ADVANCES IN IMMUNOLOGY

VOLUME 31

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Immunology

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PREFACE

Immunology has been a cyclical and opportunistic field, constantly exploiting widely divergent but developing areas. Nowhere is this better illustrated than in the diversity of experimental animal systems utilized. Several decades ago the immunized rabbit was the primary tool of the immunologist. Then, with the beginning of interest in delayed-type hypersensitivity, the guinea pig became uniquely prominent. During the era of the immunoglobulins, the human system with its large quantities of myeloma proteins dominated the picture. On the other hand, the immunologist of the decade of the seventies and the early eighties has been virtually completely preoccupied with the murine system. The development of genetics as an integral part of immunology is in considerable part responsible for the current overwhelming use of this clearly advantageous experimental animal. However, occasionally and perhaps increasingly so recently, developments originating in the human system are having a significant impact. Three of the chapters in this volume illustrate this point and cover subjects that initially arose from discoveries in the human; the autologous MLC is perhaps the most striking example.

The first chapter of this volume is written by Dr. Unanue who has become one of the leaders in macrophage research. He presents a very comprehensive review of the important regulatory role of these cells in a great variety of cellular immune reactions. The very different functions of macrophages continue to be a surprising feature of these cells. These range from the presentation of antigen to T cells to the release of prostaglandins and mediators such as LAF. Perhaps the most interesting aspect of the macrophage is its key position in *Ir* gene control; this topic and its controversial aspects are very thoroughly discussed. Other accessory cells such as the Langerhans cells, the dendritic cell, and the thymic "nurse" cells are also discussed in considerable detail, although their exact relationship to the macrophage remains to be established.

No review could be more timely than the second chapter on cloned T cells and T cell growth factor. The two authors, Smith and Ruscetti, coming from the two pioneering laboratories in this field, combine their knowledge and experience in this article. New T cell lines with different functions are appearing in publications almost daily, and this system is proving to be of tremendous aid to the cellular immunologist. It provides the much needed capacity to dissect T cell

responses in detail from a functional standpoint. The T cell growth factor or TCGF which is primarily responsible for the propagation of these clones has been isolated, and its characteristics are described in considerable detail. It is apparent that this protein is much more than just an essential for the growth of these clones and plays a most important role in T cell proliferative responses under natural conditions.

The third chapter deals with B lymphocytes, with special emphasis on their culture and precursor characteristics. The technology for continuous B cell lines has not developed to the degree described for T cells in the previous chapter. However, progress has been made and Dr. Kincade's methods show considerable promise. A very diverse literature on the relationship of B cell precursors to various known stem cells is thoroughly reviewed along with the author's observations utilizing his semi-solid agar culture system. Progress in this area has been substantially accelerated by new monoclonal antibodies which help to identify B cell precursors prior to their identification through Ig production.

There have been few areas of immunology in which so much controversy exists as for the question of the character of the Fc receptor. However, Dr. Unkeless and his associates have clarified the field markedly recently through the use of monoclonal antibodies. It still remains mystifying just how different laboratories report such different molecular weights for Fc receptors even when the same cell type is analyzed. These different results are reviewed and related to the new observations that have resolved some of the issues. It is evident from the review that much of the problem relates to the low binding affinity of IgG, aggregated IgG, and immune complexes to Fc receptors. By obtaining monoclonal antibodies to the Fc receptor directly, a much higher binding affinity is obtained that permits clear identification at the molecular level. Although most of the work concerns the macrophage, a considerable portion of the review concerns B lymphocytes and other cells with Fc receptors.

The last chapter reviews in considerable detail the important new area of immunology concerning the autologous MLC. Dr. Weksler is one of the discoverers of this unexpected reaction that appears to have profound significance in the cellular immune response. It is now evident that stimulation of autologous or syngeneic T cells results from Ia-positive non-T cells in the native state and that it is not due to modification of the stimulating cell as some critics postulated. In the mouse it is clear that the dendritic cell is the primary stimulator; in the human this question remains unsettled. Defects in this response are

striking in certain diseases, especially in patients with systemic lupus erythematosus.

