature IgM-secreting plasmablast; the second requires DNA synthesis and proliferation and constitutes the development of the mature secretory cell (PFC) containing a well developed endoplasmic reticulum.

The results of several more recent publications have essentially confirmed these observations. Gormus and Shands (1976) examined individual lymphocytes following endotoxin stimulation and found that, using hydroxyurea, thymidine incorporation could be inhibited by greater than 98%. As assessed at 48 hours, however, endotoxin-stimulated cultures had increased numbers of lymphoblasts that stained positive for intracellular IgM. Somewhat similar studies by Janossy *et al.* (1976), Askonas *et al.* (1976), and Hecht *et al.* (1976) have also described a population of endotoxin-induced plasmablasts with large amounts of intracellular Ig as well as lymphocytes with little or no intracellular Ig but relatively large amounts of surface Ig. These latter investigators indicated that, in contrast to the earlier reports of Shands *et al.* (1973) and Shohat *et al.* (1973), after endotoxin stimulation most of the cells reverted to a small lymphocyte morphology and only a small minority of the cells matured into Ig secreting cells.

The results of these studies clearly indicate that endotoxin can elicit several well defined responses within B lymphocyte subpopulations present in murine spleen cells. One of these responses leads to proliferation, another can lead to differentiation. The percentages of the B cell subpopulations that are elicited to undergo these activities in response to endotoxins appears to be somewhat variable.

b. Subsets of B Cells Responsive to Endotoxins. The data summarized above have indicated that approximately one-third of the total splenic population of B lymphocytes can respond to endotoxin and that, of those cells that do interact functionally with endotoxin, distinct cellular responses can be elicited. These results suggest the existence of subpopulations of lymphocytes that differ in their sensitivity to endotoxin stimulation. Evidence in support of such a concept has been obtained by a number of investigators using several experimental approaches. These include functional characterization of B cell responsiveness, analysis of B cell membrane-localized receptors as markers for subsets, and delineation of ontogenic responsiveness to endotoxins and other B cell activators. Although extensive interrelationships exist between these various approaches in the delineation of B cell endotoxin responsiveness, we will summarize the available data for each of these approaches separately.

The first approach has been to utilize quantitative and/or qualitative

assessment of [^{3}H]thymidine incorporation to define B cell responsiveness following stimulation with endotoxins as well as PPD and dextran sulfate, both of which also act selectively to stimulate B lymphocytes. Experiments by Diamantstein *et al.* (1974) analyzed DNA synthesis in splenic lymphocytes optimally stimulated by dextran sulfate, endotoxin, or both of these activators. The results of these studies indicated a completely additive reponse to the two stimuli, suggesting that these two molecules act independently on different subpopulations of B cells. To further probe this observation, these investigators prepared AT × BM recipients and determined responsiveness of recipient spleen cells to dextran sulfate and endotoxin at various times after reconstitution. Almost total responsiveness to dextran sulfate within 1–2 weeks after transfer was found; in contrast, endotoxin responsiveness was not detectable until 2 weeks after transfer and was not maximal until 5 weeks. On the basis of these results it was postulated that the population of B cells responding to dextran sulfate represented a less mature B cell population than the endotoxin-responsive B cells.

Similar although more extensive results were independently obtained in a comprehensive series of experiments by Gronowicz and Coutinho (1974, 1975a,b, 1976). These investigators analyzed both *in vitro* and *in vivo* responses to endotoxin, dextran sulfate, and PPD, utilized as sequential or simultaneous activators of B cell subpopulations. The majority of the results of these studies have been recently reviewed by these investigators (Gronowicz and Coutinho, 1975b). These experiments have indicated that the B cell subset responsive to dextran sulfate was distinct from that responsive to endotoxin and, in addition, demonstrated that prestimulation with dextran sulfate would *induce* B cells to responsiveness to endotoxin. Endotoxin, in contrast, induces a large fraction of B cells to differentiate to an end stage of maturation and subsequent unresponsiveness to B cell activators. Analysis of fetal liver and adult bone marrow indicated B cell responsiveness to dextran sulfate but not PPD or endotoxin (over a very wide concentration range) in accord with the results of Diamantstein *et al.* (1974). They do, however, contrast with the data of Ryser and Vassalli (1974), who reported that bone marrow cells stimulated by endotoxin had different characteristics than the splenic B cell response. Of interest, the dextran sulfate-activated bone marrow and fetal liver were reported by Gronowicz and Coutinho not to give rise to high-rate antibody-secreting cells (see also Ryser and Vassalli, 1974). Additional experiments by these investigators using the high specific activity [^{3}H]thymidine hot-pulse "suicide" type of experiment lent additional

support for the authors' conclusions. It should be noted, however, that similar experiments by Melchers and Andersson (1974a) suggested that PPD and endotoxin activate the same subpopulation of B cells to PFC maturation, although these authors cautioned that the subpopulations stimulated to PFC may not be the same as those stimulated to [^{3}H]thymidine incorporation.

More recent experiments by Bona *et al.* (1978) have used a similar experimental approach to examine Nu/nu spleen cell responsiveness to endotoxin, dextran sulfate and *Nocardia* water-soluble mitogen (NWSM). These investigators activated B cells with these stimuli in the presence of BUdR and subsequently exposed them to light. (This procedure results in irreversible inactivation or "suicide" of those cells stimulated to DNA synthesis and consequent incorporation of BUdR.) By this method they demonstrated distinct subsets of B lymphocytes responsive to these activators. Adult spleen cells depleted of cells responsive to a given activator by BUdR treatment and subsequently injected into lethally irradiated recipients maintained their selective unresponsiveness even when tested 2–3 weeks later. This suggests that a distinct B cell subpopulation existed for each activator rather than different stages of maturation of a single population. These data should be compared with those of Diamantstein *et al.* (1974), who, using untreated bone marrow lymphocytes, did demonstrate a maturational response to endotoxin in irradiated recipients.

A second approach has been to utilize surface and cytoplasmic markers to characterize B cell subpopulations responding to endotoxins. These studies have primarily focused upon the complement C3 receptor, the immunoglobulin Fc receptor, and both surface and cytoplasmic immunoglobulin (Ig). Möller (1974) demonstrated that approximately 70% of splenic B lymphocytes were Fc^+C3^+ and 30% were Fc^+C3^-. After stimulation with endotoxin there was a significant increase in the number of Fc^+C3^- cells suggesting that the endotoxin was interacting preferentially with Fc^+ B lymphocytes. Similar studies by Gormus *et al.* (1974) and Gormus and Shands (1975) also described temporal decreases in $C3^+$ B cells following endotoxin stimulation. These authors also noted that most of the $C3^+$ cells were also positive for IgM, and they concluded that endotoxins primarily stimulated $C3^+$ IgM^+ B cells. Experiments reported by Lamelin and Vassalli (1975) using spleen and lymph node cells from Nu/nu mice noted significant differences in the lymphocyte responses following endotoxin stimulation. In both cases, more than 90% of the viable cells were transformed; almost half of the splenic cells were positive for cytoplasmic IgM, but only 3–6% of the lymph node blasts had this characteristic.

More recently, Bona *et al.* (1978), using alloantisera and complement, have provided data to indicate that all the endotoxin-responsive splenic lymphocytes were positive for surface Ig as well as both I-A and I-C antigens.

As shown above, surface markers may define subsets of B cells responsive to endotoxins. Endotoxins, however, also have the capacity to modulate the expression of these B cell markers. After stimulation with endotoxin, Möller found a significant increase in the percentage of Fc^+C3^- cells, suggesting that the endotoxin was initiating differentiation of Fc^+C3^+ B lymphocytes to Fc^+C3^- cells. Similar studies by Gormus *et al.* (1974) and Gormus and Shands (1975) also described temporal decreases in $C3^+$ B cells following endotoxin stimulation. Hammerling *et al.* (1975) demonstrated that Ig^+ subpopulations of B lymphocytes separated by albumin density gradient centrifugation could be induced over a 2-hour period of culture *in vitro* to become Ia^+ and Fc^+ and that such activity could be abrogated by pretreatment with anti-immunoglobulin and complement. Further studies by these investigators indicated the following sequential appearance of B cell surface markers: Ig^+, then Ia^+, and finally $C3^+$ after endotoxin stimulation (Hammerling *et al.*, 1976). Relevant to these observations were the experiments of Fairchild and Cohen (1978), who indicated that when bone marrow cells were incubated with endotoxin the expression of Ig^+ cells from Ig^- cells was enhanced. Similarly, the ability of the population to proliferate in response to endotoxin was increased. These authors suggested that the expression of surface Ig^+ preceded the acquisition of responsiveness to endotoxin and that such expression was essential for further B cell maturation.

An interesting report by Hammerling *et al.* (1978) has correlated several of these morphologic and functional observations on endotoxin responsiveness in B cell subsets. Using several procedures to separate $C3^+$ and $C3^-$ populations of splenic B cells, these investigators clearly showed that, although both subpopulations of B cells responded equally well to endotoxins as determined by either DNA or IgM synthesis, they differed in their kinetics. The induction of both responses was approximately 24 hours more rapid in the $C3^+$ subpopulation of B cells. These combined data are consistent with the existence of distinct subpopulations of B cells, as assessed both functionally and by surface markers in response to endotoxins.

A third approach has been to examine the ontogeny of the development of responsiveness to endotoxins. As indicated above, evidence has been presented to suggest that neither fetal liver lymphocytes nor adult bone marrow lymphocytes from the mouse respond to endotoxin

(Gronowicz and Coutinho, 1975a). In a more extensive study, Gronowicz *et al.* (1974) demonstrated that primitive B cells, obtained from fetal liver, sequentially gain responsiveness to dextran sulfate, endotoxin, and finally PPD. These studies utilized fetal liver cells injected into lethally irradiated recipients with subsequent temporal analysis of DNA synthesis initiated by these B cell activators. The results essentially confirm the earlier data obtained by these investigators. It is interesting that the capacity to secrete high amounts of antibody in response to endotoxin followed the same sequential order as did DNA synthesis responses. The presence of a lag period between these two responses, however, suggested that cell replication preceded maturation into antibody-secreting cells.

Maturation of fetal liver and splenic B lymphocytes was also studied by Kearney and Lawton (1975a,b), who examined the temporal appearance of cytoplasmic immunoglobulins following endotoxin stimulation. These investigators confirmed the results of Gronowicz *et al.* (1974) showing no DNA synthesis in endotoxin-stimulated fetal lymphocytes. It should be noted, however, that the capacity of fetal lymphocytes to respond to endotoxin, as assessed by their capacity to convert a highly restrictive immunoglobulin affinity response to DNP–BGG into an "adult-type" heterogeneous antibody response, can be detected as early as 14–16 days of fetal life (Goidl *et al.*, 1976). They further found that endotoxin will stimulate the appearance of cytoplasmic μ and γ_2 in 19-day fetal cells, but γ_1 and α chains do not appear until after birth. The authors stress that the percentage of cells staining for μ, γ_1, γ_2, and α after stimulation of 1-day-old spleen cells with endotoxin is exactly what one finds after stimulation of adult splenic lymphocytes. This suggested that B cells are capable of being triggered to differentiate into mature Ig-secreting cells as soon as they can be defined by the presence of surface Ig. Very similar conclusions, based upon different experimental data, were earlier reached by Spear and Edelman (1974) and Rosenberg and Cunningham (1975).

c. Differentiation to Isotype-Specific PFC. The results of experiments summarized above have provided convincing data to indicate that endotoxins will induce B lymphocyte proliferation and differentiation into mature immunoglobulin-secreting cells. The majority of these data indicate that IgM was primarily synthesized and secreted after endotoxin stimulation. Several investigators have recently published evidence, however, to indicate that endotoxin-stimulated B cells will also differentiate into IgG- and IgA-synthesizing cells. Experiments describing these responses were first published by Kearney and Lawton (1975a,b), who stained for intracellular Ig and demon-

strated that, under the appropriate conditions of culture, endotoxins, will induce IgG_1-, IgG_2-, and smaller numbers of IgA-synthesizing cells. The ability of these cells to be elicited in cultures of lymphocytes from Nu/nu mice suggested that T cells were not essential for the observed response. Interestingly, IgG-synthesizing cells were generated only in cultures of bone marrow cells (not in cultures of thoracic duct, Peyer's patches, or mesenteric lymph node lymphocytes). In these experiments a low initial density of lymphocytes in cultures was essential for differentiation into IgG-synthesizing cells. Further studies by these investigators (Kearney *et al.*, 1976a,b) demonstrated in both newborn and adult spleen a small but significant population of cells that bear both surface IgM and IgG_2, and synthesize primarily IgG_2 in response to endotoxin. At later times of stimulation, the surface IgM tended to disappear. These studies suggested that virgin IgG_2 precursor B cells express both IgM and IgG_2 and that following endotoxin stimulation the switch from IgM to IgG involved the loss of IgM receptors.

Experiments by Zauderer and Askonas (1976) and by Andersson *et al.* (1976, 1978b) have yielded similar conclusions although some differences were noted between the various studies with regard to the conditions necessary for generation of IgG- and IgA-synthesizing cells after stimulation with endotoxin. For example, Zauderer and Askonas (1976) reported that prolonged culture times (about 18 days) with frequent media changes were required. In contrast, Andersson *et al.* (1976) demonstrated both IgG- and IgA-secreting B cells in spleen lymph node, bone marrow, fetal liver, and thoracic duct in both normal and Nu/nu mice. Culture conditions were used that allowed clonal growth and maturation into immunoglobulin-secreting cells of every endotoxin-inducible B lymphocyte (Andersson *et al.*, 1977a).

The improved culture conditions described by Andersson *et al.* (1977a), which allow proliferation and maturation of every endotoxin-inducible B lymphocyte, have allowed these investigators to apply the method of limiting dilution analysis to determine the frequency of precursor cells responsive to endotoxins. The results of these experiments indicate that, depending upon the strain and age of the donor lymphocytes, as well as the organ from which the lymphocytes were obtained, approximately $\frac{1}{3}$ to $\frac{1}{10}$ of all B cells could be induced by endotoxin to grow and mature into clones of immunoglobulin-secreting cells (Andersson *et al.*, 1977b). More recent studies of a similar nature have determined the frequency of IgG-secreting splenic B cells to be approximately $\frac{1}{30}$ to $\frac{1}{40}$, or approximately 10% of the frequency of the endotoxin-reactive precursors that develop into clones

secreting IgM (Andersson *et al.*, 1978a). Similarly, the frequency of precursor B cells with defined *V*-region gene specificity, which respond to endotoxin were estimated to be: $\frac{1}{10}$ for NIP_{12}-SRBC, $\frac{1}{50}$ for TNP_{30}-SRBC, $\frac{1}{100}$ for NIP_1-SRBC, $\frac{1}{180}$ for TNP_3-SRBC, and $\frac{1}{1000}$ for SRBC. It is of interest that virtually identical frequencies of precursor B cells were estimated after stimulation with either endotoxin or lipoprotein (LP) (Andersson *et al.*, 1977c).

The demonstration that endotoxin can give rise to IgG antibody-secreting B cells in *in vitro* culture has also been confirmed *in vivo* by Kolb *et al.* (1974, 1976). Because maternal IgG can be introduced into neonates during nursing, these authors examined the appearance in the circulation of paternal allotype-specific isotype immunoglobulin in F_1 hybrids from parents with different allotypic specificities following the injection of lipid A. There was a marked decrease in the time of onset of circulating paternal allotype IgG_{2a}, which occurred approximately 5 days after injection of lipid A and approximately 10 days after saline injection. The fact that equivalent responses were obtained in Nu/nu neonates precludes a major role of T lymphocytes in this IgG response. Control experiments verified that the enhanced levels of IgG did not have specificity for the lipid A or endotoxin. Thus, as was demonstrated *in vitro,* endotoxin can stimulate both antigen- and T cell-independent IgG synthesis and secretion in murine lymphocytes. Specific morphologic examination of spleen cells following injection of endotoxin by several investigators (Moatamed *et al.*, 1975; Peavy *et al.*, 1978; Takigawa and Hanaoka, 1978) has confirmed that the B cell responses characterized in the above *in vitro* studies have corresponding counterparts *in vivo.*

5. *Biochemical Mechanisms of Endotoxin-Induced B-Lymphocyte Triggering*

The experiments summarized above have established the profound effects that bacterial endotoxins can have upon B lymphocytes in initiating both proliferative responses and high rates of antibody secretion. These events are rapidly established following either the administration of endotoxin to experimental animals or its addition to lymphocyte cultures, and effects on protein and DNA synthesis can be detected within 7–14 hours. The triggering events for these B cell responses involve, at a minimum, an initial binding of the endotoxin to the B lymphocyte cytoplasmic membrane, a perturbational event that provides information transfer to the interior of the cell, either directly initiated by endotoxin translocation or by mobilization of one or more "second messengers," and finally the mobilization of the appropriate

subcellular components for protein and DNA synthesis. In this section we will summarize the available literature on the events coincident with or immediately following the interaction of endotoxins with B lymphocytes.

a. The Binding of Endotoxins to Lymphocytes. The studies of Adler *et al.* (1972) and Smith (1972) were the first to examine the initial interactions between endotoxins and murine lymphocytes. Using fluorescein-conjugated anti-endotoxin antibody, these authors found that endotoxin was rapidly bound to the lymphocyte membrane and that it was subsequently internalized into the lymphocyte cytoplasm over a 45–60-minute period. It was suggested that the latter internalization process may occur through micropinocytosis, as this process, but not the binding, was inhibited by low temperatures. In addition, the inhibition of internalization of endotoxin by pretreatment with neuraminidase, which also enhanced binding, further suggested that the internalization process was a more critical event in the lymphocyte-triggering mechanism than the binding.

The results of a number of later experiments provide support for this suggestion. For example, although quantitative data were not indicated, Möller *et al.* (1973a) indicated that ^{14}C-labeled endotoxin bound equally well to thymocytes and spleen cells and that no significant differences were observed at either 2 or 72 hours. Similarly Watson and Riblet (1975) demonstrated approximately linear uptake of 1% of ^{3}H-labeled endotoxin over a range of 0–10 μg after incubation at 37°C for 30 minutes with spleen cells from C3H/HeJ or C3HeB/FeJ mice (100 ng/10^6 spleen cells). Only the latter splenic B cells are triggered in response to endotoxin (see Section V) even though both cells bind endotoxin to an equal extent. Virtually identical results were obtained by Sultzer (1976) as the result of binding studies of endotoxins to spleen cells from nonresponder C3H/HeJ and responder CBA/J mice.

More recently, Kabir and Rosenstreich (1977) have examined in detail the binding of endotoxins to populations of spleen cells. These investigators confirmed the data of Watson and Riblet (1975) and Sultzer (1976) showing equal binding of ^{14}C-labeled endotoxin to endotoxin responder and nonresponder spleen cells. In addition, these authors noted the following characteristics of the binding of endotoxin: (*a*) kinetics of binding increased rapidly over a 2-hour period and was significantly slower over the next 2 days in culture; (*b*) removal of macrophages decreased by about 30% the binding of endotoxin to spleen cells; (*c*) B cells bound about twice as much endotoxin (100 ng/10^6 cells) as did T cells; (*d*) polysaccharide-deficient *S. minnesota*

R595 LPS was bound approximately 8-fold more efficiently than was boiled polysaccharide containing LPS and 40-fold more efficiently than untreated LPS; (*e*) all three LPS preparations had approximately equivalent dose-response profiles in eliciting DNA synthesis; (*f*) as earlier suggested by Adler *et al.* (1972) and Smith (1972), neuraminidase treatment, and in addition, trypsin enhanced the binding of R595 LPS; (*g*) lipid A, R595 LPS, LPS, and phosphatidylethanolamine could partially inhibit the binding of radiolabeled R595 LPS; and (*h*) endotoxin required a prolonged period of contact with lymphocytes (>24 hours) in order to elicit maximal cell responses. This latter result contrasts with the earlier results of Adler *et al.* (1972), who suggested that 1–4 hours of interaction sufficed to initiate substantial lymphocyte responses. On the basis of these combined results the authors concluded that endotoxin binding, while undoubtedly a prerequisite for lymphocyte triggering, is not by itself sufficient for activation and suggested that an additional cell alteration induced by the bound endotoxin actually determines whether activation takes place.

Several more recent reports have provided additional data to either confirm or contrast with these experiments. Zimmerman *et al.* (1977) found that radiolabeled lipid A binding to lymphocytes was essentially complete after 2 hours over a 25-μg range of lipid A. Binding was temperature dependent, and bound lipid A was noted to be associated with a lymphocyte membrane fraction. In view of the relatively high affinity of endotoxin for membranes (Shands, 1973), this result might not be totally unexpected. In addition, maximal stimulation of DNA synthesis was obtained at less than 1% of maximal lipid A binding. In the experiments of Zimmerman *et al.* (1977), virtually no binding to thymocytes or mouse RBC was detected. In spite of the minor differences in some of the experimental techniques, these results still contrast with those suggested by Möller *et al.* (1973a) and Kabir and Rosenstreich (1977) as well as those of Bona *et al.* (1976) (see below). Similar binding experiments by Washida (1978) reported temperature-independent association of endotoxin with human lymphocytes, which was of higher affinity than binding to human erythrocytes but lower than that to human granulocytes or platelets. Davies *et al.* (1978) recently presented data to support cyclical binding of endotoxin to a number of mammalian cells including erythrocytes and peritoneal lymphocytes, with a periodicity of about 60–90 minutes. This latter phenomenon was not reported in any of the earlier binding studies.

Springer and Adye and their collaborators have studied extensively

the nature of the initial interaction of bacterial endotoxins with mammalian cell membranes, primarily with respect to erythrocyte membranes but more recently with both platelets and leukocytes. In a comprehensive series of reports, these investigators described the isolation and characterization of an endotoxin receptor from human erythrocytes (Springer *et al.*, 1970, 1973, 1974). This receptor, a lipoglycoprotein was found to have a molecular weight of about 250,000. It is sensitive to Pronase, but stable to heat and alkali. The more recently published experiments have indicated, however, that the major endotoxin binding substances from human platelets, mononuclear leukocytes, and lymphocytes belong to a distinctly different class of chemical compounds than the erythrocyte receptor. These studies have indicated, similar to the results of Kabir and Rosenstreich (1977), that the endotoxin binding substances are primarily phospholipids rich in glycerophosphatides (Springer and Adye, 1975, 1977).

The prospects for significant advances in defining and characterizing the functional nature of the endotoxin receptor have been considerably advanced by the isolation of an antiserum that recognizes an endotoxin-reactive component on the lymphocyte surface. This antiserum has been prepared by Forni and Coutinho (1978a,b) using the genetically endotoxin-unresponsive C3H/HeJ mouse strain and is described in more detail in Section V,D.

The experiments of Bona and collaborators have investigated the fate of endotoxins once it interacts with cells of the immune system (see Section II,A). The authors suggested that transfer of endotoxin from the macrophage to the lymphocyte was highly relevant for the induction of antibody synthesis by lymphocytes; however, the lack of any requirement for macrophages in a number of endotoxin-induced lymphocyte responses indicates that this mechanism might not be essential for endotoxin-initiated lymphocyte activation.

More recent experiments by these investigators have examined the fate of endotoxin in murine Nu/nu and normal lymphocytes following *in vitro* stimulation (Bona *et al.*, 1976; Kaplan *et al.*, 1977; Truffa-Bachi *et al.*, 1977). These investigators detected only small differences in the initial binding of endotoxin to B or T lymphocytes. Neither actinomycin D nor cycloheximide inhibited binding, although both of these compounds inhibited incorporation of macromolecular precursors. Using radioautography, it was demonstrated that the bound endotoxin had "capped" in both B and T cells by 60 minutes. These observations are consistent with the results of Loor (1974), which demonstrated an absolute lack of correlation between capping and stimulation. Kinetic studies indicated that B, but not T, cell association

with endotoxin was biphasic with a time-dependent "unloading" of the endotoxin by the B cells. Of significant interest was the demonstration that this B cell "unloaded" endotoxin was converted to a lower molecular weight form that had the capacity to stimulate lymphocytes from the endotoxin-unresponsive C3H/HeJ mouse. The precise chemical alteration in the endotoxin molecule remains, however, to be defined.

In summary, these combined studies indicate that endotoxins can bind to both B and T cells. The total amount of endotoxin that can be bound, in general, bears little relationship to the amount required for activation, suggesting that much of the binding may be nonspecific. An association with membrane lipids is clearly suggested, but, in view of the relatively high potential for nonspecific interactions, the relationship of lipid binding and/or perturbation to the effective triggering event is unclear. Binding is, for the most part, a rapid event and the available evidence suggests that internalization of the endotoxin occurs after binding with eventual "unloading" from the B cell in an altered, but as yet undefined, form.

b. Endotoxin-Initiated "Second Signals." The experiments of numerous investigators during the past decade have provided convincing evidence for major regulatory role of "second messengers." These triggering signals are initiated by stimulus–membrane receptor interactions and may play a critical role in a diverse spectrum of cellular responses. In the particular case of the lymphocyte, this would be manifested by proliferation and differentiation. Of primary interest has been the potential role of the cyclic nucleotides, cAMP and cGMP, as well as the divalent cation calcium, in the triggering response of various B and T lymphocyte activators. While it is beyond the scope of this review to deal with this complex area in detail, it is relevant to consider briefly those studies that have examined the relationship of these factors in endotoxin-initiated B cell activation.

The experiments of Diamantstein and Ulmer (1975a) examined in detail the requirement for extracellular calcium in the initiation of proliferative responses of murine lymphocytes by endotoxin. These authors showed that the presence of ethyleneglycoltetraacetic acid (EGTA) in the culture medium completely inhibited the incorporation of [^{3}H]thymidine into DNA following endotoxin stimulation. The temporal addition of Ca^{2+} to EGTA-inhibited cultures (or the addition of EGTA to Ca^{2+}-stimulated cultures) clearly demonstrated that a gradual loss of ability to stimulate (or conversely, the ability to inhibit) endotoxin-elicited responses. The presence of calcium was not required for subsequent lymphocyte responses after 48 hours in culture.

These results suggested that the calcium-dependent period was a relatively late requirement (but preceding DNA synthesis) and might not be a regulatory participant in early endotoxin-induced triggering signals. In support of this suggestion, Raff *et al.* (1975) and Freedman *et al.* (1975) were completely unable to demonstrate any early changes in the levels of intracellular calcium by uptake of ^{45}Ca from the external medium in lymphocytes stimulated by endotoxins. Similarly, Rosenstreich and Blumenthal (1977) found no correlation between the capacity of stimuli to act as ionophores and their capacity to initiate B cell proliferative responses. Of particular relevance was the inability of purified LPS or lipid A to translocate ions in preparations of lipid bilayer membranes. In light of the indication of a late requirement for Ca^{2+} in endotoxin activation, Dumont (1975) has demonstrated that, following an initial decrease in net negative charge on the surface of lymphocytes from Nu/nu spleens (B cells) manifested 4 hours after endotoxin stimulation, there is a significant and prolonged increase in net negative charge compared with untreated controls. The relevance of this result to Ca^{2+} binding and/or translocation is currently unclear.

The potential contribution of cyclic nucleotides as intracellular mediators of B lymphocyte activation has recently been discussed by Greaves *et al.* (1974b), Watson (1975), Coutinho and Möller (1975), and Rebhun (1977). Their role in endotoxin-mediated B cell activation was first investigated by Diamantstein and Ulmer (1975b), who found that both 2′,3′-cGMP and 3′,5′-cGMP enhanced the incorporation of [^{3}H]thymidine in endotoxin-stimulated cultures whereas 3′,5′-cAMP and cdTMP inhibited these responses. These data suggested that levels of cyclic nucleotides may be important in endotoxin-induced triggering. In an extensive series of experiments, Watson (1975, 1976) examined intracellular cyclic nucleotide levels during endotoxin-elicited immune responses. Watson reported an approximate 6-fold increase in cellular levels of 3′,5′-cGMP within 15 minutes of addition of endotoxin to cultures of Nu/nu spleen cells. No changes in levels of cAMP were noted. In addition, increased incorporation of ^{32}P into cGMP was detected. Agents such as isoproterenol and dibutyryl cAMP, which raised intracellular levels of cAMP, inhibited endotoxin-elicited responses. A similar inhibition of endotoxin-induced proliferative responses of B cells was reported by Greaves *et al.* (1974b) although these authors were unable to demonstrate the reversal of cAMP inhibition by cGMP as reported by Watson. Of interest was Watson's observation that the T cell mitogen concanavalin A-inhibited endotoxin responses in cultures of Nu/nu spleen cells, but that the inhibition could be reversed by addition of cGMP. Miller and

Moticka (1977) have also demonstrated moderate effects of the cyclic nucleotides cAMP and cGMP on endotoxin-stimulated lymphocytes. A recent preliminary report by Matsui *et al.* (1977) has suggested that endotoxins initiated significant increases in intracellular levels of cAMP and cGMP in 4 of 5 cultures of unfractionated human lymphocytes, 0 of 2 cultures of purified T cells, and 1 of 2 cultures of purified B cells.

These combined data support a potential participation of cyclic nucleotides and calcium in the framework of the B lymphocyte triggering mechanisms initiated by endotoxins. Owing to the complex nature of the cellular systems involved, further work will be necessary to fully elucidate the role that these mediators play in regulating endotoxin-induced immune reactions.

c. The Role of B Lymphocyte Membrane Markers and Receptors. The evidence demonstrating that endotoxins not only interact preferentially with B lymphocyte subpopulations that express distinct cell surface characteristics, but also effectively modulate the expression of such markers on the B cell plasma membrane has been summarized above. Equally of interest, however, is the capacity of these surface markers and receptors to influence the interaction of endotoxins with the B lymphocyte surface and regulate the responses of the cell to endotoxin stimulation.

One of the earliest concepts developed in this regard was proposed by Dukor and Hartmann (1973) and Dukor *et al.* (1974). These investigators suggested that, since most T-independent antigens and B cell activators (in particular endotoxins) were also activators of serum complement, activated C3 might bind to the B lymphocyte C3 receptor and provide a triggering signal for lymphocyte activation. The postulates in support of this hypothesis have been discussed in detail by Dukor *et al.* (1974). A major role for activated C3, however, as a necessary requirement for triggering endotoxin-initiated B cell responses is not tenable on the basis of the currently available evidence. In fact, the experiments of Möller and Coutinho (1975) do not support any role for C3 in the response of B lymphocytes to endotoxin.

Additional studies by these investigators examined the potential participation of Fc receptors in the B cell response to endotoxin. Using SRBC coated with 7 S rabbit anti-SRBC to block B cell Fc receptors, no detectable effects on the capacity of endotoxin to stimulate PFC to FITC-SRBC were observed. These data suggested that the binding of antigen–antibody complexes via Fc receptors initiated neither specific nor nonspecific antibody synthesis. It is significant that the binding of such complexes also did not modify the induction of antibody synthe-

sis initiated by endotoxins. Similar results were reported by Ryan *et al.* (1975) using TNP-conjugated mouse serum albumin (TNP-MSA) and rabbit anti-TNP antibody. When such complexes were immobilized on a surface, however, profound inhibition of lymphocyte proliferative responses initiated by endotoxin (and other B cell activators) was observed. Specificity for the Fc receptor was demonstrated by the abrogation of inhibition when $F(ab')_2$ antibody was used instead of the intact antibody. Additional studies by Ryan and Henkart (1976) indicated that this inhibition was a direct effect on B cells, not the result of activation of suppressor T cells. These studies suggested that the Fc receptor-mediated inhibition may be the result of the generation of an intracellular "off" signal to the B cell. Preliminary experiments have suggested, in fact, that such a signal may involve increases in levels of intracellular cAMP (Ryan and Henkart, 1977).

A role for membrane-associated Ia antigens in endotoxin activation of B cells has also been reported in studies using alloantisera specific for Ia antigens (Niederhuber *et al.*, 1975). Pretreatment with anti-Ia antisera and complement reduced subsequent B cell proliferative responses by greater than 90%. In view of the demonstrated capacity of endotoxins to activate Ia^+ B cells, this result might not be entirely unexpected. These experiments also indicated that B cell responses to endotoxin could be inhibited by anti-Ia in the absence of complement, but inhibition did not exceed 50–75%.

The most extensively studied B cell membrane markers with respect to participation in B cell triggering by endotoxins have been surface immunoglobulins (sIg). The comprehensive experiments of Melchers and Andersson (1974b) first demonstrated a rapid and extensive decrease in the ability of radiolabeled Fab-anti-mouse Ig to bind to B cells following stimulation with endotoxin. At 37°, binding was reduced by 90% after 20 minutes. This response was shown to be an energy-dependent process which could be inhibited at 4°C or by azide. Approximately 12 hours after stimulation, sIg had reappeared (as 7–8 S IgM subunits), and by 40–60 hours it had increased to 30- to 100-fold over normal concentrations of sIg, most of which was shown to turn over with a half-life of about 5 hours. These data provide dramatic evidence for a dynamic state of sIg on B cells following endotoxin stimulation. More recently published data of Bourgois *et al.* (1977) have demonstrated specific decreases in surface IgD relative to IgM in endotoxin-stimulated B cells.

In order to examine the functional relationship between surface immunoglobulin and endotoxin-initiated B cell triggering, several investigators have probed the effects of rabbit and goat anti-mouse im-

munoglobulin (RAMIg, GAMIg) and specific anti-immunoglobulin isotype antibodies, on the proliferative and maturational responses following endotoxin stimulation. As these reagents appear to have selective effects on each of these endotoxin-elicited responses, we will consider them separately below.

As initially reported by Andersson *et al.* (1974), the addition of RAMIg (or $F(ab')_2$ fragments) to cultures of Nu/nu spleen cells enhanced the proliferative response initiated by endotoxins, although it had profound inhibitory effects on the initiation of PFC to TNP-SRBC. Experiments by Parker (1975) demonstrated that insolubilized RAMIg did not inhibit DNA synthesis but initiated a proliferative response of its own. Piquet and Vassalli (1973) had earlier reported that soluble RAMIg could induce a moderate proliferative response in splenic B cells. More recently, Sieckmann *et al.* (1978) have extensively characterized the murine B cell proliferative response to goat anti-mouse Ig. In contrast to these enhancing effects of RAMIg on endotoxin-induced responses, Schrader (1975b) showed virtually complete inhibition of endotoxin-induced proliferation by RAMIg. Inhibition required the presence of RAMIg during the first 24 hours of culture. These conflicting data have been partially clarified as the result of more recent studies. Sidman and Unanue (1976), by pretreating spleen cells with RAMIg for 1 hour, then stimulating with endotoxin for 1 hour, followed by culture in the absence of these agents, demonstrated complete inhibition of subsequent proliferation. These authors suggested that the continuous presence of endotoxin could potentially stimulate new B cells that had never seen RAMIg. A biphasic effect of rabbit anti-μ on endotoxin-induced responses was reported by Kearney *et al.* (1976a,b), who found that at low concentrations anti-μ would inhibit proliferative responses whereas high concentrations induced enhanced DNA synthesis. It is of interest that anti-γ_2 antibody had no detectable effects on proliferation. Finally, the very significant influences of culture conditions in modulating endotoxin B cell responses was recently demonstrated by Sidman and Unanue (1978), who reported that, in 5% fetal calf serum, polyvalent anti-Ig, anti-IgM, and anti-IgD all inhibited proliferation. However, in 10% fetal calf serum and 2-mercaptoethanol, anti-IgM (but not anti-IgD or anti-Ig) initiated a proliferative B cell response and enhanced the endotoxin-induced response. Thus these combined data indicate that modulation of surface IgM by multivalent ligands can produce either enhancing or inhibitory signals in regulating endotoxin-induced B cell proliferation (depending upon accessory signals, such as mercaptoethanol or treated fetal calf serum). The effect of IgM interactions *alone* always elicits a

negative triggering signal. In addition, anti-IgD, but not anti-IgG_2, can inhibit proliferative responses.

Most investigators have concluded that anti-immunoglobulin reagents inhibit endotoxin-induced differentiation to high rate antibody secretion and PFC (Andersson *et al.*, 1974, 1978b; Kearney *et al.*, 1976a,b, 1977, 1978; Sidman and Unanue, 1978). A majority of these studies demonstrate that anti-μ will inhibit maturation of IgM, IgG, and IgA precursors. Of interest, Andersson *et al.* (1978b) have shown that anti-μ and anti-κ added to endotoxin-stimulated B cells for either 30 minutes or continuously will inhibit the development of IgG precursors into PFC. In contrast, anti-γ_1 or anti-γ_{2a} will inhibit IgG-PFC in endotoxin-stimulated cultures *only* if they are maintained continuously in culture.

Several investigators have also noted that there are significant differences in the response of B cells from very young mice or neonates in comparison to adults. Thus Kearney *et al.* (1976a,b) noted that spleen cells from newborn mice were significantly more sensitive to the inhibitory effects of anti-μ on endotoxin-induced proliferation than were adult spleen cells. Sidman and Unanue (1975) showed that, following capping induced by anti-Ig, immature B cells do not reexpress surface Ig and are unresponsive to endotoxin stimulation. In contrast, sIg removed by enzymic digestion is reexpressed as is responsiveness to endotoxin. Finally, as recently shown by Kearney *et al.* (1978), adult spleen cells require simultaneous treatment with anti-μ and endotoxin in order to effect inhibition. Immature spleen cells can be inhibited with anti-μ in the absence of endotoxin.

d. Regulatory Effects of Lymphoid Cells. The regulatory role of other lymphoid cells in modulating the expression of B lymphocyte activation by endotoxins has been a subject of great interest. As will be discussed in Section VI, there is considerable evidence to indicate a suppressive effect of macrophages on endotoxin-induced B cell proliferation. The results of several recent experiments would also suggest a role for T cells (Norcross and Smith, 1977). Smith and Eaton (1976) reported a large, rapidly sedimenting cell, sensitive to anti-Thy-1 and complement, and present in spleen cells of normal and Nu/nu mice which inhibited endotoxin-induced B cell responses. Similarly, Frelinger (1977) found a population of anti-Thy-1 and complement-sensitive, nylon wool-adherent spleen cells in mice primed with minute quantities of endotoxin, which inhibited the proliferative response of normal spleen cells to endotoxin. This cell population was short-lived (7–10 days) and had several properties distinct from those of other suppressors in terms of both kinetics of induction and longev-

ity. Goodman and Weigle (1979) have recently shown that the presence of T cells (during the first 24 hours of culture) will enhance endotoxin-induced nonspecific maturation of B cells to PFC against SRBC. The lack of significant enhancement with either irradiated T cells or T cells from an endotoxin-unresponsive mouse (Section V) indicates that this response is very likely mediated by a direct T cell–endotoxin interaction. Thus, even in serum-free spleen cell cultures, the effects of endotoxins on B cells can be significantly affected by the surrounding lymphoid cell milieu.

e. Miscellaneous Effects—Enhancers and Inhibitors. It is clear from the preceding sections that a number of variables affect the capacity of B lymphocytes to respond to endotoxin. Some of these variables include age of donor tissues from which lymphocytes are obtained, culture conditions (including the concentration of tissue media supplements), source and method of preparation of the endotoxin, and the presence of metabolic inhibitors. The analysis of these variables has contributed significantly to the understanding of the mechanism of B cell triggering by endotoxins. There have been, in addition, numerous reports of additional variables that will influence B-lymphocyte responsiveness to endotoxins. Although a comprehensive analysis of such variables will contribute to our understanding of the mechanisms of B cell triggering, this information is for the most part not currently available. Nevertheless, many factors are felt to be of relevance in a summary of mechanisms of B cell responses to endotoxins, and several of these are considered briefly in the following paragraphs.

One of the potentially most important factors that may regulate B cell responses to endotoxin is isologous or heterologous serum. Bullock and Andersson (1973) showed that very low concentrations of normal mouse serum ($\sim 5 \times 10^{-4}\%$) are capable of inhibiting the PFC to NNP in cultures of Nu/nu spleen cells stimulated by endotoxin. If the mouse serum were treated with 2-mercaptoethanol, however, there was no inhibition at concentrations of serum as high as 5–25%. Similarly, Nelson and Schneider (1974) reported that normal mouse serum added to cultures of spleen cells inhibited both the background and the endotoxin-stimulated increase in RNA and DNA synthesis. Although much of the inhibition was suggested to be a "damping down" of the response, these authors did detect a specific inhibition of the endotoxin response (mediated by macrophages, see Section VI). Several reports by Poe and Michael (1976a,b) indicated that mouse serum (5%) will virtually completely abrogate the endotoxin-induced proliferative response of normal and Nu/nu spleen cells. Whereas

normal (and Nu/nu) serum was reported to enhance the endotoxin-induced PFC to SRBC of normal spleen cells, significant inhibition was obtained in cultures of Nu/nu spleen cells, as reported earlier by Bullock and Andersson (1973). Similarly, immune responses to endotoxin were unaltered in mouse serum-treated normal spleens but were abrogated in cultures of Nu/nu spleen cells. Inhibition by isologous serum is not limited to cultures of murine lymphocytes, and recent experiments by Streilein and Hart (1976) have shown similar effects in endotoxin-stimulated hamster lymphoid cells. Preliminary gel filtration chromatography of hamster serum indicated the inhibitory factor to be excluded from Sephadex G-200.

In addition to normal serum factors that can influence endotoxin-initiated B lymphocyte responses, there are also several recently reported endotoxin-initiated serum factors that can influence B cell responses. For example, Hoffmann and co-workers (1977a,b) have recently described a serum factor induced in BCG-infected mice following injection of endotoxin. This factor, termed tumor necrosis factor, has been shown to control selectively the phenotypic and functional differentiation of B cells. In addition, as its terminology implies, it has been demonstrated to induce tumor necrosis (see Sections VI and VII). A second report has described the immunopotentiating effects of serum obtained from *in vivo* endotoxin-treated mice. A factor of approximately 65,000 molecular weight has been purified that significantly enhances the immune response of mice to SRBC and does not represent endotoxin contamination (see Section V) (Tanabe *et al.*, 1977).

One of the better studied inhibitors of endotoxin-initiated B cell responses is polymyxin B sulfate. This low molecular weight cationic polypeptide has been shown to bind specifically to the lipid A region of LPS (Morrison and Jacobs, 1976) and to inhibit specifically lipid A-initiated proliferation (Jacobs and Morrison, 1976) or high-rate antibody synthesis (Smith *et al.*, 1976) in B lymphocytes. It is of interest that polymyxin B must be present during the first 12 hours of culture to obtain proximal inhibition (Jacobs and Morrison, 1977). It should also be noted that polymyxin B will inhibit endotoxin responses only in preparations of LPS that are free of lipid A-associated proteins (LAP) and that no inhibition of LAP–LPS complexes is observed (Betz and Morrison, 1977). This fact is often overlooked by investigators attempting to use polymyxin B to rule out potential contamination of reagents with endotoxins (Morrison and Curry, 1979).

Investigations by Hart and Streilein (1976, 1977) have recently examined the effects of protease inhibitors on endotoxin-initiated pro-

liferative responses of hamster lymphocytes. The results of these studies suggest that the trypsin-specific inhibitor tosyl lysine chloromethyl ketone (TLCK) (10^{-5} *M*) will specifically inhibit endotoxin-induced proliferation. This reagent is effective even if added after 8 hours of culture, but no effects are observed if it is added after 24 hours. Additional experiments indicate a trypsin-mediated response of hamster lymphoid cells to endotoxin with inhibition by soybean trypsin inhibitor. The authors suggest from these studies that a proteolytic event with a trypsinlike specificity is implicated as a relatively early event in endotoxin activation of hamster lymphoid cells.

Hart (1978) recently reported that relatively low concentrations of $ZnCl_2$ ($\sim$10–25 μM) will enhance the proliferative response of hamster lymph node cells to endotoxin. At the same concentrations $ZnCl_2$ had only a modest effect on splenocyte responses. No mechanism was proposed for the enhancement; yet the effects of Zn^{2+} as membrane-stabilizing agents and as cofactors for enzymes are well recognized. Ferguson *et al.* (1976) reported that the membrane-stabilizing agents lidocaine and chlorpromazine inhibited endotoxin-induced proliferative responses in a reversible way. It should be noted, however, that Smith *et al.* (1978) concluded that the observed inhibition by these agents could be accounted for entirely by their lymphocyte toxicity effects. Ryan and Shinitzky (1979) and Tsang *et al.* (1977) have recently reported that certain glycosphingolipids (gangliosides) and fatty acids, respectively, will inhibit lymphocyte proliferation induced by endotoxins; however, the mechanism for these effects remains to be clarified.

As indicated in Section III,A,4, there exist substantial differences in endotoxin-induced responses when lymphocytes from neonatal mice are compared with those of adult mice. Similar studies by Rabinowitz (1975) and Gerbase-Delina *et al.* (1974) have confirmed these observations. In addition these latter investigators have indicated a significantly reduced endotoxin response in lymphocytes from 2.5-year-old mice. More recent studies by Abraham *et al.* (1977) have provided data which indicate, however, that the reduced response in old mice is the result of a decreased number of responding B cells rather than an alteration in those cells that do respond to endotoxin. Kenny and Grey (1971) reported sex differences in immunologic responses of mice to endotoxins, females eliciting a better PFC response than males. Krzych *et al.* (1978) have indicated that the proliferative response of lymphocytes from female mice displayed two peaks of enhanced responsiveness to endotoxins, suggesting that female hormones may directly or indirectly stimulate immune responsiveness in adult mice.

Finally, the relative health status of the donor from whom lymphocytes are obtained can contribute to the responsiveness of B cells to endotoxins. While there exists a considerable volume of literature on this subject, it is beyond the scope of this review to more than illustrate this fact by way of several examples. Kondo *et al.* (1973) showed that spleen cells isolated from tumor-bearing mice gave abnormal proliferative responses to endotoxins, which, depending upon the method used to normalize responses, varied from slightly to significantly enhanced. In contrast, the results of Kirchner *et al.* (1974) using mice, and Veit and Feldman (1976) using rats, have established depressed lymphocyte proliferative responses to endotoxins in animals bearing Moloney sarcoma tumors. Also Garfin *et al.* (1978) have recently demonstrated transient, but significant, decreases in responsiveness of B cells to endotoxins in lymphocytes obtained from mice following infection with scrapie virus.

Of interest, several investigators have also recently described *in vitro* induction of murine C-type virus (Moroni and Schumann, 1975; Moroni *et al.*, 1975; Greenberger *et al.*, 1975) and herpes simplex virus (Kirchner *et al.*, 1976) in spleen cell cultures treated with endotoxins. In most of these experiments, generation of virus was shown to correlate well with proliferative responses, and electron microscopy revealed the presence of both budding particles and mature extracellular particles. In more recent studies, Schumann and Moroni (1976) and Phillips *et al.* (1976) have clearly shown this to be a B lymphocyte-mediated phenomenon, which was active in the absence of adherent cells and could be elicited with the isolated lipid A component of endotoxins. However, both age and strain differences have been noted in the generation of C-type viruses by endotoxins (Schumann and Moroni, 1977). The full implications of this B cell-mediated C-type viral induction by endotoxins remains to be elucidated.

B. The Interaction with T Lymphocytes

In contrast to the well characterized effects of bacterial endotoxins on B lymphocyte proliferative responses, the vast majority of the available evidence indicates that endotoxins do not directly initiate T cell proliferative responses as assessed by incorporation of [^{3}H]thymidine. This was extensively documented by the experiments of Andersson *et al.* (1972a), who showed that endotoxins did not activate murine thymocytes, cortisone-treated thymocytes, or peripheral T cells. These results were established to be independent of endotoxin concentration over a wide range of concentrations from 10^{-2} μg/ml to 10^{2} μg/ml. Additional experiments by these investigators (Möller *et al.*, 1972a,b)

documented that endotoxins failed to induce cytotoxic cells in preparations of either normal or Nu/nu spleens. Subsequent studies by numerous investigators (e.g., Peavy *et al.*, 1974) have confirmed these observations using a broad spectrum of experimental conditions. In this study, about 20 preparations of endotoxins derived from a variety of bacterial sources were tested. It therefore seems justified to conclude that endotoxins do not *induce* detectable increases in DNA synthesis or cell-mediated cytotoxicity in murine T lymphocytes.

Several interesting reports have, however, provided data that demonstrate that endotoxins can profoundly *influence* the proliferative response of T lymphocytes to selective T cell activators. For example, Schmidtke and Najarian (1975) demonstrated that, whereas endotoxins were by themselves unable to stimulate proliferative responses in human lymphoid cells (see Section III,A,1,c) they enhanced 2- to 4-fold the concanavalin A (Con A)- or phytohemagglutinin (PHA)-induced incorporation of [^{3}H]thymidine. Similar enhancing effects of endotoxins were noted by Forbes *et al.* (1975) in the proliferative response of murine thymocytes to Con A. It is of interest that the 2- to 3-fold enhancement observed by endotoxin did not result in a change in the dose response profile of the Con A, but rather an enhancement of all doses tested. These studies confirmed the report of Diamantstein *et al.* (1973) showing that endotoxins did not affect the murine T cell response to PHA (in contrast with the effect of endotoxins on *both* PHA and Con A responses in human T lymphocyte responses).

The effects of endotoxin on Con A-stimulated murine T lymphocytes were also examined by Ozato *et al.* (1975), who reported from 2- to 10-fold enhancement of [^{3}H]thymidine incorporation. Pretreatment of T cells with endotoxin prior to Con A stimulation decreased the threshold concentration at which stimulation could be detected, although simultaneous addition of these two molecules enhanced the response without a concomitant shift in the dose profile. Enhancement was dependent upon addition of endotoxin prior to, or simultaneous with, Con A. Addition of endotoxin as little as 20 minutes after Con A stimulation was without effect. Additional experiments demonstrated that endotoxin did not promote the binding of Con A to the T cells. It was postulated by these investigators that endotoxins in some way interacted with the T cell membrane and "stabilized" the membrane during its interaction with Con A leading to a triggering signal. Recent experiments demonstrating a similar endotoxin-mediated enhancement of Con A-stimulated thymocytes from the C3H/HeJ endotoxin-unresponsive mouse would be consistent with this interpretation (see Section V).

Evidence for an obligate requirement for T lymphocytes in endotoxin-induced murine Peyer's patch B lymphocyte responses was suggested by Kagnoff *et al.* (1974). These results were not confirmed by Gronowicz and Coutinho (1975b), who stressed the importance of culture conditions and endotoxin concentrations in the interpretation of *in vitro* responses. Data published by Alevy and Battisto (1976), however, have suggested that T cells, activated *in vivo* by dextran, will enhance B lymphocyte proliferation responses to endotoxin. Evidence for the participation of T cells in the enhancement was provided by the lack of such effects in Nu/nu mice.

As has earlier been documented for B lymphocyte responses, endotoxins appear to be equally capable of inducing maturation in pre-T lymphocytes. These experiments, primarily carried out by Scheid and her co-workers (1973, 1975; Hammerling *et al.*, 1975) have demonstrated that endotoxins will induce the expression of both the Thy-1 and the TL antigen in cultures of pre-T cells purified by albumin gradient centrifugation. Activation has been demonstrated to be reproduced with the isolated lipid A portion of endotoxins and thus has structural requirements similar to those required for B cell activation. As reported by Koenig *et al.* (1977), isolated LAP will also induce the expression of Thy-1 in pre-T cells. Of importance, pretreatment of lymphocytes with antiimmunoglobulin and complement had no detectable effect on the endotoxin-induced expression of Thy-1 (whereas this treatment completely abrogated the expression of Ia).

Evidence for a similar endotoxin-induced maturation of thymocytes has also been suggested on the basis of *in vivo* experiments (Baroni *et al.*, 1976; Adorini *et al.*, 1976). In these studies mice were injected with endotoxin and 4 days later their thymus cells were examined for their response to the T cell stimuli, Con A and PHA. In endotoxin-treated mice, thymocytes gained a responsiveness to PHA and also manifested a significantly increased responsiveness to Con A, as assessed by [^{3}H]thymidine incorporation. These authors also showed that these thymocytes were less sensitive to cytolysis with anti-Thy-1 and complement, suggesting that these cells expressed less Thy-1 antigen on their surface.

In summary, while there is clearly suggestive evidence for an interaction of endotoxins with both pre-T cells and with more mature T cells, the effects of such interactions are considerably less profound than those that have been documented for B lymphocyte interactions with endotoxins. There is, however, a substantial body of evidence supporting the participation of T-lymphocyte–endotoxin interactions in antigen-dependent immune responses. This evidence will be summarized in the next section.

IV. Antigen-Dependent Effects of Endotoxins on Immune Responses

In contrast to the nonspecific initiation of immune responses by endotoxins in the absence of antigen, which results in the synthesis and secretion of low levels of relatively low-affinity antibody, the presence of endotoxins prior to, during, or following immunologic challenge with specific antigen can have profound effects on the levels of antigen-specific antibody generated. In this respect, considerable evidence exists for variations in immune responses varying from almost complete suppression of antibody responses to significant enhancement, including the reversal of the induction of immunologic tolerance. Although these various immunologic manifestations of endotoxins have been recognized for a number of years, the cellular mechanisms responsible for these activities have been clarified only during the last decade. It is the purpose of this section to review the data relevant to the interactions of lymphocytes and endotoxins that result in the modulation of host immune responses to antigens distinct from those associated with the endotoxin molecule.

The results of numerous studies demonstrating many effects of endotoxins on immune responses have been summarized in earlier reviews and will not be covered in detail here. Nevertheless, several of these studies established a firm basis for the more recent investigations and are worthy of note. In a series of classic experiments, Johnson *et al.* (1956) reported a marked enhancement of the rabbit antibody response to several protein antigens when administered in conjunction with endotoxin. This enhancement was demonstrated to be a general property of endotoxins from many bacterial species. Significantly, the activity was reduced following alkaline hydrolysis of the endotoxin and could be reproduced by the lipid portion of the endotoxin following acid hydrolysis. On the basis of these studies Johnson *et al.* (1956) suggested that the enhancing action of the endotoxin was mediated through a cellular constituent concerned with the induction phase of antibody formation. Extensions of these studies by Kind and Johnson (1959) demonstrated a critical time period for endotoxin injection relative to antigen with enhancement being observed with endotoxin given as late as 2 days following antigenic doses of BSA. Significantly less effect was noted when endotoxins were given either before antigen or 3–4 days after antigen. Morphologic studies in rabbits following endotoxin and *bacillus Calmette-Güerin* (BCG) administration suggested to Langevoort *et al.* (1963) that endotoxin enhancement of antibody formation occurred through its effect on plasma cell proliferation. The extensive experiments of Franzl and McMaster (1968) and McMaster and Franzl (1968) clearly documented the bifunctional ef-

fects of endotoxins on murine immune responses to SRBC. These investigators established that endotoxins given before antigen significantly suppressed the immune response whereas endotoxin given simultaneously with or after antigen challenge significantly enhanced antibody titers. They noted virtually complete suppression when endotoxin was given 2 days prior to antigen. The cellular and biochemical mechanisms responsible for this enhancement and suppression of host responses to specific antigens will be the subject of this section.

A. Enhancing and Suppressive Effects

The effects of endotoxins on immune responses to unrelated antigens that were outlined in the early studies above have been further characterized in more recent investigations. The major points of these experiments are summarized in the following sections.

1. Humoral Immune Responses

The ability of endotoxins to enhance or suppress antigen-dependent immune responses is dependent upon the time of endotoxin injection relative to time of antigen administration. This has been confirmed by several investigators (Chester *et al.*, 1971; Diamantstein *et al.*, 1976; Nakano *et al.*, 1976). These studies all showed that administration of endotoxin to mice 2–3 days before injection of SRBC significantly inhibited the development of direct PFC to SRBC assessed on day 4. In each of these studies, injection of endotoxin with SRBC enhanced the subsequent PFC response. As demonstrated by Diamantstein *et al.* (1976), the reduced SRBC response induced by prior endotoxin injection was manifested throughout the time course of the SRBC response and thus did not reflect simply a shift in the kinetics of the anti-SRBC response. Enhanced responses to DNP-KLH induced by simultaneous injection with endotoxin were also reported by Mond *et al.* (1974). Antibody affinity was slightly increased by endotoxin administration in this study.

It was further noted by several investigators (Chester *et al.*, 1971; Tanabe and Saito, 1975) that the injection of endotoxin 2–4 days prior to injection of SRBC plus polynucleotide (IC or AU) adjuvants abrogated the enhancing effect of the polynucleotides. The mechanism of these competitive responses is presently not clear.

More recent studies by Behling and Nowotny (1977) have confirmed that the time period between preinjection of mice with endotoxin and subsequent immunization with SRBC is critical for the manifestation of either enhancement or suppression. These authors demonstrated oscillating periods of marked enhancement and suppression with a

periodicity of about 2–3 days. Enhancing effects of endotoxins could be detected as early as 12 days prior to SRBC immunization. As demonstrated by Nakano *et al.* (1975a), however, if endotoxin was injected into mice 15 days before a primary immunization with SRBC, no differences were observed from saline-injected controls. On the basis of these results the former authors proposed that the endotoxin induced a degree of synchrony in the level of maturation of cells involved in the response to SRBC. It is of interest that virtually identical results in these studies were obtained using a nontoxic polysaccharide (PS) derivative of the endotoxin (see Section IV,B).

Similar experiments performed in SRBC-primed mice, demonstrated that injection with endotoxin as early as 11 days before secondary challenge significantly inhibited the subsequent response to SRBC (Tanabe and Saito, 1975). Maximal inhibition was obtained 4 days before antigen immunization, and no significant differences in the kinetics were noted. These investigators also reported that an endotoxin serum, generated by injection of endotoxin into mice that were bled 2 hours later, reversed the inhibitory effect of the endotoxin when administered with antigen. The nature of this serum factor has more recently been characterized by Tanabe *et al.* (1977) (see also Section VI).

Walker and Weigle (1978) have also reported effects of endotoxins on secondary immune responses in mice primed to heterologous γ-globulins. Turkey γ-globulin (TγG)-primed murine spleen cells, cultured *in vitro* with antigen, were inhibited in their PFC response if endotoxins were added 12–24 hours after antigen addition. Evidence that the endotoxin was inhibiting a specific cellular response was suggested by the lack of inhibition in C3H/HeJ-primed spleen cells (see Section V). Although the mechanism was not defined, the authors suggested a possible role for B and/or T cells.

Very small quantities of endotoxin have also been demonstrated to regulate immune responses to hapten endotoxin conjugates. Both enhancing and suppressive effects have been noted. Möller *et al.* (1975) demonstrated *in vitro* that addition of endotoxin to suboptimal concentrations of hapten endotoxin conjugates enhanced the response to the hapten. Pretreatment of mice *in vivo* with minute quantities of endotoxins (7×10^{-4}ng), however, significantly inhibited their capacity to respond to TNP-conjugated endotoxin when challenged *in vitro* 3–5 days later (Waldmann and Pope, 1975). This inhibition was independent of T cells, as similar responses were observed in Nu/nu mice. Inhibition was carrier specific in that responses to TNP coupled to an endotoxin with a different serotype were unaffected. The authors con-

cluded that the unresponsiveness was the result of a lipid A-dependent activation event, resulting in the production of an endotoxin-specific product that prevents the interaction of TNP-endotoxin with hapten specific cells. *In vitro* studies by Baltz and Rittenberg (1977) have shown that pretreatment of spleen cells with nanogram quantities of endotoxins leads to inhibition of primary responses to hapten-conjugated carriers. In this case, the response appears to be carrier nonspecific and to be rapidly induced. The authors suggest that this may represent a "down regulation" by which B cells discriminate antigenic signals in deciding to respond to antigens in the environment. These *in vitro* studies have confirmed the earlier *in vivo* observations that the effects of endotoxins on antigen-dependent immune responses will vary significantly depending on the relationship between the time of endotoxin administration and time of antigen addition.

Evidence for the potential participation of B and T lymphocytes, as well as macrophages, in the enhanced or suppressive responses to antigen as mediated by endotoxins will be summarized in Section IV,C.

2. *Cellular Immune Responses*

In addition to the indirect effects that endotoxins may have in the modulation of cell-mediated immune responses by the stimulation of humoral immunity (Lagrange *et al.*, 1975), there is also evidence for more direct effects on participating cells. Skopinska (1972) showed that the injection of endotoxins into irradiated recipients 4 days after the injection of parental spleen cells accentuated the splenomegaly assessed at 21 days and increased lethality. In contrast, endotoxin treatment of recipients was shown to have no effect on the GVH reaction induced by parental thymus cells. These data suggested that endotoxin was preferentially interacting with a cell population present in donor spleen but not thymus tissue.

Evidence for a regulatory role of adherent cells was provided by the experiments of Thompson and Jutila (1974) and more recently by Thompson *et al.* (1978). In these studies, spleen cells pretreated either *in vivo* or *in vitro* with endotoxin significantly suppressed the GVH reactivity of normal syngeneic spleen cells when injected into allogeneic neonatal recipients. Activity was shown to reside in the adherent cell population. Similar results were obtained by Rose *et al.* (1976), who reported that *in vivo* treatment of donor spleen cells with endotoxin markedly enhanced the subsequent survival of allogeneic recipients. In contrast to these studies, *in vivo* treatment of thymus

cells with endotoxin 4 days prior to injection into irradiated allogeneic recipients increased significantly the mortality induced by these thymocytes in comparison to untreated controls. Additional studies by Thompson *et al.* (1978) provided data to show that serum from endotoxin-treated mice (60 μg/day for 7 days) also markedly suppressed the GVH response as well as preventing the normal rejection of allogeneic skin grafts.

In a series of experiments designed to examine the effects of endotoxins on delayed-type hypersensitivity (DTH), Lagrange *et al.* (1975) and Lagrange and Mackaness (1975) reported that treatment of mice with endotoxin and antigen (SRBC) reduced significantly the DTH response observed upon subsequent challenge with antigen. This reduction in response correlated with increased levels of circulating antibody. In contrast, mice treated with endotoxins 2 days after antigen immunization manifest more pronounced DTH when subsequently challenged with antigen. These results were confirmed recently by Skopinska (1978), who assessed the generation of MIF production. Thus, depending upon the time of antigen administration and challenge in relation to time of endotoxin treatment, responses ranging from severe depression to marked enhancement can be observed. These conclusions are similar to those reached with respect to the effects of endotoxins on humoral immune responses.

Enhancement of one-way mixed lymphocyte culture (MLC) responses was reported by Forbes *et al.* (1975), who demonstrated that endotoxins increased the proliferative response of thymocytes of allogeneic mitomycin C-treated spleen cells. Similar results were reported by Ritter *et al.* (1975) using allogeneic thymocytes as responder cells; these latter investigators reported that no response was obtained in endotoxin-stimulated cultures of macrophage-depleted mixed thymocytes. They were able, in addition, to restore activity by the addition of a small number of syngeneic, allogeneic, or even third-party macrophages. They proposed from the results of these studies that the macrophage was the primary target for endotoxin in this allogeneic T cell response.

More recent results published by Narayanan and Sundharadas (1978) and Narayanan *et al.* (1978) support a macrophage-independent enhanced MLC response of endotoxin-stimulated thymocytes. Using stimulator–responder combinations of thymocytes that differed either for the entire *H-2* region or only for the *K* or *D* subregion (which in the absence of endotoxin induces only weak MLC responses) these investigators demonstrated significantly enhanced responses in the presence of endotoxin. They further demonstrated that, unlike stimulation with poly A-U, the enhanced response due to endotoxin did not re-

quire the participation of macrophages. The enhancement could be detected even when the endotoxin was added as late as 48 hours after the initiation of response. Although most of the enhanced responses were assessed by cytotoxicity measurements, similar results were obtained for T cell proliferation.

It is evident that endotoxins can profoundly affect immune responses in cell-mediated immunity by direct interactions with T cells as well as by interactions with macrophages that then regulate T cell responses (see also Section V,A,2).

3. *Allergic Responses*

Fewer studies have been performed to examine the effect of endotoxins on antigen-induced stimulation of IgE antibody responses. This is, in part, due to the inherent difficulty in assessing IgE responses. Nevertheless, available evidence supports the concept that endotoxins, when administered with an IgE-inducing antigen, can enhance the host allergic response to that antigen. The experiments of Perini and Mota (1973) first addressed this question by immunizing guinea pigs simultaneously with ovalbumin and endotoxin. Endotoxin promoted an early and sustained production of IgE as well as IgG_1 antibody. Relatively low amounts of endotoxin and/or antigen favored IgE whereas higher amounts of either favored IgG_1. No responses were observed if endotoxin was given 24 hours before or 48 hours after injection of antigen. Further studies by these investigators (Mota and Perini, 1975) demonstrated endotoxins to be significantly better adjuvants for IgE antibody synthesis than poly IC.

To examine the cellular mechanisms responsible for the adjuvant properties of endotoxins in IgE antibody production, Newburger *et al.* (1974) employed adoptive transfer of DNP-conjugated *Ascaris* antigen (DNP–Asc)-primed spleen cells into sublethally irradiated syngeneic recipients. When these mice were challenged with DNP–Asc in the presence of endotoxin, increased levels of IgE and IgG were observed relative to saline-treated controls. Evidence that the enhanced response was not the result of direct stimulation of B cells was suggested by the observation that no antibody responses were observed when mice were challenged with DNP–ovalbumin (DNP–OVA) plus endotoxin. Additional proof for this concept was provided by the demonstration that adoptive transfer of endotoxin-stimulated carrier-primed (OVA) cells and hapten (DNP)-primed cells led to significant enhancement of IgE and IgG responses as compared to untreated carrier-primed cells. As enhanced responses were obtained with B cells that had never "seen" endotoxin, it was argued that T cells were the target for the adjuvant effects of endotoxin.

Results obtained using Nu/nu mice and reported by Danneman and Michael (1976) also support a primary role for T cells. The injection of OA and endotoxin into +/nu but not Nu/nu mice led to the production of both IgE and IgG_1 antibody. Of significant interest, using a variety of experimental conditions, these investigators were unable to stimulate an anti-endotoxin IgE response, even though these mice made normal IgM responses. These combined data are consistent with a T cell requirement in the endotoxin-enhanced antibody-dependent initiation of IgE antibody responses.

B. Endotoxin Requirements for Adjuvant Effects

As with most of the biologic activities of endotoxins, a prominent role for the lipid A region of the molecule has been demonstrated for adjuvant activity. There are, however, several modifications of endotoxins which, while markedly altering a number of the toxic properties, have been shown to leave the adjuvant properties intact.

The experiments of Johnson *et al.* (1956) first suggested the lipid A dependence of endotoxin-mediated adjuvant activity (see Section IV,A). They found that adjuvant activity was abrogated by alkaline hydrolysis and could be found in the lipid portion following acid hydrolysis. The demonstration by Nakano *et al.* (1971a,b) that adjuvant effects, assessed by enhancement of 7 S and 19 S PFC, could be reproduced by endotoxin from the polysaccharide-deficient *S. minnesota* R595 mutant, provided support for this concept. Additional experiments by Chiller *et al.* (1973), Nowotny *et al.* (1975a), and Nakano *et al.* (1975b), using isolated lipid A, established conclusively that lipid A had the capacity to enhance immune responses to both soluble and insoluble antigens. Experimental evidence in support of this conclusion has been subsequently provided by numerous investigators. An interesting aspect of the latter study by Nakano *et al.* (1975b) was that isolated lipid A manifested its enhancing activity on the SRBC response in mice only when complexed with BSA or, to a lesser extent, with RSA. Evidence in support of the active participation of such complexes was provided by the demonstration that enhancing activity could be abrogated by treatment of mice with antibody to the BSA. The results of the other studies, however, suggest that complexing of lipid A with soluble carriers is not essential for enhancement of antibody formation (Chiller *et al.*, 1973).

The association of endotoxin with antigen has also been examined by Schenck *et al.* (1969) in relation to adjuvant activity. These investigators covalently coupled HSA to endotoxin prior to immunizing rats and found that the HSA–endotoxin complexes were significantly better in promoting antibody synthesis than the two molecules injected

separately. These data suggested to the authors that one of the main effects of adjuvants such as endotoxins is direct and highly localized and that the antigen linked to the endotoxin reaches critical target cells with a greater efficiency than antigen alone.

These investigators also established that nontoxic polysaccharides derived from the parent endotoxin molecule by deacylation or by treatment with succinic anhydride were potent enhancers of antibody formation in rats. Additional studies by McIntire *et al.* (1976) and Chédid *et al.* (1975) confirmed these results. In addition, it was reported that endotoxin derivatized by treatment with hydroxylamine or with sodium phthalate, which was essentially nontoxic and nonpyrogenic, retained potent adjuvant activity. Of significant interest, these substituted endotoxins were also markedly less active in inducing proliferative B cell responses, suggesting to these investigators that the capacity of endotoxins to act as immunologic adjuvants may not be related to their capacity to initiate B cell proliferation. Evidence is available, however, that does support a correlation of B cell proliferative responses and immune adjuvanticity of endotoxins (Skidmore *et al.*, 1975a).

Modification of endotoxins by treatment with potassium methylate or by acid hydrolysis to prepare water-soluble low molecular weight polysaccharides (PS) has also been demonstrated to affect significantly the adjuvant properties of these molecules. In comparison with the native endotoxin, the acid-hydrolyzed nontoxic PS was of comparable activity in enhancing the *in vitro* PFC to a number of heterologous erythrocytes. Treatment with potassium methylate destroyed activity. As reported earlier for the succinylated and phthalated derivatives, the PS was also found to be inactive in initiating proliferative B cell responses. Similar effects of potassium methylate treatment were more recently reported by Elekes *et al.* (1978).

One final interesting observation is worthy of comment. A review of the available literature has indicated that many investigators utilize a boiling procedure for the preparation of endotoxins for immunologic studies. Although all the effects of such a procedure have not been investigated in detail, Dresser and Phillips (1974) have documented that boiling and sonication can have significant effects on the adjuvant properties of endotoxins by enhancing the primary (IgM and IgG) immune response to SRBC. The differences in properties of endotoxins determined by their method of preparation may well contribute to the differences observed by various investigators in analyzing the effects of these molecules on immune responses. As indicated earlier (Section II), these procedures can also have equally profound effects on the intrinsic immunogenicity of the endotoxin molecule.

C. Endotoxin Lymphoid Cell Interactions in Humoral Responses to Antigen

In Section II, the data documenting the T lymphocyte and macrophage independence of the host immune response to endotoxins has been discussed. Endotoxins, however, may interact with both T cells and macrophages, as well as B cells. This suggests that these accessory cells may play an integral role in endotoxin modulation of antibody responses to unrelated antigens. There is considerable evidence to support this conclusion and the available data indicate that the function of each of these lymphoid cell components is affected by endotoxin. For the sake of convenience, we will deal with each cell type separately.

1. T Lymphocytes in Adjuvant Effects of Endotoxins

a. Evidence for T Cell-Independent Antigen Responses. Many experiments have provided evidence that, as was earlier demonstrated for antibody responses to antigenic determinants on the endotoxin molecules themselves, endotoxins will circumvent the requirement of most antigens for T-helper cells in the initiation of immune responses. As demonstrated by Möller *et al.* (1972a), endotoxin coupled to SRBC permitted this T-dependent antigen to elicit a primary immune response in adult thymectomized, lethally irradiated, and bone marrow-reconstituted (AT × BM) mice. That such a response was unrelated to antigenic determinants on the endotoxin molecule itself was shown by the equivalent (and even enhanced) response in mice previously made tolerant to endotoxin. These authors concluded that the presence of endotoxin on the erythrocyte substituted for the presence of T-help in the immune response to the SRBC. Very similar results were obtained independently by Jones and Kind (1972), who were able to generate PFC to SRBC in thymectomized-irradiated and bone marrow-reconstituted mice injected with endotoxin, or with mice reconstituted with bone marrow cells and T cells. Reconstitution with both cell types plus endotoxin, however, led to significantly enhanced PFC to SRBC. Very similar results were reported by Watson *et al.* (1973a) using Nu/nu mice. Endotoxin again was shown to substitute for T cells in the response to SRBC. A requirement for coupling the endotoxin to the SRBC was, however, not established in these experiments. Using another experimental system, Schmidtke and Dixon (1972) showed that TNP coupled to mouse erythrocytes (TNP-MRBC) when administered to syngeneic recipients in the presence of endotoxin, elicited PFC to the TNP hapten. As helper T cells are nonexistent under these experimental conditions, the ability of endotoxin to bypass

the requirement for T cells was proposed. Similar experiments were also described by Watson *et al.* (1973b) using TNP coupled to single amino acids or haptens where endotoxins also elicited an antihapten PFC response. The binding of the TNP peptides to either endotoxin or indigenous serum proteins could not be rigorously excluded in these experiments. A strict requirement for particulate antigens (i.e., SRBC, MRBC) was also excluded by the results of Schrader (1973a,b, 1974a,b), who demonstrated that endotoxins would substitute for T-helper function in an *in vitro* and *in vivo* antibody response to fowl γ-globulin (FγG). These data have since been confirmed by numerous investigators and indicate that antibody responses to normally T-dependent antigens (or antigens for which T-help does not exist) can be elicited in the presence of endotoxins and the absence of T-helper cells.

As an alternative to examining the immune response to haptens coupled to nonimmunogenic carriers, several investigators prepared covalent conjugates of low molecular weight haptens, e.g., 4-hydroxy-3,5-dinitrophenol (NNP) (Coutinho *et al.*, 1974) or 2,4,6-trinitrophenol (TNP) (Fidler, 1975; Jacobs and Morrison, 1975b) with endotoxins. These complexes were found, when tested in AT × BM or Nu/nu mice, to yield immune responses which shared the T-independent property of the endotoxin carrier. Several lines of evidence (e.g., concentration, antibody avidity) indicated that the antibody responses elicited were the result of antigen specific interactions and were thus distinguishable from the nonspecific antigen-independent antibody responses also elicited by endotoxins. Also, as with primary antiendotoxin responses, the spleen cell responses were prompt, with a peak antibody response on day 3 (Fidler, 1975). As suggested by Quintans and Lefkovits (1976), B cells activated by TNP–LPS are stimulated to undergo limited cell division without sustained clonal expansion.

The experiments of Jennings and Rittenberg (1976), using a number of T-dependent and T-independent hapten-conjugated antigens suggested that there exist separate subpopulations of B cells responding to the T-dependent and T-independent antigens. More recently, Diaz-Espada *et al.* (1978) have shown that spleen cells, primed *in vivo* with either T-dependent or T-independent hapten–antigen conjugates undergo a short period of hyperreactivity to TNP–LPS followed by a sustained period of hyporesponsiveness. These authors suggest that B cell differentiation precludes the establishment of immunologic memory to TNP–LPS, as the TNP–LPS-activated B cells become insensitive to subsequent stimulation by the same stimulus (see also Section

III,A,2, terminal differentiation of B cell subpopulations by endotoxin.)

Specific antibody responses have also been obtained with hapten–endotoxin conjugates prepared from polysaccharide-deficient endotoxins. These immune responses were demonstrated to be both hapten specific and T cell independent, thus obviating a necessary structural requirement for repeating oligosaccharide units in the T-independent response (Skelly *et al.*, 1979b). A functional requirement for lipid A was demonstrated by several investigators, who showed that treatment of hapten–endotoxin conjugates with mild alkali abrogated the capacity of these molecules to serve as T-independent carriers for induction of specific antibody (Jacobs and Morrison, 1975b; Jacobs, 1975a; Slowe and Waldmann, 1975). As such procedures also destroyed the capacity of these preparations to initiate proliferative B cell responses (see Section III), a specific role for such activity in T-independent responses was suggested. Support for this concept was established by experiments demonstrating that the C3H/HeJ endotoxin-unresponsive mouse also did not make either proliferative responses or T-independent immune responses to hapten–endotoxin conjugates (Jacobs, 1975b; Slowe and Waldmann, 1975; Skidmore *et al.*, 1975a; Coutinho, 1976; see also Section V).

The experiments reported by Jacobs and Morrison (1975a), however, provided evidence suggesting that the nonspecific B cell-activating properties of endotoxin–hapten conjugates could be completely abrogated under conditions where antihapten responses were in fact significantly enhanced. These responses were obtained by the use of the cationic polypeptide polymyxin B, which was demonstrated (Morrison and Jacobs, 1976) to bind to the active lipid A portion of the endotoxin molecule. These data indicated that the property of endotoxins that allowed initiation of nonspecific B cell proliferative responses could be dissociated from the property that allowed endotoxin to act as a T cell-independent carrier. Completely contrasting results were reported by Smith *et al.* (1976), who found that polymyxin B abrogated both the proliferative response and the specific immune response to NNP–endotoxin conjugates. They further reported that polymyxin B neither cross-reacted with NNP nor interfered with the expression of NNP determinants on NNP–endotoxin complexes. These authors indicated that their data were consistent with the concept that the nonspecific B cell-activating properties of the hapten–endotoxin conjugate were essential for its T-independent immunogenic properties.

Several postulates have been put forth to explain these discrepan-

cies. Möller *et al.* (1976a) extrapolated the data of Jacobs and Morrison (1975a) using a series of dose response curves that predicted a shift in the dose response as a result of polymyxin B, as was found experimentally by Smith *et al.* (1976). This was found not to be the case for the PFC response to TNP–LPS [although it was true for the proliferative responses (Jacobs and Morrison, 1976)]. The peak PFC response was identical (although significantly enhanced) in the presence of polymyxin B. Differences in culture conditions were also postulated to account for this conflict, but experiments using conditions similar to those used by Jacobs and Morrison did not confirm the results obtained with PB–TNP–LPS using a system employing CM–NNP–LPS (Smith and Hammarström, 1976). Potentially selective alterations in the endotoxin structure during the hapten conjugation procedures utilized to prepare the NNP and TNP derivatives remain to be explored to resolve this question.

Thus, although these combined data leave questions unanswered as to the precise mechanisms involved in endotoxin-mediated bypass of T cell function in the initiation of immune responsiveness, the potent capacity of this molecule to generate these T-independent responses is clear.

Haptens covalently conjugated to endotoxins have served as extremely useful model antigens for the delineation of host immune responsiveness to T-independent antigens. Implicit in experiments employing these conjugates is the assumption that the immune response to the conjugated hapten reflects the host immune response to the endotoxin antigen. It is, therefore, unexpected to find that the presence of the hapten, under some experimental conditions, profoundly affects the host immune response to the endotoxin O-antigen (O-Ag). As recently demonstrated by Skelly *et al.* (1979a), some murine strains (e.g., C57BL/6J) but not others (e.g., BALB/c) make normal antibody responses to unsubstituted endotoxins but make significantly reduced responses to the O-Ag when immunized with hapten–endotoxin conjugates. These altered responses are not the result of alteration in O-Ag, dose, method of assay, kinetics of response, or type of hapten and/or endotoxin employed. They do not appear to be dependent upon the presence of mature (suppressor) T cells. Mice pretreated with hapten–endotoxin conjugates respond similarly to those pretreated with endotoxin alone upon subsequent challenge with unsubstituted endotoxin. These data suggest that the haptenated endotoxins are not inhibited in their interaction with O-Ag-specific B cells. Regulation occurs subsequent to this interaction or by another mechanism that is not yet defined.

These results are highly reminiscent of the regulation of immune responsiveness to the common enterobacterial antigen (CEA) first described by Kunin (1963). This antigen, which is present in a number of different strains of gram-negative organisms is highly immunogenic in isolated form. When present in bacterial suspensions, however, or when mixed with isolated purified endotoxins or lipid A, no antibody against CEA is produced. Unresponsiveness requires that these two bacterial antigens be physically mixed and/or injected simultaneously in order to effect suppression (Whang *et al.*, 1965). It is significant that, although no CEA antibody is produced, such mice are immunologically primed for a secondary challenge with CEA alone (Neter *et al.*, 1966). Virtually identical results are obtained when the antigen common to gram-positive bacteria (Whang and Neter, 1967) is used. Whang and Neter concluded that the presence of the lipid A interfered nonspecifically with the productive phase of antibody formation without altering the establishment of immunologic memory. This regulation of immune recognition of one bacterial product by another may have significant relevance to host immune responses to bacteria in general.

b. Endotoxins and T Cell-Dependent Antigen Responses. As indicated above, under the appropriate conditions endotoxins can provide immunologic triggering to bypass the T cell requirement of some antigens in eliciting the synthesis and secretion of antibody. In the normal immune response to most antigens, however, the requirement for T cells is apparent. The available evidence indicates that endotoxins manifest their profound enhancement of antigen-induced antibody formation by, at least in part, an interaction with T cells. A requirement for endotoxin interaction with antigen-specific T-helper cells is also suggested.

The experiments of Allison and Davies (1971) provided the first indication of T cell participation in the adjuvant effects of endotoxins in a study of the primary and the secondary immune response of mice to BSA. These investigators demonstrated that mice depleted of functional T cells by either adult thymectomy or antilymphocyte serum, and reconstituted with bone marrow cells, were unable to respond to BSA in the presence of endotoxin unless the mice were also reconstituted with syngeneic thymus cells. These investigators concluded that T lymphocytes were an essential element for the potentiation of antibody formation against BSA by endotoxins and other adjuvants.

A similar approach was taken by Nakano *et al.* (1971a), who explored the mechanism of enhancement of the PFC response to SRBC by endotoxin. Treatment of mice with endotoxin was shown to increase both

the 7 S and 19 S PFC after primary and secondary immunization with endotoxin. A potential role for T cells in these responses was suggested by the fact that endotoxin acted primarily on nonadherent cells that were not antibody-forming precursor cells and exerted antagonistic effect on the immune suppression caused by antilymphocyte serum or cortisone. An extension of these studies, using *in vitro* treatment of cell populations with endotoxins followed by transfer into irradiated recipients, supported these authors' conclusions (Nakano *et al.*, 1973) and, in addition, suggested a role for endotoxin–B lymphocyte interactions.

Considerable support for this concept was generated by the comprehensive series of experiments of Katz and his collaborators. Using an adoptive transfer system, Hamaoka and Katz (1973) demonstrated that endotoxins affect T-helper activity in the absence of detectable increases in specific B lymphocyte responses. Thus, irradiated recipients, reconstituted with DNP–KLH primed spleen cells and challenged with DNP–BGG responded with anti-DNP antibody only in the presence of either endotoxins or BGG-primed helper T cells. The adjuvant effect of endotoxin was abrogated by anti-Thy-1 and complement but could be restored by BGG-primed helper T cells. To indicate that the endotoxin-induced adjuvant effect was on specific helper cells, these investigators immunized mice with the synthetic polymer D-GL-DNP (for which few if any helper T cells exist) along with either allogeneic T cells or endotoxin. Whereas the former regimen yielded a good anti-DNP response (due to the allogeneic effect), there was little response in the presence of endotoxin, suggesting that this latter molecule executed its effect on specific T cells. These authors proposed that the endotoxins serve to facilitate expansion of such helper T cells in response to antigenic stimulation.

The effects of endotoxins on *in vitro* enhancement of antigen-induced immune responses was examined by Armerding and Katz (1974) using similar protocols. The results of these studies essentially confirmed the earlier conclusions of these investigators. Using a variety of experimental conditions, they first documented the capacity of endotoxin to enhance the nonspecific antibody response to DNP–KLH in the absence of added antigen. Antigen (DNP–KLH) had no significant effect on the primary PFC response obtained in the presence of endotoxin. [This latter characteristic of *in vitro* endotoxin-stimulated cultures has been extensively analyzed by Möller *et al.* (1975c).] In contrast, significant antigen-dependent enhancing effects of endotoxins were noted in secondary responses. This was restricted to experiments in which the T cells had been appropriately carrier primed.

Additional experiments using mixtures of primed and unprimed spleen cells provided convincing data to implicate a significant influence of endotoxins on specific helper T-cell function in the adjuvant effects of endotoxins on antigen-dependent immune responses. These conclusions were similar to those summarized above Section IV,A) for endotoxin-induced enhancement of IgE antibody production (Newburger *et al.*, 1974).

More recent experiments by Ness *et al.* (1976) have examined the effects of endotoxins on murine immune responses to the genetically regulated polypeptide antigen (T,G)-A–L. High-responder (C57BL/10) and low-responder (B10.BR) mice both make T-independent primary immune IgM responses to this antigen, but only the high-responder strain is induced to a secondary IgG response upon antigen challenge. Examination of the effects of endotoxins on these responses indicated a T cell-independent enhancement of the primary IgM response in all strains of mice tested. In contrast, secondary IgG responses were strictly T cell dependent. Virtually identical conclusions were reached independently by Schrader (1975a) using FγG as an antigen. These combined studies provided strong evidence for both a T cell-dependent and T cell-independent mode of action of endotoxins in enhancing antibody formation to antigens, although, as Katz and co-workers earlier cautioned, these data did not distinguish between a direct effect of endotoxins on T cells or indirect effects on T cell function mediated by endotoxin interactions with other lymphoid cells.

In addition to supporting an effect of endotoxins on specific T cell helper function in enhancing immune responses, evidence also exists for nonspecific perturbation of T cells by endotoxins, which may lead to either enhancing or suppressive effects. Experiments by Baroni *et al.* (1976) suggested that thymocytes from mice primed with endotoxin 4 days prior to transfer into irradiated recipients had increased helper activity in the adoptive immune response to SRBC.

Hoffmann *et al.* (1975) reported that cultures of normal, Nu/nu, or AT × BM spleen cells in the presence or the absence of SRBC antigen responded equally to endotoxin stimulation. The T cell-dependent *in vitro* SRBC response was significantly suppressed by the addition of endotoxin at the initiation of culture. If endotoxin was added at later times, however, significant enhancement ensued, but only if endotoxin had not been added at the start of culture. It is of interest that the addition of *in vitro* primed T cells did not reverse the inhibition.

More recently, Uchiyama and Jacobs (1978b) have confirmed and extended these studies. They reported that endotoxins acting on sepa-

rate T cell subpopulations can both enhance and suppress the *in vitro* immune response to hapten-coupled erythrocytes (TNP–SRBC). Although both the enhancing and suppressive cell populations obtained from carrier-primed spleen cells were shown to be sensitive to anti-Thy-1 and complement, the endotoxin-induced enhancement was demonstrated to be resistant to irradiation and not adherent to nylon wool (characteristics of helper T cells). In contrast, the endotoxin-responsive cell that depressed PFC to TNP–SRBC was sensitive to irradiation and was glass wool adherent (characteristic of suppressor T cells). Whether the endotoxin was acting only on those T cells specific for the antigen carrier was not determined.