The Editors wish to express their gratitude to the authors for the fine chapters they have written and thank the publishers for their constant cooperation.

HENRY G. KUNKEL
FRANK J. DIXON

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The Regulatory Role of Macrophages in Antigenic Stimulation Part Two: Symbiotic Relationship between Lymphocytes and Macrophages

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I. Historical

In 1972, I reviewed for this series the regulatory role of phagocytes in antigenic stimulation, i.e., in immune induction (Unanue, 1972). The information gathered at that time clearly pointed to a highly significant role of accessory phagocytic cells in the multiple cellular interactions taking place during the early development of an immune response. To recapitulate briefly, a number of *in vivo* experiments had indicated a direct relationship between the uptake of antigens by the phagocyte system and the extent of the immune response. Thus, changes in antigen molecules that resulted in enhanced or decreased uptake by the phagocytes resulted in higher or lower immune responses, respectively. After the development of methodologies for obtaining live exudate cells rich in phagocytes and for pulsing these with antigen, the immune response to macrophage-associated antigens was

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possible to assay, using combinations of *in vivo* and *in vitro* methods. This approach led to the result that the presentation of antigen bound to live macrophages to the lymphocytes was a highly efficient mode of generating an immune response. Invariably, a strong antibody response was produced to antigen molecules transferred to a host in syngeneic, live macrophages. Concurrently with this approach, the analysis of cellular interactions employing culture methods pointed to a requirement for adherent phagocytic cells in order for lymphocytes to proliferate or to make antibody. In 1972, the basis for the strong immunogenicity of macrophage-associated antigens and for the requirements of macrophages for *in vitro* immune responses were not clear, nor were the cellular and molecular events understood. We felt strongly, based in part on our own studies on antigen handling, that antigens associated with macrophage surface were involved in antigen presentation, yet how, in what form, and with what class of lymphocyte remained unexplained. Whether this antigen simply served as a device to focus T and B cells or played a more essential role was not known. The role of macrophages in directly regulating growth and differentiation of lymphocytes could only be speculated, based on suggestive experiments on the effects of adjuvants on macrophages. Although great steps had been taken in our basic knowledge of the biology of phagocytes, many issues were still to be resolved, most notably those of macrophage differentiation, antigen handling, and secretion.

The 8 years after the earlier review witnessed an incredible number of studies on phagocyte biology and on macrophage-lymphocyte interactions. These studies have placed the macrophage, now more than ever, as a critical regulatory cell with functions never before suspected. Our progress in this field came as the result of improvement in tissue culture methods, in techniques for isolating and identifying lymphocytes, in procedures to probe various cellular interactions, plus major advances in macrophage biology, and, most notably, in basic cellular immunobiology. Progress in immunobiology developed in parallel in the 1970s in various areas, i.e., transplantation genetics, immune response genetics, T-B cell collaboration, cytolytic T cell–tumor effects, and macrophage–lymphocyte interactions. The major findings and discoveries are now beginning to be integrated with each other, but it is obvious that the advances in immunogenetics and in histocompatibility have been of major impact in all of immunology. Indeed, in the course of a brief period of time, three basic cellular interactions, that of macrophages with T cells (Rosenthal and Shevach, 1973), T cells with B cells (Kindred and Shreffler, 1972; Katz *et al.*,

1973), and cytolytic T cells with their targets (Zinkernagel and Doherty, 1974), were found to be regulated by the major transplantation locus of the species. I consider the series of studies by Alan S. Rosenthal and Ethan M. Shevach published in 1973 the most important study on macrophage–lymphocyte interaction in the 1970s and perhaps one of the most seminal ones regarding the genetic control of immune responses. These studies indicated a role of macrophages in events controlled by the *I* region of the major histocompatibility gene complex, a feature never previously suspected, and signaled an *essential* interaction between macrophages and the thymus-derived T lymphocyte. The explosive series of studies in macrophage biology that followed represented, to a great degree, extensions of their basic findings. It may turn out that the common denominator for all the phenomena of cell-to-cell interaction is the process of antigen handling regulated by the transplantation gene locus.