Further studies have demonstrated that T cell replacing factor (TRF) markedly enhances the ability of endotoxin to stimulate the response of B cells to SRBC antigen under conditions where endotoxin alone is only marginally stimulatory. Jacobs (1979) proposed from these experiments that the mechanism of endotoxin adjuvant activity was actually equivalent to the synergy observed with TRF. By this mechanism the endotoxin served primarily to induce B cells to become more sensitive to T help and that T cells served only their normal cooperating cell function. While this conclusion is consistent with the endotoxin-TRF synergy, it does not explain the adjuvant responses described by Katz and co-workers, where significant enhancement was documented under conditions where the B cells were never exposed to the endotoxin.

2. *B Lymphocytes and Regulatory Effects of Endotoxin*

The cellular target for the adjuvant effect of endotoxins was initially suggested to be the B lymphocyte by the experiments of Nakano *et al.* (1973) and Dresser and Phillips (1973, 1974). In an extensive series of adoptive transfer experiments, Nakano *et al.* (1973) demonstrated that macrophage-depleted spleen cells, bone marrow, lymph node, or thymus cells treated *in vitro* with endotoxin and subsequently transferred either separately or together, all enhanced the capacity of recipients to respond to SRBC in comparison with untreated controls. These studies suggested that the enhancing effect of endotoxin depended upon its effect on immunocompetent cells of both thymus and bone marrow origin. Dresser and Phillips (1973, 1974) defined an index of interaction to assess relative adjuvant effects in both T cell-depleted and T cell-containing mice. On the basis of the relative response obtained with and without endotoxin in these mice, the authors concluded that the endotoxins acted primarily through B cells.

A somewhat different approach was taken by Skidmore *et al.*

(1975a,b, 1976) to examine the relationship between the B cell-activating capacity of endotoxin and its capacity to act as an adjuvant of antibody formation. Using several situations in which B cells were known to be unresponsive to endotoxins (mild alkaline hydrolysis, which abrogated the ability of endotoxin to initate B cell proliferative responses, and lymphocytes from the endotoxin unresponsive C3H/HeJ mouse) Skidmore and co-workers demonstrated a direct correlation between these two immunologic manifestations of endotoxin activity. Additional experiments analyzed inbred backcross mice of C3H/HeJ and endotoxin responder mice. These results provided evidence for an exact segregation of the capacity of the endotoxin to elicit B cell proliferative responses and its ability to enhance immunologic responsiveness to a secondary challenge with BSA. B cell responses also correlated with the ability to modulate the induction of immunologic tolerance to a normally tolerogenic antigen (see Section IV,D). These data clearly demonstrated that the relevant factors that regulate endotoxin B lymphocyte interactions also regulate the capacity of endotoxins to act as adjuvants.

Several lines of evidence indicate that endotoxin-activated B lymphocytes can also manifest suppressive effects on responses to other antigens. This was demonstrated by Braley-Mullen *et al.* (1977), who showed that endotoxin given simultaneously with pneumococcal polysaccharide (SIII) suppressed the primary IgM response to the SIII antigen. No suppression was observed in C3H/HeJ spleen cells or following alkaline hydrolysis of endotoxin. Evidence for the lack of a role for T cells was provided by the demonstration of equivalent suppression in Nu/nu mice. It is of interest that there was no suppression of primed B cells; nor did the endotoxin suppress the development of IgG memory B cells to the SIII.

The ability of endotoxin to alter B cells *in vivo* was reported by Persson (1977) and more recently by Uchiyama and Jacobs (1978a). These investigators found that spleen cells from mice primed 2 days earlier with endotoxins contained a population of B cells that were functionally abnormal. Persson, on the basis of several lines of evidence indicated that the *in vivo* endotoxin-treated spleen cells contained a suppressor B cell. Additional experiments indicated that the suppressor cell could be induced in the absence of macrophages and was resistant to treatment with anti-Thy-1 and complement. On the basis of these experiments, Persson concluded that the endotoxin-induced suppressor cell was indeed a B cell. Uchiyama and Jacobs also reported that spleen cells from endotoxin-treated mice were abnormal in their capacity to cooperate in the initiation of immune responses to T-dependent antigens. These data indicated that both the B

cells and the T cells were defective, but that the suppressor activity present in such populations was probably of T cell origin.

3. *Macrophages and Regulatory Effects of Endotoxins*

Evidence for a pivotal role for macrophages in the endotoxin-induced enhancement of immune responses was first provided by the experiments of Spitznagel and Allison (1970). Mouse peritoneal exudate cells were exposed to BSA and/or endotoxin *in vitro* and then transferred into syngeneic recipients. Cells treated with endotoxin and antigen gave consistently higher antibody responses than cells which had taken up antigen in the absence of endotoxin. Experiments also documented that endotoxins had no detectable effects on either the uptake or degradation of antigen. Significantly, treatment of lymph node cells with endotoxin before transfer to irradiated recipients had no detectable effects on the subsequent immune responses to endotoxin. These data suggested that the adjuvant effects of endotoxins were, in part, mediated through their interactions with macrophages.

Two recent reports, using lymphoid cell subpopulations from the endotoxin unresponsive C3H/HeJ strain (see Section V) in coculture with lymphoid cells from histocompatible endotoxin-responsive C3H strains have provided convincing evidence for a macrophage role in the adjuvant effects of endotoxins. Hoffmann *et al.* (1977a) examined the *in vitro* responses to TNP-conjugated mouse erythrocytes (TNP–MRBC), which were previously shown by Schmidtke and Dixon (1972) to be capable, in the presence of endotoxins, of bypassing T cell help. These authors demonstrated that, although anti-TNP antibody could not be elicited in cultures of C3H/HeJ lymphocytes with TNP–MRBC and endotoxin, the addition of responder macrophages to the culture restored the capacity to make antibody. Additional experiments using cultures of responder macrophage-depleted spleen cells and C3H/HeJ macrophages (and/or vice versa) showed a clear dissociation between the endotoxin-induced B cell proliferative response and the capacity to bypass T cell help.

Similar experiments by McGhee *et al.* (1979) have examined the adjuvant effects of endotoxins on the *in vitro* immune response of subpopulations of endotoxin-responsive and nonresponsive cells to SRBC. These experiments indicated that both macrophages and T lymphocytes were required to be endotoxin responsive to manifest an enhanced immune response to SRBC. These investigators confirmed the observations of Hoffmann *et al.* (1978) that the B cell population need not be responsive to endotoxin, nor was B cell responsiveness to endotoxin alone sufficient for the expression of the endotoxin adjuvant

activity. The requirement for T cells in the experiments of McGhee *et al.* (1979), which was not apparent in the adjuvant experiments of Hoffmann *et al.*, may well reflect the immunologic nature of the antigen employed, as in the latter experiments using TNP–MRBC, helper T-cells do not exist.

D. Endotoxins and Immunologic Tolerance

The capacity of an agent to regulate the state of immunologic tolerance has been viewed as a major manifestation of its ability to act as an adjuvant in antibody formation. The capacity of any such agent to reverse the state of immunologic tolerance, in fact, has been defined as one of the more stringent definitions of an immunologic adjuvant (Dresser, 1961). In a broader sense, the mechanisms of induction and regulation of an immunologically tolerant state as well as its reversal, with the resulting initiation of autoimmunity, have presented one of the most intriguing questions of immunology during the last decades. It should come as little surprise that endotoxins, in view of the potent adjuvant activities described above, have the capacity both to interfere with the induction of immunologic tolerance and to induce the formation of antibody against self-antigens. It is, however, beyond the scope of this review to discuss in detail the multiple mechanisms that may be responsible for the induction or reversal of immunologic unresponsiveness and the potential mechanisms by which endotoxins may regulate these responses. These topics have been reviewed in a recent volume "Mechanisms of B-Cell Tolerance" (Möller, 1979) to which the interested reader is referred (see especially Fernandez, *et al.*, 1979; Parks and Weigle, 1979; Scott *et al.*, 1979). We will, however, in the following subsections briefly summarize a number of the relevant factors with regard to the effects of endotoxins on these immunologic responses.

1. Tolerance to Foreign Antigens

Claman (1963) first showed that endotoxins administered to mice shortly after a tolerogenic dose of monomeric or disaggregated bovine γ-globulin (dBGG) blocked the induction of a tolerant state to that antigen. Similar results were obtained by Brooke (1965) with respect to tolerance induction to a polysaccharide antigen (pneumococcal polysaccharide SIII). This investigator noted that no effects were detectable if the endotoxin was injected 1 day before antigen. More extensive temporal relationships were established by Golub and Weigle (1967) using dHGG as antigen. These investigators showed that the time period from 3 hours before to as late as 2 days after

antigen administration were, to varying extents, effective. Control experiments showed no physical–chemical effects on the antigen and no effects on the phagocytic capacity of the reticuloendothelial system.

In these studies, Golub and Weigle (1967) reported that they were not able to reverse the unresponsive state established at birth by subsequent injection of endotoxin into adult mice. Somewhat different results were obtained by Mader *et al.* (1973) using rats neonatally tolerized to SRBC antigens. These authors reported an almost complete reversal of tolerance using endotoxins and antigen 3 weeks after the final tolerizing dose of antigen. More recent experiments by these investigators (Mader *et al.*, 1975) described similar results using HSA antigen and neonatal tolerance of rabbits. These investigators also noted that antibody responses to HSA in chickens made previously tolerant to this antigen were not increased by injection of endotoxin. However, in these studies, endotoxin was also unable to act as an adjuvant to increase the antibody response of control chickens.

The experiments performed by Chiller and his colleagues (Chiller and Weigle, 1973; Louis *et al.*, 1973; Chiller *et al.*, 1974) first established a cellular basis for the effects of endotoxins on the induction and/or maintenance of the tolerant state. Their experiments demonstrated that, at a time following induction of tolerance to dHGG when T cells were still tolerant, the injection of antigen and endotoxin together, but neither alone, induced antibody to the HGG. When both B and T cells were tolerant, however, no antibody could be generated by endotoxin. These investigators also showed that endotoxin would induce a primary response to the dHGG tolerogen but that the kinetics of the response were markedly different than those to Agg HGG. Their data suggested that endotoxin did not alter the induction of tolerance at the T cell level. Furthermore, primed spleen cells from mice treated 30 days previously with dHGG and endotoxin, treated *in vitro* with anti-Thy-1 and complement, and transferred to irradiated recipients, made apparently normal responses to AggHGG upon subsequent challenge. A correlation between the capacity of endotoxin to initiate B cell proliferative responses and to regulate the induction of tolerance was noted in these and other (Chiller *et al.*, 1973; Skidmore *et al.*, 1975a, 1976) studies. Similar results on the reversal by endotoxin of tolerance induction to dHGG in athymic Nu/nu mice were more recently reported by Parks *et al.* (1977). Unlike the Nu/+ littermates, mice primed with endotoxin plus dHGG did not respond to a subsequent challenge with AggHGG unless T cells were present, indicating that, although unresponsive in the absence of T help, the B cells were not tolerized. These data, in addition to demonstrating endotoxin in-

duced reversal of tolerance in the absence of T cells suggest a possible defect in Nu/nu B cell function.

Ornellas *et al.* (1974) reported that endotoxins would also reverse immunologic tolerance in rats at the B cell level to TNP-conjugated rat γ-globulin (TNP–RGG). Using deaggregated sheep γ-globulin and TNP-conjugated SGG (dSGG) as tolerogen and (TNP–SGG) as immunogen, these authors confirmed that endotoxins did not interfere with tolerance induction at the T cell level. In some experiments, however, complete tolerance was not observed if the time between tolerance induction and challenge was delayed. The authors attributed this to putative helper effects of anticarrier antibody.

Möller *et al.* (1976b) made the interesting observation that high concentrations of endotoxins would induce antibody formation in populations of spleen cells previously made tolerant to a give hapten. Although completely unresponsive to either a T-dependent or independent form of the haptenic determinant, responses initiated by endotoxins in the absence of specific antigen were indistinguishable from untreated controls. In contrast to these results, Parks and Weigle (1980) have recently reported no evidence for endotoxin-induced antibody responses in mice made tolerant to dHGG until such time that the B cells regained the equivalent capacity to cooperate with nontolerized T-helper cells in response to antigen. Such tolerized B cells were completely incapable of antibody responses to the tolerized antigen unless both antigen and endotoxin were present; neither alone was sufficient. These data would not be consistent with the concept that tolerance can never be completely established at the B cell level, nor that endotoxins have the capacity to circumvent tolerance by direct activation of B cells.

There are several situations in which endotoxins have been noted to be unable to reverse the induction of immunologic tolerance. As pointed out by Nossal and Pike (1975), antigen-induced tolerance in cultures of murine bone marrow cells could not be reversed by the addition of endotoxins, suggesting a different mechanism of establishment of tolerance in these systems. Similarly, Scott and Diener (1976) demonstrated that endotoxins were incapable of either preventing or reversing the induction of tolerance to polymerized flagellin (POL), suggesting that the B cell-activating properties of endotoxin were insufficient to reverse induction to this T-independent antigen. Finally, as reported recently by Scott *et al.* (1979) and Etlinger and Chiller (1979), and in contrast to the earlier studies with adult mice, endotoxins administered to newborn (1- or 8-day) mice 3 hours after a tolerizing dose of antigen did not reverse the induction of im-

munologic tolerance. As B cells from such mice are fully capable of responding to endotoxins as assessed by proliferation and/or immunoglobulin synthesis (Section III), these data would support the concept that such activities are not sufficient for reversal of tolerance induction. Moreover, in the latter studies, as the acquisition of responsiveness to tolerance reversal correlated with the ability of endotoxin to act as an adjuvant in these mice, similarities in the cellular mechanisms of these responses to endotoxins was suggested.

As recently concluded by Scott *et al.* (1979), however, there is currently no unitarian hypothesis for B cell tolerance that will accommodate all the available data. In view of the multiple effects that have been documented for endotoxin–lymphoid cell interactions, many of which have been summarized in this review, it would not appear unreasonable to anticipate that multiple endotoxin-initiated responses may contribute to these varied facets of immunologic tolerance.

2. *Autoimmunity*

One of the highly relevant manifestations of the capacity of bacterial endotoxins to modulate the state of immunologic unresponsiveness is their ability to initiate the generation of antibodies against self components. The evidence discussed above demonstrates that endotoxin may break the tolerant state by interaction with lymphoid cells. There have, in fact, been a number of recent reports that indicated the capacity, both *in vivo* and *in vitro*, of endotoxins to induce antibody directed against serum and cellular antigens.

As first reported by Fournié *et al.* (1974), the administration of endotoxins to mice led to the rapid appearance of DNA within the circulation and the subsequent appearance of anti-DNA antibody. Injection of DNA with the endotoxin led to significantly higher levels of anti-DNA antibody. This phenomenon was demonstrated in a variety of mouse strains including the athymic Nu/nu mouse, and a number of endotoxin preparations including purified lipid A. The amount of DNA found in the circulation was directly related to the dose of endotoxin injected. An extension of these studies by Izui *et al.* (1977a,c) explored the immunologic basis of the relationship between the release of DNA initiated by endotoxins and the formation of anti-DNA antibody. These experiments utilized two murine strains that have been described as having abnormal responses to endotoxins, the CBA/N mouse, which expresses an X-linked B lymphocyte defect in response to endotoxin, and the C3H/HeJ. Using the (CBA/N × DBA/2)F_1 male (low responder) and female (high responder), Izui demonstrated equal amounts of DNA release in both strains, yet

anti-DNA antibody was restricted to the high endotoxin responder. In the C3H/HeJ mouse, neither DNA release nor anti-DNA antibody were found. Of significant interest, using adoptive transfer of C3H/FeJ (responder) spleen cells into C3H/HeJ (nonresponder) recipients followed by endotoxin challenge, it was shown that normal amounts of anti-DNA antibody were found even though no circulating DNA was detected. Additional studies showed a clear dissociation between the "DNA releasing" capacity of these molecules and their ability to stimulate anti-DNA antibody, thus confirming the studies using the mutant mouse strains.

Other studies by these investigators (Izui *et al.*, 1977b) have further characterized the DNA release following injection of endotoxin and found it to be a mixture of single- and double-stranded DNA of about 4–6 S. These investigators reported that DNA–anti-DNA antibody complexes were not found in the circulation but that immune complexes (DNA–anti-DNA), which correlated directly with circulating levels of anti-DNA antibody were found in tissues, particularly in glomerular capillary walls. Fischbach *et al.* (1978) found IgM anti-polyadenylate [poly(A)] responses of a number of murine strains to endotoxins. As activity was abrogated by polymyxin B and as no responses were detected in C3H/HeJ mice, a nonspecific stimulation of B lymphocytes by endotoxins was suggested. The similarities between these murine responses to endotoxins and the classic features of systemic lupus erythematosus are apparent.

Another manifestation of endotoxin administration to mice has been the appearance of IgM rheumatoid factors in the circulation (Izui *et al.*, 1979a). The kinetics of the IgM anti-IgG response were found to be similar to that observed for anti-DNA antibody. No significant strain or endotoxin differences were noted with the exception of the lack of responsiveness in the C3H/HeJ mouse. The serum of mice treated with endotoxins expressed antigen binding activity against mouse, guinea pig, goat, human, and bovine IgG. This latter observation confirms the earlier observations of Dresser and Pophan (1976), who earlier also detected significant levels of rheumatoid-like IgM antibody against bovine IgG.

Experiments by Esquivel *et al.* (1977) and by Primi *et al.* (1977c, 1978b) have documented that antibodies against serum proteins other than immunoglobulins can be elicited in mice following the administration of endotoxins. In the extensive studies reported by Esquivel *et al.* (1977), endotoxins, in conjunction with mouse thyroglobulin (MTg) but not in its absence, were shown to induce an IgG antibody response to MTg. The generation of an IgG anti-MTg implicated an obligatory

role for T cells in the response. Evidence for this was suggested by the demonstration that antibody responses were absent in Nu/nu but not Nu/+ mice. Similar results were obtained using AT × BM mice. It was significant that relatively high titers of anti-MTg could be elicited by endotoxins, yet no thyroid lesions were detected. These authors proposed that endotoxins promoted the development of anti-MTg antibody by utilizing T cells rather than by circumventing the need for T cells. In the experiments reported by Primi *et al.* (1977c, 1978b), the *in vitro* stimulation of mouse spleen cells with endotoxins generated the production of IgM antibody with specificity for autologous serum proteins and/or albumin. Unlike the experiments of Esquivel *et al.* (1977), however, there was no apparent requirement for T lymphocytes in the response, suggesting that suppressor T cells do not play a primary role in the maintenance of tolerance to self. These data would not be consistent with the active role for suppressor T cells proposed by Cunningham (1975) for maintenance of tolerance to self.

There also exists a growing body of evidence that endotoxins induce antibody against isologous cells. Experiments reported by Primi *et al.* (1977a,b,d), established that the *in vivo* injection of endotoxins into mice generated serum factors that, in the presence of complement, caused lysis of autologous and syngeneic spleen cells. Activity was not generated in the endotoxin unresponsive C3H/HeJ mouse. In addition, the serum activity could be absorbed on anti-Ig columns. The results of additional studies using adoptive transfer of fetal liver cells into irradiated recipients showed that immature B cells, unresponsive to endotoxin did not generate this activity when immediately challenged with endotoxin but did so if allowed to mature *in situ* for several weeks. The antigenic specificity of the antibody generated in response was found not to be directed primarily against H-2 determinants. It was also of interest that *in vitro* studies using human tonsil cells stimulated with endotoxin-produced supernatants which, in the presence of complement, lysed autologous cells.

Similar studies by McHugh and Bonavida (1977) and more recently by Izui *et al.* (1979b) demonstrated that both *in vitro* and *in vivo* stimulation of murine lymphocytes with endotoxins led to the production of IgM antibody with a relatively high affinity for both syngeneic and allogeneic thymocytes as assessed by cytolytic capacity in the presence of complement. As suggested in the experiments of Primi, the antibody was not primarily directed against H-2, or in the present studies TL, antigens. Izui *et al.* (1979b) reported that the antibody was absorbable by thymocytes, spleen, and brain tissue but not kidney or liver. As reported by McHugh and Bonavida (1977) endotoxins can

stimulate approximately 1 to 2×10^3 PFC/10^8 spleen cells against syngeneic thymus cells. It would not be unreasonable to anticipate that, under the appropriate circumstances, such a population of endotoxin-reactive precursor cells might be stimulated to become a relevant factor in the initiation of autoimmune phenomena.

V. C3H/HeJ—The Endotoxin-Unresponsive Mouse Strain

In 1968, Sultzer published a brief report in *Nature* describing the responses of a number of inbred strains of mice to several preparations of endotoxin isolated from *S. typhosa* O-901 and *E. coli* 0127:B8. Each of the strains examined showed an increase in extravascular peritoneal polymorphonuclear leukocytes 24 hours after the injection of endotoxin. Surprisingly however, whereas most strains showed a decrease in mononuclear cells, the C3H/HeJ strain consistently displayed a rather striking 2-fold increase in these cells at 24 hours. Furthermore, when the two strains that displayed the most divergent responses were compared for their susceptibility to the lethal effects of endotoxin, an approximate 20- to 40-fold increase in LD_{50} of the C3H/HeJ mouse was obtained (~1–2 mg) relative to the endotoxin responsive A/HeJ strain (~40–60 μg). Sultzer concluded from this study that "the C3H/HeJ strain thus offers a convenient tool for the investigation of the mechanism of resistance to endotoxin toxicity."

This observation has, indeed been fulfilled during the ensuing decade in terms of furthering our understanding of the underlying mechanisms of the role of endotoxins in toxicity. Equally as impressive, if not more so, however, has been the information gained from experiments performed using the C3H/HeJ mouse as a tool to examine the mechanisms of immune responses to endotoxins, in particular the mechanisms and interrelationships of B cell activation in general. The following subsections will summarize briefly the data that have contributed to our current understanding of the mechanism(s) of unresponsiveness of this unique mouse strain.

A. Immunopathologic Responses

As indicated above, the C3H/HeJ response to endotoxin was first characterized by an abnormal peritoneal inflammatory response (Sultzer, 1968). In an extension of these studies, Sultzer (1969) examined the kinetics of the peritoneal cellular infiltrate following injection of endotoxin. These experiments clearly demonstrated a more rapid polymorphonuclear leukocyte (PMN) response, which rapidly terminated by 24 hours, as well as a significantly more rapid rise in

mononuclear cell infiltrations in the C3H/HeJ. In a more recent report (Sultzer and Goodman, 1977), these peritoneal inflammatory responses of the C3H/HeJ mouse to endotoxin were reviewed in order to emphasize the point that, in terms of these responses, this mouse strain could not truly be classified as a "nonresponder."[3] These abnormal inflammatory responses were further characterized by Moeller *et al.* (1978), who confirmed Sultzer's data defining the cellular infiltration patterns. It was further established that the chemotactic activity of the C3H/HeJ serum was normal and that the PMNs were normal in their ability to chemotact. In addition, their *in vivo* responses to other inflammatory stimuli were equal; thus these mice were selectively abnormal in their inflammatory responses to endotoxins. Recent experiments by Curry and Morrison (1979) have established *in vitro* and *in vivo* that serum complement from C3H/HeJ and the genetically related, but endotoxin-sensitive C3H/St mice, are equally capable of being activated by both the alternative and the classical pathways following antibody-independent interactions with endotoxins. Further, Musson *et al.* (1978) have characterized both the rate of disappearance of endotoxin from the circulation and its accumulation in various tissues of C3H/HeJ and C3H/St mice (see Section II). These data suggest that the unusual feature of the C3H/HeJ mouse is not its inability to handle endotoxin on a physiological level, but its inability to handle it at the cellular level. This concept has been firmly established by a number of investigators.

An interesting manifestation of the resistance of C3H/HeJ mice to endotoxin is their sensitivity to infection with gram-negative organisms. To explore this question, Vas *et al.* (1973) compared several strains of mice for susceptibility to infection with live *Klebsiella* organisms as well as resistance to endotoxin lethality. These authors observed that the C3H/HeJ strain, which was highly endotoxin resistant, was surprisingly the least resistant to the effects of infection. It was concluded from these studies that these two traits are inherited separately. In contrast, Sultzer and Goodman (1977) reported that C3H/HeJ mice were significantly more resistant to *E. coli* infection than A/HeJ mice. It would thus appear that relative resistance may depend upon the type of infecting organism. Very similar results to those of Vas *et al.* (1973) were more recently reported by von Jeney *et*

[3] Although Sultzer and Goodman are entirely correct in this interpretation, the inability of the lymphoid cells from this strain to respond to LPS continues to be one of the major features that distinguishes the C3H/HeJ mouse. For the purpose of this review therefore, we will continue to employ the terminology "endotoxin nonresponder" to refer to the C3H/HeJ mouse.

al. (1977) following infection with *S. typhimurium.* The C3H/HeJ mice were by far the more susceptible strain in comparison with the other strains examined. This concept has recently been discussed by Rosenstreich (1979). These combined data make apparent the important conclusion that resistance to lethal effects of endotoxin does not necessarily confer upon the strain equal resistance to gram-negative infection.

Resistance to endotoxins, to infection, and to the lethal effects of X-irradiation are also known to be enhanced in mice by pretreatment with endotoxins. (The experiments summarizing these data will be reviewed in Section VII). It is significant that pretreatment of C3H/HeJ mice with endotoxin did not, unlike the endotoxin-sensitive strains, increase their resistance to endotoxin or infection with *Klebsiella pneumoniae* (Chédid *et al.*, 1976) or to lethal X-irradiation (Urbaschek *et al.*, 1977). However, if the C3H/HeJ mice were lethally irradiated and reconstituted with spleen or bone marrow cells from a histocompatible but endotoxin-responsive strain (e.g., C3H/HeN), then significant effects in terms of decreases in LD_{50} (Glode *et al.*, 1976a) and increases in resistance (Galelli *et al.*, 1979) could be detected. Conversely, transfers of C3H/HeJ cells into lethally irradiated C3H/HeN recipients increased resistance to endotoxin (Glode *et al.*, 1976a), but only temporarily decreased the resistance to infection (Galelli *et al.*, 1979). These latter data suggested that the failure of C3H/HeJ mice to respond to endotoxin with nonspecific immunity was due to a defect in two types of bone marrow-derived cells, a radioresistant and a radiosensitive population.

Recent experiments of Sultzer and Goodman (1977) have also suggested a role for serum in the unresponsiveness of C3H/HeJ mice. These authors demonstrated that treatment of endotoxin with C3H/HeJ serum, but not A/HeJ serum, reduced its subsequent lethality. Another unusual characteristic of C3H/HeJ serum was recently reported by Hoffmann (1978) who indicated that this serum did not support the fixation of C3 on sensitized erythrocytes. These latter observations were not however, completely confirmed in experiments reported by Curry and Morrison (1979). The nature of this putative serum factor remains, however, to be fully characterized.

These combined data have already provided considerable information with regard to the relevant immunopathologic events that regulate the deleterious events in endotoxin-initiated lethality. The use of this endotoxin-resistant mouse should continue to yield valuable information on the biochemical nature of these endotoxin-elicited responses. The generation of serum factors in the C3H/HeJ mouse in response to

endotoxin also appears to be defective. Thus endotoxin does not induce the production of serum colony-stimulating factor (CSF) following *in vivo* administration to C3H/HeJ mice (Apte and Pluznik, 1976a,b; Russo and Lutton, 1977). These results have been confirmed following *in vitro* stimulation of lymphoid cells with endotoxin (Apte *et al.*, 1977a,b; Russo and Lutton, 1977). Finally, endotoxins do not elicit high levels of the circulating acute phase serum amyloid protein (SAA) in C3H/HeJ mice (McAdam and Sipe, 1976; Watson *et al.*, 1978b).

B. Immunologic Responses to Endotoxins

The first demonstration of the altered immunologic recognition of endotoxin by the C3H/HeJ mouse was reported by Brooke (1965). This investigator, in experiments designed to examine the capacity of endotoxins to alter the induction of immunologic paralysis to pneumococcal polysaccharide (see Section IV,D), found that endotoxins were highly effective in CAF_1 mice but were virtually without effect in C3H/HeJ mice. Although not specifically recognized by Brooke at the time, such immunologic unresponsiveness of the C3H/HeJ mouse to endotoxins has subsequently been documented to be one of the hallmark features that distinguish this strain. The following paragraphs will attempt to summarize briefly the evidence in support of this conclusion.

In a series of experiments designed to characterize murine lymphocyte responses to PPD, Sultzer and Nilsson (1972) reported that the C3H/HeJ mouse displayed a specific B lymphocyte deficiency in response to endotoxin. This was suggested by its relatively low proliferative response *in vitro* to endotoxin, while responding normally to PPD. This report established the basis for an extensive series of investigations that have subsequently characterized the cellular, and indeed most recently, the molecular basis of this selective defect. Experiments designed to define the B cell immunologic unresponsiveness to endotoxin were reported by Watson and Riblet (1974, 1975), Skidmore *et al.* (1975a), Coutinho and Gronowicz (1975), Coutinho *et al.* (1975a), Rosenstreich and Glode (1975), Jacobs (1975b), Slowe and Waldmann (1975), Talcott *et al.* (1975), Rudbach and Reed (1977), and Goodman *et al.* (1978). The basic conclusions of these collective studies were that the C3H/HeJ mouse was selectively unresponsive both *in vitro* and *in vivo* to virtually all the immunologic properties that endotoxins normally manifest in their interactions with lymphoid cells. These activities included initiation of B cell proliferative responses or maturation to PFC against hapten-conjugated erythrocytes, inability to

mount a high-titer or sustained primary IgM antibody response to endotoxin or hapten-conjugated endotoxin, inability to be primed by endotoxin for an enhanced secondary immune response to the endotoxin molecule, inability to act as an adjuvant of antibody formation against an unrelated antigen (see Section IV,A) and finally, inability to reverse the induction of immunologic tolerance to a protein antigen (as demonstrated earlier for a polysaccharide antigen by Brooke, 1965). The lack of responsiveness of the C3H/HeJ mouse to endotoxin as assessed by these various immunologic activities was established to be independent of a variety of different protein-free endotoxins and lipopolysaccharides prepared from various gram-negative organisms. The dose of endotoxin, the kinetics of induction of the response, selective loss of lymphocyte survival in culture, selective unresponsiveness limited to only one or a few lymphoid organs, or any of the other trivial explanations were not shown to account for the lack of responsiveness. It was thus concluded that the C3H/HeJ mouse possessed a true lymphocyte defect in its capacity to respond to the B-lymphocyte activator, endotoxin, but not a variety of other activators previously shown to act selectively on the B cell population.

On the basis of a majority of these experiments, however, it was not possible to establish unequivocally that the observed unresponsiveness was directly linked to the C3H/HeJ B lymphocyte or that this lack of response to endotoxin might be alternatively explained on the basis of a putative suppressor or auxiliary helper cells. (This would appear unlikely on the basis of earlier experiments establishing the lack of a requirement for these cells in many of the normal B-cell responses to endotoxin.) To further explore this possibility, several investigators performed the appropriate cell-mixing experiments and/or adoptive transfer experiments to rule out suppressor/helper effects, and to demonstrate that the observed responses could be directly attributable to the B lymphocyte (Watson and Riblet, 1975; Slowe and Waldmann, 1975; Coutinho, 1976; Glode *et al.*, 1976b; Skidmore *et al.*, 1977). The results of these studies clearly demonstrated that the endotoxin unresponsiveness of the C3H/HeJ was a specific defect of the B lymphocytes themselves and was not due to the presence of suppressor cells or the absence of helper cells.

With the delineation of the B cell unresponsiveness to endotoxins firmly established, two comments appear to be warranted. First, this unresponsiveness of C3H/HeJ B cells only extends to the nonspecific interactions with the lipid A portion of endotoxins. In this respect, a recent report by Freudenberg (1977) has demonstrated that hyperimmunization of C3H/HeJ mice with isolated lipid A leads to antibody

responses that are indistinguishable from other endotoxin-sensitive strains (e.g., CBA/H). Thus the C3H/HeJ mouse is not defective in its capacity to respond antigenically to lipid A. Second, because of this apparent selective unresponsiveness of the C3H/HeJ B-lymphocyte to these diverse nonspecific immunologic effects of lipid A, it was attractive to postulate that there must be a necessary functional interrelationship between these various B cell activities. The ability of endotoxin to initiate proliferative responses was postulated as being responsible for its being a T cell-independent immunogen as well as an immunologic adjuvant of antibody formation (see, e.g., Coutinho and Gronowicz, 1975; Skidmore *et al.*, 1977; Slowe and Waldmann, 1975). Whereas this type of approach would appear to have merit in immunologic responses that are regulated strictly by B cells, the demonstration that other cell types were also unresponsive would tend to introduce additional complicating factors into such an analysis.

Several investigators have established that the C3H/HeJ B-lymphocyte unresponsiveness to endotoxins also extends to T cells, as evidenced by an inability of either of these cells to be induced by endotoxin to express surface markers. For example, Watson (1977), showed that lipid A will induce expression of Ia^+ antigens in a subpopulation of albumin separated bone marrow cells from C3H/DiSN, but not from C3H/HeJ, mice. Similar differential results were found when cells were examined for ability to be induced to express the T cell antigen Thy-1.2. Essentially similar results were obtained by Koenig *et al.* (1977) when responder and nonresponder mice were examined for induction of expression of the C3 receptor and membrane surface expression of IgG in pre-B cells as well as Thy-1.2 antigen in pre-T cells. Thus the C3H/HeJ mouse defect in response to endotoxin appears to be expressed at all levels of B cell maturation and, in addition, appears to be reflected in lymphoid cells other than B cells as well.

Another lymphoid cell in which endotoxin unresponsiveness has also been demonstrated is the C3H/HeJ macrophage. As indicated earlier (and in Section VI), both *in vitro* and *in vivo* studies have indicated a lack of responsiveness of C3H/HeJ mice to endotoxins in the generation of colony-stimulating factor, which the available evidence has suggested is derived from mononuclear leukocytes. In addition, Chédid *et al.* (1976, 1977) have reported that C3H/HeJ peritoneal macrophages were unable to be activated *in vitro* by endotoxins to inhibit the growth of a mastocytoma cell line (see Section VI,D). Additional manifestations of this selective macrophage unresponsiveness have been described, on the basis of both *in vivo* (Bianco and Edelson,

1977) and *in vitro* (Ryan *et al.*, 1979) stimulation by endotoxins. In the former report, endotoxin induced reduced numbers of peritoneal macrophages at 4 days, which in addition had significantly reduced activity. *In vitro,* endotoxins did not increase metabolic responses of C3H/HeJ macrophages as assessed by glucose metabolism. Extensive experiments by Rosenstreich and his collaborators have documented the unresponsiveness of C3H/HeJ macrophages to endotoxin as assessed by *in vitro* cytotoxicity (Glode *et al.*, 1977), generation of the prostaglandins PGE_2 and $PGF_{2\alpha}$ (Rosenstreich *et al.*, 1977), tumor cytotoxicity (Ruco and Meltzer, 1978a,b; Ruco *et al.*, 1978), and generation of lymphocyte-activating factor (LAF) (Rosenstreich *et al.*, 1978b).

The suggestion that endotoxin unresponsiveness of the C3H/HeJ mouse at the cellular level can be manifest to a number of different cells including B lymphocytes, T lymphocytes, and macrophages is highly suggestive of a global defect in the capacity of all cells from this mouse to recognize and/or respond to the lipid A portion of endotoxin. Several points with regard to this point are worthy of note. First, the lipid A unresponsiveness has recently been documented to extend to C3H/HeJ fibroblasts. Thus, Ryan and McAdam (1977) demonstrated that the stimulation of glucose transport and/or metabolism in cultures of embryonic fibroblasts of responder C3H/HeN mice by endotoxin was not manifest in cultures of C3H/HeJ fibroblasts. These data support the concept of a generalized membrane defect in the response of all cells of the C3H/HeJ strain.

Several very recent lines of evidence, however, have established that the C3H/HeJ mouse is not totally unresponsive to the lipid A portion of endotoxins. Dumont (1978) has examined the capacity of endotoxins to enhance the T cell proliferative response to concanavalin A (Con A) (see Section III). Surprisingly, he observed that the endotoxin-mediated enhancement of Con A stimulated thymidine incorporation in T cells from a responder strain (98% ± 15%) was manifest to an equal extent in thymocytes from the unresponsive C3H/HeJ strain (94% ± 16%). A second endotoxin-elicited regulatory response in spleen cells from the C3H/HeJ mouse has also recently been described by Haas *et al.* (1978). In experiments designed to examine the effects of endotoxin on suppression of the primary immune response (see Section IV) of mice to SRBC, these investigators also demonstrated that endotoxin-treated spleen cells from *both* the responder C3Heb/FeJ and nonresponder C3H/HeJ mice, treated either *in vivo* or *in vitro* 2 days prior to antigen challange, were suppressed in their subsequent PFC to the SRBC antigen. It is of interest that the suppres-

sive effect could be transferred by endotoxin-treated spleen cells. In addition to providing evidence that the mechanisms of immune response may be different, these data provide additional evidence that cells from C3H/HeJ mice are not totally refractory to the modulatory effects of the lipid A region of endotoxins.

There is, however, substantial evidence to indicate that lymphoid cells from the C3H/HeJ mouse are capable of eliciting completely normal responses to the lipid A-associated proteins (LAP) of bacterial endotoxins, which are firmly associated with the lipid A region of the LPS portion of endotoxins. It was initially suggested by Coutinho *et al.* (1975a) that the proliferative activity of some preparations of lipopolysaccharide was due to contaminating materials as complete unresponsiveness was obtained when highly purified LPS preparations were tested in C3H/HeJ spleen cells. This suggestion was considerably clarified by the demonstration by Skidmore *et al.* (1975b) that the ability of endotoxins to elicit proliferative responses in C3H/HeJ spleen cells depended critically on the method used to purify the endotoxin. Using several physical chemical procedures to isolate endotoxins extracted by treatment of *E. coli* with aqueous butanol, these investigators demonstrated that the observed activity was indeed associated with the endotoxin molecule, and that subsequent extraction of positive preparations of endotoxin with phenol removed its capacity to initiate proliferative responses in C3H/HeJ spleen cells. The observation that the method used to prepare the endotoxin regulated its capacity to initiate proliferation in nonresponder lymphocytes was independently confirmed by Chédid *et al.* (1976), who postulated that the activity in such positive endotoxin preparations might be due to the fact that the active preparations were complexes of protein and LPS.

The recently reported experiments of Goodman *et al.* (1978) have, however, provided convincing data that indicated that endotoxins containing the active LAP component will not initiate, in the C3H/HeJ mouse, all of the immunologic responses normally attributable to the lipid A region of the LPS. Thus although LAP will stimulate B cell proliferation and antibody synthesis in C3H/HeJ lymphocytes it will not act as an adjuvant of antibody formation to unrelated antigens. Similarly, whereas LAP will inhibit the induction of immunologic tolerance to a protein antigen, it will not substitute for tolerant T cells in generating immune responses to the tolerogen. These experiments, in addition to distinguishing the differential activities of LAP and lipid A, provide important information on the interrelationships between the various immunologic activities of endotoxins in their interactions with lymphoid cells.

As indicated earlier, the experiments of Sultzer and Goodman (1976) and Morrison *et al.* (1976) independently established that the active component(s) of endotoxins that elicited responses in C3H/HeJ lymphocytes was, in fact, the LAP to which the C3H/HeJ lymphocyte response is comparable to all other strains. Thus the endotoxin unresponsive nature of the C3H/HeJ mouse is a selective defect limited to the lipid A portion of endotoxins. It is, therefore, essential when defining endotoxin responses in the C3H/HeJ mouse to distinguish between highly purified preparations of endotoxic lipopolysaccharides and endotoxic complexes of LPS and LAP.

C. The Genetic Basis of Endotoxin Unresponsiveness

The demonstration by Sultzer of the unresponsiveness of the C3H/HeJ mouse to endotoxin challenge evoked considerable interest in the genetic basis for such unresponsiveness. As an initial approach to define the patterns of inheritance of the endotoxin defect, Sultzer (1972) examined the F_1 progeny of C3H/HeJ × A/HeJ as well as backcrosses of F_1 with the parental strains. Responses measured included resistance to the lethal effects of endotoxins as well as the peritoneal leukocyte infiltrates following injection of endotoxins. On the basis of these initial studies, Sultzer proposed that the peritoneal inflammatory cellular responses elicited by endotoxins were consistent with the role of multiple gene action in controlling this response.

The suggestions of Sultzer (1972) and Sultzer and Nilsson (1972) that spleen cells from the C3H/HeJ mouse were also altered in their proliferative responses to endotoxin as assessed by incorporation of [^{3}H]thymidine allowed more quantitative evaluations of the genetic pattern of inheritance of the altered endotoxin response. The experiments published by Watson and Riblet (1974) were designed to examine such proliferative responses in F_1 hybrids and parental backcrosses. These studies suggested that, as assessed by this parameter, endotoxin responsiveness was inherited as a dominant trait, which was not associated with either H-2 or heavy-chain allotype and was not sex-linked. Watson and Riblet concluded, on the basis of studies to examine immune responsiveness to the endotoxin O-antigen and the proliferative B cell responses, that these endotoxin properties were inherited as a single gene product.

These initial studies were extended by a number of investigators to examine immune responsiveness to the endotoxin O-antigen and the the properties summarized above and to examine the interrelationships between these properties, including B cell proliferative and maturation responses (Watson and Riblet, 1975; Coutinho *et al.*,

1975b; Glode and Rosenstreich, 1976; Skidmore *et al.*, 1976; Sultzer, 1976; Watson *et al.*, 1977; Kelly and Watson, 1977), enhanced secondary responses to endotoxins (Rudbach and Reed, 1977), adjuvant effects (Talcott *et al.*, 1975), reversal of tolerance induction (Skidmore *et al.*, 1976), generation of serum interferon (Apte *et al.*, 1977a), enhancement of nonspecific resistance to infection (Parant *et al.*, 1977), and association with the LPS receptor (Coutinho *et al.*, 1978). Virtually all these studies concluded that the inheritance of responsiveness to endotoxin was the product of a single, autosomal gene that segregated independently of both H-2 antigens or heavy-chain allotype. Depending upon the method used to assess endotoxin responsiveness, various investigators concluded that the gene expression for endotoxin responsiveness was either dominant or codominant. The majority of the available evidence would, however, strongly favor the concept that responsiveness is the result of a single codominantly expressed autosomal gene.

The recent experiments of several investigators have provided suggestive evidence that the gene responsible for endotoxin responsiveness may be subject to allelic exclusion. On the basis of radioautographic analysis of thymidine incorporation into endotoxin-stimulated splenic lymphocytes of F_1 hybrids of responder–nonresponder parents, Kelly and Watson (1977) suggested that approximately half of the number of spleen cells were stimulated to proliferate as were observed in the parental responder. Although these investigators suggested that these data would be consistent with allelic exclusion, they cautioned that firm conclusions could be drawn only after an analysis of individual cells for the endotoxin-responsive gene product. The experiments of Coutinho *et al.* (1978) have provided, in part, such an analysis using the fluorescein-conjugated anti-endotoxin receptor (LPSR) antibody (see Section V,D). These studies also found, on the basis of 12 different experiments, approximately half of F_1 spleen cells staining for LPSR in comparison with the parental control. They also reported that results yielding equivalent conclusions were obtained in preliminary experiments using limited dilution analysis. More precise conclusions must, however, await the unequivocal demonstration of the defective gene product on those B cells negative for LPSR.