Phagocytes are now viewed as cells capable of exerting a fine control on their environment, particularly on the lymphoid system. This control is exerted not only by carrying out antigen presentation, an essential function, but also by regulating growth and differentiation of lymphoid cells by way of a number of secretory products. Clearly, immune induction depends on a critical interrelationship between the phagocytes and the lymphocytes, the former being the nonspecific cell probably descendent of the primitive amebocytes, whereas the latter are cellular elements that give specificity to the immune response. Both cell types control and regulate each other, and the key in this regulation are the *I*-region products.

This chapter is organized into two major sections. The first reviews the various experimental systems in which macrophages have been involved, such as T-cell proliferation to antigen and lectins, T-B cell interactions, cellular immunity reactions. Some of these I have described more extensively than others. For example, T-cell proliferation and T-B cell interaction are analyzed in depth inasmuch as they were the basic systems for study of macrophage–T cell effects. In contrast, the involvement of macrophages in the development of the cytolytic T-cell response to viruses and tumors is treated briefly, this being a highly complex response involving a multiplicity of interactions and still very much under current study. The second part analyzes the biology of the phagocytes, the synthesis and expression of *I*-region-associated antigen, the issue of macrophage heterogeneity, and the function of antigen presentation and secretion of active products.

II. Analysis of the Regulatory Role of Macrophages

A. Interactions with T Cells in Antigen-Induced Proliferation

The proliferative response of T cells to antigen has been one of the systems most extensively employed for studying macrophage–lymphocyte interactions in culture. The first series of analyses in man (Hersch and Harris, 1968; Cline and Sweet, 1968) and in the guinea pig (Seeger and Oppenheim, 1970) on lymphocyte proliferation have already been reviewed (Unanue, 1972). Most recent experimental studies have used the guinea pig and mouse as the species of choice. Populations of lymphoid cells rich in T lymphocytes were obtained from lymph nodes, draining the depot of antigen, spleen, or the peritoneal cavity. The cells from the peritoneal cavity were an excellent source of T cells developing a very strong and notable antigen-specific response much higher than that shown by lymph node T cells (Rosenstreich *et al.*, 1971; Rosenstreich and Rosenthal, 1973). This may be because the immune T cell may migrate selectively into sites of inflammation as indicated by the studies of Koster and associates (1971) in antibacterial T-cell immunity carried out at the Trudeau Institute. Accordingly, in our own studies examining the proliferative response of T cells to *Listeria monocytogenes*, we found that an injection of proteose peptone intraperitoneally was *essential* in order to induce the exudate rich in strong T cells (Farr *et al.*, 1979a). Other studies in the mouse used thioglycolate broth (Schwartz *et al.*, 1978). Mineral oil was the choice inflammatory agent in experiments using the guinea pig (Rosenstreich and Rosenthal, 1973). It is worth recalling that the first successful transfer of contact sensitivity, the classical experiments of Landsteiner and Chase (1942), utilized lymphoid cells harvested from the peritoneal cavity.

Regardless of the source, the T cells have been isolated by separating out the phagocytes and B cells using combinations of brief culture on dishes to remove the bulk of adherent cells, followed by passage through glass beads or, preferably, nylon-wool columns as per the technique described by Julius *et al.* (1973). The technique of Julius requires a critical amount of nylon wool per input number of cells; if carried out correctly, it results in yields of about 25–35% of lymph node or splenic lymphocytes, the bulk of which bear T-cell markers. A good separation results in about 95% T cells, without phagocytes, and, at the most, 2–3% B cells.