Because of the multiple biologic activities that may be elicited by endotoxins, it would be anticipated that these responses should segregate together in the appropriate F_1 backcrosses into parental strains. The functional co-expression of such endotoxin responses has been confirmed with regard to T-independent responses to endotoxin and nonspecific stimulation of immunoglobulin synthesis (Coutinho *et al.*,

1975b) and B cell proliferation and reversal of the induction of immunologic tolerance (Skidmore *et al.*, 1976). More recently several investigators have demonstrated that endotoxin unresponsiveness, which is manifest in different cell types, also cosegregates in recombinant strains. Rosenstreich *et al.* (1978b) showed that the B cell proliferation response to endotoxin was expressed in the same backcross population as were macrophage activation leading to generation of LAF. Watson *et al.* (1978b) examined three distinct endotoxin-defective responses; namely hypothermia, serum colony-stimulating factor, and serum levels of SAA in a number of recombinant inbred strains. These investigators demonstrated that all three responses segregated together. As it was postulated that these responses probably represent distinct manifestations of endotoxins on different cell types, it was concluded that the single-locus genetic defect may be expressed in a variety of cell types.

Watson *et al.* (1977, 1978a) have also performed experiments to localize the genetic defect responsible for endotoxin unresponsiveness in the C3H/HeJ mouse. These authors have confirmed that endotoxin unresponsiveness segregates independently of H-2 and Ig allotype but did segregate with the allelic class that defines the major urinary protein (*Mup*) previously demonstrated to be localized to chromosome 4 (and linked to brown coat color). This concordant inheritance of the endotoxin unresponsive gene locus (*Lps*) and *Mup-1* were found to have a recombination frequency of about 0.06 ± 0.02. Additional genetic analyses using the polysyndactyly marker (*Ps*) demonstrated the *Lps* locus to be between the *Mup-1* and the *Ps* loci.

D. The Lipopolysaccharide-Membrane Receptor

The experimental evidence summarized above has provided considerable evidence for a selective defect in the responsiveness of C3H/HeJ B-lymphocytes to the triggering response to the lipid A portion of bacterial endotoxins. Most of the available evidence has, however, indicated little if any difference in the binding of LPS to B cells from LPS-responsive and unresponsive mice. This suggests that the inability to respond may be due to a B cell component that can recognize the bound LPS molecule and convert this interaction into an initiation signal for the cascade of events that regulate B cell responses. This concept of a specific defect in a putative "LPS receptor" has been one of the earliest proposals to explain the unresponsiveness of the C3H/HeJ mouse and is consistent with the bulk of the genetic studies summarized above. Evidence that such a defect may be a characteristic of the B cell surface membrane was suggested by the

experiments of Dumont and Barrois (1976). These investigators examined the electrophoretic characteristics of splenic lymphocytes from several strains of mice, including those of the C3H/HeJ strain. Their results indicated that, although all strains had identical numbers of low electrophoretic mobility (B) lymphocytes (54 ± 1%) and high mobility T cells (46 ± 1%), the electrophoretic mobility of the C3H/HeJ B cells (but not T cells) was slightly but significantly less than that of the other strains tested.

Recent experiments by Forni and Coutinho (1978a,b) and Coutinho *et al.* (1978) have provided solid experimental evidence supporting the existence of the LPS receptor. These investigators prepared a rabbit anti-C3H/TiF (responder B cell) antisera, which they extensively absorbed with tissue from the C3H/HeJ (nonresponder) strain. The resulting antiserum (AS) had the following characteristics:

1. In the presence of complement it lysed C3H/TiF but not C3H/HeJ spleen cells.
2. The fluoresceinated AS stained 30% of C3H/TiF spleen cells but less than 0.1% of C3H/HeJ spleen cells (selective staining was also observed in lymph node, bone marrow and thoracic duct, and peripheral blood lymphocytes).
3. Ontogeny of AS^+ cells over a 5-week period following birth paralleled proliferative responses to LPS.
4. AS binding to lymphocytes was blocked by prior treatment with LPS or lipid A, but not PPD or lipoprotein.
5. The IgG fraction of AS stimulated proliferation and PFC in responsive spleen cells.
6. In populations of F_1 hybrids, AS stained approximately half as many cells as in the parental responder strains.
7. In F_1 × parental backcross progeny, the functional proliferative response correlated with the serological staining with AS.

These combined data are consistent with, and highly suggestive of, a direct correlation between the antigenic determinants recognized by AS and the membrane-localized molecular entity that recognizes specifically the lipid A portion of LPS. The authors have termed this putative lipid A interaction structure on the B cell membrane, which is recognized by AS, the LPSR (for LPS receptor).

Using specific rhodamine- and fluorescein-conjugated antisera to IgD, IgM, Fc, and LPSR, these investigators have more recently examined the interrelationships between these various surface markers following stimulation with LPS. The results of extensive cocapping experiments have suggested that there is an integral functional relation-

ship between the LPSR and the Ig receptors that results in relevant interactions between these two receptors when either of the surface molecules interacts with its specific ligands. These data confirm morphologically the functional results of inhibition of LPS-induced proliferative responses by pretreatment of B cells with anti-Ig (see Section III).

E. Additional Murine Strains with Abnormal Endotoxin Responses

Although the C3H/HeJ strain has been by far the most extensively studied with respect to its apparent total unresponsiveness to endotoxins, several other strains have also received considerable attention during the last several years and will be discussed briefly in this section.

The first of these strains is the CBA/N. As initially reported by Amsbaugh *et al.* (1972), both BALB/c and CBA/J mice elicit a significant day 5 antibody response to *E. coli* endotoxin; however, no response was detected in the CBA/N mice. This observation was found not to be uniquely characteristic with respect to the *E. coli* antigen, and defective immune responses were also defined with a number of additional T cell-independent antigens. Extensive analyses of the CBA/N mouse have established (reviewed in Scher *et al.*, 1977) that this mouse, as well as (CBA/N × DBA/2) F_1 male progeny, display a specific B lymphocyte maturational defect to many T-independent antigens which is expressed on the X chromosome. As the primary focus of this manuscript is to summarize the relevant information specifically with respect to endotoxins, it is beyond the scope of our review to deal with this interesting mouse strain in detail. There are, however, several points that should be mentioned. As reported by Zaldivar and Scher (1977) the (CBA/N × DBA/2) F_1 female mice demonstrate good antibody titers in response to protein–LPS complexes (1 : 6830), which is somewhat enhanced in mice injected with protein-free LPS (1 : 10,240). In contrast, a low response (1 : 340) to protein–LPS complexes was observed in (CBA/N × DBA/2) F_1 male mice, which was entirely abrogated if mice were injected instead with protein-free LPS. It is significant that both male and female mice preinjected with protein-free LPS (100 μg) and subsequently challenged with 2× an LD_{50} of LAP–LPS displayed equal protection generated by the first LPS injection. Thus both mice were protected (12/32 vs. 12/33) even though only the females were capable of anti-LPS antibody.

An additional point with respect to this strain is that the defect

appears to be strictly limited to B cell responses. As reported by Coutinho *et al.* (1977) the LPSR, as assessed by fluorescence (see above), appeared to be positive. Thus, although (CBA/N × DBA/2) F_1 males have abnormal proliferative responses to LPS, the response of macrophages isolated from this strain, as assessed by LAF production, generation of prostaglandins, or lethality, would appear to be normal (Rosenstreich *et al.*, 1978a). As the F_1 males are sensitive to both the lethal and the adjuvant effects of LPS *in vivo*, the experiments using this mouse would support the concepts that mature B cells are not required for either of these immunologic or immunopathologic manifestations of endotoxins.

The recent discovery of a second endotoxin-unresponsive strain of mouse was the result of the screening of a wide spectrum of splenic lymphocytes using fluorescein-conjugated antisera (AS) against the putative LPS receptor (Coutinho *et al.*, 1977). As was earlier described for the C3H/HeJ mouse, lymphocytes from C57Bl/10 ScCr lymphocytes were examined *in vitro* for their capacity to be stimulated by LPS. They were found not to generate proliferative responses or PFC against hapten-conjugated erythrocytes; however, they were found to respond normally to LP. These results were confirmed by McAdam and Ryan (1978) using an assay of increased glucose utilization. These authors demonstrated that the B lymphocyte defect was specific for LPS and that B cells responded normally to 8-Br-cGMP and LAP. A similar defect was observed by McAdam and Ryan in the C57Bl/10 ScCr peritoneal cells, which were unresponsive to LPS. These authors also noted that the *in vivo* response to LPS in terms of SAA production was defective. These data suggested that the defect in the C57Bl/10 ScCr mouse that renders it unresponsive to endotoxin is similar, if not identical, to that found in the C3H/HeJ mouse. The results of recent genetic analyses of these two strains have provided data in support of this concept (Coutinho and Meo, 1978). Analysis of F_1 hybrids demonstrated to complementation of LPS responsiveness. Thus these authors concluded that the C57Bl/10 ScCr mice carry a defective allele at the same LPS locus, previously identified by the mutation responsible for the defect in the C3H/HeJ mouse.

There are in addition, a number of additional mouse strains that are defective in their immunologic responsiveness to endotoxin. Many of these strains, however, express an immunologic defect for which the altered responsiveness to endotoxins is but an incidental (although perhaps important) manifestation. Two examples will serve to illustrate this point. The first is a mutant murine strain, termed "motheaten." This strain, bred onto the C3Heb/FeJ background is

characterized immunologically by normal levels of T cells but reduced levels of B cells. Those B cells that are present demonstrate a considerably reduced proliferative response to endotoxin and do not respond to T-independent antigens, in particular endotoxin (Sidman *et al.*, 1978). The second is the well studied (NZB × NZW) F_1, which at older ages does not stimulate PFC to haptenated erythrocytes in response to endotoxin even though its proliferative responses appear normal (Cohen and Ziff, 1977; Primi *et al.*, 1978a). This contrasts with the responses obtained after stimulation of lymphocytes from 5-week-old mice with endotoxin. This result is not due to the presence of suppressor T cells. While there are undoubtedly numerous additional strains that demonstrate altered responses to endotoxins, it is not with the scope of this review to elaborate them. Nevertheless, it would appear certain that multiple genetic variables regulate potential responses to endotoxins.

VI. The Effects of Endotoxins on Macrophages/Monocytes

The critical role of mononuclear phagocytes in regulating host immune responses is well documented (reviewed in Möller, 1978; Allison, 1978). In view of the multiple profound effects of endotoxins on B and T lymphocyte function, reviewed in the preceding sections, it should not be surprising to find equally profound effects of endotoxins on macrophage function, particularly with respect to immunoregulation. In this section we will review the data which have defined these endotoxin-mediated macrophage dependent immune responses. We will also summarize the results of recent experiments which have documented a number of additional characteristics of endotoxin-macrophage interactions. While such responses may not currently have direct relevance to immune responses per se, they clearly play a highly relevant role in the immunopathologic host response to endotoxin.

A. Endotoxin–Macrophage Interactions and Lymphocyte Responses

The influence of macrophages on host immune responses may be manifest directly via interaction with antigen, or indirectly through the synthesis and secretion of immunoregulatory molecules. These highly potent secretory products may serve either to mediate macrophage effector function or they may regulate lymphocyte function by both activation and/or suppression. These macrophage-secreted effector molecules have been collectively defined as monokines. Endo-

toxins, as will be documented in this section, have been demonstrated to be one of the most potent stimuli of macrophages leading to synthesis and secretion of monokines. The analysis of the mechanism of macrophage responses to endotoxins has provided a valuable tool in defining the role(s) of monokines in immune responses.

As indicated, following appropriate stimulation *in vitro*, macrophages secrete effector monokines which are released into the culture medium. These culture supernatants have been demonstrated *in vitro* to affect lymphocyte function in a number of systems, including proliferative responses to mitogens, antigen-stimulated antibody production, generation of specific subsets of T lymphocytes, mixed lymphocyte reactions, and the production of some lymphokines (reviewed in Möller, 1978). As will be discussed below, endotoxins have been suggested to render many of these processes macrophage independent, that is, endotoxins will substitute completely for certain macrophage functions. Although this conclusion is, in many instances, justified, a note of caution would appear to be indicated. First, as it is extremely difficult to remove completely macrophages from cultures of mixed lymphoid cells, the possibility of amplification of a residual macrophage effect by stimulation with endotoxin must always be considered (e.g., Ritter *et al.*, 1975). Further, macrophage supernatants generated by endotoxins usually contain residual amounts of endotoxins. Although undetectable by very sensitive assays, such endotoxin, either alone or acting synergistically with monokines, may be highly effective in modulating immune responses. This would be particularly true for macrophage-dependent immune responses, since these cells appear to be exquisitely sensitive to stimulation by endotoxins.

Recently, a subpopulation of nonphagocytic esterase-positive adherent lymphoid cells has been defined in the peritoneal cavity of mice by Nathan *et al.* (1976, 1977). These cells have many of the properties of B lymphocytes, including the capacity to be stimulated to proliferate in response to endotoxin. As these cells have been suggested by the authors to contaminate most preparations of peritoneal macrophages, the potential contribution of such cells to macrophage responses to endotoxins may well have to be considered. Additional experiments will, however, be required to fully assess the effects of these cells (see especially, Section VI,A,3).

1. Effects on B-Lymphocyte Responses

Evidence has been reported that suggests a prominent role for macrophage–lymphocyte interactions in the cellular responses of B lymphocytes to endotoxins (Bona *et al.*, 1971, 1972, 1973; Bona, 1973).

In these studies it was suggested, on the basis of radioautographic analyses, that the formation of macrophage–lymphocyte islands preceded the cellular transfer of endotoxin to the lymphocyte. The presence of the endotoxin in the lymphocyte was postulated to be associated with the synthesis of specific antibody, presumably by endotoxin-specific B lymphocytes. Similar macrophage–lymphocyte islands were reported by Shands *et al.* (1974), who did not, however, speculate on their relationship to endotoxin-initiated cell responses. The results of more recent physical–chemical investigations (Stoecker *et al.*, 1978) have not, however, confirmed these conclusions. Although activation of T cells with specific mitogens led to significant association of T cells with macrophages, no detectable association of endotoxin-activated B cells with macrophages was ever noted.

Most, by far, of the available evidence, therefore, would provide strong support for the conclusion that the majority of the direct effects of endotoxins on B lymphocytes do not require the presence of macrophages. In terms of immune responses to endotoxins, this was clearly demonstrated by Poe and Michael (1974) (see Section II). The lack of a requirement for macrophages in the B cell proliferative and antibody secretion responses to endotoxins was established by Lemke *et al.* (1975). As assessed by [^{3}H]thymidine incorporation, or synthesis of antibody to SRBC, or the haptenic determinant NNP, virtually identical responses were obtained with both normal and adherent cell-depleted spleen cell cultures from either normal or athymic Nu/nu mice. These results were confirmed in a more recent study by Takigawa and Hanaoka (1977).

Although macrophages are not essential for the antigen-specific and antigen-independent activation of B lymphocytes by endotoxins, there is considerable evidence to indicate that both unstimulated and *in vivo* or *in vitro* activated macrophages profoundly affect B cell responses in general, and endotoxin-induced B cell responses in particular (see, e.g., Keller, 1975; Veit and Feldman, 1976; Persson *et al.*, 1977a,b; Möller, 1978; see also Section IV,C,3).

The experiments of Yoshinaga *et al.* (1972) first reported the marked suppressive effects of macrophages on the proliferation of rat splenic B lymphocytes in response to endotoxin. In these experiments the addition of as few as 1–5% macrophages to cultures of adherent-cell-depleted spleen or lymph node cell cultures markedly inhibited proliferative responses. Inhibition could not be reversed by increased concentrations of endotoxin. The authors proposed that inhibition was mediated by both direct effects of macrophages as well as indirect macrophage-dependent T-suppressor cells. In these experiments,

however, it was not ascertained whether the endotoxin effect resulted from a direct interaction with macrophages. In other experiments, *in vivo* activation of murine peritoneal macrophages by endotoxins, followed by *in vitro* culture for 24–48 hours was shown to result in the generation of a macrophage supernatant factor(s) that inhibited *in vitro* proliferative responses to endotoxins (as well as several T cell stimuli) (Nelson, 1973). Similar suppressive factors were shown to be generated by *in vitro* culture of peritoneal cells with endotoxins, although the degree of suppression was less extensive. This was suggested by the authors to be due to the presence of residual endotoxin in the macrophage culture supernatants.

Recent experiments have strongly implicated a prominent role for prostaglandins in mediating the suppressive effects of endotoxin-activated macrophages on B cell responses. Prostaglandins are efficiently synthesized by macrophages (reviewed in Allison, 1978), and their production has been documented in cultures of stimulated murine and human macrophages (Fischer *et al.*, 1977; Rosenstreich *et al.*, 1977; Kurland and Bockman, 1978). These latter authors also have demonstrated prostaglandin production in a number of macrophage and monocyte cell lines following endotoxin activation; however, no prostaglandin synthesis was detected in two nonmacrophage cell lines. The ability of indomethacin, an inhibitor of prostaglandin synthetase, to reverse the endotoxin-induced macrophage suppression of B cell responses (Kurland, 1978), would suggest a role for these macrophage products in the modulation of B cell proliferation.

The activation of macrophages by endotoxins also results in the release of additional factors that regulate B cell responses. Many of these factors are not, however, biochemically well defined. Endotoxin-stimulated macrophages release a factor that enhances the formation of B lymphocyte colonies in agar, an effect that is distinct from the direct stimulation of B cells by endotoxins (Metcalf, 1976; Kincade, 1977). The available evidence would indicate that this effect is also mediated via prostaglandins (Kurland, 1978). Indeed, many additional active products are released by endotoxin-activated macrophages (e.g., Moore *et al.*, 1976); however, their effects on host immune responses remain to be defined.

One area of particular interest has been the endotoxin-induced release of macrophage factors that have the capacity to support antigen-induced antibody synthesis. As reported by Schrader (1973b), supernatants obtained from cultures of peritoneal cells stimulated with endotoxins supported PFC responses to the T cell-dependent antigen FγG in cultures of Nu/nu spleen cells. Similarly Wood and Cameron

(1976) described the release of factors from cultures of human peripheral blood monocytes following stimulation by endotoxins. This B lymphocyte activating factor (BAF), generated in response to subnanogram quantities of endotoxin (Wood and Cameron, 1978) also stimulated antigen-dependent antibody synthesis in cultures of Nu/nu spleen cells. Dimitriu and Fauci (1978) have described a monokine factor (MK) produced by human monocytes following stimulation with either MLC supernatants (and to a lesser extent) endotoxins that enhance pokeweed mitogen-induced PFC responses. Its relationship to BAF, however, is at present unclear.

A third factor, with properties very similar to those of BAF, has also very recently been described. This factor, termed TRF-M (for T-cell replacing factor from macrophages), may be generated from *C. parvum*-elicited peritoneal cells cultured with endotoxin for 4 hours (Hoffmann and Watson, 1979; Hoffmann *et al.*, 1979). Preliminary biochemical characterization of TRF-M has suggested the presence of at least two active components, which differ significantly in their apparent molecular weight (150,000 and 15,000). This biochemical approach should be extremely beneficial in the eventual elucidation of the relationships between these various macrophage-derived monokine factors.

2. *Effects on T-Lymphocyte Responses*

The available evidence summarized in Section III,B indicates that the direct effects of endotoxins on T lymphocytes are considerably less profound than those on B lymphocytes. Nevertheless, there is substantial evidence to indicate that endotoxins can regulate T cell activity indirectly via monokines released from endotoxin-activated macrophages. The most thoroughly characterized monokine in this respect has been lymphocyte-activating factor (LAF) first described by Gery and his collaborators (1971). As reported by Gery *et al.* (1972a), the *in vitro* culture of human peripheral blood mononuclear cells with endotoxin generated a supernatant factor that enhanced markedly the proliferative response of murine thymocytes to the T lymphocyte activator PHA. Direct proliferative responses of T cells were also detected in response to LAF; however, no detectable effects on B lymphocytes were noted.

Fractionation of human peripheral blood lymphoid cells over nylon wool columns provided evidence that the LAF activity was the result of endotoxin stimulation of adherent cells (Gery and Waksman, 1972). Biochemical analyses of supernatant fractions containing LAF have

suggested an approximate molecular weight of about 15,000 for the active material (Gery and Handschumacher, 1974). Subsequent experiments have indicated that LAF activity is sensitive to chymotrypsin, thus suggesting a protein component of LAF (Blyden and Handschumacher, 1977). Temporal analysis of mononuclear cells stimulated by endotoxin by these investigators has indicated that activity can first be detected at 6 hours, with maximal activity between 12 and 24 hours after stimulation.

Factors released from endotoxin-stimulated macrophages with properties similar to LAF have subsequently been described by numerous investigators. Unanue *et al.* (1976) found that 3-day peptone-elicited murine peritoneal cells cultured *in vitro* with endotoxin for 24 hours generated a factor that stimulated T-lymphocyte proliferative responses. In these experiments, macrophage responses to endotoxin were found to be dependent critically upon the concentration of endotoxin. It is interesting that when peritoneal cells were elicited by endotoxin rather than peptone 3 days before harvest no active supernatants were generated upon subsequent *in vitro* stimulation with endotoxin. The relationship between this immunostimulatory factor and the LAF reported earlier by Gery *et al.* (1971, 1972a) was not discussed in this report. More recent experiments, however, have suggested that lymphostimulatory activity could be generated by endotoxins after stimulation *in vitro* of murine peritoneal exudate cells elicited 7 days previously with intraperitoneal injection of endotoxin (Meltzer and Oppenheim, 1977). These authors also reported the generation of LAF following stimulation of cultures of the macrophage tumor line P388D_1 with endotoxin. These data have since been confirmed in a second macrophage tumor line J774.1 (Okada *et al.*, 1978). Of interest in the latter study, the endotoxin-elicited LAF was also shown to induce splenic T cell cytotoxic responses.

The results of recent biochemical analysis of the LAF generated by murine peritoneal cells in response to endotoxins have suggested a molecular weight of about 16,000 (Mizel *et al.*, 1978). This value is remarkably close to that described earlier by Gery and Handschumacher (1974) for human monocyte-derived LAF. The experiments of Mizel *et al.* (1978) have also effectively precluded a direct role for contaminating endotoxin in the thymocyte responses, by using thymocytes from the endotoxin unresponsive C3H/HeJ mouse (see Section V). These studies documented that, as with the numerous other defective responses of the C3H/HeJ mouse to the lipid A portion of endotoxins, LAF activity was not detectable in supernatants of C3H/HeJ peritoneal exudate cells after stimulation with protein-free preparations of endotoxin.

The generation of T-lymphocyte activating factors from supernatants of macrophages of other species has also been reported. In particular, rabbit alveolar macrophages were reported to generate LAF-like activity, although in this system, high concentrations were reported to be suppressive (Ulbrich, 1977). Another stimulatory factor has recently been reported to be generated by guinea pig adherent peritoneal cells in response to endotoxin stimulation. This factor has also been defined by its capacity to enhance antigen specific activation of sensitized T cells by the production of macrophage inhibition factor MIF (Ohishi and Onoue, 1976). These studies suggested the generation of an immunostimulatory factor with molecular weight between 15,000 and 100,000, which was distinguished from endotoxin by its heat lability (85°, 30 minutes). Further experiments (Yamamoto *et al.*, 1978) have indicated that, as for LAF, the activity of this factor was sensitive to proteolytic enzymes. The molecular weight was suggested to be considerably larger than that reported for LAF (80,000 vs. 15,000). Its precise relationship to human and murine LAF will await further biochemical analysis.

3. *Macrophage Activation by Endotoxin-Stimulated Lymphocytes*

In view of the multiple activities, both direct and indirect, of endotoxins in their interaction with lymphoid cells, it might be suggested that endotoxin-activated lymphocytes would be effective in modulating macrophage function. This is not to suggest that endotoxins may not directly stimulate macrophages, as there is considerable evidence available from numerous experimental systems to support this concept. A particularly compelling argument is the recent demonstrated effects of endotoxins on macrophage tumor cell lines (e.g., Mizel *et al.*, 1978).

Nevertheless, the results of a number of experiments support the concept that B lymphocytes play a role in mediating the endotoxin activation of macrophages. Using preparations of guinea pig peritoneal exudate cells, Wilton *et al.* (1975) suggested a critical role for endotoxin-activated B lymphocytes in the uptake of glucosamine by macrophages. Activity was found in the supernatant of the activated lymphocytes. Confirmation of these results was suggested by Fernandez *et al.* (1977), who reported that removal of nonadherent cells from preparations of guinea pig peritoneal exudate cells abrogated the ability of the latter cells to incorporate glucosamine. Using a murine system, Ryan *et al.* (1979) have reported enhanced consumption of glucose by adherent peritoneal cells in the presence of splenic lymphocytes stimulated by endotoxins. Thus there appear to be conditions under which macrophages are activated directly by endotoxin, and

others where lymphocytes may mediate the endotoxin activation. This point may be of particular relevance in experiments that utilize washed adherent monolayers as a source of purified macrophages; these preparations often contain adherent lymphocytes (Nathan *et al.*, 1976, 1977).

The direct assessment of lymphocyte products (lymphokines) that may be elicited by endotoxins and subsequently activate macrophages has not been extensively analyzed. Such biologically potent lymphokines have been demonstrated to have a number of activities in their interaction with macrophages (reviewed in Blanden *et al.*, 1976; David and Remold, 1976). A major technical problem that has beset investigators with regard to endotoxin-elicited lymphokines has been the exquisite sensitivity of macrophages to the endotoxins themselves. Thus, the contamination of lymphocyte supernatants with minute amounts of endotoxins would complicate the subsequent assessment of lymphokine activity. The use of genetically unresponsive strains of mice (see Section V) as sources of purified lymphoid cell subpopulations in conjunction with protein-free LPS preparations should, however, allow these problems to be addressed directly.

B. Endotoxin-Elicited Macrophage Effector Molecules

In the preceding section the secretion of factors from macrophages (monokines) after stimulation by endotoxin was described. These monokines primarily affect immune responses by interactions with both B and T lymphocytes. There are also, however, a number of additional potent effector molecules secreted by endotoxin-activated macrophages whose primary target cell is not the lymphocyte. In this section we will summarize the experimental data relevant to several of these effector molecules.

1. Colony-Stimulating Factor

The differentiation of myeloid precursor cells into mature monocytes and granulocytes is enhanced by a glycoprotein of about 45,000 molecular weight, termed colony-stimulating factor (CSF). This factor is normally found in low levels in urine of many species. As reported by Metcalf (1971) the injection of small quantities of endotoxin into mice resulted in enhanced CSF activity detected in urine. In addition, bone marrow cells obtained from such mice were shown to mature into colonies of granulocytes and monocytes when subsequently cloned in soft agar. Evidence of CSF activity was also detected in serum within 30 minutes of endotoxin administration; however, such activity was transient and was essentially returned to control levels

after 24 hours. Repeated doses of endotoxins to mice were reported to induce tolerance to CSF production (Quesenberry *et al.*, 1975) (see also Section VII,B). It is of interest that similar CSF activity has been detected in human serum within 60 minutes after the injection of minute quantities of endotoxin into healthy human volunteers (Golde and Cline, 1975). This peak of activity was suggested to correlate with the nadir of the granulocytopenia observed in these subjects.

It was originally suggested that granulocytes were the target of endotoxins which resulted in generation of CSF (Chervenick, 1972). In this respect, both the granulocytopenia and the subsequent granulocytosis that occurs after administration of endotoxin might be explained by a direct action of endotoxins on these cells (Chervenick, 1972; Quesenberry *et al.*, 1972). The results of *in vitro* experiments, however, using isolated subpopulations of peripheral blood cells, provided strong evidence for the monocyte as the principal cell responsible for CSF production after endotoxin stimulation (Moore *et al.*, 1973). Similar conclusions were reached by Eaves and Bruce (1974) using murine peritoneal cells. These investigators reported that resident peritoneal cells incubated *in vitro* with endotoxins released increasing amounts of CSF over a 6-hour period. In these experiments, evidence for macrophage participation was suggested by association of CSF production with the adherent cell subpopulation. In addition, Ficoll–Hypaque purified human adherent mononuclear cells were reported to release CSF after *in vitro* stimulation with extremely low concentrations of endotoxins (Ruscetti and Chervenick, 1974).

Other studies, however, suggested that the generation of CSF by lymphoid cells in response to endotoxin was more complex. For example, it was reported by Eaves and Bruce (1974) that cultured liver cells, which contain a high percentage of macrophages, did not generate CSF in response to endotoxin. In addition, Cline *et al.* (1974) have provided data indicating that purified human monocytes did not respond to endotoxins by increased secretion of CSF. After approximately 10 days in culture, when a majority of the monocytes have assumed the morphological and biochemical characteristics of macrophages, significantly increased production of CSF in response to endotoxin was noted. Murine spleen cells were also reported to release CSF in response to endotoxins and other lymphocyte activators (Parker and Metcalf, 1974). Although removal of adherent cells had no effect on CSF activity secreted in response to some stimuli (suggesting that lymphocytes may also be a source of CSF) such studies were not reported using endotoxin as a stimulus.

The extensive experiments recently published by Apte *et al.* (1977b)

and Staber *et al.* (1978) have, however, provided convincing evidence for direct macrophage-mediated lymphocyte-independent CSF production in response to endotoxins. Both these groups of investigators utilized a number of experimental techniques to dissociate lymphocyte responses to endotoxins from CSF stimulation. In the former study, inhibition of proliferative responses by polymyxin B or inhibitors of DNA synthesis had no effect on CSF stimulating activity. In addition, spleen cells from the CBA/N mouse, which display a B cell maturational defect in response to endotoxin (see Section V,E) had normal CSF activity responses to endotoxins. The latter investigators utilized separation of phagocytic and nonphagocytic murine lymph node cells, analysis of adherent peritoneal cells, treatment of cell populations with either anti-immunoglobulin or antimacrophage antiserum and complement, and irradiation to effect a complete functional dissociation between the ability of endotoxins to elicit CSF activity and their ability to initiate proliferative responses. These studies have provided strong evidence that endotoxin-induced CSF activity in lymphoid cell populations is the result of a direct interaction of endotoxin with macrophages that does not require the participation of lymphocytes.

There is, however, some question as to the active moiety of the endotoxin complex responsible for the generation of CSF by macrophages. The experiments published by Nowotny and his co-workers (Chang *et al.*, 1974; Nowotny *et al.*, 1975a,b; Butler and Nowotny, 1976) have provided evidence to indicate that a nontoxic polysaccharide (PS) prepared by acid hydrolysis of purified LPS was responsible for CSF stimulating activity. Such PS preparations displayed none of the toxic properties of the intact LPS, yet were as active as the intact LPS in inducing CSF activity in mice. These authors also reported that the LPS derived from the heptose-deficient *S. minnesota* R595 mutant was without detectable activity; purified lipid A was indicated to have low but significant activity. Further evidence for activity in PS preparations was suggested by the demonstration that activity was destroyed by mild treatment with periodate, indicating a prominent role for carbohydrate in the response.

Experimental results that contrast significantly with these data have been reported by Apte and collaborators (Apte and Pluznik, 1976a,b; Apte *et al.*, 1976). These investigators found significant CSF stimulatory activity using purified LPS preparations from a number of polysaccharide-deficient mutants. In these experiments a PS preparation prepared by acid hydrolysis was virtually completely inactive over a wide range of concentrations. These studies also indicated a

lack of CSF generating activity in response to endotoxin in the C3H/HeJ endotoxin-unresponsive mouse strain (see Section V,E). Since the progenitor cells from the C3H/HeJ mouse did respond to endotoxin-induced CSF from a responder strain, the data indicated that the unresponsiveness resided in the generation of CSF rather than the ability to respond to CSF. Similar results have been reported by Russo and Lutton (1977).

It would therefore appear that under the appropriate experimental conditions, both the PS and the lipid A regions of endotoxins may have the capacity to stimulate CSF activity. It is noteworthy that the C3H/HeJ mouse has been shown to be unresponsive to endotoxins, specifically in this case with respect to CSF stimulation. However, a vast majority of the reported endotoxin unresponsiveness has been associated with lipid A-mediated activity. Therefore, the demonstration that this mouse strain was also defective in responsiveness to the active PS fractions described by Nowotny would be a highly significant finding with respect to mechanisms of endotoxin unresponsiveness in this mouse strain.[4]

More recent experiments by Apte *et al.* (1977b) have examined the cellular biochemical events required for stimulation of CSF activity by endotoxins. As indicated earlier, inhibitors of DNA synthesis and mitosis had no effect on CSF activity. In contrast, inhibitors of both protein and RNA synthesis almost completely abrogated the capacity of spleen cell cultures to generate CSF activity in response to endotoxin. Thus these data would indicate that CSF most probably does not exist preformed in macrophages, but rather that stimulation with endotoxins initiates the synthesis and subsequent secretion of CSF.

2. *Interferon*

Interferon molecules (IF) are proteins with a rather heterogeneous range of molecular weights, which upon appropriate stimulation are secreted from a number of cell types including lymphoid cells. Although these molecules were first recognized for their capacity to inhibit viral replication *in vitro*, subsequent experiments by numerous investigators have demonstrated multiple additional biological activities of these molecules in their interactions with lymphoid cells. The induction of interferon production may be accomplished by a wide variety of molecules that have been grouped loosely into nonspecific inducers (type I) and immune cell-specific (type II) in-

[4] A. Nowotny (personal communication) has recently confirmed that C3H/HeJ mice do not respond to PS by the formation of CSF.

ducers (reviewed in Ho and Armstrong, 1975). Considerable evidence has indicated that the types of interferon produced in response to these inducers may be distinguished on the basis of molecular weight, heat and acid stability, and immunogenicity.

Endotoxins have been recognized for some time for their capacity to induce interferon production, although they are moderate to poor inducers in comparison to certain viruses and double-stranded RNA (Ho and Armstrong, 1975). These authors, as well as several additional reviews (De Clercq, 1973; Allison, 1978) have summarized in detail much of the early literature that has defined the parameters of induction of IF by endotoxins. Many studies have provided convincing evidence that macrophages are a principal target of endotoxins for IF production. Some early evidence, however, also suggested lymphocytes derived from thymus tissue as an additional source of endotoxin elicited IF. The temporal dependence of the *in vitro* response of macrophages to endotoxins differs from that of some other IF inducers, with endotoxins eliciting a characteristic "early IF response." Maximal activity is expressed with 1–6 hours and may remain elevated for up to 24 hours. As with virtually all the other properties of endotoxins (see, however, Section VI,B,1), IF induction has been demonstrated to reside in the lipid A region of the endotoxin molecules (Feingold *et al.*, 1970). It would thus be anticipated that the endotoxin-unresponsive C3H/HeJ mouse would be refractory to the induction of IF following injection of endotoxin, and this has recently been documented (Apte *et al.*, 1977a).

A majority of the experimental data that defined the characteristics of the IF produced in response to endotoxin stimulation of macrophages indicated that the IF was primarily of the nonimmune type I class. The increasing recognition of endotoxins as potent activators of B lymphocytes prompted a critical evaluation of the potential contribution of such interactions to the production of "immune-specific" type II IF production. In this respect, Ho *et al.* (1976) and Maehara and Ho (1977) have recently provided evidence for macrophage-dependent (type I) and B cell-dependent (type II) production of IF in the response of murine spleen cells to endotoxin. Using the parameters of temperature and time dependence of the production of IF in peritoneal and spleen cells, as well as analysis of purified splenic cell subpopulations these authors demonstrated significant differences in IF production. Further distinguishing characteristics of the two IF moieties produced were defined on the basis of heat and acid stability as well as inhibition by specific antibody. Thus it would appear that B lymphocyte-induced IF may contribute to the total IF observed fol-

lowing *in vitro* culture of lymphoid cells with endotoxin; its relevance to *in vivo* responses, however, remains to be further defined.

3. *Additional Factors*

In addition to those effector molecules already described, there are many additional factors released by macrophages in response to endotoxin. Two such molecules, which serve as pathophysiologic landmarks for host responses to endotoxins, include endogenous pyrogen and tissue factor. The former molecule is responsible for the febrile response, which is characteristic of many disease states including gram-negative bacteremia, whereas the latter molecule provides one of the critical coagulation factors leading to disseminated intravascular coagulation, one of the most serious consequences of gram-negative sepsis. The available evidence indicates that both of these effector molecules may be derived from macrophages and/or monocytes after stimulation with endotoxins. The production of fever and coagulation by endotoxins is a topic that is not directly relevant to this review. There exists a considerable volume of literature dealing with each of these topics. Endogenous pyrogen and tissue factor production from monocytes, however, will be briefly considered.

After the appropriate stimulation of peripheral blood leukocytes from most mammalian species, endogenous pyrogen (EP) is produced by both granulocytes and mononuclear cells (Atkins *et al.*, 1967; Nordlund *et al.*, 1970; Atkins and Bodel, 1974). The biochemical characterization of EP has shown this molecule to be a protein of about 38,000 molecular weight (Dinarello *et al.*, 1974). The EP produced by both rabbit and human monocytes is heat labile and subject to inactivation by treatment with proteolytic enzymes (Atkins and Bodel, 1974; Bodel and Miller, 1976). As reported by Bodel (1974), incubation of Ficoll-Hypaque purified human mononuclear cells with endotoxins for periods of time as short as 15 minutes results in detectable EP production, maximal activity being detected at about 45–90 minutes. In confirmation of the earlier studies of Nordlund *et al.* (1970), these experiments also demonstrated a requirement for protein synthesis in the generation of EP. Although lymphocytes themselves do not produce detectable levels of EP in response to endotoxins, their effects on monocyte production of EP appears variable, both enhancement (Atkins and Francis, 1973) and no effect (Bodel, 1974) having been reported.

Tissue factor (TFa), produced by mononuclear cells in response to endotoxin, participates in the extrinsic pathway of blood coagulation by promoting the association of factor VII with calcium. This factor

exists as a complex of phospholipid and protein. The experimental evidence supporting the monocyte as the major source of leukocytic TFa has recently been reviewed in detail (Morrison and Ulevitch, 1978). These studies have revealed that, unlike the production of EP, TFa is produced only by mononuclear cells. As with most of the other properties of macrophage–endotoxin interactions, activity has been demonstrated to be the result of an interaction with the lipid A portion of the endotoxin molecule. It is significant that mononuclear cell production of TFa in response to endotoxin appears to be exquisitely sensitive (Rickles *et al.*, 1977a,b). As reported by Niemetz and Morrison (1977), based upon an approximate molecular weight of 10^6, approximately 200 molecules of endotoxin per cell are sufficient to induce detectable TFa production.

Several recent reports have described markedly increased levels of serum amyloid protein (SAA) in both mink and mice following injection of endotoxin (Anders *et al.*, 1976; McAdam and Sipe, 1976; Sipe, 1978). SAA is an acute-phase protein with an approximate molecular weight of 160,000. After a 2-hour lag period, SAA production and release into the circulation increases markedly, reaching milligram levels, which then return to normal levels by 48 hours. Recent experiments have provided both *in vitro* and *in vivo* evidence for a major role for endotoxin activation of macrophages in the generation of SAA (Sipe *et al.*, 1979). These studies have reported that, after endotoxin stimulation of macrophages, but not of splenic lymphocytes, a factor (SAA inducer) is rapidly released, which, the authors suggest, provides the appropriate stimulus for SAA synthesis by other cells. Trypsin sensitivity of the SAA inducer has indicated that, similar to many of the other mediator molecules secreted by macrophages in response to endotoxin, this factor also consists in part of protein.

C. Endotoxin-Elicited Macrophage Effector Functions

In the preceding paragraphs of this section we have summarized the information relating to the production of specific mediators generated by macrophages in response to endotoxin. In general most of these molecules have specific target cells upon which they act to effect their regulatory role. Endotoxins also interact with macrophages to bring about morphologic changes in the macrophage itself as well as to induce release of macrophage products that do not have specific cellular targets. Macrophages that display these altered morphologic and biochemical characteristics have been termed "activated macrophages." However, it is becoming increasingly clear that macrophage activation is a complex multistep process, and the assessment of macrophage responses to various stimuli, including endotoxins, depends critically

on the degree of prior activation of the macrophage as well as the parameters utilized to assess activation (reviewed in North, 1978; Cohn, 1978; Karnovsky and Lazdins, 1978).

1. Morphologic Responses

In contrast to their effects on B lymphocytes, where one of the early profound changes in response to endotoxins is the appearance of B-cell blasts and initiation of DNA replication, there is considerably less evidence to support the concept of endotoxin-induced macrophage mitosis. Until recently, the most notable report of macrophage proliferation in response to endotoxin was published by Forbes (1965). In this study the subcutaneous administration of endotoxin to mice resulted in the subsequent stimulation of DNA synthesis in peritoneal macrophages and lymphocytes. These results were not confirmed by Shands *et al.* (1974), who detected no DNA synthesis in peritoneal macrophages stimulated either *in vitro* or *in vivo* with endotoxin. If anything, a cytotoxic effect on the macrophages was noted (see Section VI,C,3). More recently, however, Dienstman and Defendi (1978) have reported that a percentage of endotoxin-elicited murine peritoneal macrophages will proliferate *in vitro* in response to a secondary stimulation with endotoxin. Significant differences were noted in these experiments in the agents used to elicit the macrophages versus their ability to respond *in vitro* to stimulating agents. This presumably reflects different degrees of *in vivo* activation of the macrophages.

The primary morphologic change that is highly characteristic of the activated macrophage is enhanced spreading on surfaces (Rabinovitch and DeStefano, 1973). Further experimental support for enhanced spreading in response to endotoxin has been presented by Rabinovitch *et al.* (1977) and Bianco and Edelson (1977). In both these studies, intraperitoneal injection of endotoxin into mice led to high proportions of the resident or elicited macrophages manifesting the spreading response, with maximal activity detected at 24–48 hours. It is of interest that in the former study, neither X-irradiation nor splenectomy was demonstrated to influence the response to endotoxin. Both studies reported defective spreading responses of C3H/HeJ macrophages to injected endotoxin. More extensive morphologic analyses of murine macrophages following *in vitro* and *in vivo* stimulation with endotoxin were reported by Morland and Kaplan (1977), who noted, in addition to spreading characteristics, alterations in cell size, intracellular granule contents, and degree of "ruffling" of the cytoplasmic membrane. Significant effects of fetal calf serum on the *in vitro* responses to endotoxins were also noted in these studies.

In addition to the morphologic changes induced in macrophages,

endotoxins also induce significant functional changes in these cells. Enhancement of phagocytosis by cells of the reticuloendothelial system in fact, was probably one of the earliest effects reported for endotoxin interactions with mononuclear phagocytes (reviewed in Landy and Braun, 1964, and more recently in Allison, 1978). The results of several recent reports, however, have provided direct evidence for enhanced phagocytosis by macrophages treated either *in vitro* or *in vivo* with endotoxins. Hamburg *et al.* (1978) provided data that demonstrated marked *in vitro* enhancement of phagocytosis of antibody-coated erythrocytes by peritoneal macrophages exposed 24 hours earlier to endotoxin. Similar results were reported by Morland and Kaplan (1977). In these latter studies, much greater relative increases in phagocytosis of C3-coated particles relative to Fc particles were observed in macrophages from endotoxin-treated mice. As normal untreated macrophages manifest a greater capacity to phagocytose particles coated with Fc, this relative difference in the endotoxin-treated cells may reflect the background level of specific membrane receptors relative to maximal obtainable stimulation. In macrophage cultures treated *in vitro* with endotoxin, Morland and Kaplan (1977) reported little effect on Fc-mediated phagocytosis; however, serum-dependent complement-mediated phagocytosis of C3-coated particles were markedly enhanced with endotoxins in the presence of serum. More recent results by these investigators (Kaplan and Morland, 1978) have established similar results using the macrophage tumor line J774.1. Treatment with endotoxin significantly increased C3 binding while having no effect on Fc receptor expression. These data are consistent with the earlier studies of Bianco *et al.* (1975) which established a central role for C3 receptors in the phagocytic process of activated macrophages.

2. *Synthesis and Secretion of Enzymes*

Macrophage lysosomes are richly endowed with a variety of hydrolytic enzymes. Stimulation of macrophages by a number of activator molecules, including endotoxins, results in secretion of these hydrolytic enzymes to the external environment. Early *in vivo* studies with endotoxins were important in suggesting mechanisms by which tissue damage may occur through the release of these enzymes (e.g., Weissmann and Thomas, 1962; Janoff *et al.*, 1962). As indicated above for phagocytic responses mediated by endotoxins, enzyme secretion from macrophages may also be mediated indirectly by complement components generated by the interaction of endotoxins with serum complement, particularly C3b and factor B. The multiple studies that

have defined the macrophage responses to endotoxins resulting in enzyme synthesis and secretion have recently been reviewed in detail (Morrison and Ulevitch, 1978). The following paragraphs, therefore, will summarize a number of the significant points relevant to these studies.

The experiments of Cohn and Benson (1965) described increased secretory activity of lysosomal enzymes in murine macrophages following *in vivo* stimulation with endotoxins. Evidence for the equivalent *in vitro* macrophage response was reported by Wiener and Levanon (1968), who demonstrated increased acid phosphatase secretion in cultures of murine monocytes following endotoxin stimulation. Subsequent experiments by Allison *et al.* (1973) and Page *et al.* (1974) confirmed and extended these results. These authors demonstrated increased levels of murine macrophage enzymes, both contained within the cytoplasm (e.g., LDH, leucine-2-napthamidase) and secreted to the external milieu (e.g., *N*-acetyl-β-D-glucosaminidase, acid phosphatase). Significantly, no effects, either in the medium or within the cell, were detected in the levels of β-glucuronidase, another cytoplasmic enzyme marker. The mechanism(s) responsible for this apparently exquisite selective induction of enzymes within the macrophage remains unknown.

In confirmation of these studies, a number of investigators have more recently described the results of experiments to define enzyme secretory responses of macrophages following endotoxin stimulation (e.g., McGivney and Bradley, 1977; Bruley-Rosset *et al.*, 1976; Morland and Kaplan, 1977). In contrast to earlier results, however, these recent investigations have suggested increased levels of secretion of cytoplasmic β-glucuronidase; however, the requirement for fetal calf serum to elicit this response is currently not clear. Endotoxin-induced enzyme secretion has also been reported using the macrophage tumor line J774.1 (Morland and Kaplan, 1978). It is apparent, however, that, as reported in the earlier investigations, not all enzyme levels are increased after endotoxin activation. Thus plasma membrane-associated alkaline phosphatase levels remain unchanged (Edelson and Erbs, 1978), and 5′-nucleotidase levels are actually decreased in response to endotoxin.

Proteins with the potential for activation of plasma zymogens or degradation of tissues have been some of the more extensively characterized enzymes secreted by macrophages. One such enzyme is plasminogen activator (PA), a serine protease of approximately 48,000 molecular weight. Interestingly, although thioglycolate-elicited peritoneal macrophages secreted high levels of PA, endotoxin-elicited

macrophages were able to do so only in response to a phagocytic stimulus. If the phagocytic stimulus was nondigestible, increased levels of secreted PA could be detected for as long as 7 days (Unkeless *et al.*, 1974; Gordon *et al.*, 1974). Since secreted PA can generate plasmin from plasminogen, both fibrinolysis and activation of factor XII of the intrinsic coagulation pathway may result. Such a mechanism, in addition to generation of tissue factor of the extrinsic coagulation pathway, provides multiple mechanisms for activation pathways leading to inflammatory responses (reviewed in Morrison and Ulevitch, 1978; Allison, 1978).

One of the most well characterized enzymes secreted by endotoxin-activated macrophages is collagenase (Wahl *et al.*, 1974). Wahl *et al.* reported that oil elicited guinea pig peritoneal macrophages, stimulated *in vitro* with either endotoxins or purified lipid A, secreted high levels of collagenase within 24–48 hours of incubation. Inhibitors of protein synthesis abrogated collagenase secretion, indicating that endotoxins induced the active synthesis of the enzyme. Subsequent studies by these investigators (Wahl *et al.*, 1977) established that the stimulation was inhibited by indomethacin and was enhanced by prostaglandin E_2. These studies also documented enhanced secretion of prostaglandins within 4 hours after exposure to endotoxins. It is clear from these studies that prostaglandin intermediates play a prominent role in the modulation of endotoxin-macrophage interactions. As indicated earlier (Section VI,A,1) such prostaglandins may well exert modulatory influences on B lymphocyte function. Further, the regulation by macrophages of a spectrum of lymphoid cell functions may be critically dependent upon prostaglandin intermediates.

3. *Cytotoxicity and Detoxification*

As we have indicated earlier, and will reemphasize in Section IV,D, macrophages appear to be exquisitely sensitive to the presence of endotoxins, with concentrations in the range of nanograms per milliliter or less, able to elicit detectable responses. This contrasts with their effects on lymphocytes (Sections II and III) where tens to hundreds of nanograms per milliliter are required for the generation of specific immune responses and concentrations in the range of 10 to 100 μg yield maximal responses in terms of proliferation and high-rate immunoglobulin synthesis. It might not be totally unexpected, therefore, to find that this exquisite sensitivity to the perturbational effects of endotoxins which leads to cell activation is also reflected by an increased cytotoxic effect of endotoxins on macrophages.

Experimental evidence to support this concept was first suggested by the experiments of Heilman (summarized in Heilman, 1964, 1965). It was demonstrated that *in vivo* administration of endotoxins to rabbits induced extensive cytotoxic changes that were rapidly manifest in macrophages as assessed morphologically. Similarly, Kessel and Braun (1965) reported that *in vitro* treatment of guinea pig macrophages with low (10 μg/ml) concentrations of endotoxin in serum-free medium caused extensive cytotoxicity of these cells, as assessed by their failure to exclude trypan blue 6 hours later. An excellent series of experiments using murine peritoneal macrophages was reported by Wiener and Levanon (1968). The results of these studies provided a firm basis for the concept that endotoxin–monocyte interactions leading to either stimulation or cytotoxicity were critically dependent upon the concentration of endotoxin, the presence of serum, and the age of the cultures. Relatively low endotoxin concentrations, high serum concentrations, and more differentiated monocytes all favored stimulation rather than cytotoxicity. This latter effect was postulated to be due, at least in part, to be the result of degradative effects of macrphage hydrolytic enzymes on the endotoxin (see below).

These results have more recently been confirmed and extended by a number of investigators (Shands *et al.*, 1974; Glode *et al.*, 1977; Peavy *et al.*, 1978). As reported by Shands *et al.* (1974), incubation *in vitro* of nonadherent peritoneal cells (containing 10% phagocytic cells) with endotoxins caused a significant decrease in the percentage of these cells over a 3-day period, which the authors suggested was in part due to cytotoxic effects. More extensive experiments by Peavy *et al.* (1978) confirmed *in vivo* and *in vitro* destruction of macrophages by endotoxins that was dependent upon the lipid A portion of the molecule. In this respect, neither alkaline-hydrolyzed endotoxin nor native protoplasmic polysaccharide (NPP) (see Section II) were able to elicit cytotoxic responses.

These studies as well as those reported earlier by Glode *et al.* (1977) have reported the inability of endotoxins to initiate cytotoxic events in macrophages from the endotoxin-unresponsive C3H/HeJ mouse strain. This conclusion was originally postulated by Chédid *et al.* (1975). Of interest in these studies was the fact that endotoxins containing LAP were significantly less cytotoxic than those preparations that were protein free. As suggested by experiments with (CBA/N $\times$ DBA/2N) F_1 hybrids (the males of which express a maturational B-lymphocyte defect which limits their response to endotoxin, see Section V,E), stimulation and cytotoxicity of macrophages in response to endotoxins are independent of B lymphocyte activity (Rosenstreich

et al., 1978a). These experiments lend strong support for B cell independence of at least some endotoxin–macrophage interactions. The fact that both the stimulatory functions of endotoxins and the cytotoxic effects of endotoxins in C3H/HeJ macrophages are defective would be consistent with a similar molecular mechanism for these two cellular processes, both of which are dependent upon lipid A. In this respect cytotoxicity could be brought about by an overstimulation of the macrophage and the release of excess amounts of hydrolytic enzymes, which causes the "self-destruction" of the cell. This postulate was suggested by Wiener and Levanon (1968). Alternatively, lipid A may perturb the macrophage membrane, leading to activation with excessive perturbation resulting in dissolution of membrane integrity and consequent cytolysis. Such a mechanism was, in part, suggested by Peavy *et al.* (1978). Both mechanisms would be consistent with a lack of responsiveness of C3H/HeJ macrophages to these dual effects of endotoxins. Future experiments will undoubtedly define the contribution of one or both of these mechanisms to macrophage activation/cytoxicity.

As indicated above, the capacity of endotoxins to be either stimulatory or cytotoxic for macrophages may be counteracted by the ability of the macrophages to detoxify endotoxins. Evidence in support of the latter mechanism was first provided almost 20 years ago by Rutenburg *et al.* (1960). In these studies, endotoxins incubated *in vitro* with rabbit peritoneal macrophages in serum were significantly less potent in their subsequent ability to elicit Shwartzman reactions or kill chick embryos. Detoxification was accompanied by substantial release of ^{32}P from the endotoxin. Similarly, Filkins (1971) and Trejo and Di Luzio (1973) reported that macrophage sonicates from liver, lung, spleen, and/or peritoneal exudates were highly active in decreasing the lethal potential of endotoxins, as assessed by subsequent lethality in lead-sensitized rats. Of interest in the former study, activity was further localized to the macrophage large granule fraction. Extensive experimental investigations as to the biochemical mechanisms by which macrophage granule products accomplish the actual endotoxin detoxification have not, however, been performed to date.

D. Tumoricidal Activity of Endotoxin-Activated Macrophages

One of the most important functions of activated macrophages is in the defense of the host against the invasion of infectious microorganisms or neoplastic cells. The classic experiments reported by Alexander and Evans (1971) established a solid foundation for the concept

that endotoxin-activated macrophages markedly inhibited the growth of murine lymphoma cells. In these experiments *in vitro* exposure of murine thioglycolate elicited adherent peritoneal cells to 10–100 ng of endotoxins or purified lipid A per milliliter for periods of time as short as 30 minutes rendered these macrophages capable of inhibiting the growth of L5871Y lymphoma cells *in vitro*. Similar results were reported after *in vivo* activation of macrophages with endotoxin prior to isolation. These investigators found no evidence for the presence of a soluble factor released from the endotoxin-activated macrophages. In contrast, morphologic observations suggested that the lymphoma cells firmly adhered to the activated macrophages.

It has subsequently been documented by a number of investigators that, under the appropriate circumstances in a variety of experimental situations, endotoxins will induce in macrophages changes that allow these cells subsequently to kill tumor cells but not normal cells. What has emerged from these studies is the increased awareness of the critical importance of the state, or degree, of "activation" of the macrophage that will allow it to respond to endotoxin (or other stimuli) in the acquisition of its tumoricidal activity. The following paragraphs will summarize the results of a number of these studies. An additional concept that has become apparent from these combined studies and that we have noted earlier, is the unique sensitivity of macrophages to the presence of endotoxins, particularly with respect to tumoricidal effects. As a consequence, particular attention must be devoted to the use of endotoxin-free reagents in analyses of endotoxin–macrophage interactions. The need for critical concern in such situations has recently been emphasized (Martin *et al.*, 1978; Weinberg *et al.*, 1978), particularly with respect to cytotoxic responses of "normal" macrophages.

Based upon the studies of Alexander and Evans, Chédid *et al.* (1976) demonstrated that resident peritoneal macrophages from C3Heb/FeJ mice could be stimulated *in vitro* to kill mastocytoma cells. Of interest, however, these authors also reported that peritoneal macrophages from the endotoxin-unresponsive C3H/HeJ mouse could not be stimulated by either protein-free or protein-containing endotoxin preparations. These data would indicate a major role for lipid A in the activation leading to killing. More recent experiments published by Ruco and Meltzer (1978a,b) and Ruco *et al.* (1978) have confirmed the results of Chédid *et al.* (1976) and have extended these findings. These authors also reported the inability of LAP to stimulate tumoricidal responses in C3H/HeJ macrophages. Surprisingly, however, these macrophages were equally incapable of responding to BCG. Genetic analysis has

indicated that unresponsiveness to tumoricidal responses also maps with the LPS gene (see Section V).

The experiments reported by Doe and Henson (1978) and Doe *et al.* (1978) have also examined murine thioglycolate-elicited peritoneal macrophage responses to endotoxin. In these studies, cytotoxicity for P815 mastocytoma target cells could be detected 24 hours after stimulation with microgram quantities of a variety of endotoxins. Evidence for independent modes of action of lipid A and LAP was suggested from these experiments. In support of the results of Alexander and Evans (1971), no evidence for the presence of a soluble factor was found. Further, these data indicated that the macrophage responses appeared to be independent of B and T lymphocytes, although a possible contribution of nonphagocytic adherent lymphocytes was not excluded.

A recent well designed series of experiments reported by Weinberg *et al.* (1978) have reaffirmed the potent role of lipid A in mediating the tumoricidal activity of macrophages and more quantitatively documented the relative insensitivity of the C3H/HeJ macrophages to the potential stimulatory activity of endotoxin. These investigators have also provided further convincing evidence that the relative sensitivity of macrophages to endotoxin stimulation is a function of the state of activation of the cell prior to endotoxin stimulation. Equally important, however, in these studies was the demonstration that cloned macrophages, in the complete absence of B or T lymphocytes could be stimulated by lipid A to become tumoricidal. This represents the strongest evidence that endotoxins may directly stimulate macrophages without lymphocytes playing a role.

Studies reported by Hibbs *et al.* (1977) and Chapman and Hibbs (1977) have also investigated the capacity of endotoxin-stimulated murine macrophages to effect killing of tumor cells. In these experiments, endotoxin concentrations as low as 1–5 ng/ml would stimulate BCG-elicited macrophages (as well as macrophages from *Toxoplasma gondii* infected mice) to become cytotoxic for tumor target cells. Serum factors were shown to regulate the tumoricidal capacity of macrophages with significant (but reversible) inhibition manifest by a high molecular weight lipoprotein component. In experiments designed to explore the mechanism responsible for activation by endotoxin, these authors reported that prior treatment of macrophages with trypsin abrogated the endotoxin effects. Similarly, when a broad spectrum of protease inhibitors was examined, only tosyl lysine chloromethyl ketone (TLCK) was able to inhibit endotoxin-induced macrophage killing. These studies provide highly suggestive and promising evi-

dence for the participation of a serine protease in the elicitation of a macrophage activation mechanism.

The analysis of macrophages isolated directly from sites of tumor growth has also provided considerable information on the potential of these cells to be activated to kill tumor cells. Such studies were originally reported by Evans (1973), who demonstrated that endotoxins would enhance the cytotoxic activity of macrophages recovered from mouse or rat fibrosarcomas. These observations were considerably expanded by the experiments of Russell *et al.* (1977), who isolated macrophages from both progressing and regressing murine sarcomas. These investigators made the important observation that macrophages isolated from progressing tumors did not have the capacity to kill target cells. Incubation of such cells with minute (picogram per milliliter) amounts of endotoxins, however, stimulated these cells to become cytotoxic. Macrophages isolated from regressing tumors could kill target cells immediately after isolation from the tumor. Upon culture *in vitro,* these macrophages lost their cytotoxic capacity, which could, however, be restored by treatment with endotoxin. These experiments indicate the primed nature of the progressing tumor macrophage and suggest possible mechanisms for the *in vivo* effects of endotoxins on tumor necrosis and/or regression (see Section VII,C).

More recent experiments by Esser and Russell (1979) have explored the biochemical nature of the signal delivered by endotoxins to the macrophage to render it cytotoxic. Using electron spin resonance, these authors have reported a significant perturbational event in the cytoplasmic membrane of macrophages following stimulation with endotoxins. Of importance, this effect was noted only in macrophages that subsequently were demonstrated to elicit target cell killing. The relationship between this membrane event (4 hours after endotoxin) and initiation of killing (6 hours) remains to be elucidated. Nevertheless, these data suggest exciting new approaches for the analysis of endotoxin–macrophage interactions.

An interesting series of studies designed to investigate mechanisms of endotoxin-initiated macrophage killing has been reported by Currie and Basham (1975). These authors reported that supernatants of endotoxin-treated cells were active in killing transformed target cells. Analysis of the supernatant fraction by Currie (1978) has documented the inhibitory factor to be the enzyme arginase. Inhibition in this instance was therefore primarily the result of depletion of arginine from the culture medium which the author suggests, may be a relevant factor in a number of systems where endotoxin (or other stimuli) activated macrophages have been reported to be effective.

There are clearly numerous additional mechanisms by which endotoxin (or other stimuli) activated macrophages may bring about killing of tumor cells. Particularly with respect to endotoxins, a potential role for induction of natural killer (NK) cells by endotoxin-induced interferon production (Section VI,B,2) would be an attractive candidate (Dieu *et al.*, 1979). Tumor cells may be killed by H_2O_2 release from macrophages (Nathan *et al.*, 1979a); however, these experiments have not provided support for endotoxin activation of H_2O_2 production in macrophages (Nathan *et al.*, 1979a,b). A possible role for superoxide anion release by macrophages in response to endotoxin has been demonstrated (Johnston *et al.*, 1978), but its role in mediating macrophage cytotoxicity remains to be defined. One potentially exciting mechanism is suggested by the findings of Carswell *et al.* (1975) of a tumor necrosis factor (TNF) which is released from activated macrophages following endotoxin triggering (see Section VII,C).

The role of macrophages in general and TNF in particular in the initiation of tumor necrosis and/or regression in the compromised host following the therapeutic administration of endotoxins will be considered in Section VII,C.

VII. Biomedical Applications of Endotoxin-Activated Immune Systems

The data summarized in the previous sections underscore the unique immunologic activities of bacterial endotoxins. These are manifest by potent intrinsic immunogenicity as well as a profound capacity to potentiate or to suppress immune responses to unrelated antigens. As noted above, each of these immunologic activities of endotoxins has been noted by scientists and physicians for many years. Indeed, immunity to bacterial antigens, including endotoxins has played an essential role in the systematic development of protective immunoprophylaxis against infectious microbial agents. The last decade has, however, witnessed several important advances in the use of endotoxins in the therapeutic control of host susceptibility to disease. Several lines of evidence suggest that endotoxins may play a critical role in regulating host responsiveness to infections and neoplasia. Equally important has been their use as immunogens in the development of protective antisera for control of gram-negative infections.

We will review in this section the recent research that has explored the potential biomedical uses of endotoxins and antiendotoxin antibodies. While a majority of these studies are still in the developmental stage, several clinical trials have already been completed. At this time both the careful administration of endotoxin as an im-

munopotentiating agent, as well as the active immunization of human beings or experimental animals with endotoxin appear to hold great promise as therapeutic modalities in oncology and infectious disease. Whether this promise will be realized in fact should become apparent during the forthcoming decade.

A. Resistance to Infection: Antibody to Endotoxin

Owing to the multiplicity of potential immunogenic determinants present on the outer surface of gram-negative bacteria, humoral immune responses to these organisms normally result in antisera with complex antibody specificities (Robbins and Hill, 1977). As the endotoxin complex constitutes a major component of the cell wall of gram-negative bacteria, and as endotoxins are highly immunogenic, it is not unexpected to find antibody to endotoxins playing a major role in host responses to these bacteria. In this section we will review the role of antibody to endotoxin, either actively elicited or passively administered, in reducing or abrogating the pathophysiologic effects of endotoxins and gram-negative bacteria. Our discussion will be limited to a consideration of those antibodies directed against the lipopolysaccharide component of endotoxins, which we will consider separately on the basis of the three major structural regions of the LPS: (*a*) the "O-antigen" polysaccharide; (*b*) the "core" polysaccharide; and (*c*) lipid A. Antibodies to each of these distinct subregions of LPS have, under the appropriate conditions been documented to provide considerable protection to the host following challenge with either isolated endotoxins and/or infectious organisms.

It should be noted that there are clearly enormous clinical benefits that would accrue to the approximately 300,000 yearly hospitalized patients who may become at risk due to gram-negative bacteremia (McCabe, 1976). As a consequence, there is a considerable volume of literature that has dealt with this important topic. It is much beyond the scope of this review to discuss these accumulated data in detail. Rather we will focus on a number of recent reports that illustrate several promising approaches to the use of antibody in the clinical intervention in the control of gram-negative bacteremia.

1. Antibody to O-Antigen Polysaccharide Determinant

Endotoxins and purified LPS normally elicit antibody responses with specificities directed at least in part against the O-antigen polysaccharide (see Section II). The classic studies of Boivin and Mesrobeanu (1938), using passive transfer of immune serum, first explored the potential use of antisera against O-antigens in the protection of

recipient animals against the lethal effects of endotoxins. In the 30 years following these early studies, numerous investigators have explored a variety of systems designed to test the efficacy of antibody to endotoxin in protecting against endotoxin-induced lethality, pyrogenicity, Shwartzman reactivity, and leukocyte alterations as well as gram-negative bacteremia (reviewed in Greisman *et al.*, 1969). The results of these combined studies illustrate a number of relevant points. First, and perhaps of primary importance, antibody to endotoxin in many of the studies provided significant protection against subsequent endotoxin challenge. Second, the observed protection in many instances did not correlate directly with the serum titer of antibody to the O-antigen. Third, antibody raised against endotoxin isolated from one organism often provided some protection against challenge with an unrelated endotoxin.

In light of these and other observations, several considerations are worthy of note. It seems reasonably clear that, although antibody directed specifically against O-antigen determinants can mediate protection (see, e.g., Greisman *et al.*, 1969), it is often irrelevant to protection (see, e.g., Kim and Watson, 1965; Tate *et al.*, 1966). In addition, the results of an extensive clinical study have found no correlation between the levels of antibody to O-antigen determinants and survival of patients with gram-negative bacteremia (McCabe *et al.*, 1972). More recently, the presence of high-titer serum antibody to O-antigen did not correlate with prevention of disease in patients with recurrent urinary tract infection (Kaijser and Olling, 1973).

In addition to the lack of consistent evidence favoring a major protective role for antibody to O-antigens is the important practical consideration that gram-negative infections can be caused by a broad spectrum of gram-negative organisms (and their equally broad spectrum of uniquely defined O-antigenic polysaccharide determinants). Therefore, it might be anticipated that antibody directed against specific O-antigen determinants would be of limited therapeutic value in treatment of patients with gram-negative bacteremia. The difficulties inherent in this approach have recently been enumerated by McCabe (1976). This author has pointed out that, among 84 cultures of *E. coli* isolated from patients with bacteremia, 27 different O-serotypes were characterized. This diversity of species and strains that may initiate gram-negative bacteremias would of necessity preclude the general use of O-specific antibody in the control of infection by these organisms.

However, in situations where infections can be limited to a select number of antigenic serotypes, O-antigen immunotherapy may be of considerable value. One such organism would be *Pseudomonas*

aeruginosa, in which only seven serotypes have been antigenically defined. The propensity of this organism to invade the immunosuppressed or burned patient further underscores the need for effective therapeutic control. An additional problem with *Pseudomonas* is that many strains are resistant to serum killing and antibiotic therapy. As a consequence, a great deal of effort has been devoted to the development of immune protection mechanisms against *Pseudomonas* infection both by active immunization as well as passive transfer of antisera to *Pseudomonas* subfractions. These comprehensive studies have been the subject of a recent symposium [*J. Infect. Dis.* **130**, SI-S166 (1974)] as well as numerous recent publications (e.g., Lieberman *et al.*, 1979; Pollack and Young, 1979), to which the reader is referred for additional references and information. These combined studies have established that antibody to *Pseudomonas* can, indeed, be of significant protective value both in experimental animals and in man following infection with this organism.

Of particular relevance to this section of the review is the unequivocal demonstration of the effective role of antibody directed against the type-specific O-antigen component of the LPS in conferring the observed protection. In this respect a number of reports that provide strong experimental support for this conclusion should be cited. Bass and McCoy (1971) clearly showed that antisera directed against *Pseudomonas* strains of the same serotype conveyed high levels of cross-reactive protection in mice against infection with the corresponding organisms, but little cross-protection against different serotypes. Harvath *et al.* (1976) demonstrated that dogs pretreated with serotype-specific antibody to a given *Pseudomonas* sp. were significantly less susceptible to a subsequent infectious challenge with that specific organism, as assessed by resulting bacteremia and tissue infection as well as increased survival times. In contrast, dogs pretreated with antiserum against an unrelated serotype behaved as did normal controls in response to infectious challenge. The elegant experiments recently published by Fisher (1977) have established that the polyvalent γ-globulin (PG) prepared by hyperimmunization of human subjects with *P. aeruginosa* LPS antigens (which has been used extensively in clinical trials) confers protection by serotype-specific antibody. Using a checkerboard type of protocol, Fisher demonstrated that preabsorption of PG with serotype-specific *Pseudomonas* removed selectively the capacity of the preabsorbed PG to provide protection to mice subsequently infected with that particular serotype but not the other six serotypes. No cross-protection was detected against infection with unrelated gram-negative organisms.

It is clear from the experiments summarized here, as well as others

too numerous to cite in this review, that immune responsiveness to O-antigenic determinants on the LPS molecule can be used as an effective therapeutic approach in the treatment of infection by *Pseudomonas* sp. as well as numerous other gram-negative species (e.g., *Salmonella, Yersinia, Klebsiella*).

2. *Antibody to Core Polysaccharide Determinants*

As an alternative approach to the generation of antisera effective in the treatment of gram-negative infections, several investigators have utilized antibody directed against core polysaccharide determinants of the LPS. This subregion of the LPS molecule displays considerably less chemical and antigenic diversity between strains and species in comparison to O-antigenic determinants, and thus might be expected to obviate many of the potential drawbacks of these latter immunogens. The development of this conceptual approach has benefited significantly from the early studies of D. Watson and Kim (1963) and Kim and Watson (1965) as well as the experimental demonstrations by Tate *et al.* (1966) and Chédid *et al.* (1968) of the protective capacity of antibody directed against rough (O-antigen-deficient) mutant gram-negative bacteria. The primary establishment of the clinical feasibility of this approach has resulted from the extensive studies of Braude and his collaborators in La Jolla (reviewed in Braude *et al.*, 1977a,b) and McCabe and his co-workers in Boston (reviewed in McCabe *et al.*, 1977).

The results of early experiments reported by Braude demonstrated a marked increase in the 50% lethal dosage of endotoxin in mice pretreated with hyperimmune serum directed primarily against core antigen determinants. In an extension of these studies, Braude and Douglas (1972) reported that antiserum against the J-5 (rough) mutant of *E. coli* 0111:B4 afforded protection against the localized Shwartzman reaction elicited by several unrelated O-antigen-containing endotoxins. Similar protection against the generalized Shwartzman reaction was reported, and both 7 S and 19 S antibody were found to be effective (Braude *et al.*, 1973). Using a laboratory model of experimentally induced infection in agranulocytic rabbits, Ziegler *et al.* (1973a) established that the J-5 antisera provided impressive protection against lethal bacteremias following challenge with either *Klebsiella* or *E. coli*.

Based upon the results of these highly promising studies, Braude and his collaborators prepared human antisera to the J-5 organism. This human antiserum was shown to provide protection against lethality in mice and the local Shwartzman reaction in rabbits (Ziegler *et al.*, 1973b). More recent studies have extended these results to include

protection against lethal *Pseudomonas* bacteremia (Ziegler *et al.*, 1975; Braude *et al.*, 1977a,b), and reduction of *Neisseria meningitidis* endotoxin-induced systemic effects (Davis *et al.*, 1978). Currently, this antiserum is being examined in prospective double-blind fashion for its protective capacity against the lethal effects of gram-negative sepsis in hospitalized patients. Preliminary data indicate no toxicity associated with treatment as well as a highly promising, increased incidence of recovery from shock and decreased mortality accompanying the administration of this antiserum (Ziegler *et al.*, 1978).

A similar experimental approach has been taken by McCabe and collaborators using antiserum to the (rough) heptose-deficient Re595 mutant of S. *minnesota*. This LPS contains only lipid A and the LPS-specific sugar KDO. These studies were premised, in part, upon the observation by McCabe *et al.* (1972) that, in patients with gram-negative bacteremia, decreased incidence of shock and death correlated with titers of antibody to Re antigen, but not with O-antigen antibody or common enterobacterial antigen. Analysis of the serum antibody of these patients suggested that survival was primarily associated with levels of IgG antibody (Zinner and McCabe, 1976). Using both active immunization as well as passive transfer of hyperimmune serum, McCabe *et al.* (1973) and McCabe (1976) demonstrated protection in mice against the lethal effects of heterologous bacteria, including both *Klebsiella* and *Proteus* sp. Specificity of the protection for the Re determinants was suggested by the demonstration that activity could be absorbed with Re bacteria. Immunization of rabbits with Re organisms afforded protection in a subsequent challenge of these granulocytopenic rabbits with either *E. coli* or *Enterobacter aerogenes* (Bruins *et al.*, 1977; McCabe *et al.*, 1977), results similar to those obtained by Braude. It would thus appear that antibody directed specifically against the lipid A-KDO region of the core polysaccharides of the LPS is sufficient to elicit protection against gram-negative infections.

More recent experiments by several investigators have confirmed and extended the observations of Braude and McCabe to additional models of gram-negative infections. Experiments by Young and Stevens (1977) have provided evidence that active immunization of dogs with isolated LPS from Re mutants protected them from the systemic effects normally elicited by injection of endotoxin, which, the authors suggest, is due to the ability of the antibody to function as an antitoxin rather than its capacity to promote phagocytosis (Young *et al.*, 1975). Using another experimental model, immunization with endotoxin isolated from a rough strain prevented the subsequent intracerebral

infection with *Neisseria gonorrheae* (Diena *et al.*, 1978). Similarly embryonated eggs from hens that had been immunized with rough *Neisseria* endotoxin were protected from the lethal effects of *Neisseria* infection.

It should be noted, however, that not all investigators have achieved protection against endotoxins, or gram-negative infection with antisera to R595 endotoxin. Evidence that would indicate a minor protective role for these antisera has derived from the experiments of Mullan *et al.* (1974). These investigators found that such antisera did not prevent enteropathogenic *E. coli*-induced fluid loss in isolated piglet gut, suggesting that, in these organisms, the lipid A-KDO determinants were present in the cell wall in cryptic locations. Similarly Ng *et al.* (1976) found that, whereas a high level of antigenic cross-reactivity existed between antisera to R595 endotoxin and polysaccharide-containing endotoxins from a number of species, no protective effect against infection was ever noted. These authors also concluded that the lipid A-KDO groups may not be accessible to the potentially protective antisera. Thus, although in some instances protection may be conferred by antibody to core determinants, such protection may well depend upon multiple variables, including antibody titer and infecting organism.

3. *Antibody to Lipid A*

The experimental success achieved with antibodies directed against core polysaccharide determinants, where limited antigenic diversity is expressed, might suggest a similar, if not enhanced, protective capacity of antibody directed solely against the lipid A region of LPS, where virtually no structural heterogeneity is found among a number of gram-negative organisms[5] (Galanos *et al.*, 1971a; Rietschel *et al.*, 1977; Johns *et al.*, 1977). However, the success of this approach, as was also indicated for the experiments cited above, would again depend critically upon the potential accessibility of the lipid A to the antibody. While this might not be expected to pose a major problem in the case of isolated endotoxins, the cell wall of the intact organism may not allow free access of the anti-lipid A antibody.

With regard to this latter point, the available data do not provide a general consensus as to the expression of lipid A antigenic determinants on the cell surface of gram-negative bacteria, and considerable variation may exist within strains. As suggested by Kim and Watson

[5] While this statement is true for many gram-negative bacteria, it should be recognized that both subtle and profound differences in lipid A structure and antigenicity can exist in some strains of gram-negative bacteria such as, e.g., *C. violacieum* (see the references cited in text).

(1965) humoral factors may also play a role in exposing lipid A "sites." Galanos *et al.* (1971a) found that immunization with Re mutants generated antibody to lipid A. However, in these studies immune responses to lipid A could be significantly enhanced by passive absorption of free lipid A onto Re organisms prior to immunization. Nevertheless, these studies suggest that some lipid A antigenic sites are exposed in rough mutants. In contrast to these results, Johns *et al.* (1977) suggested that the presence of KDO (or O-antigen polysaccharide) effectively masked the expression of lipid A antigenic determinants, a conclusion based in part on the inability of these investigators to protect against infections using antibody to lipid A (Bruins *et al.*, 1977). Similarly, Mattsby-Baltzer and Kaijser (1979) have provided data to indicate that neither intact rough or smooth strains of gram-negative bacteria have exposed lipid A antigenic determinants on their surface, although the O-antigen extracts of most rough mutants did possess lipid A antigenicity. An interesting recent finding of Westenfelder *et al.* (1977) however, has demonstrated antibody to lipid A in sera of patients with chronic pyelonephritis. As earlier reported by Ryan *et al.* (1973), rough mutants are a frequent cause of recurrent urinary tract infections. However, the possibility that endotoxins released from the cell wall may have served as the immunogens for the generation of antibody to the lipid A cannot be excluded by these data.

The lack of a direct role of antibody to lipid A in protecting against the pyrogenic responses elicited in rabbits by either purified LPS or lipid A was also reported by Rietschel and Galanos (1977). These investigators found that neither pretreatment of lipid A with antibody nor passive administration of antibody to rabbits prior to injection of lipid A significantly decreased the 3-hour fever response. In contrast, almost complete protection was afforded by antibody to lipid A in rabbits pretreated with lipid A 2 days earlier, and antibody to lipid A 1 day before a second pyrogenic dose of lipid A. In this experiment, purified LPS was found to substitute for lipid A for either injection. Of interest, antibody to O-antigens did not protect against LPS-induced fever in this system, suggesting that the mechanisms for protection by these two antisera may not be related. The precise mechanism responsible for the observed protective effect of the lipid A antiserum remains, however, to be defined. Nevertheless, additional experiments by these investigators have also reported marked protection against skin necrosis in the local Shwartzman reaction if antibody to lipid A was given 2 hours before the challenge injection. Significantly, protection was noted against both lipid A and heterologous LPS preparations given in various combinations in the preparation and challenge injec-

tions. These data establish that, under certain circumstances, lipid A antibody can be of considerable protective value in mediating the deleterious effects of endotoxins.

These combined data would therefore, indicate that the *in vivo* effects of a spectrum of gram-negative organisms, as well as their isolated endotoxins may be effectively reduced or even abrogated by antibody directed against antigenic determinants present on the various subregions of the LPS compound of bacterial endotoxins. The results of clinical trials reported in the last 5 years appear highly encouraging and are suggestive of a promising future for this immunotherapeutic approach to the control of gram-negative bacteremias.

B. Resistance to Infection: Antibody Independent

The capacity of endotoxins and other bacterial products to influence nonspecifically host responsiveness to infectious microbes has been recognized for many years (Metchnikoff, 1893, reviewed by Shilo, 1959). As originally shown by Rowley (1955) using isolated *E. coli* cell walls, and shortly thereafter by several investigators with partially purified endotoxins (Landy, 1956; Dubos and Schaedler, 1956; Kiser *et al.*, 1956), the appropriately timed administration of these bacterial products prior to infectious challenge with gram-negative bacteria will afford significant protection to mice. A period of 24 hours in most of these studies was found to be optimal, and doses of endotoxin as low as 10 μg were found to be effective. In the years following the publication of these observations, similar experimental protocols have established that endotoxins will equally affect the pathogenicity of a wide variety of infectious agents in addition to bacteria, including parasites, fungi, viruses, and mycobacteria. These studies have recently been reviewed by Cluff (1971), who has proposed that the similarities in host responses to these different agents effected by endotoxins would suggest phagocytes as a major target for endotoxin action.

Similar alterations in responses to endotoxins themselves can be engendered in the host by pretreatment with one or more injections of endotoxin (reviewed in Chédid and Parant, 1971). This latter phenomenon of endotoxin-induced hyporesponsiveness has been classically termed "endotoxin tolerance." However, this type of endotoxin unresponsiveness should not be confused with immunologic tolerance to endotoxin (see Section II). The extensive experiments by Greisman *et al.* (1969) and later by Milner (1973) showed that endotoxin tolerance can be characterized by an early (<48 hours) endotoxin hyporesponsiveness that is independent of antibody to endotoxin and

shows interendotoxin cross-reactivity. This is followed by a late (>48 hours) response, which is mediated by circulating antibody and is usually specific for the endotoxin (see Section II). The latter, but not the former, phase of endotoxin tolerance may be passively transferred to confer tolerance to recipient animals. We have discussed several aspects of the antibody-dependent phase of protection against gram-negative infections in Section VII,A. Similarly the data documenting these early nonspecific effects of endotoxins is also extensive and will not be reviewed in detail here. Nevertheless, as these endotoxin-induced antibody-independent protective responses hold promise of being of potential therapeutic value, we will consider selected topics.

The mechanisms by which host resistance to lethal doses of bacteria may be enhanced by pretreatment with endotoxins have similarly been shown to be characterized by an early nonspecific phase, which can be clearly distinguished from a later antibody-dependent phase. This was convincingly demonstrated by Parant (1968), whose experiments showed that pretreatment of mice with 1μg of endotoxin 24 hours prior to challenge with *Klebsiella pneumoniae* reduced significantly the numbers of infectious organisms recovered in several tissues. To contrast this mechanism of protection with that provided by immune serum, she demonstrated that the rate of disappearance of organisms from the circulation in mice immune to *K. pneumoniae* was critically dependent upon the number of organisms injected. In contrast, the rate of clearance in endotoxin-treated mice was virtually independent of the number of circulating organisms.

In experiments designed to examine the mechanisms of this early antibody-independent protection induced by endotoxins, Chédid and his collaborators have examined a number of parameters of the response to *K. pneumoniae*. Even though antibody synthesis was not involved in the protection, Chédid (1973) showed that if spleen cells were administered to newborn mice (which are normally highly sensitive to the lethal effects of endotoxin) at the same time as endotoxin pretreatment, they became significantly more resistant to subsequent lethal challenge with endotoxin. These results support a role for endotoxin-activated spleen cells in mediating the protective response. Additional experiments by Chédid *et al.* (1976) using the C3H/HeJ endotoxin unresponsive mouse, as well as the histocompatible C3Heb/FeJ mouse, have shown no protection in the former mouse strain by pretreatment with either highly purified LPS or with endotoxin containing lipid A-associated protein (LAP) (see Section V). As B lymphocytes from the C3H/HeJ mouse are known to be activated by LAP but not LPS, a role for B cell activation in the observed protection

would not be implicated. Further, as the available data do not support the concept of a major defect of the C3H/HeJ humoral mediation systems in response to endotoxins (Curry and Morrison, 1979), a cellular response is implicated. Additional support for this conclusion has derived from the more recently published data of Parant *et al.* (1976), who found no correlation between the capacity of various endotoxins or endotoxin derivatives to initiate B cell proliferative responses and their ability to confer protection against *K. pneumoniae* infection. Of interest in these studies was the observation that the congenitally athymic Nu/nu mouse could also manifest protection against infection in response to endotoxin. Thus a major role for T lymphocytes would not be implicated.

These combined data underscore the profound effects that endotoxins can manifest on host responses to infection with gram-negative bacteria. A major effect on lymphoid cells, presumably macrophages, is implicated as being responsible for the observed protection. The adaptation of these impressive experimental models of protection against gram-negative bacteremia to clinical situations remains, however, as an intriguing challenge for future research.

C. Endotoxin Effects on Tumor Necrosis and/or Regression

Probably the first systematic exploration of the potential use of mixed bacterial toxins, including endotoxins, in the therapeutic treatment of inoperable malignant tumors were the extensive clinical trials reported by Coley (1896). Based upon the apparent remarkable correlation between erysipelas infection and neoplasm regression, Coley prepared culture filtrates of *Streptococcus erysipelatis* for administration to patients with various inoperable tumors. These early bacterial extracts were later supplemented with extracts of *Serratia marcescens* and were employed by numerous physicians. Their early promise as antitumor agents was suggested by the fact that for many years such toxin extracts were commercially available (Nauts *et al.*, 1946). However, considerable questions as to the efficacy of these various preparations precluded their general acceptability as useful therapeutic agents (reviewed in Nauts *et al.*, 1946).

More recently, however, interest in the use of bacterial products has been revived (reviewed in Hersh *et al.*, 1977; Terry, 1976). This has been in part the result of extensive chemical characterization of these various bacterial products and a recognition of their profound capacity to perturb host defense systems. Of particular interest has been the recent recognition of the multiple profound activities of endotoxins on

cells of the immune system, the results of which have been summarized above. In this respect, the profound capacity of endotoxins to induce macrophages to become cytotoxic for tumor cells, as discussed in Section VI, has provided a significant stimulus for the further exploration of endotoxins in the therapeutic treatment of tumors. The recent results obtained by a number of investigators using *in vivo* experimental models of tumor growth would support a role for endotoxin-induced lymphoid cell activation in tumor necrosis and/or regression.

The results of several studies (Shear and Turner, 1943; Shear, 1943) provided the first evidence that at least one of the components active in tumor necrosis and present in the "Coley toxin" preparations was indeed endotoxin. Following these studies, Lemperle (1966), in a series of experiments designed to examine several agents known to stimulate RES function, showed that purified endotoxins would retard the growth of a sarcoma tumor in mice. He found that weekly intraperitoneal injections of endotoxin reduced significantly tumor growth, which the author attributed to both sensitized lymphoid cells as well as humoral antibody formation. Similar results were reported by Mizuno *et al.* (1968) and Kato *et al.* (1973) on growth of a solid Ehrlich carcinoma in mice. These latter investigators concluded that intracutaneous endotoxin at 2 days after tumor injection gave optimal results. Both direct cytotoxic effect on tumor cell viability and a stimulation of the RES by endotoxin were suggested.

An interesting study reported by Tripodi *et al.* (1970) demonstrated that spindle-cell sarcoma cells preincubated with endotoxins were significantly reduced in their capacity to grow when subsequently injected into mice. Growth rates were reduced in a dose-dependent fashion and inhibiting activity was abrogated by treatment of the endotoxin with alkali. These investigators reported, however, that generalized stimulation of nonspecific resistance was not the mechanism responsible, in that pretreatment of mice with endotoxin by intraperitoneal injection did not inhibit tumor growth. No direct cytotoxic effects of endotoxin on the tumor cells were detected.

The results of several more recent studies have essentially provided support for these earlier studies although the relative efficacy of such treatment with endotoxin has not always been entirely consistent. Somewhat variable protection of rats by pretreatment with endotoxin 5 days before challenge with carcinosarcoma ascites cells as assessed at 10 days was noted by Keller and Hess (1972). The experiments of Parr *et al.* (1973) suggested a role for hemorrhagic necrosis of tumors by endotoxins. In addition, however, the demonstration by these authors that endotoxin-induced tumor regression, but not necrosis, could be

abrogated by treatment of mice with antithymocyte serum also suggested that different mechanisms may be operable in these two effects of endotoxins on tumors. In a more recent study, some antitumor effects of endotoxins observed in mice, although of interest, showed no significant enhancement of survival (Ikekawa *et al.*, 1975). Saito *et al.* (1978) described the results of experiments that indicated that endotoxin was able to act with syngeneic antitumor serum in enhancing significantly the survival of mice implanted with an ascites tumor. These combined data would support a role for endotoxin in tumor necrosis and regression, although it would appear that a number of potential variables remain to be further defined.