Using stringent procedures to remove the macrophages and to en-

rich for T cells invariably resulted in T cells that did not respond to antigen in culture even with media that contained growth-promoting components, such as fetal calf serum and 2-mercaptoethanol (Section II,C). In the guinea pig, the studies of the proliferative response were the first to indicate clearly the essential role of phagocytes. Seeger and Oppenheim (1970) first found that passing lymph node cells through glass-bead columns resulted in the total loss of antigen-induced DNA synthesis. These analyses were then extended by Waldron *et al.* (1973), studying the response to purified protein derivative (PPD). The lack of response of macrophage-depleted lymph node cells could be reconstituted fully by adding peritoneal macrophages; in fact, the degree of reconstitution was proportional to the amounts of phagocytes added, i.e., from 1 to 30%, the last dose used. I accentuate this point because, in contrast to the studies using murine cells, guinea pig phagocytes showed little inhibitory effect even at high doses. Waldron *et al.* also used macrophages pulsed briefly with PPD. The response of the lymphocytes to PPD administered macrophage-bound was identical to that induced by PPD in soluble form, in the presence of optimal amount of phagocytes. These studies, therefore, confirmed the efficiency of antigen presentation by way of the macrophage but added the important new point that this step was essential in order for the lymphocyte to respond.

In the murine system, the requirement for phagocytes or other accessory cells was such that as little as 1% contamination with phagocytes still enabled a T-cell proliferative response to develop. Thus, depletion procedures had to be extremely efficient. A single passage of lymphoid cells through nylon wool, for example, or a single cycle of adherence, did not completely eliminate macrophages. Most investigators are now employing removal by one or two cycles of adherence, together with one or two passages through nylon wool. Under such procedures, the T-cell proliferation to antigen, as in the guinea pig, was strictly macrophage dependent (for example, Rosenwasser and Rosenthal, 1978b; Richman *et al.*, 1979; Farr *et al.*, 1979a; Kammer and Unanue, 1980).

Many of the analyses of antigen presentation using the mouse have employed accessory cells pulsed with antigen, which are then added to the lymphocytes, thus bypassing the need for extensive macrophage depletion of the lymphocyte populations. One important consideration in the mouse, however, is the amounts of macrophages added to the cultures. Too many macrophages invariably inhibited proliferation (Section III,D). This was particularly striking if the macrophages were activated. In most instances, the reasons for the suppressive effects of

macrophages were not investigated. Some experiments, however, attribute the inhibition to prostaglandins and other soluble molecules (Section III,C). Major representative results in experimental animals are shown in Table I.

There are few analyses of an antigen-driven proliferation in man, but these have clearly shown the requirements for monocyte-macrophages (Hersh and Harris, 1968; Cline and Sweet, 1968; Rodey *et al.*, 1979; Bergholtz and Thorsby, 1977, 1978, 1979; Breard *et al.*, 1979).

For effective T cell–macrophage collaboration, two essential features are required: (*a*) that the proliferating T cell and the macrophage be histocompatible, sharing part of the *I* region; (*b*) that the phagocyte bear the *I*-region-associated antigens (Ia) of the major histocompatibility complex (MHC) of the species.

The *I* region of the major histocompatibility gene complex was discovered by McDevitt and associates as they attempted to map the locus within the mouse *H-2* that controlled the immune response to the branched polypeptides poly(Tyr,Glu)-poly(DL-Ala)-poly(L-Lys) [(T,G)-A-L] and poly(His,Glu)-poly(DL-Ala)-poly(L-Lys) [(H,G)-A-L] (McDevitt *et al.*, 1972). They identified, using a number of congenic strains of mice, a gene segment between *H-2K* and *S* which was termed *I*. This observation followed their initial study relating the capacity to make an immune response to the *H-2* type of the responding strain (McDevitt and Chinitz, 1969). Subsequently, more *Ir* genes have been linked to this segment, which was subsequently subdivided into various subregions, i.e., *I-A*, *-B*, *-J*, *-E*, *-C* (reviewed by Shreffler and David, 1975; Klein, 1975; Klein and Hauptfeld, 1976; Benacerraf and Germain, 1978; Snell, 1978). After this study, five different laboratories within a brief period of time raised antibodies to the *I*-region products using appropriate *I*-region congenic strains of mice. These antibodies identified a new surface antigenic system, that of the "*I*-region-associated antigens" (Sachs and Cone, 1973; David *et al.*, 1973; Gotze *et al.*, 1973; Hauptfeld *et al.*, 1973; Hammerling *et al.*, 1974). The Ia antigens were identified on B cells and in a few activated T cells and also in macrophages (Section III,A). Ia antigens have now been identified corresponding to *I-A*, *I-J*, and *I-E/I-C* regions. The I-A and I-E/C products were characterized as two polypeptide chains of about 33,000 (α chain) and 26,000 (β chain) molecular weight (Cullen *et al.*, 1974, 1976; B. D. Schwartz *et al.*, 1976). (Whether *I-E* and *I-C* are two distinct regions is in dispute; hence the notation now in use: *I-E/C*.) I-A and I-E/C show extensive serological polymorphism with various allelic forms. The Ia antigens are the main surface proteins responsible for the mixed leukocyte reaction. The HLA-D region in