Similar results have been obtained with plasmacytoma cells in experimental models using mice. In these studies, nanogram amounts of endotoxin give intraperitoneally prevented tumor growth of the MOPC 315 cell line (Bober *et al.*, 1976). More recent results from this laboratory have suggested that these low doses of endotoxin may, under some circumstances, enhance the growth of plasma cell tumors (Platica and Hollander, 1978). In these studies, it was found that small but significant amounts of endotoxins could be detected in peritoneal fluids of mice injected with plasmacytoma cells in the presence of mineral oil. It was postulated that the mineral oil-induced peritonitis could have given rise to the release of endotoxin from the gut, which subsequently might have participated in the pathogenesis of the plasma cell tumors. Further work on this interesting experimental model should resolve this apparent Janus role of endotoxins in regulating the growth of tumor cells.

In addition to the direct effects of endotoxins on the growth of tumors *in vivo*, these agents have also been utilized in conjunction with other bacterial products as immunopotentiators of tumor regression. The comprehensive experiments of Ribi and his co-workers have established that both BCG cell wall extracts, as well as the purified active component, trehalose dimycolate, will suppress line 10 hepatoma growth in guinea pigs (Ribi *et al.*, 1975, 1976). It is of interest that, when purified endotoxic lipopolysaccharides from polysaccharide-deficient mutant bacteria were included in the immunization, significant enhancement of the suppressive capacity was observed. Studies using deproteinized BCG cell walls yielded a similar enhancing effect of endotoxin; however, moderate toxicity of these bacterial products was noted (McLaughlin *et al.*, 1978). Importantly, in these studies the intradermal injection of the mixture of these agents eradicated metastatic lymph node lesions in animals from which the primary tumor had been surgically removed (Kelly *et al.*, 1978). These

highly promising studies may well provide fundamental information on the potential use of a combined bacterial product regimen in immunotherapeutic treatment of tumors.

The combined studies summarized above suggested that enhanced nonspecific RES function, cellular immunity, humoral factors, and direct cytotoxic effects of endotoxin on tumor cells may all participate in endotoxin-induced alterations in tumor cell growth. The results of recent experiments of several investigators have provided additional strong support for one or more of these factors. Using the murine TA3 carcinoma strain nonspecific (TA3-Ha) tumor line, Nowotny and his co-workers established that injection of as little as 1.0 μg of endotoxin into C57B1/10Sr mice 24 hours before challenge with tumor significantly reduced the development to fatal tumor as compared to development in untreated mice (Grohsman and Nowotny, 1972). Additional studies demonstrated that 10 μg of endotoxin given from 3 days before tumor challenge to the day of tumor challenge yielded optimal results. No direct cytotoxic effects on the tumor cells were detected; however, local intraperitoneal administration of endotoxin to the site of the ascites tumor cells was found to be more effective than systemic injections (Yang and Nowotny, 1974). Of importance in these studies was the demonstration by these investigators that suppression of tumor growth could be transferred with lymphoid cells previously activated either *in vivo* or *in vitro* with endotoxin. As the activity of the endotoxin-stimulated cells was found to reside in the adherent cell population, a prominent role for macrophages was suggested. More recent experiments have provided evidence that suppression can be demonstrated in Nu/nu mice, but not in the endotoxin-resistant C3H/HeJ mouse (Nowotny, 1977). These data suggested a possible role for endotoxin-induced B cell activation as a significant factor in tumor resistance. The capacity of serum from these treated mice to transfer resistance, to subsequent challenge with tumor cells as well as cause necrosis of established tumors would indicate a protective role for a serum factor as well. This author noted the potential relationship between CSF activity (see Section VI) in the serum and its ability to transfer serum resistance. These experiments also indicated that detoxified preparations of endotoxins were also highly effective in protecting mice from the lethal effects of tumor growth.

An excellent series of studies has also been recently reported by Berendt and his collaborators. Using the TA3-Ha tumor cell line also employed by Nowotny (see above), Berendt and Saluk (1976) demonstrated significant protection against tumor growth by injection of endotoxins from 1 day before to as late as 3 days after administration of

tumor cells. These authors also reported no detectable effects on tumor cell viability, and transfer of suppressive activity with *in vivo* endotoxin-activated peritoneal cells. Additional studies by these investigators (Berendt *et al.*, 1978a) have provided convincing evidence for participation of two lymphoid cell populations, one of which appears to be the macrophage and the other a nonadherent radiosensitive lymphoid cell.

To establish more precisely the relationship between endotoxin-induced necrosis of tumors and tumor regression, Berendt and his colleagues (1978b,c) administered endotoxin intravenously to groups of mice 7 days after the intradermal initiation of four different tumors. It is important that, whereas necrosis of tumor tissue occurred in all four tumors, regression of the tumor occurred only in those tumors classically defined as being immunogenic. Considerable evidence for a primary role for cell-mediated immunity in the endotoxin-induced regression was provided by experiments using adult thymectomized lethally irradiated and bone marrow-reconstituted mice, where no endotoxin-induced regression was detected. Further evidence was provided by passive transfer of protection with spleen cells from endotoxin-induced tumor regressed donor mice. Such passive transfer was abrogated by treatment with anti-Thy-1 and complement. Temporal studies have suggested that tumor regression follows the acquisition of specific effector T cells in response to endotoxin stimulation. Further, the hemorrhagic necrosis that precedes tumor regression was suggested to facilitate specific T cell effector function.

The mechanism of the endotoxin-induced tumor necrosis, as originally observed by Shear and Turner (1943) and Shear (1943), has received considerable attention during the last several years. As suggested by Mizuno *et al.* (1968), endotoxins may affect directly the viability of some tumor cells. Other investigators, however, have found no cytotoxic effects of endotoxins on tumor cells (e.g., Tripodi *et al.*, 1970; Yang and Nowotny, 1974; Berendt and Saluk, 1976). Inhibition of growth of transformed, but not of normal, rat fibroblasts by endotoxins was reported by Brailovsky *et al.* (1973) to correlate with binding of endotoxin to the cell membranes of such cells and subsequent increases in levels of intracellular cyclic AMP. Bara *et al.* (1973) suggested a role for complement-mediated lysis of tumor cells following passive sensitization with endotoxin. As assessed by both DNA and protein synthesis, relatively high concentrations of endotoxin (100 μg/ml) were found by Ralph and Nakoinz (1977) to be cytostatic when incubated *in vitro* with several murine monocyte cell lines. More recently, Raschke *et al.* (1978) indicated a significant effect of minute quantities (1 ng/ml) on the growth of an Abelson leukemia virus-

transformed macrophage cell line. In addition, a recent brief report (Jones, 1977) has suggested an indirect effect of endotoxins on tumor necrosis. In this study, the release of fatty acids following endotoxin injection was suggested to inhibit oxidative metabolism in tumor cell mitochondria thus leading to tumor cell death.

Probably the most extensively studied mechanism by which endotoxins bring about tumor necrosis is via the induction of tumor necrosis factor (TNF). This factor is generated *in vivo* by injection of endotoxin into BCG (or *C. parvum*) primed mice (Carswell *et al.*, 1975). Optimal activity, as assessed by the capacity to cause necrosis of sarcoma Meth A tumors was observed at 2 hours after endotoxin injection. TNF was shown to be distinct from residual endotoxin by a number of criteria and was cytotoxic *in vitro* for neoplastic, but not for untransformed, cell lines. Partial purification of TNF has resulted in its characterization as a sialic acid- and galactoasmine-containing glycoprotein of approximately 150,000 MW (Green *et al.*, 1976). It is of interest that cytotoxic activity was not restricted to murine tumor lines but was active also against a human melanoma cell line. Although the available evidence would indicate the macrophage as the source of TNF, the recent report of Hoffmann *et al.* (1978) that *C. parvum*-primed athymic Nu/nu mice cannot be induced to generate TNS in response to endotoxin would suggest a possible role for T cells. It should be cautioned, however, that Parks *et al.* (1977) have also suggested a possible B cell defect in Nu/nu mice (see Section IV,D,1); therefore, the lack of TNF generation in such mice may be more complex.

In addition to causing cytolysis of transformed cells, which contrasts with the lack of such effects of endotoxins, it is clear that TNF has a number of properties that are similar to those of endotoxin in its ability to activate lymphoid cells. These activities have been summarized by Hoffmann *et al.* (1978), who predicted that the further elucidation of the various biological activities of endotoxin-initiated TNF would clarify significantly the mechanisms of the immune response-independent necrosis and immune response-dependent rejection of malignant tumors. These efforts may well provide a firm foundation for the reinitiation of Coley-toxin therapy as an effective means of affecting tumor necrosis and/or regression.

VIII. Summary and Conclusions

The accumulated data that we have summarized in the preceding sections underscore the profound multiple pathways by which bacterial endotoxins influence host immune responses. Major direct interac-

tions of endotoxins with B lymphocytes have been documented, leading to synthesis and secretion of antibody directed not only against antigenic determinants on the endotoxin molecules themselves, but also with specificities characteristic of the complete repertoire of variable region gene products. In neither of these situations is there a requirement for either T lymphocytes or macrophages. Nevertheless, equally impressive interactions of endotoxins with both these lymphoid cells have been demonstrated. The primary consequences of these latter interactions appear to be manifest in the regulation of immune responses to unrelated antigens, where both enhancement and suppression of antibody responses may be predictably controlled. It would appear fair to conclude that these combined highly impressive major advances in our understanding of endotoxin–lymphoid cell interactions, as summarized in this review, have contributed significantly to the current degree of sophistication of the role of lymphoid cells in host immune responses.

The advances made in the delineation of the mechanisms of direct B lymphocyte and macrophage activation by bacterial endotoxins, as characterized by Möller, Melchers, Andersson, and their colleagues, provide a firm foundation for major advances in defining the molecular biology of lipid A–membrane interactions in general. Lipid A is known to perturb a spectrum of mammalian cells, presumably through interactions with the cell membranes of these cells. Knowledge gained from studies of lymphoid cell responses to lipid A should provide valuable information for the analysis of mechanisms of response of other cell types to lipid A. Of great potential significance in this respect is the now well-defined murine gene locus for lipid A responses (*Lps*) characterized by Watson and his colleagues, its phenotypic expression, characterized by Mergenhagen and Rosenstreich and their colleagues, as well as its gene product (presumably identical with the membrane receptor for lipid A), recently described by Coutinho and his co-workers. Indeed, the contribution of endotoxin-unresponsive mutant mouse strains (particularly the C3H/HeJ mouse), pioneered by the experiments of Sultzer, will undoubtedly prove as invaluable to the elucidation of endotoxin immunobiology as has been the contribution of mutant bacterial strains to the elucidation of endotoxin immunochemistry.

Knowledge of the immunobiology of endotoxins has already prompted investigations in their therapeutic uses in man as both antigenically distinct and cross-reactive immunogens in protection against gram-negative infections. There is, in addition, cause for optimism in their potential use as immunostimulatory and/or immunosuppressive

agents in the therapeutic intervention of infection and neoplasm in man. These objectives will be realized when all the potential interactions of endotoxins with host immune systems are defined. The data summarized in this review indicate that this goal is well on the way to being accomplished.

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ADVANCES IN IMMUNOLOGY, VOL. 28

Responses to Infection with Metazoan and Protozoan Parasites in Mice[1]

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I. Introduction

Immunoparasitology, the study of the immunologic aspects of the relationship between hosts and parasitic protozoa, metazoa, and ectoparasites (Warren, 1977a), is a discipline in a state of expansion. This activity is paralleled by an upsurge in interest in tropical immunology

[1] Abbreviations used: BCG, Bacillus Calmette-Guérin; B cell, lymphocyte precursor of antibody-secreting cells; B^{μ}, $B^{\gamma 1}$, and B^{ε} cells, B cell precursors of IgM, IgG_1, and IgE secreting cells, respectively; DNP, 2,4-dinitrophenyl; DTH, delayed-type hypersensitivity; EO-CFC, eosinophil colony-forming cell; ES antigens, excretory and/or secretory antigens; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; H-2, major histocompatibility complex (MHC) of the mouse; HCG, human chorionic gonadotropin; Ig, immunoglobulin; L3, third-stage larvae; Ly, Ia, Thy-1, TL, antigenic markers on lymphocytes; PC, phosphorylcholine; SPF, specific pathogen-free; T cell, thymus-influenced (-derived) cell.

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(e.g., Alpers, 1978; Goodman, 1978; Minners, 1977; Nossal, 1977) and stems from at least five sources: (*a*) the belief that parasitic infections and diseases of medical and veterinary importance should be amenable to vaccine-based control; (*b*) the expectation that studies on the mechanisms of evasion of potentially aggressive host defenses by parasites will provide information of broad biological significance; (*c*) the innate fascination of parasites and parasitism; (*d*) the challenge of defining and exploiting the functions of certain, almost invariable, accompaniments of chronic metazoan parasitic infection, such as eosinophils and IgE antibodies; and (*e*) the increasing availability of funds for basic and applied research in immunoparasitology.

This article attempts to provide an analysis of recent studies on immunological and "paraimmunological" responses to various metazoan and protozoan parasites in mice. Reference will be made to the important point of whether the host–parasite relationship under discussion is natural or unnatural. Like most reviewers in an area that cuts across a large number of parasites, and in which the literature is growing rapidly, I apologize in advance to those colleagues whose work receives scant coverage. Systems that have been neglected, but for which there are several recent reviews, are the mouse/*Schistosoma mansoni* (Phillips and Colley, 1978; Sher, 1977; Smithers *et al.*, 1977; Warren, 1977b) and the mouse/*Trichinella spiralis* models (Catty, 1976; Despommier, 1977). Moreover, the intracellular protozoal infections, murine malaria, toxoplasmosis, and *Trypanosoma cruzi* infection, have not been discussed in detail (Jones, 1974; Trager, 1974). The objectives of this review are to highlight the potential of research on the immunology of model laboratory parasitic infections and, in particular, the potential of the comparative immunoparasitological approach. Several recent overview articles are especially relevant to the subject matter under consideration, examples being Clegg and Smith (1978), Cohen (1974, 1976), Cox (1978), Hudson (1973), Ogilvie and Wilson (1976), Sinclair (1970), and Wakelin (1978a,b).

It must be stated at the outset that no comprehensive analysis of the spectrum of specific immune responses induced to any life cycle stage of any parasite has been undertaken. The principal reason for this is that methods for the quantitative isolation and characterization of parasite antigens, unmodified by the method of procurement, are at a primitive stage of development; without parasite antigens, no specific immune reponse can be analyzed. Only with antigens can the immunoparasitologist hope to study antibody responses of various isotypes, specific B cell frequencies, delayed-type hypersensitivity, the subpopulations of T cells activated, anatomical locations of antibody production, and the specific immune responses not induced or

actively suppressed. Moreover, the rewards of research in immunoparasitology, such as identification of immune responses correlating with host protection or immunopathology, new vaccines and new skin-testing reagents, are also dependent upon the availability of parasite antigens. Thus, it is the parasite immunochemist/biochemist who, at the present time, is such a critical member of the immunoparasitology research team. The structural and life-cycle complexity of both metazoan and protozoan parasites will certainly provide a challenge to the molecular immunoparasitologist. Moreover, such a person cannot work without well defined parasitological starting material, and reliable immunological readout systems must be used for assessing the immunopotency of isolated antigens, for example. In addition, input from persons familiar with actual clinical problems and field conditions is essential when new tools for the control or detection of parasitic infection and disease are being developed. It is for these reasons that collaborative immunoparasitological research programs of the type established at this Institute and elsewhere, consist of immunologists, biochemists, epidemiologists, and parasitologists, and they cannot function without extensive linkages with other specialist research groups and field investigators.

There is no doubt that new tools for the control and detection of parasitic infection and disease will be developed over the next 10–15 years, and the rewards of research in immunoparasitology can be expected to include: (*a*) vaccine-based control of infection and disease; (*b*) immunodiagnostic reagents of high sensitivity and specificity; (*c*) identification of immunologically mediated, genetic variation in susceptibility to infection or disease; (*d*) identification of immunopathologic host responses; and (*e*) an understanding of the mechanisms of survival of foreign living entities in potentially hostile host environments, the information being relevant to the question of the survival of other persistent infectious agents as well as tumors and the conceptus. The value of analysis of model host–parasite relationships in achieving some of these outputs from research will be the principal focus of this article. However, the scope of the article has been broadened by inclusion in Section II of a discussion on several current concepts in immunoparasitology.

II. Some Concepts in Immunoparasitology

A. Immunology in Host–Parasite Relationships

Parasites in their natural hosts are of particular fascination to immunobiologists simply because they generally infect the young in pre-

TABLE I
STAGES IN THE EVOLUTIONARY DEVELOPMENT OF HOST–PARASITE RELATIONSHIPS[a]

	Stage				
	1	⇄	2	⇄	3
Principal characteristic	Exaggerated hypersensitivity		Adaptation tolerance		No antigens, no immunity
Consequence	Host pathology, threat to host survival		Survival of both parasite and host		Large parasite burdens, threat to host survival

[a] After Sprent (1959).

reproductive life. Severe restraints are therefore imposed on the parasite in terms of the life-threatening consequences of infection and, in this regard, parasites differ from disease entities characteristic of old age, such as tumors. No successful parasite kills a large proportion of its susceptible hosts. The host immune system, with its array of closely regulated effector cells and molecules, must be an important restraining influence on parasites; one can expect successful parasites to be immunogenic and immune responses to be involved in maintaining parasite burdens at tolerable levels (Burnet and White, 1972; Mims, 1977; Sprent, 1959). A scheme proposed by Sprent (1959) to describe the evolutionary development of balanced host–parasite relationships is outlined in Table I. In a state of "adaptation tolerance," parasites induce immune responses that prevent parasite numbers from overwhelming the host but the responses are generally ineffective at eliminating parasites from those hosts (or at least all individuals in the host species before transmission to the next generation or other susceptibles). Of course, immune responses may also be responsible for the development and manifestations of immunopathology in chronic infections. They may also be responsible for parasite protection, and chronicity of antigenic exposure per se can be expected to influence profoundly the types of immune response induced by resident parasites.

Resistance to homologous parasitic reestablishment is often seen in already parasitized individuals. The term "concomitant immunity" has been used to describe this situation, where already parasitized hosts are highly or absolutely resistant to reinvasion or reinfection with the same parasite (Smithers *et al.*, 1969). Concomitant immunity makes "biological sense" since the life cycle of the parasite is perpetuated without prejudice to the survival of the host through superin-

TABLE II
IMPORTANT QUESTIONS IN THE IMMUNOLOGICAL DISSECTION OF A HOST–PARASITE SYSTEM

1. What is required of a vaccine?
2. Would an immunodiagnostic reagent be useful?
3. Is the disease caused by immunopathology?
4. Does concomitant immunity exist, and is host protection mediated by antibodies?[a]
5. What life cycle stages are, or are potentially, vulnerable to immune attack?
6. What immunological effector mechanisms are likely to be of little consequence to parasite survival?
7. What responses are counterproductive in terms of host resistance?
8. What method(s) does the established parasite use to thwart potentially sterilizing host-protective immunities?
9. Is immunologically mediated, genetically based variation in susceptibility demonstrated readily in hosts?[b]
10. Will the project founder because of parasitological shortcomings and parasite supply problems?
11. What methods are available for the production, identification, isolation, and characterization of parasite antigens?

[a] When antibodies are responsible for host protection, immunoprecipitation and affinity-chromatography techniques can be used in antigen isolation.

[b] Such "experiments of nature" provide a powerful analytic tool for the comparative study of protective immunity in a particular host species (Section III,A).

fection. This type of immunity, in which *establishing* parasites are susceptible to immune responses which are ineffective against *established* parasites, has also been termed nonsterilizing immunity (Cohen, 1974) or premunition (Sergent, 1963), and can obviously be exploited in vaccine development. The existence of such a state of resistance augurs well for the development of a vaccine that is effective only against *first* infection, and an immunological strategy for elimination of *established* parasites will need to be different from one that simulates naturally occurring concomitant immunity.

Many parasitic infections in man are zoonoses. Thus the study of the immunological aspects of unnatural infections, often scorned, can be a valid area of immunoparasitological investigation. For example, studies on an immune response to a parasite antigen that militates against the survival of that parasite in an unnatural host may provide important clues on the nature of responses that are potentially host protective, but are actively suppressed by the parasite, in natural hosts. However, it is in this area of choice of system and the aspect of parasitism to be dissected (Table II) where the immunoparasitologist is absolutely dependent upon the parasitologist and epidemiologist for guidance. Even though it is dangerous to predict outcomes and ramifi-

TABLE III
MECHANISMS OF EVASION OF EXTANT OR POTENTIAL HOST–PROTECTIVE RESPONSES BY PARASITES IN THEIR HOSTS[a,b]

A. Reduced net parasite antigenicity
   1. Antigenic variation
   2. Blocking antibodies
   3. Non-Ig masking of antigens
   4. Modulation of antigens
   5. Molecular mimicry
   6. Loss of MHC antigens on parasitized cell
   7. Anatomical inaccessibility

B. Modification of intramacrophage environment
   1. Inhibition of digestive enzymes
   2. Escape from phagolysosomes
   3. Inhibition of vacuole fusions

C. Modulation of host immune responses
   1. Immunosuppression
      a. Lymphoid tissue disruption
      b. Cytotoxic parasite molecules
      c. Effector cell blockade with antigen or immune complexes
      d. Nonspecific suppressor cells
      e. Specific active suppression
      f. Clonal deletion
      g. Mitogens and antigenic competition
   2. Anticomplementary and anti-inflammatory effects
   3. Degradation of antibodies

[a] Many of the demonstrated or postulated mechanisms are interrelated.

[b] Several relevant references are: A1, Brown, 1974; Cross, 1978; Vickerman, 1974. A2, Mitchell *et al.*, 1977a; Rickard, 1974. A3, Sher *et al.*, 1978; Smithers *et al.*, 1969. A4, Aust-Kettis and Sundqvist, 1978; Barry, 1975; Biagi *et al.*, 1966; Doyle *et al.*, 1974; Dwyer, 1976. A5, Damian, 1978; Dineen, 1963. A6, Handman *et al.*, 1979. A7, Porter and Knight, 1974. B1 to B3, Hirsch *et al.*, 1974; Nogueira and Cohn, 1976; Porter and Knight, 1974. C1a, Greenwood, 1974; Wedderburn, 1974. C1b, Barriga, 1978; Faubert, 1976. C1c, Wilson, 1974. C1d, Corsini *et al.*, 1977; Coulis *et al.*, 1978; Jayawardena *et al.*, 1978b; Warren and Weidanz, 1976. C1e and C1f, Porter and Knight, 1974. C1g, Hudson *et al.*, 1976. C2, Hammerberg and Williams, 1978; C3, Eisen and Tallan, 1977. Other references pertaining to several of these mechanisms are provided in the text (see also Cohen, 1976; Mims, 1977; Ogilvie and Wilson, 1976).

cations of research in any system, regardless of how artificial the system seems, one is perhaps justified in expecting little to be achieved from an in-depth dissection of at least the immunological aspects of laboratory host–parasite relationships that could not possibly exist in nature.

B. Chronicity of Infection and Mechanisms of Evasion of Host-Protective Immunities

One key feature of most metazoan and protozoan parasitic infections is chronicity of infection. Persistence of the parasite presumably requires that the parasite evolve mechanisms to subvert, thwart, or coexist with potentially host-protective immune responses. As alluded to above, chronicity of parasite antigen stimulation may itself result in induction of host immune responses that are somewhat bland in terms of immunological aggression (see Section V,B). However, the dictates

of concomitant immunity are that the established parasite maintain a level of aggressive immunity sufficient to prevent successful reinvasion by *large numbers* of homologous parasites.

Mechanisms of evasion of host protective responses that have been demonstrated or postulated are listed in Table III. All the mechanisms fall into three broad categories: (*a*) reduced net antigenicity of the parasite, (*b*) modification of intracellular environment, and (*c*) modulation of the host immune response by the parasite. As mentioned in the footnote to Table III, many of the listed mechanisms are closely interrelated, and several will be examined in some detail below. In all probability, the two mechanisms likely to be consistently effective at thwarting extant host-protective immunities will be antigenic variation and anatomical inaccessibility—i.e., "relative sequestration" within cells or cysts and "relative exclusion" on mucous membranes and skin.

C. Parasite Vaccines and Candidate Target Molecules for Host-Protective Immunities

Several methods of vaccination against parasites have been usefully employed, but the list of safe, effective, and quality-controlled antiparasite vaccines is very short indeed. Resistance against disease and/or infection has been achieved by controlled exposure to fully pathogenic organisms [in cutaneous leishmaniasis caused by *Leishmania tropica* in man (e.g., Stauber, 1963)], controlled exposure to attenuated parasites [in bovine babesiosis caused by *Babesia* spp. (e.g., Callow, 1977; Mahoney, 1977)], or injection of irradiated larvae [in ruminant lungworm infections caused by *Dictyocaulus* spp. and in canine hookworm caused by *Ancylostoma caninum* (Jarrett *et al.*, 1960; Miller, 1971; other literature cited in Cox, 1978)]. Vaccines consisting of parasite antigen preparations are at an advanced stage of development in the case of veterinary taeniids (Rickard and Adolph, 1976, 1977), and much hope is held for a malaria vaccine in the next 5–10 years (Brown *et al.*, 1970; Mitchell *et al.*, 1975; Nussenzweig, 1977; Reese *et al.*, 1978; Siddiqui *et al.*, 1978; Simpson *et al.*, 1974).

The most effective and safest strategy of vaccine-based control of parasitic infection and disease is presumably one that simulates naturally occurring host-protective immune responses to prevent infection or to eliminate parasites (Table IV). However, in those situations where immunopathology is responsible for disease, a useful strategy of vaccine-based control of disease (rather than infection) would be to "desensitize" infected individuals against the antigens responsible for the induction of the immunopathological immune responses. In addi-

TABLE IV
VACCINE-BASED CONTROL OF PARASITIC INFECTION OR DISEASE[a]

Strategy	Comment
1. Vaccine simulating concomitant or sterilizing immunity induced during natural infection ("Natural" antigen approach)	Use of such a vaccine should present relatively few difficulties
2. Vaccine based on an immune response not normally induced during natural infection ("novel"antigen approach)	Stringent safety testing required
3. Vaccine that desensitizes susceptible individuals ("negative vaccine" approach)	Theoretically useful where immunopathology is the cause of disease or where parasites are "utilizing" products of immediate hypersensitivity reactions, for example

[a] A "host-protective antigen" may be one that induces an immune response which is itself host-protective, or which enables existing immune responses, or immune responses induced by subsequent infection, to eliminate or prevent establishment of the parasite.

tion, if it can be proved that immediate hypersensitivity reactions and associated secretions are used by parasites (e.g., ectoparasites or mucous membrane-located parasites) for feeding purposes, then desensitization may again be a useful outcome of vaccination. There is another aspect of the parasite vaccine approach that must be emphasized, and this is the question of efficacy. In those situations where: (*a*) the disease is proportional to the parasite burden; (*b*) the parasite is endemic; (*c*) the presence of a few parasites is of no consequence to the host; (*d*) immunity to reinfection wanes rapidly after parasite elimination; and (*e*) regular vaccinations are logistically impossible, a 90–95% effective vaccine may be more useful than a 100% effective vaccine. With many intestinal parasitic infections (human hookworm in certain geographic locations, for example), the desirable outcome of irregular vaccination may be to preserve a low level of infection which is capable of regularly boosting the state of immunity induced by the vaccine. Useful new tools may be lost therefore if the applied immunoparasitologist strives only for the absolute "sterilizing" vaccine in all situations. Related to this point is the further notion that a host-protective vaccine need not necessarily include the full complement of "host-protective antigens" of the parasite. A particular immune response to a particular antigen included in a vaccine may enable other immune responses to be induced more efficiently subsequent to natural exposure to the

parasite. These immune responses *in toto* may effect host protection (Table IV and Mitchell, 1979).

In the search for relevant parasite molecules for inclusion in an antiparasite vaccine, it is necessary to view the *entire* spectrum of the host-parasite relationship, not only the immunological aspects. By isolating only those antigens that induce very obvious immune responses during infection, novel antigens that are more suitable targets for host-protective immunity may be missed. The survival of the allogeneic conceptus in the female and the development of immunological methods of fertility regulation provides a reasonable example of this point (Mitchell, 1978a). One may study, in exquisite detail, the antigens of the embryo and early placenta responsible for inducing maternal immune responses, all the time looking for the antigen that may be exploited in the development of a fertility-regulating vaccine. However, the most promising approach at the present time is based on an immune response to a hormone (HCG; in particular, a portion of the β chain of HCG) that is normally nonimmunogenic but can be "engineered" so as to become immunogenic (Griffin, 1978; Harper, 1975). In the case of parasites, an immune response to an equivalent essential parasite molecule may well neutralize the biological activity of that molecule and thus prejudice the survival of the parasite in the immunized host. Clearly, the most important overriding consideration in the use of innately nonimmunogenic molecules in vaccines is that of safety (Table IV).

Relatively few discussions have appeared in the literature on actual or hypothetical molecules, with certain biological functions, which are attractive candidates for immunological intervention or neutralization (Soulsby, 1963; Clegg and Smith, 1978). Several target molecules are indicated in Table V, but the value of such a listing is severely handicapped by scanty information on the biochemistry and molecular biology of parasitic protozoa and metazoa. This aspect of immunoparasitology, like most others in fact, is one that is ripe for intense investigation.

The candidacy of parasite recognition structures (for target host cells) as molecules for host-protective immunity (Table V) has received the greatest attention in the case of hemoprotozoa, e.g., *Plasmodium* and *Babesia* spp. An antibody that interferes with recognition of host cell surface molecules by merozoites should increase the duration of the extracellular existence of the merozoite and thus expose it for longer periods to other immunological effector molecules and cells (Butcher *et al.*, 1978; Chapman and Ward, 1977; Cohen, 1974; Miller *et al.*, 1977; Trager, 1974).

TABLE V
CANDIDATE TARGET MOLECULES FOR IMMUNOLOGICALLY MEDIATED INTERRUPTION OF SUCCESSFUL HOST–PARASITE RELATIONSHIPS

1. Parasite recognition structures for target host cells
2. Parasitized cell surface antigens
3. Tissue penetration and feeding enzymes
4. Parasite protective molecules
   a. Immunosuppressive molecules
   b. Anticomplementary molecules
   c. Anti-inflammatory molecules
   d. Blocking antibodies
5. Parasite antigens in the blood-sucking vector
6. Differentiation antigens expressed transiently in the host
7. Antigens of the early invasive forms of the parasite

Antigenic parasite, or parasite-dependent, molecules on the surface of infected cells (Table V) are attractive candidate molecules for cellular and humoral elements of immune aggression (Brown, 1974). Such antigens may or may not be expressed for prolonged periods. This raises the question of transiently expressed (differentiation) antigens of parasites (Table V) as target molecules, an example being the molting antigens of larval nematodes (e.g., Guerrero and Silverman, 1971; Stromberg and Soulsby, 1977). At first glance, such antigens may seem to be unattractive candidates for immunological attack simply because of their limited duration of expression. However, a preexisting high state of host sensitivity to such antigens (induced by vaccination with purified antigen preparations) may be relatively unmodified by subsequent exposure to infection. Under these circumstances, the host-protective qualities of the state of immunity induced by the vaccine may be preserved. The point here is that chronic exposure to parasite antigens that are present in parasites throughout their life cycle may modify an existing state of sensitization through high-dose antigen effects. Thus, it has been demonstrated that *high-dose, prolonged* exposure to histocompatibility antigens (Basch, 1974; Harris and Harris, 1975) and sheep erythrocytes (Chapman *et al.*, 1979b) preferentially leads to induction of murine IgG_1 antibodies that appear to be relatively inert in terms of complement fixation and opsonization abilities (Spiegelberg, 1974) (see Section V,B). Parasite antigens that are expressed transiently may be exposed to the host system for long enough to be targets of immune aggression, but not exposed for long enough to modify the type of immunity induced by the vaccine.

As in the case of host-cell recognition structures of parasites, the surface antigens of parasitized cells are receiving increasing attention

in hemoprotozoal infections. Antigens recognized by rhesus monkey antibodies have been demonstrated on the schizonts of *Plasmodium knowlesi* (Brown, 1974). Antibodies with functions other than complement-dependent cell lysis are presumably required for any antiparasite effects, since rupture of the schizont is its natural developmental fate. Several recent studies have focused on the use of surface molecular radiolabeling probes for the analysis of structural changes on infected red cell membranes (e.g., Trigg *et al.*, 1977; Wallach and Conley, 1977). The power of this technology can be increased when fluorescence-activated cell sorting is used to isolate parasitized cells on the basis of their DNA content (Howard *et al.*, 1979a). The studies of Howard *et al.* (1979b) have demonstrated another dramatic change in cell surface molecules of red cells taken from the blood of mice heavily infected with lethal murine *Plasmodium* and *Babesia* protozoa. Sialoglycoproteins are either absent or refractory to the incorporation of label using the periodate-tritiated borohydride reaction, and both infected and uninfected red cells appear to be involved. Whether these changes result in a decreased life-span of red cells and therefore exacerbate the anemia caused by cell loss through direct parasitization, remains to be determined (Ristic, 1970; Zuckerman, 1970).

Tissue penetration enzymes (Chandler, 1935) and enzymes utilized in feeding at mucous membranes or in systemic sites (Table V) may be susceptible to neutralization by host antibodies. These molecules rank as attractive candidates, especially when the bulk of the worm appears to be refractory or inaccessible to many of the conventional forms of immune attack. A comprehensive review, and excellent discussion, on secretory antigens, enzymes, and molecules such as the stichosome antigens of trichuroid nematodes, can be found in Clegg and Smith (1978). Parasite-protective molecules, such as immunosuppressive, anticomplementary, and anti-inflammatory substances (Table V) may also be susceptible to immunological neutralization of their biological effects. Inhibition of the formation of blocking antibodies should also have the effect of swinging the balance in favor of the host rather than the established parasite. Recently, Carter and Chen (1976), Gwadz (1976), and Mendis and Targett (1979) have shown that transmission of malaria by mosquitoes can be reduced by immunization of the host against parasite antigen contained in (if not unique for) the parasite stages in the vector (Table V).

Regardless of their biological functions, the antigens of early invasive forms of parasites (Table V) would seem to be useful targets for immune intervention. The parasite mass can be expected to be low, and the establishing parasite has a limited time available to it for

modification of host immune responses in its favor. For example, good evidence exists that the early invasive larvae of *Taenia taeniaeformis* in rats and mice, and *Fasciola hepatica* in rats, are the targets of concomitant immunity (see Sections III,A and B) although no information is available on the biological activities of the "host-protective antigens" involved as targets for this immune aggression.

D. Immunodiagnosis

Serodiagnostic immunological methods for the detection of antiparasite antibodies, as well as parasite antigens for skin testing, have found wide application in human and veterinary medicine; this aspect of immunoparasitology has received considerable attention in the past couple of decades (e.g., Cohen and Sadun, 1976; Kagan, 1976; Ruitenberg *et al.*, 1977a; Welch and Dobson, 1978). Since the assays depend on the detection of a heterogeneous population of antibodies with, in most cases, a heterogeneous population of parasite antigens, serious limitations in terms of specificity and sensitivity are predictable. Although the chemotherapeutic kit is far from satisfactory, there are several effective "one-shot" drugs available for many parasitic infections. In such situations, immunodiagnostic reagents of high sensitivity and specificity would be particularly useful. In addition, such reagents would find application in the monitoring of parasite control programs. It must be borne in mind that the detection of circulating or urinary antigens will often be more useful for diagnostic purposes than detection of antibodies.

New immunodiagnostic reagents based on the use of hybridoma-derived monoclonal antibodies will be added to the diagnostic kit of the clinician or epidemiologist within the next couple of years. The extraordinary specificity and sensitivity of a prototype immunodiagnostic reagent for a model parasitic infection has already been demonstrated (Mitchell *et al.*, 1979a). One advantage of this new technology in immunodiagnosis is that crude parasite antigens can be used in the assay system; the prototype detection system employed is to inhibit the binding, with sera from infected mice, of a reaction between a labeled hybridoma antibody and a crude parasite antigen extract in a solid-phase competitive radioimmunoassay. Only limited success has been achieved in attempts to convert the assay to one not requiring parasite antigens and based on the inhibition of binding of the labeled hybridoma antibody with a large pool of anti-hybridoma idiotypic sera (Mitchell *et al.*, 1979a). The other principal use of the hybridoma technology in immunoparasitology will be for the isolation of specific parasite antigens. As in many other branches of immunology,

hybridoma-derived monoclonal antibodies will add a new dimension to the study of the immunological aspects of host-parasite relationships.

III. Resistance to Infection

The expression of resistance to parasitic infection can be grouped into the broad categories of (*a*) nonpermissiveness of the host; (*b*) concomitant or nonsterilizing immunity; and (*c*) parasite elimination (combined with resistance to reinfection) although a "sterilizing" immunity is virtually impossible to demonstrate since the onus is on the investigator to prove the absence of a single parasite in the body, a futile task with all parasitic protozoa and some parasitic metazoa. Alterations in parasites in immunized hosts include reductions in growth rate, proliferative rate, and reproductive rate and changes in migratory behavior, tissue localization, antigenic constitution, and morphology. Varying degrees of stage- and species-specific immunity can also be expected. As is well known, there is no reason to assume *ab initio* that expression of resistance to infection is in any way immunologically based (discussed in Mitchell, 1979). In this section, three topic areas have been chosen for discussion: mouse strain variations in resistance, intestinal parasite rejection, and susceptibility of the nude mouse. Emphasis will be placed on natural mouse–parasite relationships.

A. Mouse Strain Variation in Resistance to Infection

Very obvious mouse strain variation exists in susceptibility of mice to the natural rodent parasites *Taenia taeniaeformis,* a larval cestode; *Leishmania tropica,* an intraphagocytic protozoan; *Giardia muris,* an extracellular intestinal protozoan; and the intestinal nematodes, *Nematospiroides dubius* and *Trichuris muris.*

1. Taenia taeniaeformis

The variation in resistance of young mice exposed to eggs of *T. taeniaeformis* (Dow and Jarrett, 1960; Mitchell *et al.,* 1977a; Olivier, 1962; other literature cited in Wakelin, 1978b) is expressed in the number and rate of development of cysts in the liver after oral administration of eggs. C3H/He mice are particularly susceptible, BALB/c mice are relatively resistant, and C57BL/6 mice are highly resistant. This genetic resistance can be abolished by injection of cyclophosphamide, just prior to egg administration, in doses sufficient to induce a profound but temporary inhibition of antibody production. Moreover, genetic resistance is not seen in hypothymic nude mice (Mitchell

et al., 1977a). Variation in resistance to infection may reflect the rate at which host-protective antibodies appear after oral egg administration. Thus, host-protective effects of sera in passive serum transfer experiments (Campbell, 1938; Miller, 1932) are demonstrable earlier in infection in (resistant) C57BL/6 mice than in (susceptible) C3H/He mice (Mitchell *et al.*, 1979b). In such experiments, and as demonstrated very clearly in rats by Musoke and Williams (1975b; see also Ito, 1977; Kwa and Liew, 1978; Leonard and Leonard, 1941; Lloyd and Soulsby, 1974), serum must be given to recipients at about the time of egg administration. In addition, decomplementation of recipients of serum, including nude mice, abolishes an otherwise resistance-inducing effect of serum (Mitchell *et al.*, 1977a; Musoke and Williams, 1975b). Apparently, a race against time exists for the establishing parasite—the development of anticomplementary activity in the growing parasite (Hammerberg and Williams, 1978) is time dependent, as is the development of antiparasite, complement-fixing antibodies by the host (Leid and Williams, 1974; Mitchell *et al.*, 1979b). Yet to be dissected are any mouse strain variations in the persistence of established cysts, resistance in taeniid infections being long considered to operate at two levels—resistance against establishment of infection and resistance against persistence of infection (Campbell, 1938; Ito, 1978; also reviewed in Gemmell and MacNamara, 1972).

Taenia taeniaeformis infections in mice and rats provide an excellent example of antibody-mediated concomitant immunity although experiments designed to establish this fact must carefully take into account age-dependent resistance to infection (Dow and Jarrett, 1960; Greenfield, 1942). As discussed in Section II, unequivocal evidence for resistance to homologous reinfection in already parasitized hosts is of particular interest to the immunoparasitologist wishing to develop vaccines against *first* infection. Provided it is logistically possible to vaccinate the susceptible host prior to first exposure to the parasite, the existence of concomitant immunity indicates that vaccination using the "natural antigen" approach (Table IV) is feasible. In the *T. taeniaeformis*/mouse system, absolute protection can be afforded to nude or intact mice by injections of sera harvested from either genetically susceptible or genetically resistant mice (Mitchell *et al.*, 1977a, 1979b). Some evidence suggests that complement-fixing IgG antibodies (presumably IgG_2 in the mouse) which bind to, and are eluted from, protein A–Sepharose by low pH buffers, are necessary (but not sufficient) for host protection in passive transfer experiments (Fig. 1). However, IgA antibodies in the intestinal tract have also been shown to be host protective (Lloyd and Soulsby, 1978), and, in systemic sites,

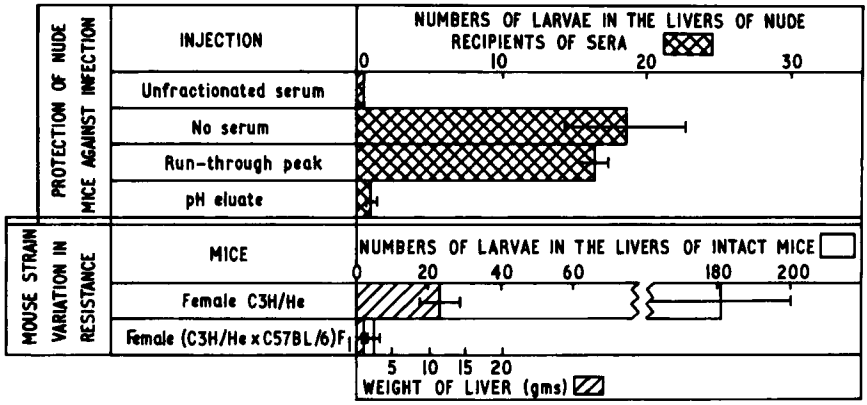

FIG. 1. Aspects of *Taenia taeniaeformis* infection in mice. BALB/c.nu/nu mice can be protected against infection by 0.4 ml of serum harvested from 40-day-infected mice when injected at the time of oral egg administration; the protective activity is retained on a protein A–Sepharose column and can be eluted with low-pH buffer. Young (7 weeks old) C3H/He mice are highly susceptible to *T. taeniaeformis* infection as assessed by the number and size of larvae in the liver, whereas C57BL/6 and (C3H/He $\times$C57BL/6)F_1 mice are highly resistant. For details see Mitchell *et al.* (1977a, 1979b).