man codes for the same stimulatory protein and is thus regarded as the equivalent of the *I* region. The major differences between the two inbred lines of guinea pigs, the strains 2 and 13, lies in the *I* region, so that cross-immunization with cells, i.e., strain 2-anti-strain 13 lymphoid cells results in antibodies that are essentially anti-Ia reagents (Geczy *et al.*, 1975; B. D. Schwartz *et al.*, 1976).

1. Histocompatibility Requirements

The requirements for *I*-region histocompatibility between phagocytes and T cells in order for both cells to interact were found both for antigens under *Ir* control as well as for those with no obvious *Ir* gene effects. Antigens under *Ir* gene control are those antigens that induce high immune responses in some strains and low responses in others, the state of responsiveness being linked to the *I* region of the MHC strain. These antigens include synthetic polypeptides of limited antigenic heterogeneity, some weakly immunogenic alloantigens or conventional proteins administered at low doses (reviewed by Katz, 1977; Benacerraf and Germain, 1978). The first observations on the histocompatibility requirements for macrophage–T cell interactions were made by Rosenthal and Shevach (Rosenthal and Shevach, 1973; Shevach and Rosenthal, 1973). They obtained T cells—depleted of phagocytes—from the peritoneal cavity or lymph nodes of immunized inbred guinea pigs, either strain 2 or strain 13, and cultured them with macrophages of either strain pulsed briefly with the antigen under study (Table II). The optimal response to PPD or dinitrophenyl (DNP)-albumin, antigens to which there was no obvious *Ir* gene effect, was elicited only if the T cells from one strain were challenged with the antigen in the macrophages from the same homologous strain. While T cells from an F_1 cross [$(2 \times 13)F_1$] could respond perfectly well to PPD in either strain 2 or strain 13 macrophages, T cells from strain 2 responded only to PPD associated with macrophages from strain 2, or $(2 \times 13)F_1$, but not from strain 13. Rosenthal and Shevach went on to show that this lack of responsiveness to allogeneic macrophages could not be ascribed to differences in the kinetics of the response nor to a suppressor-type effect: excellent proliferation could be elicited from T cells mixed with syngeneic and allogeneic antigen-pulsed macrophages.

Further experiments indicated that antibodies to the MHC gene products could block the macrophage–lymphocyte interaction [such antibodies had been previously found to block antigen-driven T-cell proliferation (Shevach *et al.*, 1972)]. Macrophages from $(2 \times 13)F_1$ pulsed with PPD stimulated proliferation of T cells from strain 2, but

TABLE I

EXPERIMENTS SHOWING ANTIGEN PRESENTATION BY MACROPHAGES FOR T CELL PROLIFERATION[a]