IgG_1 may have host-protective effects (Musoke and Williams, 1975a) through postulated activities such as increasing tissue permeability (Musoke *et al.*, 1978) or neutralizing anticomplementary parasite molecules (Mitchell *et al.*, 1977a). Protection of established *T. taeniaeformis* cysts may involve the operation of anticomplementary factors (Hammerberg and Williams, 1978) and/or "blocking antibodies" (Rickard, 1974). There is some suggestion that the pH of *T. taeniaeformis* cyst fluid may be high (G. F. Mitchell, unpublished observations), and this in itself would presumably be anticomplementary.

A mechanism of mouse strain variation in resistance based on the rate at which host-protective antibodies appear during first infection should not hinder the development of a model vaccine effective in *all* mouse strains. A prototype vaccine, dependent for its action on the type of response shown to be host protective, and with antigen from the parasite stage known to be particularly susceptible to immune aggression (the early larvae or oncosphere) is at an advanced stage (Rajasekariah *et al.*, 1979b). Successful vaccination has already been achieved in rats using *T. taeniaeformis* strobilocercal antigen (Ayuya and Williams, 1979; Kwa and Liew, 1977) and in ruminants using appropriate taeniid oncospheral culture fluids (Rickard and Adolph, 1976, 1977). Since the economic importance of veterinary taeniid infections results from carcass condemnation at abattoirs, a vaccine to be

useful will need to be 100% effective at preventing tissue establishment of larvae (see Section II,C).

Genetic analyses of mouse strain variation in resistance to *T. taeniaeformis* infection have not been pursued vigorously because of substantial variation between experiments. This is suspected to be related to variation in *T. taeniaeformis* egg batches and is seen in strains such as CBA/H, A/J, and BALB/c rather than the two "type" strains C3H/He and C57BL/6, which are invariably susceptible and resistant, respectively. It is conceivable that the variable amount of parasite material other than *viable* eggs contained in oral inocula may influence the rate at which host-protective antibodies are induced early in the infection (discussed in Mitchell *et al.*, 1979b). Resistance in (C3H/He × C57BL/6)F_1 mice is invariably dominant and, in most experiments, males have been more susceptible than females. Clearly, there are many parasite factors as well as host factors, such as environmental experience, age, and sex, that must be identified and/or controlled in genetic analyses before meaningful data are obtained on the genetics of mouse strain variation in resistance to *T. taeniaeformis*.

2. *Leishmania tropica*

Variation in resistance to *L. tropica* in mice (Handman *et al.*, 1979; Preston and Dumonde, 1976a,b) is expressed as differences in the size of the cutaneous lesions that develop and the fate of the mice, after intradermal injection of large numbers (e.g., 1×10^6) of *in vitro*-derived promastigotes. SPF-derived mice in this laboratory can be grouped into four categories: highly resistant (C57BL/6 and NZB), transiently infected (CBA/H, C3H/He, and A/J), chronically infected (BALB/c and DBA/2), and highly susceptible (nude mice). Intact C57BL/6 and NZB never really develop any lesion characteristic of human cutaneous leishmaniasis ("oriental sore") and their macrophages *in vitro* are "relatively nonpermissive." Lesions in C3H/He, A/J, and CBA/H mice are expressed transiently (for 6–8 weeks), and recovered mice are highly resistant to reinfection. In some experiments, CBA/H mice have approached C57BL/6 mice in their degree of resistance (G. F. Mitchell, unpublished observations). In the case of mice of the highly susceptible strain BALB/c, large lesions develop that may kill the mice at 15–20 weeks of infection (Handman *et al.*, 1979; Weintraub and Weinbaum, 1977). The majority of BALB/c mice injected with large numbers of promastigotes certainly die, but an occasional mouse recovers. In DBA/2 mice, the disease, although chronic, is less severe than in BALB/c mice. Macrophages from strains of mice of the transient and chronic infection groups readily support *L.*

tropica growth *in vitro*. SPF-derived hypothymic nude (nu/nu) mice of CBA/H and BALB/c genotypes, but also of C57BL/6 genotype, are highly susceptible: lesions progress to cover much of the dorsum of the mouse, cutaneous metastases are common, and visceralization is very obvious at the time of death.

Using antigen-pulsed syngeneic infected and uninfected macrophages, a difference in the ability to induce delayed-type hypersensitivity (DTH) was seen in naive BALB/c and CBA/H mice. The antigen preparation used in these studies for pulsing of macrophages and for elicitation of DTH was a crude mixture from *L. tropica* promastigote culture supernatants. Infected BALB/c macrophages differed from uninfected BALB/c macrophage and either infected or uninfected CBA/H macrophages in being relatively inefficient at sensitization. When used as "cold targets" to inhibit cytotoxic reactions between alloreactive T cells and ^{51}Cr-labeled tumor target cells, infected BALB/c macrophages again differed from the other three preparations in being relatively poor inhibitors. Defective expression of self H-2 antigens required for efficient parasite antigen recognition by T cells may account for differences in susceptibility to disease in CBA/H and BALB/c mice. However, it must be emphasized that the results are merely suggestive as yet and quantitative analyses of H-2 antigens and *L. tropica* antigens on infected macrophages of various genotypes are required. Interestingly, *increased* expression of an H-2 antigen on cells has been implicated as the basis of increased resistance in certain mouse strains to radiation-induced leukemia (Meruelo *et al.*, 1978), and decreased H-2 expression on virus-infected cells (Blanden and Pang, 1978; Haspel *et al.*, 1977; Hecht and Summers, 1972; Koszinowski and Ertl, 1975) may reduce T cell-mediated recognition (Doherty *et al.*, 1976).

The genetics of susceptibility are complex in *L. tropica* infections and results with some congenic combinations have shown variations within groups and between experiments (G. F. Mitchell, unpublished observations). Despite this variation, it has been shown that BALB/c.H-2^{k} mice are relatively resistant and thus differ from BALB/c mice. F_1 hybrids between CBA/H and BALB/c mice are also clearly resistant. Since BCG has been shown to protect BALB/c mice against the lethal effects of *L. tropica* infection (Weintraub and Weinbaum, 1977), concurrent infections and the activation status of macrophages may markedly influence the course of cutaneous leishmaniasis in murine, as well as human, hosts. Cross protection using organisms that infect macrophages is well known (literature cited in McLeod and Remington, 1977; Mahmoud *et al.*, 1976; Mauel *et al.*, 1974), and genetic

analyses of *L. tropica* infection will need to be conducted with carefully matched mice. Graded numbers of infective promastigotes for injection and proved absence of residual heterozygosity in congenic mice will also be important. The genetics of mouse strain variation in susceptibility to *Leishmania donovani* have been examined and evidence presented for a major effect of a single, non-H-2 linked, locus (Bradley and Kirkley, 1977; Bradley, 1977). Interestingly, the strains of mice found to be highly resistant to *L. tropica* are also the strains that are relatively resistant to another intraphagocytic organism, *Listeria monocytogenes* (Cheers and McKenzie, 1978). In summary, genetics of resistance in the *L. tropica*/mouse system is likely to operate at two levels at least: (*a*) in the phenomenon of "relative nonpermissiveness" of macrophages; and (*b*) in the events leading to spontaneous resolution of infection.

3. *Nematospiroides dubius*

Prominent mouse strain variations in susceptibility to *N. dubius* have been described (Behnke and Wakelin, 1977; Cypess and Zidian, 1975; Leuker and Hepler, 1975; Liu, 1966; Mitchell and Prowse, 1979; Prowse *et al.*, 1979; Spurlock, 1943). Sex effects are also apparent (Dobson, 1961; Prowse *et al.*, 1979). As in the *L. tropica*/mouse system, at least three types of mouse strain variation in resistance exist in infection with this intestinal nematode. Oral administration of a relatively high dose of third-stage infective larvae (L3) will kill NZB mice (Mitchell and Prowse, 1979), terminal signs being massive intestinal hemorrhage (Baker, 1954). The life cycle of *N. dubius* involves a period of intestinal wall encystment, after which time (8 days or so) the parasites move back into the lumen of the intestine and, in particular, the duodenum. Mortalities in NZB mice are seen at about the time of reemergence of *N. dubius* from the intestinal wall and the mice presumably die of blood-loss anemia and damage to the intestinal lining. There is a suggestion that blood clotting in NZB is defective, since individuals of this strain will often continue to bleed and will die after routine blood collection from the tail (M. C. Holmes, personal communication). (NZB × BALB/c)F_1 mice are not killed by exposure to relatively high doses of L3 nor are NZC mice.

Female BALB/c and male CBA/H represent two extremes of a spectrum of resistance seen among various mouse strains (Prowse *et al.*, 1979). No striking differences in susceptibility to first infection are seen in terms of the numbers of intestinal wall cysts that develop or the numbers of adults that reside in the intestines for many weeks or months (see Section III,B). However, a marked difference exists in the

consequences of multiple administrations of L3. A third oral administration of L3 virtually fails to establish in female BALB/c mice and resident intestinal adults arising from previous L3 doses are slowly flushed from the intestines. Bartlett and Ball (1974) and Behnke and Wakelin (1977) have also demonstrated resistance to infection in previously exposed BALB/c mice. In CBA/H male mice, this resistance to reinfection is much less obvious, worm burdens can reach high levels, and weight gains of young infected mice are impaired (Mitchell and Prowse, 1979; cf. Bartlett and Ball, 1974). Development of resistance to reinfection is defective, but not absent, in BALB/c.nu/nu mice, and the rejection of adults from the intestines appears to be highly T cell dependent (Prowse *et al.*, 1978b).

Although the analogy is not precise in all respects, the three consequences of infection of NZB, female BALB/c, and male CBA/H mice—death, relatively high resistance to infection, and high parasite burdens, respectively—resemble stages 1, 2, and 3 in the evolution of balanced host–parasite relationships discussed by Sprent (1959) (Table I).

4. *Giardia muris*

Mouse strain variation in resistance to infection with the extracellular intestinal protozoan parasite, *G. muris*, is expressed in the duration of infection, i.e., the rate at which resolution of intestinal infection is achieved (Roberts-Thomson and Mitchell, 1978, 1979). C3H/He male mice and BALB/c female mice represent two ends of a susceptibility spectrum. C3H/He mice develop a chronic giardiasis, whereas in BALB/c the infection apparently resolves approximately 6 weeks after oral administration of fecal cysts (Fig. 2). Infection may not resolve entirely, and a flare-up of infection in late pregnancy and during lactation in an outbred mouse strain has been demonstrated (D. Stevens, personal communication). Periparturient relaxation in resistance to intestinal parasitic infection has been well documented (Gordon, 1973; Ogilvie and Jones, 1973). Strains of mice such as CBA/H are of intermediate susceptibility, infection taking longer to resolve than in BALB/c mice. Resistance to reinfection in those strains which have apparently eliminated the infection is very obvious. Hypothymic BALB/c.nu/nu mice resemble C3H/He mice in developing chronic giardiasis, although fecal cyst counts in nude mice of resistant genotype exceed those in intact mice of susceptible genotype. An injection of syngeneic lymphoid cells to BALB/c.nu/nu mice leads to apparent resolution of infection (Roberts-Thomson and Mitchell, 1978; see also Stevens *et al.*, 1978).

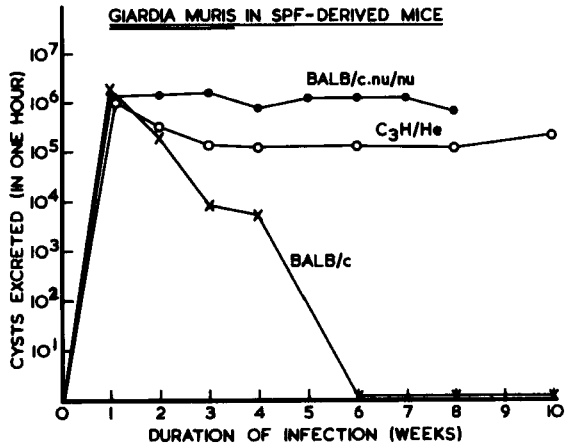

FIG. 2. Output of *Giardia muris* fecal cysts in intact BALB/c (×——×) and C3H/He mice (○——○) plus hypothymic BALB/c.nu/nu (nude) mice (●——●) given 10^3 cysts orally at time 0. Giardiasis resolves spontaneously in BALB/c mice but not in C3H/He mice over a 10-week period, and the time course of infection in hypothymic nude mice of relatively resistant genotype resembles that in intact C3H/He mice. Data from Roberts-Thomson and Mitchell (1978).

BALB/c mice can be protected against infection by injection of purified *G. muris* intestinal trophozoites in Freund's complete adjuvant (FCA). The same vaccination protocol fails completely in C3H/He mice, and Freund's incomplete adjuvant (FIA) is without effect in BALB/c mice (Roberts-Thomson and Mitchell, 1979). Whether C3H/He mice lack T cells reactive to the "host-protective antigens" of *G. muris* trophozoites remains to be established. It is also not known as yet whether C3H/He mice differ from BALB/c mice and most other strains in producing lower titers of intestinal antibodies capable of preventing strong attachment of trophozoites to the intestinal epithelium. Unlike the case of *T. taeniaeformis*/mouse and *L. tropica*/mouse systems, we have no evidence as yet that increased susceptibility (of C3H/He mice) is immunologically based. A comprehensive dissection of the immunological response in BALB/c mice versus C3H/He mice vaccinated with trophozoites in FCA versus FIA, and that in naturally infected BALB/c versus C3H/He mice, should provide clues as to whether immune responses are involved. Results to date have not shown any differences in titers of IgG and IgA circulating antibodies directed against a crude mixture of trophozoite antigens in a solid-phase radioimmunoassay using sera from naturally infected BALB/c and C3H/He mice (R. F. Anders, I. C. Roberts-Thomson, and G. F. Mitchell, unpublished observations). Moreover,

infected or uninfected C3H/He and BALB/c mice do not differ in the intensity of DTH reactions using a crude *G. muris* trophozoite antigen mixture. Thus, the results have yet to shed light on differences in immunoresponsiveness of mouse strains showing acute versus chronic giardiasis. More defined antigen preparations are required before the search shifts from immunological to nonimmunological mechanisms of variation in resistance. In addition, detailed examination of T cell-dependent cellular events (Ruitenberg and Elgersma, 1976) in the intestinal wall (Miller, 1972) have not yet been performed in this mouse/*G. muris* model (Ferguson and MacDonald, 1977; Roberts-Thomson and Mitchell, 1978).

5. *Plasmodium yoelii*

Like many studies on *Trypanosoma* spp. in mice, those using some species of laboratory-adapted *Plasmodium* and *Babesia* parasites involve highly unnatural host–parasite relationships since the parasites are uniformly lethal. Some isolates of *P. yoelii* are nonlethal and with such isolates a pronounced mouse strain variation is seen in the ability of *P. yoelii* infection to protect against a subsequent and otherwise lethal, *P. berghei* infection (Barker, 1971; Mitchell *et al.*, 1978; Wedderburn, 1974; Weinbaum *et al.*, 1976a). Although partial host protection can be demonstrated in naive recipients of serum from *P. yoelii*/*P. berghei* exposed BALB/c mice (Mitchell *et al.*, 1978), or mice exposed to multiple *P. yoelii* challenge (Freeman, 1978; Jayawardena *et al.*, 1978a), no evidence exists that the strain variation in development of resistance is immunologically based. Since *Plasmodium* parasites generally prefer reticulocytes rather than mature erythrocytes, one of several events contributing to mouse strain differences in susceptibility may be the rate at which reticulocytosis appears during infection. The complexity of malaria is well illustrated by the extraordinarily diverse effects of serum transfers in mice (e.g., Freeman, 1978; Jayawardena *et al.*, 1978a; Parashar *et al.*, 1977) and the fact that, after protein A fractionation of host-protective serum in the *P. yoelii*/*P. berghei* system using differential pH elution to provide immunoglobulins of different isotypes (Chapman *et al.*, 1979a; Ey *et al.*, 1978), *all* fractions have some degree of host-protective activity (G. V. Brown and G. F. Mitchell, unpublished observations). Some variation exists in the time course of lethal *P. berghei* infections (Greenberg and Kendrick, 1956; Most *et al.*, 1966; Whitelaw *et al.*, 1977; other literature cited in Wakelin, 1978b), but again, no evidence is available on whether immune responses are involved.

The human plasmodia are exquisitely adapted parasites, and the complexities of the host–parasite relationship are somewhat bewildering. It is for this reason of complexity of the natural diseases that those murine malaria models which share features in common with the various human malarias take on great significance (Carter and Diggs, 1977). Immunological events in the mouse can be dissected with some precision although the lack of availability of parasite antigens restricts analyses of specific antiparasite responses. Because of the amenability of the mouse to immunological analysis, it is something of a disappointment that, in malaria, mice of various genotypes have not yet proved to be very useful in the search for, and examination of, antigenic parasite variations and the immunological aspects of parasite persistence, whether this be in liver or blood. The availability of mouse strains that show variations in liver persistence, parasite crisis, or fluctuating chronic infection, for example, would be of enormous value. One aspect of malaria in which mouse models are finding application is in the study of parasite crisis reactions using immunotherapeutic biologicals such as BCG or *C. parvum*, (Clark *et al.*, 1976), and some mouse strain variation has been found in the capacity of BCG to afford protection against *P. berghei* (Mitchell *et al.*, 1978).

6. *Parasitic Infections in Biozzi Mice*

Mice have been genetically selected, from an outbred population, for high (Ab/H) and low (Ab/L) responsiveness against sheep erythrocytes by Biozzi *et al.*, 1972, 1979; Stiffel *et al.*, 1974). Such mice differ with respect to Ig levels and antibody responses of various types. Cell-mediated immunities do not differ markedly (Mouton *et al.*, 1974), and macrophages are "activated" in Ab/L relative to Ab/H mice (Wiener and Bandieri, 1974). Ab/L mice have been shown to be more susceptible to *Trypanosoma cruzi* of two strains with different tissue tropisms (Kierszenbaum and Howard, 1976), Ab/L and Ab/H mice do not differ markedly in the number of *Trichinella spiralis* larvae present in muscles (Perrudet-Badeux *et al.*, 1975) although resistance to second infection is greater in Ab/L than Ab/H mice (Perrudet-Badeux *et al.*, 1978), and Ab/L mice are marginally less suceptible than Ab/H mice to *Schistosoma mansoni* (Blum and Cioli, 1978). The reported resistance of Ab/L mice to *Leishmania tropica* (referred to in Biozzi *et al.*, 1979; Blum and Cioli, 1978) may reflect their activated macrophage status (see Section A,2). Ab/H, but not Ab/L, mice, have been protected against lethal *Plasmodium berghei* infection by prior vaccination with heavily irradiated infected blood cells (Biozzi *et al.*, 1979).

Increased resistance of Ab/H versus Ab/L mice suggests a role for antibodies in host protection; conversely, increased resistance of Ab/L versus Ab/H mice suggests a role for activated macrophages in host protection. Clearly, the Biozzi mice, like anti IgM-treated (B cell deficient) and hypothymic (T cell deficient) mice will be extremely useful in the dissection of the contributions of T cells, B cells, and macrophages in resistance to, and manifestations of, parasitic infection (see also Section IV,C).

7. *Other Infections*

In terms of numbers of lung larvae appearing after oral administration of embryonated eggs, mouse strains vary in susceptibility to the pig parasite *Ascaris suum*. Thus, C57BL/6 mice contain many more larvae in their lungs at day 7 of infection compared with other common inbred mouse strains. Numbers of larvae in the lungs of C57BL/6 mice dosed for a second time with *A. suum* eggs resemble those in the lungs of other strains not previously exposed to infection. Natural and acquired resistance appears to be expressed somewhere between the liver stage (day 3) and the lung stage (day 6–7) (Mitchell *et al.*, 1976a). Mouse strain variation in susceptibility is also seen in lethal *Trypanosome congolense* infections (Morrison *et al.*, 1978), *Trypanosoma cruzi* (Cunningham *et al.*, 1978), *Toxoplasma gondii* (Araujo *et al.*, 1976), *Leishmania donovani* (Bradley and Kirkley, 1977; Bradley, 1977), *Trichinella spiralis* (literature cited in Wakelin and Lloyd, 1976a; Rivera-Ortiz and Nussenzweig, 1976), *Echinococcus multilocularis* (Ali-Khan, 1974; Lubinsky, 1964), and *Schistosoma mansoni* infections (Stirewalt *et al.*, 1965; Sher, 1977). Other literature and further discussions on genetic control of resistance to parasitic infection can be found in Wakelin (1978b).

Very obvious mouse strain variation in resistance is also seen in infection with the natural intestinal nematode of mice, *Trichuris muris* (Wakelin, 1975b). This variation is expressed in the time at which expulsion is initiated; early expulsion is dominant in F_1 mice, and good evidence supports the notion that the genetically based variation is immunologically mediated (discussed in Ogilvie and Wilson, 1976; Wakelin, 1978b). No striking mouse strain variation in resistance has been seen in mice infected with *Fasciola hepatica* (Andrews and Meister, 1978; Rajasekariah *et al.*, 1979a), *Nippostrongylus brasiliensis* (Mitchell *et al.*, 1976b), *Mesocestoides corti* following injection of larvae (G. F. Mitchell, unpublished observations), and lethal *Babesia rodhaini* (G. F. Mitchell, unpublished observations).

8. *General Comment*

Studies of the mechanisms underlying genetic variation in susceptibility are capable of highlighting problems to be faced by the immunoparasitologist aiming to produce antiparasite vaccines. For example, in the *Leishmania tropica*/mouse system it is difficult to imagine a vaccination strategy that would overcome a consequence of cell infection in BALB/c mice, such as loss of self H-2 antigen expression (Handman *et al.*, 1979). Regardless of the degree of sensitization of the T cell population in the BALB/c mouse, sensitized effector cells will be constantly frustrated by a low efficiency of recognition of the infected target cell, where the immune aggression needs to be focused. Likewise in the *Giardia muris*/mouse system we have yet to detect an immune response that is defective in the C3H/He mouse, which develops chronic giardiasis and is refractory to vaccination using trophozoites in adjuvants (Roberts-Thomson and Mitchell, 1979). Although we have by no means exhausted the list of specific anti-trophozoite immune responses theoretically feasible, there is the distinct possibility that nonimmunological factors underlie the chronicity of giardia infections in C3H/He mice. Thus in cutaneous leishmaniasis in BALB/c mice and chronic giardiasis in C3H/He mice, and by analogy in the equivalent minority of human hosts of the appropriate parasites, chemotherapy or nonspecific immunotherapy may offer greater promise for disease control than vaccination. It is for the above reason that dissection of variation in resistance in model host–parasite relationships has focused on the *mechanisms* rather than the *genetics* of these "experiments of nature" in several laboratories. Emphasis on the actual strains showing varying manifestations of resistance should certainly take lower priority than exploiting the variations to study mechanisms of resistance. Genetic drift and different methods of husbandry (SPF versus conventional origins having a profound influence in *Taenia taeniaeformis* and *Giardia muris* infections, for example), will ensure that mouse strains with the same designation differ markedly in different laboratories. Since parasites are in a state of evolution there is every reason to believe that genetic variation in the parasite population will be as great as that in the *natural* host population and numerous selection procedures have been employed to obtain parasites with very different infection characteristics in the host (e.g., Desowitz, 1963; Dobson and Owen, 1977). Analyses of host factors influencing resistance must therefore attempt to take into account the additional aspect of genetic variation in the parasite population.

B. Intestinal Parasite Rejection

Extremely complex mechanisms can be expected to operate in the resolution of intestinal parasitic infection as evidenced by rejection of parasites from the intestines. First, florid immune responses against many living and nonliving antigenic entities in the intestines would clearly be counterproductive for the host. Second, lumen-dwelling parasites must be sequestered away from many of the body's immunological effector cells and molecules. Thus, both induction and expression of intestinal immune responses will have unique features (e.g., Rowley, 1978; Wakelin, 1978a). Major questions in this area of intestinal immunoparasitology relate to:

1. The transport route of antigen across intestinal epithelia (e.g., Joel *et al.*, 1978; Owen, 1977; Walker *et al.*, 1972) and the partitioning of transported antigen into the portal system versus the lymphatics
2. The influence of intestinal antibodies in preventing access of antigen to systemic sites (discussed in Soothill, 1977, and David, 1977) and the role of the liver in removing antigen present in the portal system (discussed in Thomas and Vaez-Zaheh, 1974, and Mitchell, 1978b)
3. The events of T and B cell sensitization in anatomically organized lymphoid tissues (Peyer's patches) and more disorganized submucosal lymphoid aggregates (Porter and Knight, 1977)
4. The consequences for the parasite, as well as for the fate of parasite antigen, of different types of immunologically initiated inflammatory responses in the intestinal wall, goblet cell increases, and production of mucus, mast cell, and eosinophil accumulations, "leaky" membranes, increased intestinal tract motility, etc. (e.g., Barth *et al.*, 1966; Mayrhofer, 1977; Miller, 1972; see discussion in Nawa and Miller, 1978, and Wakelin, 1978a)
5. The sequence of pharmacological and immunological events responsible for temporarily converting the intestinal tract from a net absorptive organ into a net secretory organ under the impact of various insults.

In several immunization studies, systemic injections followed by a local boosting dose of antigen lead to better IgA responses and host protection against intestinal infections than two local administrations of antigen (Pierce and Gowans, 1975; Husband, 1978). These observa-

tions have important implications not only in the design of vaccination protocols, but in studies on the modulation of host immune responses by continuous antigenic exposure at mucous membranes.

Rejection of parasites from the intestines of mice is a highly T cell-dependent process as judged by persistence of parasite infections in hypothymic nude mice (Section III,C). A cautionary note is that nude mice suffer from a generalized epithelial defect that may extend to the intestinal tract; complete reconstitution of resistance to infection must be demonstrated in nude mice before a T cell-based interpretation of the data is warranted (Prowse *et al.*, 1978a). Perhaps another cautionary note is in order, since in several systems a positive transfer of an effect with one injection of a mixed cell population, and a negative effect with a serum injection, is used as evidence for a role for cell-mediated immunity in resistance. Especially when the readout system involves a long period of time, such an emphasis is unjustified (discussed in Mitchell, 1977c, 1978c).

Rejection of intestinal nematodes, in models in which the duration of infection is innately short, is achieved *most efficiently* by the operation of (T cell-dependent) antiparasite antibodies combined with T cell-dependent inflammatory (secretory) responses in the intestinal tract (Ogilvie and Love, 1974; Ogilvie and Parrott, 1977; Wakelin, 1975a). The final expulsion phase is mediator dependent (Dineen *et al.*, 1974; Rothwell, 1975; discussed in Askenase, 1977). Thus, like so many T cell-dependent effects, the final effector mechanism has no immunological specificity. The *Nippostrongylus brasiliensis*/rat model has been used extensively in the analysis of this sequence of events, this parasite being rejected from the intestines within 2–3 weeks although rejection is delayed in young and lactating rats. Resistance to reinfection after expulsion of the primary worm burden is very obvious though complex in its manifestations (discussed in detail by Ogilvie *et al.*, 1977; Love, 1975). The *Trichinella spiralis*/rat (Love *et al.*, 1976) and *T. spiralis*/mouse models (Wakelin and Lloyd, 1976b) and the *Trichuris muris*/mouse model (Wakelin, 1975a) have similarities with the *N. brasiliensis*/rat system although cell-mediated immunities may play the dominant role in expulsion of *T. spiralis* (Despommier *et al.*, 1977; Larsh and Race, 1975; Wakelin and Wilson, 1977). In the *N. brasiliensis*/mouse model, no good evidence exists for the involvement of antibodies in rejection of intestinal adults (Jacobson *et al.*, 1977; see also Mitchell *et al.*, 1976b).

The "self-cure" phenomenon, in which resident parasites are flushed from the intestines in a time course that resembles an immediate hypersensitivity reaction, is seen in infected sheep reexposed to

infective larvae (Jarrett and Urquhart, 1971; Stewart, 1953; Turner *et al.*, 1962). Dineen *et al.* (1977) have clearly demonstrated that other resident parasites can be "caught up" in the rejection process in sensitized sheep exposed to both homologous and heterologous infections. Behnke *et al.* (1977) and Bruce and Wakelin (1977) have referred to this phenomenon as "interactive expulsion," an event that appears to be T cell dependent. However, another possible consequence of double parasite exposure, protection from rejection of one by the other, has also been demonstrated when *Nematospiroides dubius* is one of the participants (Behnke *et al.*, 1978; Colwell and Westcott, 1973; Della Bruna and Xenia, 1976; Jenkins, 1975; Jenkins and Behnke, 1977) and immunosuppression may be a factor responsible for the observations (literature cited in Behnke *et al.*, 1978).

Although several studies have been performed with isolated *N. brasiliensis* antigens (literature cited in Day *et al.*, 1979), a lack of characterized antigens from intestinal parasites (including nematodes, cestodes, trematodes, and protozoa) has handicapped dissection of *specific* host immune responses. Excretory/secretory (ES) antigens from adult intestinal worms are easily harvested and provide a convenient source of products that, theoretically, should contain antigens available for both host sensitization and expression of immunological effector mechanisms. In a comparative study of the immunopotency of ES products of *N. brasiliensis* and *N. dubius* intestinal worms, no marked differences were observed in T and B cell mitogenicity *in vitro*, allergenicity, capacity to induce and elicit DTH responses in naive and infected mice, respectively, rates of *in vitro* production of ES proteins, and complexity of proteins (Day *et al.*, 1979). One difference between ES products from these parasites with very different time courses of infection (Fig. 3) was that *N. brasiliensis* ES products in Freund's complete adjuvant (FCA) could induce a high degree of protection against homologous infection whereas *N. dubius* ES (at the dosages chosen) plus FCA was without effect even in the mouse strain that develops impressive resistance to infection after intraperitoneal injection of adult worms or after multiple challenges with third-stage larvae (i.e., BALB/c mice). In double intestinal adult worm transfers in SPF-derived mice, the rejection of *N. brasiliensis* was neither influenced by, nor did it affect, the persistence of *N. dubius* transferred adults. Differences between this result and those of others remain unexplained (see above). A hypothesis to account for the differences in duration of infection with these two nematodes in mice is that *N. brasiliensis*, but not *N. dubius*, is dependent upon the uncompromised functional integrity of intestinal worm ES products and that *N. dubius*

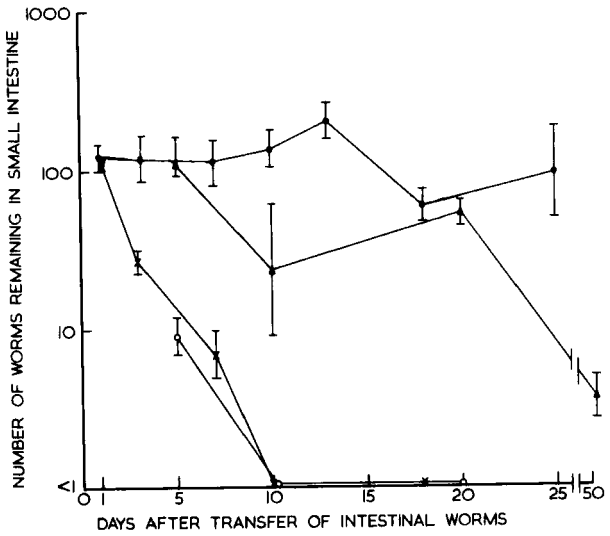

FIG. 3. Time course of rejection from the small intestines of 200 transferred *Nippostrongylus brasiliensis* intestinal worms (×——×, ▲——▲, ○——○) and persistence of 200 transferred *Nematospiroides dubius* intestinal worms (●——●) in female CBA/H.nu/nu (▲——▲) and male or female CBA/H intact mice (limits of the standard error of the geometric mean indicated). *Nippostrongylus brasiliensis* are eliminated slowly from nude mice (and also nude rats, M. E. Rose and B. M. Ogilvie, personal communication). Persistence of *N. dubius* and rapid rejection of *N. brasiliensis* are seen in intact mice during natural infection as well as after transfer of intestinal adult worms (literature cited in Day *et al.*, 1979).

worms are relatively resistant to the nonspecific components of the intestinal rejection process. Chronicity of *N. dubius* infection, and nonpersistence of *N. brasiliensis* can certainly not be explained readily in terms of differences in innate immunopotency (and perhaps availability) of intestinal worm ES products (discussed in Day *et al.*, 1979). The *N. dubius*/mouse system would appear to be a particularly suitable model for dissection of the immunological aspects of *chronic* intestinal parasitic infection and for elucidation of mechanisms of evasion of potentially aggressive host-protective responses. Chronic intestinal parasitic infections are of greater clinical and economic importance than transient intestinal infections, but analyses of the latter are important simply because they will ultimately provide clues on what types of *specific* immune responses are necessary or sufficient to initiate rejection of parasites from the intestines.

As judged by duration of intestinal infection, the tapeworm *Hymenolepis diminuta* resembles the nematode *Nippostrongylus brasiliensis* in being a more natural parasite of rats rather than mice. In

mice, *H. diminuta* is rejected within 2 or 3 weeks (e.g., Hopkins *et al.*, 1972; see also Befus, 1977), this rejection being delayed or absent (depending on the infecting cysticercoid dose) in nude mice (Andreassen *et al.*, 1978; Bland, 1976; Isaak *et al.*, 1975). Virtually all that is known about rejection of this and the more persistent, and autoinfecting, intestinal cestode, *Hymenolepis nana* (Freidberg *et al.*, 1967; Isaak *et al.*, 1977; Okamoto, 1970), is that the process appears to be immunologically based and T cell dependent. Good evidence exists that it is the tissue phase of infection with *H. nana* that induces resistance to reinfection (Heyneman, 1962; literature cited in Isaak *et al.*, 1977). Resistance against intestinal cestodes which are exclusively lumen dwellers is poorly developed in *natural* hosts (Weinmann, 1970) although sensitization definitely occurs (Harris and Turton, 1973).

The intestinal wall, as a barrier to penetration by metazoa, is a site where immunological mechanisms may operate to effectively limit access of invasive larvae (Section II,C). This does not appear to be a site where natural or acquired resistance to *A. suum* is mediated, at least in the mouse (Mitchell *et al.*, 1976a) but may well be a location in which resistance to reinfection with *F. hepatica* is mediated (e.g., Doy *et al.*, 1978; Hayes and Mitrovic, 1977; Rajasekariah and Howell, 1977). Good evidence for concomitant immunity exists in the *F. hepatica*/rat model (literature cited in Rajasekariah *et al.*, 1979a) but no comparable evidence for such resistance has been found in mice in this laboratory (cf. Lang, 1974). An important series of experiments will be to compare intestinal reactions and immune responses induced in rats versus mice infected with *F. hepatica*. Just as comparative studies in different mouse strains have provided information on mechanisms of resistance to various parasites (Section III,A), similar studies in two host species of different susceptibilities are potentially capable of providing the same type of data.

C. Susceptibility of the Hypothymic Nude Mouse

The nude mouse provides a ready means of assessing the consequences of a gross T cell defect on resistance to infection. Because of striking mouse strain variations in susceptibility to parasitic infection (see Section III,A), the value of the nude mouse has been increased by the availability of nude mice of various genotypes. Before T cell dependence of an effect is emphasized, reconstitution experiments are required, and before specific T cells are implicated, properties such as specific memory and specific tolerance must be demonstrated in reconstitutive cellular inocula (discussed in Mitchell, 1977c, 1978c).

At least two review articles are available on changes in parasitic

infection characteristics in T cell-deficient mice (Mitchell, 1978c; Targett, 1973). In terms of parasite burdens, congenitally hypothymic nude (nu/nu) mice may be more susceptible (the usual observation), of comparable susceptibility, or even more resistant than intact mice of appropriate genotype. A listing of the susceptibility of nude mice to more than 20 parasites is given in Table VI; of course, "susceptibility" is a crude term when applied to such diverse parasite systems, and the table merely serves to make the point that the nude mouse has found wide application in the study of parasitic infections.

Many of the studies on the parasites listed in Table VI involve highly artificial or unusual host–parasite relationships; artificial either because of the use of parasites of other host species or because of laboratory alteration of natural parasites. The pinworms (*Aspicularis tetraptera* and *Syphacia obvelata*), *Hymenolepis nana*, *Giardia muris*, *Hexamita muris*, *Taenia taeniaeformis*, *Nematospiroides dubius*, *Mesocestoides corti*, and at least some laboratory strains of *Trypanosoma musculi*, *Babesia microti*, *Plasmodium yoelii*, and *Leishmania tropica*, can be considered as "natural" parasites of mice. For all these parasites, infections (± disease) are more severe in nude mice than in intact mice: worm burdens are higher, infection persists longer, resistance to reinfection is not seen, mice are killed by the infection, or genetically based resistance is abrogated. These observations (plus demonstrations of amelioration of increased susceptibility by injection of T cells) provide strong support for the idea that *T cell-dependent* immunological and/or "paraimmunological" responses (Mitchell, 1979) have been important in the evolution of balanced host–parasite relationships.

Nude mice have proved to be particularly valuable in assessing and analyzing the T cell dependence of various manifestations of parasitic infection, one useful parasite for this purpose being the larval cestode, *Mesocestoides corti*. *M. corti* is a parasite which can be propagated readily in the laboratory by intraperitoneal or oral administration of larvae from infected donors. The parasites (larvae or tetrathyridia) proliferate in the liver and spill over into the peritoneal cavity. In intact mice, passive and active immunization manipulations to reduce establishment or the proliferative rate of injected larvae have marginal effects (Kowalski and Thorson, 1972a,b; Kazacos, 1976; Niederkorn, 1977a; see also Novak, 1974) although vaccination can have dramatic effects in rats (Niederkorn, 1977b; W. L. Nicholas, personal communication). In the *M. corti*/mouse system at least eight features of infection are T cell dependent, i.e., absent or reduced in nude mice and largely or completely restored by injections of T cells. These are:

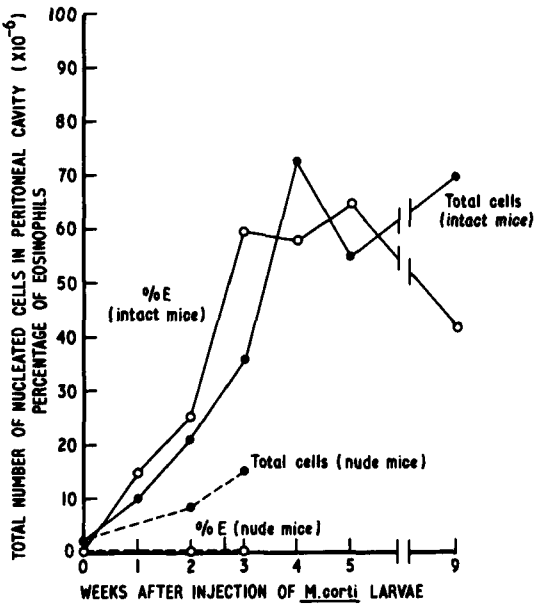

FIG. 4. Cellular inflammatory responses in the peritoneal cavities of intact BALB/c.nu/+ mice (solid lines) and hypothymic BALB/c.nu/nu (nude) mice (dashed lines) infected with *Mesocestoides corti*. Eosinophils (E) are prominent in the peritoneal cavity of infected intact mice but absent in that of infected nude mice. Data from Johnson *et al.* (1979).

1. Peritoneal eosinophilia (Johnson *et al.*, 1979) (Fig. 4)
2. Fibrotic encapsulation of the parasites in the liver (Pollacco *et al.*, 1978)
3. Peritoneal malabsorption of injected cells and molecules (Mitchell and Handman, 1978) (see Fig. 5 in Section IV,A),
4. Antibody responses to haptenated larvae (Mitchell *et al.*, 1977b)
5. IgG_1 antibody responses to *M. corti* surface antigens (Mitchell *et al.*, 1977b) including an antibody response involving an IgG_1 hybridoma idiotypic determinant (Mitchell *et al.*, 1979a)
6. IgG_1 hypergammaglobulinemia (Chapman *et al.*, 1979a; Mitchell *et al.*, 1977b)
7. Restrained parasite proliferation (Mitchell *et al.*, 1977b; Pollacco *et al.*, 1978)
8. Survival of the host (Mitchell *et al.*, 1977b)

This model should be particularly useful for assessing the contribution of various T cell subpopulations to these manifestations of infection. Ly 1^+ T cells appear to be more efficient at promoting peritoneal

TABLE VI
SUSCEPTIBILITY OF HYPOTHYMIC NUDE MICE TO FIRST INFECTION WITH VARIOUS PARASITES (RELATIVE TO INTACT MICE)

Susceptibility	Parasite[a]	Parameter[b]
Persistent infection, delayed rejection, uncontrolled infection, or higher parasite numbers	1. *Nippostrongylus brasiliensis*	Number of intestinal worms
	2. *Trichinella spiralis*	Number of intestinal worms
	3. *Hymenolepis diminuta*	Number of intestinal worms
	4. *Aspicularis tetraptera*	Number of intestinal worms
	5. *Syphacia obvelata*	Number of intestinal worms
	6. *Hymenolepis nana*	Number of intestinal worms and cysticercoids
	7. *Giardia muris*	Number of intestinal parasites
	8. *Hexamita muris*	Number of intestinal parasites
	9. *Trypanosoma musculi*	Parasitemia
	10. *Babesia microti*	Parasitemia
	11. *Plasmodium yoelii*	Parasitemia
	12. *Leishmania tropica*	Size of cutaneous lesions and visceralization
	13. *Taenia taeniaeformis*	Number and size of liver cysts
	14. *Mesocestoides corti*	Proliferative rate of larvae
	15. *Nematospiroides dubius*	Development of resistance to reinfection
Comparable or slightly more susceptible	16. *Ascaris suum*	Number of lung and liver larvae
	17. *Fasciola hepatica*	Number of liver larvae, mortality
	18. *Schistosoma mansoni*	Number of adults
	19. *Trypanosoma* spp.	Mean survival time, parasitemia
Comparable or slightly more resistant	20. *Plasmodium berghei*	Mean survival time, parasitemia
	21. *Babesia rodhaini*	Mean survival time, parasitemia
	22. *Trypanosoma rhodesiense*	Mean survival time, parasitemia

[a] References: 1, Jacobson and Reed, 1974a, 1976; Mitchell *et al.*, 1976b; 2, Ruitenberg *et al.*, 1977b; Ruitenberg and Steerenberg, 1974; 3 and 6, Bland, 1976; Isaak *et al.*, 1977; Reed *et al.*, 1977; cf. Andreassen *et al.*, 1978; 4 and 5, Jacobson and Reed, 1974b; 7, Roberts-Thomson and Mitchell, 1978; Stevens *et al.*, 1978; 8, Boorman *et al.*, 1973; Kunstyr *et al.*, 1977; 9, Brooks and Reed, 1977; Rank *et al.*, 1977; see also Pouliot *et al.*, 1977; 10, Clark and Allison, 1974; 11, Clark and Allison, 1974; Roberts *et al.*, 1977; Weinbaum *et al.*, 1976b; 12, Handman *et al.*, 1979; 13, Mitchell *et al.*, 1977a; 14, Mitchell *et al.*, 1977b; Pollacco *et al.*, 1978; 15, Prowse *et al.*, 1978b; 16, Mitchell *et al.*, 1976a; 17, Rajasekariah *et al.*, 1979a; 18, Phillips *et al.*, 1977; 19, Jayawardena and Waksman, 1977; Morrison *et al.*, 1978; B. M. Ogilvie, personal communication; 20, Waki and Suzuki, 1977; 21, Mitchell, 1977b; 22, Campbell *et al.*, 1978. Nude mice are likely to be more susceptible than intact mice to *Trichuris muris* (Wakelin and Selby, 1974).

[b] Defective resistance to reinfection has been demonstrated using nude mice in the *S. mansoni* (Phillips *et al.*, 1977), *N. brasiliensis* (Mitchell *et al.*, 1976b), *N. dubius* (Prowse *et al.*, 1978b), *A. suum* (Mitchell *et al.*, 1976a), *H. diminuta* (Andreassen *et al.*, 1978), *H. nana* (Isaak *et al.*, 1977), *P. yoelii* (Roberts *et al.*, 1977), and *G. muris* (Stevens *et al.*, 1979) systems.

eosinophilia than Ly 2^+ cells although difficulties in interpretation have arisen (Johnson *et al.*, 1979). The T cell dependence of hepatic fibrosis around resident larvae is presumably akin to the T cell dependence of the granulomatous reaction round *S. mansoni* eggs in mice (Byram and von Lichtenberg, 1977; Warren *et al.*, 1967; Warren, 1974). There are examples of pathological changes that are of greater intensity in normal and reconstituted T cell-deficient mice than in non-reconstituted T cell-deficient mice, including nudes (Bartlett and Ball, 1974; Ferguson and MacDonald, 1977; Giger *et al.*, 1978; Phillips *et al.*, 1977; Roberts-Thomson and Mitchell, 1978; Ruitenberg and Elgersma, 1976). Conversely, direct toxic effects of parasite infection may be more intense in nude mice, which presumably possess limited means of detoxifying or sequestering noxious substances (von Lichtenberg, 1977).

IV. Immunosuppression

The daunting complexity of the mammalian immune system, with its multitude of identified regulatory checks and balances, provides a comparable multitude of potential means by which parasites can modify host immune responses. Reduced responses to nonparasite antigens are readily demonstrated in many host–parasite combinations and range from gross immunosuppression, fully anticipated in a mouse about to die from an unrestrained and overwhelming unnatural parasitic infection, to far more subtle changes. Depressed immune responses have been documented in protozoan infections such as malaria, babesiosis and trypanosomiasis (e.g., Callow and Stewart, 1978; Goodwin *et al.*, 1972; Greenwood, 1974; Hazlett and Tizard, 1978; Mansfield, 1978; Murray *et al.*, 1974a; Terry, 1976; Wedderburn and Dracott, 1977; Strickland and Sayles, 1977) and in metazoan parasitic infections (literature cited in Dessaint *et al.*, 1977; Good and Miller, 1976).

In natural host–parasite relationships, there would appear to be no advantage to the parasite in effecting severe immunosuppression: the life of the host (and thus the parasite) will be threatened by intercurrent infection, particularly when a suboptimal nutritional state also prevails. Immunosuppression makes "biological sense" if it reflects (e.g., McBride *et al.*, 1977), or results in, partial inhibition of certain selected antiparasite immune responses. For example, a consequence of a degree of immunosuppression in protozoan infections, in which the parasite is capable of rapid proliferation with attendant differentiation (to other antigenic variant forms), will be to reduce the rate at which variant-specific immunity develops. A consequence of this is

that the parasite will keep ahead of the "floundering" immune response yet parasite burdens will be maintained at tolerable levels. [An interesting point is raised by the nature of antigens that engage in antigenic variation in parasitic protozoa and are potentially "host-protective antigens" (see Table IV). For hydrophobic determinants, suppressed levels of high-affinity (high cross-reactivity, Little and Eisen, 1969; see also Mitchell, 1977a) host-protective antibodies, and unaltered or high levels of low-affinity (more specific) host-protective antibodies, can be expected to allow minority antigenic variants in the parasite population to proliferate selectively (e.g., Laver and Webster, 1968). For hydrophilic determinants, which generally induce only low combining-site affinity antibodies (Eisen, 1973), a prediction is that no amount of immunosuppression would be required for the selection of antigenic variants.] Another consequence of immunosuppression may be to restrict induction of autoantibody production (Greenwood and Voller, 1970) when parasite and host share antigenic determinants (see Section V).

A. Architectural Disruption of Lymphoid Organs

The raison d'être for lymphoid tissue organization is presumably to optimize both the circumstances of induction and opportunities for regulation of immune responses to localized antigens. Thus any parasitic infection that markedly disrupts organized lymphoid tissue can be expected to alter immune responses induced in that tissue. Architectural disruption as a cause of immunosuppression has received most attention in the case of malaria and trypanosome infections involving splenomegaly (Albright *et al.*, 1977; Greenwood, 1974; Greenwood *et al.*, 1971; Hazlett and Tizard, 1978; Moran *et al.*, 1973; Murray, 1974; Wedderburn, 1974; Wyler and Gallin, 1977; see also Veress *et al.*, 1977). Numerous aspects of architectural disruption resulting from chronic infection can be expected to be T cell dependent (Roberts and Weidanz, 1978).

A profound (i.e., $> 1 \log_{10}$) decrease in the response to antigen injected intraperitoneally is seen in mice infected with the larval cestode *Mesocestoides corti,* a natural parasite of mice. The proliferating larvae of this parasite induce a violent inflammatory response involving a T cell-dependent, macrophage- and eosinophil-rich exudate in the peritoneal cavity (Johnson *et al.*, 1979) and a T cell-dependent deposition of fibrous tissue around the parasites in the liver (Pollacco *et al.*, 1978) that may extend to the lining of the peritoneal cavity. The rapid absorption of injected cells and molecules from the peritoneal cavity, normally seen in uninfected mice, is absent in infected intact

mice (Mitchell and Handman, 1978). This impaired absorption from the peritoneal cavity is also T cell dependent in that it is not seen in infected nude mice (Fig. 5; see also Section III,C). The immunosuppression seen after intraperitoneal, but not after intravenous, injection of antigen presumably reflects local sequestration and destruction of antigen in the inflamed peritoneal cavity, and thus minimal access of antigen to organized lymphoid tissues such as the spleen. Of course, this mechanism of immunosuppression could operate only in certain parasitic infections, but the system is mentioned in order to highlight the need to examine the trivial explanations for immunosuppression in addition to events pertaining to lymphocyte triggering. In a similar vein, recent studies by Freeman (1978) have demonstrated that the analysis of immunosuppression in murine malaria using isolated spleen cell from infected mice must carefully take into account the dilu-

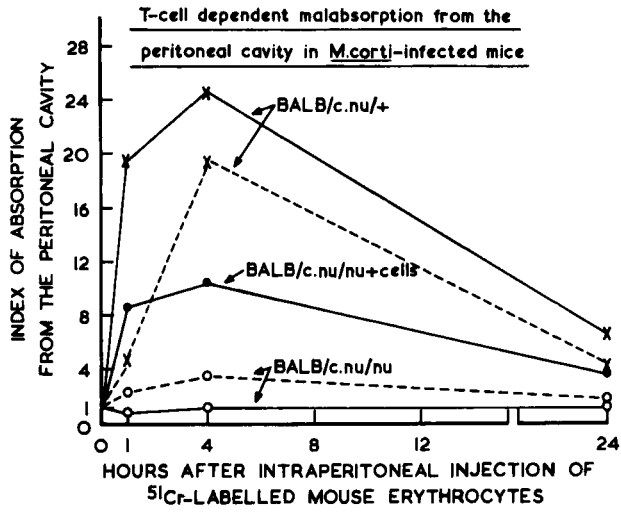

FIG. 5. Ratios of the geometric mean levels of radioactivity estimated to be in the bloodstream (Mitchell and Handman, 1978) following an intraperitoneal injection of ^{51}Cr-labeled mouse erythrocytes in noninfected versus 20 or 25-day *Mesocestoides corti*-infected female BALB/c.nu/nu (○——○, ○---○) (two experiments), BALB/c.nu/+ (×——×, ×---×) (two experiments), and BALB/c.nu/nu mice given a pool of thymus plus mesenteric lymph node cells from BALB/c mice at a dose of 2.5 donors per recipient (●——●). The index of absorption is the ratio of absorption from the peritoneal cavity in uninfected versus infected mice: thus, there is little difference in absorption between uninfected and infected nude mice, but absorption is much greater in uninfected intact mice than in infected intact mice. Defective absorption from the peritoneal cavity is one of at least eight manifestations of *Mesocestoides corti* infection that is T cell dependent.

tional effects of mononuclear red cell precursors (normoblasts) and the toxic effects these cells may have in *in vitro* cultures.

B. Toxic Parasite Products

Metazoan parasites are complex multicellular organisms. It is not surprising therefore that extracts of parasites have cytotoxic or inhibitory effects on host lymphoid cells (Barriga, 1975, 1978; Dessaint *et al.*, 1977; Faubert, 1976). Digestive enzymes and cell membrane active molecules of the lysolecithin type are good candidates for the molecules that mediate these effects. Of course, a demonstration of cytotoxicity with a disrupted parasite is not a sufficient reason to implicate toxic parasite molecules as being important in protecting parasites *in vivo* from aggressive cellular immune attack. A search for toxic surface and/or secreted molecules may be rewarding in the case of the liver migrating stages of *Fasciola hepatica* (Goose, 1978) since tissue destruction is very obvious in larval fascioliasis. Similar studies with pathogenic *Entamoeba histolytica* trophozoites should also be illuminating.

A high antiphosphorylcholine response has been recorded in mice infected with the larval ascarid *Ascaris suum*, an unnatural larval nematode infection. The phosphorylcholine molecule (PC) is of some interest as it is related to lysolecithin (e.g., Weltzien, 1973); it appears to be present in high amounts in *A. suum* larvae (Crandall and Crandall, 1971; Gutman and Mitchell, 1977; Péry *et al.*, 1974; von Brand, 1973), and it usually induces antibody responses of markedly restricted heterogeneity, which are largely, if not exclusively, of the IgM isotype (literature cited in Mitchell and Lewers, 1976). Suggestive, but far from conclusive, evidence has been obtained for the notion that restriction of anti-PC responses reflects a degree of inhibition of B cell expression (B^{γ} cell tolerance?). Thus PC, when conjugated in large amounts to a dinitrophenyl (DNP)-containing antigen, and when used freshly prepared, reduces the adoptive secondary anti-DNP response of DNP-primed B cells (Mitchell and Lewers, 1976). Conceivably, parasites such as larval ascarids may "utilize" PC-like molecules to effect partial suppression of high-affinity antiparasite antibody responses in their hosts (Fig. 6).

C. T Cell-Dependent Suppression and Other Mechanisms

In addition to the effects of toxic parasite antigens, there are at least five consequences of antigen exposure that are capable of effecting *specific* suppression of antiparasitic immune responses: clonal abortion of specifically reactive T and B cells, induction of anti-idiotype

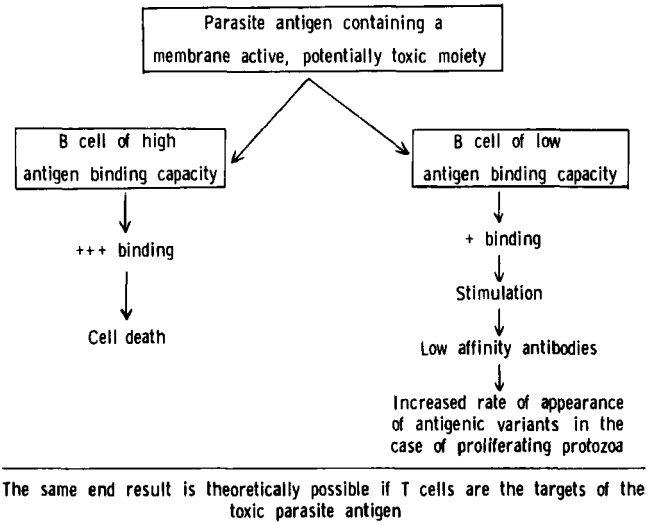

FIG. 6. Postulated means by which toxic parasite antigens may result in suppression of high-affinity specific antibody responses (in metazoan and protozoan infections) and thereby lead to favorable conditions for the selective proliferation of (protozoan) antigenic variants.

reactivity, lymphocyte blockade by antigen, "feedback inhibition" by antibody, and suppressor T cell activities. Analyses of suppression of specific antiparasite immune responses (e.g., Adams, 1978) are severely handicapped by a lack of defined parasite antigens (see Section I). One very useful means of assessing T cell responses to parasite antigens is through the use of haptenated parasites with antihapten antibody production as the readout system (Mitchell *et al.*, 1977b; Playfair *et al.*, 1977; Ramalho-Pinto *et al.*, 1976).

Few systematic studies have been performed on the consequences of early exposure of the host to parasite antigens, i.e., neonatal tolerance experiments (Bryceson *et al.*, 1972; Jarrett, 1971; Moriarty, 1966; Urquhart, 1961; also discussed in Hudson, 1973; Sinclair, 1970). Early exposure to parasite antigens can be expected in endemic areas, and the effects of this on subsequent immunoresponsiveness of hosts is largely unknown. There is no doubt that vaccination may be totally unsuccessful in early life compared with later life (Benitez-Usher *et al.*, 1977; Duncan *et al.*, 1978; Gregg *et al.*, 1978). In addition, antigen exposure in early life has been shown to modulate granuloma formation in *Schistosoma mansoni* infections in mice (Lewert and Mandlowitz, 1969; Warren *et al.*, 1972; Warren, 1974).

A clear distinction must be made between nonspecific suppression

resulting from T cell-dependent effects in parasitized mice, and the activities of suppressor T cells with parasite antigen specificity; several examples of the former exist (Corsini *et al.*, 1977; Coulis *et al.*, 1978; Eardley and Jayawardena, 1977; Jayawardena *et al.*, 1978b), *no* examples of the latter are available to date. Trypanosome infections in mice have been widely used to study depressed T and B cell responses (Corsini *et al.*, 1977; Hazlett and Tizard, 1978; Pearson *et al.*, 1978; literature cited in Terry, 1976). A cautionary note in the interpretation of *in vitro* and adoptive cell and serum transfer experiments has been sounded by Albright *et al.* (1977, 1978; see also Cunningham *et al.*, 1978), and hypotheses must take into account the possible inhibitory effects of residual *Trypanosoma* antigens in serum and particularly in lymphoid cell suspensions.

Mitogenic effects of parasite products (see Section V), antigenic competition, and phagocyte activation can all be expected to contribute to reduced immune responses to both parasite and nonparasite antigens in parasitized mice. In vaccination studies, the complexity of crude parasite antigen mixtures can be guaranteed to lead to antigenic competition effects, and responses to important (e.g., "host protective") minority antigens in the mixture may be minimal. The immunosuppressive consequences of mitogen injection are well known (e.g., Diamantstein *et al.*, 1976), and B cells are presumably capable of being "pushed" along abortive differentiation pathways (Corsini *et al.*, 1977; Hazlett and Tizard, 1978; Hudson *et al.*, 1976) rather than along more "productive" clonal expansion pathways. However, no purified B or T cell mitogen derived from a parasite has been isolated and characterized to date (but see Freeman and Parish, 1978; Hazlett and Tizard, 1978; Weinbaum *et al.*, 1976a).

Phagocyte activation in parasitic infections will have various effects on immune responses. Defects in macrophage presentation of antigen and rapid clearance of antigen may contribute to reduced immune responses (Coulis *et al.*, 1978; Greenwood *et al.*, 1971; Loose and diLuzio, 1976; Loose *et al.*, 1972; Warren and Weidanz, 1976). As has been emphasized previously (Mitchell, 1977a), there are presumably not only limited opportunities for, but little point in, production of antibodies when macrophages and other phagocytic cells are capable of eliminating antigen from the body. In the low-responder (Ab/L) mice produced by Biozzi *et al.* (1979), for example, macrophage activation may reduce the availability of antigen for lymphocyte triggering. Thus a parasitized mouse which is "immunosuppressed" in terms of antibody production may still be fully able to achieve one of the dominant purposes of the immune system, i.e., elimination of injected foreign antigen.

V. Hypergammaglobulinemia

Increases in levels of serum immunoglobulins (Igs) have been well documented in human parasitic infections (Cohen, 1974; Greenwood, 1978; Houba *et al.*, 1969). Macroglobulinemia is common in malaria and African trypanosomiasis in man, and serum IgG levels are elevated in malaria and visceral leishmaniasis (kala azar). Two of the most dramatic increases in circulating Ig in experimental parasitic infections are the recently described IgG_1 hypergammaglobulinemias in mice chronically infected with various metazoan parasites (Section V,B) and the macroglobulinemia of murine trypanosomiasis (Terry, 1976).

In those cases where IgM is elevated, "direct" B cell mitogenicity (Greenwood and Vick, 1975; Wyler, 1974) or "indirect" T cell-dependent mitogenicity of parasite products (Clarkson, 1977; Rosenberg, 1978) may be involved. With non-antigen-specific triggering of B cells, IgM can be expected to predominate simply because of the predominance of B^{μ} cells in lymphoid organs. Numerous reports have appeared recently on the increase in IgM-secreting cells in the spleens of *Plasmodium-*, *Babesia-*, and *Trypanosoma*-infected mice (Clayton, 1978; Corsini *et al.*, 1977; Cox *et al.*, 1977; Freeman and Parish, 1978; Hudson *et al.*, 1976; Morrison *et al.*, 1978; Murray *et al.*, 1974b; Rosenberg, 1978). Splenomegaly (Roberts and Weidanz, 1978; Wyler and Gallin, 1977) and violent proliferative T cell responses (Freeman and Parish, 1978; Jayawardena *et al.*, 1975; Weinbaum *et al.*, 1978) are common in such infections, many of which are lethal. Presumably, an increase in T cell-dependent (if not T cell-derived) mediators leads to recruitment of bystander B cells into Ig synthesis. This effect alone may have severe immunosuppressive consequences (Section IV,C). Macroglobulinemia is a feature of the peculiar tropical splenomegaly syndrome (literature cited in Crane, 1978; Greenwood, 1978) and may well be associated with a genetically based production of low-affinity IgM antibodies to (and complexing with) malaria antigens.

The principal unknown in discussions on parasite-dependent hypergammaglobulinemia is whether all, some, most, or none of the increased amounts of serum Ig represent specific antiparasite antibodies. If mitogens are present and if immunosuppression leads to a flare-up of concurrent infections (Cox, 1975), much of the Ig is likely to be non-parasite specific. Concerning the ratio of parasite-specific to nonspecific Igs, IgG_1 hypergammaglobulinemias may consist of a high proportion of antiparasite antibodies (Section V,B) whereas in macroglobulinemias, non-parasite specific Ig may predominate (Freeman *et al.*, 1970; Houba *et al.*, 1969; Hudson *et al.*, 1975). However, since methods for obtaining *all* antigens from *all* life-cycle stages of para-

sites are not available it may be unwise to assume that most of the serum Ig in any hypergammaglobulinemia associated with parasitic infection is non-parasite specific. This applies even if autoantibodies are identified, since (*a*) demonstrations of *in vitro* binding of Ig to autoantigens must take into account the age-old problems of substrate antigen modification in tissue processing for the assay, and the possibility that antibodies of very low combining-site affinity for autoantigens are being detected; and (*b*) molecular mimicry may operate to reduce antigenic disparity between hosts and parasites (Damian, 1978; Dineen, 1963) and induction of autoantibodies is a predictable consequence of such a mechanism of immune evasion: linked antigenic recognition (Mitchison, 1971) through parasite antigen-reactive helper T cells and autoreactive B cells (Weigle, 1973) being responsible for some degree of autoantibody production.

A. Potentiated Reagin Responses

Elevated IgE responses to nonparasite antigens have been demonstrated in several experimental parasitic infections, and high IgE levels have long been considered a feature of chronic metazoan parasitic infection (literature cited in Ishizaka *et al.*, 1976; Jarrett and Bazin, 1974, 1977; Kojima and Ovary, 1975; Ogilvie and Jones, 1969). Potentiated IgE responses associated with parasitic infection in previously sensitized laboratory animals are T cell dependent (Jarrett and Ferguson, 1974). The underlying reasons for potentiated IgE antibody responses remain unknown, and any proposal must take into account the fact that *protozoan* infections have not yet been shown to lead to increased IgE levels or potentiated responses. Based on several studies on allergenicity, we have emphasized the possible contribution (to potentiated IgE responses) of antigen persistence in, and antigen accessibility to, mucous membranes and subcutaneous tissues and disruption of such structures by migrating or resident metazoa. If such sites are the preferred location of B^{ϵ} cells, then localized inflammation and T cell activation may lead to induction of IgE synthesis by resident B^{ϵ} cells (Mitchell, 1976; Mitchell and Clarke, 1979; O'Donnell and Mitchell, 1978). Striking host genetic factors can also be expected in this phenomenon (Marsh *et al.*, 1974; Willcox and Marsh, 1978).

In the area of IgE production, in particular, any increase or decrease in circulating IgE antibody titer is often ascribed to activities of suppressor versus helper T cells. Such an emphasis on inductive events at the lymphocyte level is clearly unwarranted, particularly if changes in mast cell numbers influence serum titer, if persistent undigested antigens act as a sink for high-affinity minority antibodies, if plaque-

forming cell analyses of lymphoid and nonlymphoid organs have not been performed, and if helper T cell effects lead to increased production of antibodies of other Ig isotypes, which in turn have feedback influences on the availability of antigen for recruitment of minority B cell types such as B^e cells. This is not to deny the influence of triggered T cells of different subpopulations in regulating antibody responses of any isotype, but it seems that simpler, more anatomical, explanations based on antigen availability have been neglected (discussed in Mitchell and Clarke, 1979).

The question of whether IgE antibodies play any role in host protection against parasites remains unresolved. Possible modes of action, which are related to one another, include (*a*) a contribution to secretory responses in the intestinal tract which may flush parasites from this site (Barth *et al.*, 1966; Ogilvie and Parrott, 1977); (*b*) a contribution to increased tissue permeability and thus accessibility of aggressive antiparasite effector cells and molecules (Leid, 1977; Musoke *et al.*, 1978); and (*c*) a contribution to modification of cutaneous environments that may militate against persistence of ectoparasites (Askenase, 1977; Benjamini *et al.*, 1961). A role for IgE as cytophilic antibody in macrophage-mediated schistosomicidal effects has also been suggested (Capron *et al.*, 1977). A general impression is that IgE-mediated immediate hypersensitivity reactions fall far short of being an indispensable component of host-protective immunity (Ogilvie and Parrott, 1977). In addition, and as mentioned previously, it is conceivable that mucous membrane-located parasites and ectoparasites may have evolved mechanisms to exploit immediate hypersensitivity reactions by utilizing secretions rich in serum proteins for feeding purposes.

B. IgG_1 Hypergammaglobulinemia

A striking consequence of chronic parasitic infection in mice is an IgG_1 hypergammaglobulinemia. This has been documented in infection of mice with *Schistosoma mansoni* (Sher *et al.*, 1977), *Nematospiroides dubius* (Prowse *et al.*, 1978a,b) and *Mesocestoides corti* (Mitchell *et al.*, 1977b) and elevations of lower magnitude have been demonstrated in *Taenia taeniaeformis* and *Leishmania tropica* infections (Chapman *et al.*, 1979a; Fig. 7). The studies of Chapman *et al.* (1979a) have demonstrated that IgG_1 hypergammaglobulinemia is most evident in the case of parasites or mouse strains which exhibit chronic high-level infections and the magnitude of the response is much lower in infected hypothymic nude mice.

Concentrations of IgG_1 in the serum of *Mesocestoides corti*-infected mice may be 50 times above normal levels of 1–2 mg/ml.

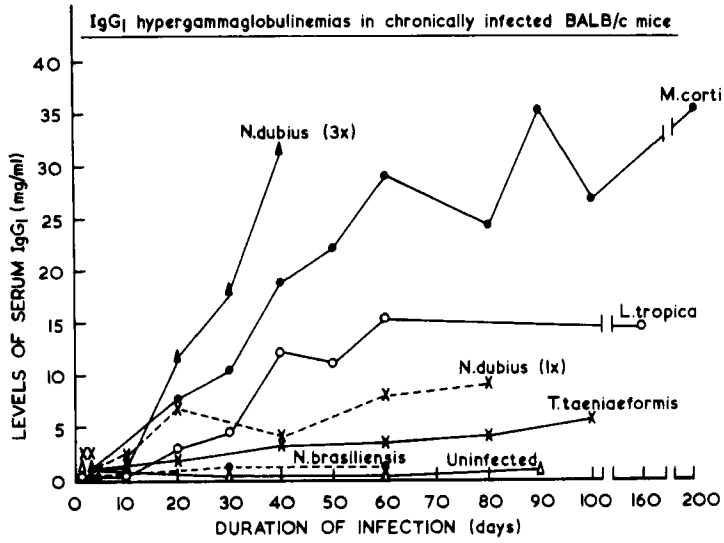

FIG. 7. IgG_1 hyperglobulinemias in chronically infected BALB/c mice. Levels of serum IgG_1 in male BALB/c mice dosed on day 0 with *Mesocestoides corti* larvae intraperitoneally (●——●), *Nematospiroides dubius* larvae orally (×----×), *N. dubius* larvae orally in three administrations over a 20-day period (▲——▲), *Taenia taeniaeformis* eggs orally (×——×) (low infection level), *Leishmania tropica* promastigotes intradermally (○——○), *Nippostrongylus brasiliensis* larvae subcutaneously (●----●) (transient infection), or left as age- and sex-matched control uninfected mice (△——△). Details in Chapman *et al.* (1979a).

These amounts of IgG_1 in the circulation are well in excess of the amounts seen with most myelomas in mice. It must be emphasized that apart from a slightly distended abdomen, mice heavily infected with *M. corti* are in apparent good health although liver pathology and peritoneal cellular exudates are severe. Parasites from the peritoneal cavity are coated with immunoglobulins with IgG_1 antibodies predominating. At least a portion of the IgG_1 in the circulation has specificity for parasite surface antigens (Chapman *et al.*, 1979b; Mitchell *et al.*, 1977b, 1979a). However, the ratio of specific antibody to nonspecific Ig in the serum cannot be determined because all antigens of the parasite are not available for absorption analyses. As mentioned above, this is a recurring problem in studies designed to determine whether a majority or a minority of Ig in hypergammaglobulinemic states has specificity for the antigens of the causative agent. If IgG_1 antibodies are as bland as currently believed (Spiegelberg, 1974; but see Table VII) then they may act as "blocking antibodies" (Hellström and Hellström, 1970; Voisin, 1971) in larval

TABLE VII
POSSIBLE FUNCTIONS FOR APPARENTLY "INERT" ANTIBODIES[a] IN PARASITIC INFECTIONS[b]

Host protection
1. Inhibition of the release of infective particles from infected host cells
2. Inhibition of entry of infective particles to host cells
3. Prolongation of the half-life of infective particles in the circulation, thereby increasing their vulnerability to other effector molecules when the preferred location of the parasite is an intracellular site
4. Neutralization of anticomplementary parasite molecules

Parasite protection
1. Inhibition of the access of more aggressive effector molecules and cells by antigen masking or steric hindrance ("blocking antibodies")
2. Interference with the triggering of at least some lymphocytes via immune complex-mediated blockade
3. Activation of specific suppressor T cells via immune complexes

[a] The "type" antibody may be IgG_1 in the mouse if this Ig isotype is indeed non-complement fixing and non-macrophage binding. If immediate hypersensitivities have antiparasite effects through increasing tissue permeability or rendering mucous membranes or cutaneous sites less favorable for parasitization (see text), then the binding of IgG_1 to mast cells may have some host-protective consequences.

[b] The mechanisms listed are *purely hypothetical* at present, and *none* have been shown to be operative or mediated by a particular antibody isotype.

cestode infections (Mitchell *et al.*, 1977b; Rickard, 1974). It must be emphasized, however, that this and other postulated roles for IgG_1 are purely hypothetical and no experiment has yet been performed to test the various possibilities (Table VII).

Although high levels of serum IgG_1 may be obtained in mice exposed to a single dose of *Nematospiroides dubius* L3 (Prowse *et al.*, 1978a), the levels are boosted markedly by reexposure of infected mice to L3 (Chapman *et al.*, 1979a). Since the larvae encyst in the intestinal wall, it is assumed that sytemic access of antigen (or mitogen?) is greater during larval encystment compared with intraluminal residency of intestinal adults. Biosynthetic Ig labeling analyses have demonstrated that the lymph nodes draining the small intestine are principally involved in IgG_1 synthesis (Chapman *et al.*, 1979a). A high proportion of the IgG_1 in hypergammaglobulinemic sera from *N. dubius*-infected mice appears to be specific for parasite antigens (Molinari *et al.*, 1978). Since the intestines are damaged by *N. dubius* infection (Baker, 1954; Mitchell and Prowse, 1979; Spurlock, 1943), some of the IgG_1 may be directed toward nonparasite antigens coming from the intestinal tract. IgG_1 has affinity for mast cells (Nussenzweig *et al.*, 1964; Ovary *et al.*, 1970), and in the case of intestinally located

parasites, IgG_1 antiparasite antibodies may have some host-protective effects if, indeed, immediate hypersensitivity reactions have antiparasite effects (see Section V,A). Much of the IgG_1 in *S. mansoni*-infected mice has antiparasite specificity (Sher *et al.*, 1977).

We have attempted to determine whether IgG_1 hypergammaglobulinemia is confined to chronic metazoan parasitic infection by examining IgG_1 levels in BALB/c mice chronically infected with the protozoan *Leishmania tropica*. Results obtained by Chapman *et al.* (1979a) clearly demonstrate that an IgG_1 hypergammaglobulinemia is seen in *L. tropica*-infected mice although the levels attained are certainly lower than those seen in *M. corti*- or *N. dubius*-infected mice (Fig. 7). Unfortunately, we do not have in this laboratory *Trypanosoma, Plasmodium,* and *Babesia* spp. that establish chronic, nonlethal infections in mice. Thus on the basis of the result with a single chronic protozoan infection, IgG_1 hypergammaglobulinemia seems to be common to both protozoan and metazoan chronic infections. It will be important to determine whether *blood-borne* protozoan parasites lead to IgG_1 hypergammaglobulinemia during chronic infection.

The IgG_1 isotype appears to be more responsive to antigenic stimulation in the mouse than other isotypes (Barth *et al.*, 1965). In addition, chronic exposure to antigens of allogeneic cells (Basch, 1974; Harris and Harris, 1975) and sheep erythrocytes in high doses (Chapman *et al.*, 1979b) will preferentially result in IgG_1 antibody production. An important question is whether this reflects a peculiarity of $B^{\gamma 1}$ cells in the mouse, a peculiarity not shared with other murine B cells but also not shared with any B cells of other species (discussed in Chapman *et al.*, 1979a,b).

The commitment of various organs in *N. dubius*- and *M. corti*-infected mice to IgG_1 synthesis has been demonstrated using biosynthetic labeling analyses with organ fragments *in vitro*. With a serum level of > 30 mg/ml and a half-life of $[^{125}I]IgG_1$ in parasitized mice of < 2 days, and assuming a high rate of production of 10,000 molecules per second per plasma cell (Nossal and Mäkelä, 1962; Salmon and Smith, 1970; Woodland, 1974), the number of plasma cells engaged in IgG_1 synthesis is in excess of 2×10^8 per mouse (Chapman *et al.*, 1979a). A major challenge in this area is to determine whether this apparently exaggerated host response is counterproductive for host-protective immunity. If it is, then any vaccination strategy will need to be designed such that it militates against induction of the type of response that occurs during natural infection and is being "utilized" by the established parasite to direct the immune system away from more host-protective types of response.

VI. Eosinophilia

Until recently, the eosinophil remained the last clearly identifiable cell type of the blood with no clearly identified functions (Archer, 1968; Beeson, 1977; Zucker-Franklin, 1974). Eosinophils are a common feature of metazoan parasitic infection and immediate hypersensitivities; their mere presence might suggest an involvement in antiparasite responses and anti-immediate hypersensitivity responses. Current interest in the eosinophil in immunoparasitology centers on its possible functions as a cell type capable of (*a*) antibody-dependent parasitocidal activity (Butterworth *et al.*, 1977; Sanderson *et al.*, 1977); and (*b*) mediating tissue repair by neutralizing the products of mast cell degranulation in immediate hypersensitivity reactions (Butterworth, 1977; Goetzl and Austin, 1977; Hubscher and Eisen, 1973; Kay, 1976) and, conceivably, by limiting the extent of fibrotic encapsulation, wound repair, and granuloma formation during parasitic infections. Concerning the antiparasite activities of eosinophils, some questions remain as to whether antibody simply focuses the "killer" eosinophil or whether the eosinophil acts to increase the parasitocidal potency of the antiparasite antibodies.

Eosinophils bind to antibody-coated metazoan parasites of various types and life cycle stages (early literature cited in Butterworth, 1977; Higashi and Chowdhury, 1970; MacKenzie *et al.*, 1977; McLaren *et al.*, 1977) and damage has been demonstrated in the case of *Schistosoma mansoni* schistosomules and eggs (Glauert *et al.*, 1978; James and Colley, 1977, 1978; MacKenzie *et al.*, 1977; Ramalho-Pinto *et al.*, 1978) and *Trichinella spiralis* newborn larvae (Kazura and Grove, 1978). The demonstrations of eosinophil potency in antibody- and complement-dependent parasitocidal activities do not in any way exclude the involvement of other cell types, such as neutrophils (Dean *et al.*, 1974) and macrophages (Capron *et al.*, 1977) as effector cells (particularly in rats). The only experiments that bear on the question of whether eosinophils have antiparasite effects *in vivo* are those in which antieosinophil sera have been used in mice. Chronically treated mice are reported to be more susceptible to reinfection with *S. mansoni* and are more susceptible to larval *T. spiralis* infection (Grove *et al.*, 1977; Mahmoud *et al.*, 1975).

Eosinophils are commonly seen in situations of tissue damage by tracking parasites and in fibrotic granulomatous reactions to parasites and their products. Part of their postulated repair function may therefore be to limit the pathological consequences of (T cell-dependent) chronic inflammation or even the extent of acute inflammation resulting from tissue penetration and damage by parasitic larvae. Although it

is most unwise to draw conclusions on the nature of eosinophil involvement from time-course studies on circulating eosinophil numbers, eosinophilia is seen in larval *Ascaris suum* infection in mice only after the bulk of the parasites have completed their destructive migration through the lungs and when the organ is presumably in a state of repair (Mitchell *et al.*, 1976a; Nielsen *et al.*, 1974; also Fig. 8). The affinity of eosinophils for uterine tissues (Tchernitchin *et al.*, 1974) may also reflect physiological tissue repair functions.

The T cell dependence of eosinophilia and tissue eosinophil accumulation during murine parasitic infections has been demonstrated in numerous systems (Fine *et al.*, 1973; Hsu *et al.*, 1976; Johnson *et al.*, 1979; Mitchell *et al.*, 1976a; Nielsen *et al.*, 1974; Phillips *et al.*, 1977). Eosinophils are present in nude mice, but such mice fail to mount a response to infection involving eosinophilia or accumulation in localized sites. One important and unresolved question is whether T cell-dependent effects in eosinophilia are mediated indirectly via T cell-dependent antibodies, at least in part (Askenase, 1977b; Beeson, 1977; Pepys *et al.*, 1977; Sher, 1977). Also unknown is the nature of the effect of complement deficiency in increasing lung eosinophil accumulations in *A. suum* infections (Leventhal *et al.*, 1978). The techniques for growing colonies of eosinophils *in vitro* (Metcalf *et al.*, 1974; Rabellino and Metcalf, 1975) provide a powerful tool for analysis of events at the eosinophil precursor level in tissues from parasitized mice. Using mice infected with *M. corti*, a parasite that induces a striking T cell-dependent peritoneal eosinophilia (Johnson *et al.*, 1979), increases in the numbers of eosinophil colony-forming cells (EO-CFC) have been found in the spleens of infected mice, a response that is defective in infected nude mice (D. Metcalf, G. R. Johnson, and G. F. Mitchell, unpublished observations). Ly 1^+ T cells appear to be more efficient at promoting peritoneal eosinophilia than Ly 2^+ cells in *M. corti*-infected nude mice (Johnson *et al.*, 1979), but changes in eosinophil precursor frequency in tissues of reconstituted mice have yet to be examined.

As the number of postulated functions of eosinophils increases, a search for heterogeneity within the eosinophil population and purification of various subpopulations, should be rewarding (Kay and Butterworth, 1977). Using Percoll-purified peritoneal eosinophils from *M. corti*-infected mice of 95% purity (Burgess *et al.*, 1979), we have not found good evidence for eosinophil heterogeneity (at least in this particular population) using a range of alloantisera with specificity for lymphocyte markers such as Ly, Ia, Thy-1, TL, Ig, etc. (M. Hogarth, I. F. C. McKenzie, K. M. Cruise, and G. F. Mitchell, unpublished observations).

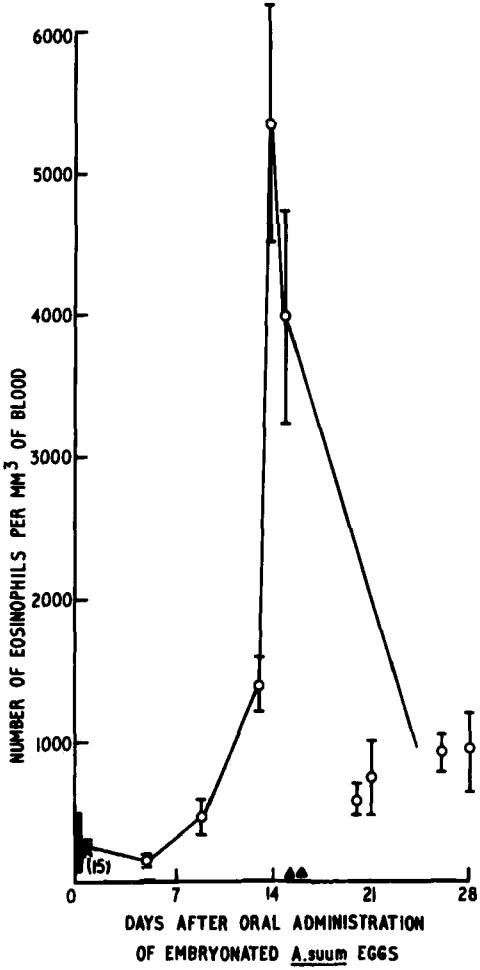

FIG. 8. Peripheral blood eosinophilia in female C57BL/6 mice given *Ascaris suum* eggs orally (O——O), but not in female C57BL/6.nu/nu mice (▲) given *A. suum* eggs. Standard error of the arithmetic mean is indicated, the blackened area representing the blood eosinophil number in 15 normal mice with the mean indicated (x). Eosinophilia in *A. suum*-infected mice is T cell dependent and appears several days after larvae have migrated through the lungs (Mitchell *et al.*, 1976a).

The eosinophil will continue to be a cell type of particular fascination to the immunoparasitologist. Dissection of its antiparasitic functions will lead to attempts to exploit the activities of the cell in host resistance against parasites. However, possible tissue repair functions should not be overlooked in studies on the influence of this cell in host–parasite relationships. There are as yet no *in vivo* systems de-

vised to look at this postulated function of eosinophils, and the cell engaged in this activity may or may not be identical to the cell involved in interacting with antibodies in mediating antiparasite effects. Heterogeneity is not only a biological necessity of parasites and hosts, but the inevitable result of intensive study on any morphological cell type. Since the eosinophil has the appearance of a specialized "end cell," its range of activities will presumably be less than that of another morphological cell type with extreme biological heterogeneity, the lymphocyte.

VII. Concluding Comments

Immunoparasitology is a rich area for collaborative application of the skills of the parasitologist capable of providing defined parasitological material, the epidemiologist who understands field and clinical problems, the biochemist capable of providing defined parasite antigens, and the immunologist capable of detecting (and inducing) specific antiparasite immune responses of various types. Factors such as the potential rewards of research mentioned in Section I, the biological fascination of parasites, and various new sources of research funding, will ensure that the field of immunoparasitology evolves into a major area of biomedical research. Not that this is a new field of research endeavor. The danger for the student of the "new immunology," in particular, is in assuming that "nothing is known" and all that has gone before is "low key." In point of fact, it is often something of an embarrassment for an immunologist to be asked by a parasitologist how to ensure that, after vaccination, a particular type of immune response is induced by a particular parasite antigen preparation—this despite the enormous research effort over the past 15 years or so on the induction of immune responses and the abundance of accumulated information on regulatory immune mechanisms. Of course, the existence of such deficiencies says more about the intricacies of the immune response than the aims and abilities of the experimental immunologist. The opportunities for the "new biology" lie in building on a wealth of background parasitological information. Veterinary parasitology, for example, is a discipline with a long history of excellence in applied research, and the veterinary parasitologist will undoubtedly have a central role to play in the expansion of, and the recruitment of workers into, the field of experimental immunoparasitology.

It will be obvious that in several discussions throughout this article, in particular in the sections on hypergammaglobulinemia and immunosuppression, an attempt has been made to emphasize the more

"architectural" and parasite-oriented aspects of infection. This has been done in an attempt to balance out the interpretations, common in the recent literature, that emphasize events at the level of immune induction, i.e., the triggering of lymphocytes of different subpopulations. Since the latter is one of the most exciting and rapidly moving areas in contemporary immunology, there is a temptation to ascribe all alterations in immune response levels, for example, to lymphocyte cellular events. Such an emphasis is not justified in most, if not all, cases. However, one of the growth areas in immunoparasitology will certainly be the cellular and molecular events of induction and regulation of *specific* antiparasite immune responses in hosts: detailed studies will commence when relevant parasite antigens are in hand.

Numerous model host–parasite systems are now in widespread use, and this availability of systems (although they are of varying relevance to clinically important parasitic diseases) augurs well for the rapid accumulation of knowledge in immunoparasitology. Every host–parasite relationship has its unique aspects, and the comparative immunoparasitological approach should be capable of identifying broad biological principles as well as the intricacies of each relationship. Many systems, including unnatural host–parasite relationships, have been used simply because they display some manifestation of infection that is worthy of study in its own right, examples being mechanisms of induction of eosinophilia, reaginic antibody production, restricted IgM antiphosphorylcholine responses, hypergammaglobulinemia, and granuloma formation.

There are several gaps in the catalog of immunoparasitological materials and methods that will handicap progress. Obvious ones are (*a*) *in vitro* culture systems for various parasites; (*b*) technologies for the isolation of relevant and unmodified antigens from complex metazoan and protozoan parasites; and (*c*) type organisms such as *Escherichia coli* and *Drosophila melanogaster* for both metazoan and protozoan immunoparasitology. However, several new technologies and reagents have already been used and can be expected to form the basis of much of the research activity in the immediate future, e.g., fluorescence-activated cell sorting (of malaria-infected blood) and other cell separation technologies (for production of pure populations of eosinophils, for example), hybridoma-derived monoclonal antibodies (for antigen isolation and immunodiagnostic assays), recombinant mouse strains (for analysis of the genetics of variation in resistance to infection), judiciously reconstituted hypothymic and other immunodeficient mice (for assessing the effects of various T cell subpopulations and antibody isotypes in resistance and in the various

manifestations of infection), surface and biosynthetic labeling probes (for tagging parasite and parasitized cell antigens), *in vitro* cultivation of parasites (for *Plasmodium, Leishmania,* and *Trypanosoma* spp.) and recombinant DNA technologies (for analysis of the genetics of expression of antigens in *Trypanosoma* variants). Thus the movement of the analytical tools of the "new biology" to experimental immunoparasitology has commenced and will accelerate.

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