Antigens	Species	Investigators	Main results
Tetanus toxoid and PPD	Guinea pig	Seeger and Oppenheim, 1970	Poor response in macrophage-depleted cultures; reconstitution with PEC
PPD	Guinea pig	Waldron *et al.*, 1973	Complete absence of response without phagocytes; reconstitution with PEC
Horseradish peroxidase	Guinea pig	Rosenstreich and Rosenthal, 1973	1–2% of macrophages bind the antigen and serve as antigen-presenting cells
PPD, DNP-albumin	Guinea pig	Rosenthal and Shevach, 1973	Antigen presentation by macrophages requires histocompatible macrophages; anti-Ia antibodies inhibit
DNP-GL, GT, PPD	Guinea pig	Shevach and Rosenthal, 1973	Macrophages from "responder" strains are required to present antigen
GT, GL	Guinea pig	Shevach, 1976	*I*-region control of macrophage T cell interactions in outbred guinea pigs
TNP	Guinea pig	Thomas *et al.*, 1977; Thomas and Shevach, 1977	Macrophages are required. Primary sensitization can be elicited if allo-reactive T cells are eliminated; anti-Ia blocks the macrophage presentation
Ovalbumin	Guinea pig	Thomas and Shevach, 1976; Paul *et al.*, 1977	Two independent sets of F_1 T cells responding to antigen in macrophages from either of the parental strains
DNP-ovalbumin, GAT, etc.	Mouse	Yano *et al.*, 1977, 1978; Schwartz *et al.*, 1978	*I*-region restrictions mapped to *I-A* in the interaction between spleen antigen-presenting cells and T cells; macrophages from responder strains are required
Insulin	Guinea pig	Barcinski and Rosenthal, 1977; Rosenthal *et al.*, 1977	Determinant selection by the macrophage
DNP-GL, PPD DNP-albumin	Guinea pig	Yamashita and Shevach, 1977	*Ia*-positive macrophages are required for antigen presentation
PPD, GAT, OVA	Mouse	Suzuki *et al.*, 1978	Macrophages present the antigen; non-*Ir* genes modulate the response

DNP-OVA, hemocyanin, PPD and GLT	Mouse	Rosenwasser and Rosenthal, 1978a,b	The response to multideterminant antigen requires macrophages; allogeneic macrophages can present; a macrophage factor can replace the macrophages
PPD, hemocyanin	Mouse	Cowing *et al.*, 1978b	Spleen accessory cells present antigen and bear I-A and I-E/C determinants
PPD, TNP	Guinea pig	Stingl *et al.*, 1978a,b	Langerhans cells of the skin present antigen
Fibrinopeptide	Guinea pig	Thomas *et al.*, 1979a,b	Determinant selection by the macrophage
Insulin, DNP, ovalbumin	Guinea pig	Yokomuro and Rosenthal, 1979	T cells from guinea pigs can be selected by *in vivo* immunization with DNP-ovalbumin transferred in one or the other parental macrophage; only "responder" macrophages present insulin
Listeria monocytogenes	Mouse	Farr *et al.*, 1979a; Beller and Unanue, 1979; Weinberg and Unanue, 1980	Presentation shows *I*-region restrictions at *I-A* and requires Ia-positive macrophages; thymic macrophages and alveolar macrophages present *Listeria*
GLPhe	Mouse	Schwartz *et al.*, 1979	Two complementing *Ir* genes must be expressed in the same macrophage
PPD	Mouse	Lee *et al.*, 1979	Ia-bearing macrophages present antigen; differences found among Ia-positive cells
Myoglobin	Mouse	Richman *et al.*, 1980	T cells proliferate to antigen presented in Kuppfer cells from responder strains
TNP-OVA, PPD	Guinea pig	Rogoff and Lipsky, 1980	Kuppfer cells present antigen
Hemocyanin	Mouse	Kammer and Unanue, 1980	The response to hemocyanin requires syngeneic macrophages and is not replaced by a macrophage factor

[a] PEC, peritoneal exudate cells; PPD, purified protein derivative; DNP, TNP, di-, trinitrophenyl; GT, GL, GAT, copolymers, respectively of L-glutamic acid and L-tyrosine; L-glutamic acid and L-lysine; and L-glutamic acid, L-alanine, and L-tyrosine.

TABLE II
THE REQUIREMENT FOR HISTOCOMPATIBLE MACROPHAGES IN ANTIGEN-MEDIATED DNA SYNTHESIS IN IMMUNE GUINEA PIG LYMPH NODE LYMPHOCYTES[a]

Macrophage		Lymphocyte DNA synthesis: [^{3}H]TdR incorporation (cpm × 10^{-3})		
Strain	Antigen pulse	Strain 2	Strain 13	(2 × 13)F_1
2	0	0.92 ± 0.28	5.68 ± 1.08	1.60 ± 0.30
2	+	26.38 ± 8.27	8.61 ± 2.08	6.98 ± 0.80
13	0	4.63 ± 1.86	1.66 ± 0.37	1.78 ± 0.47
13	+	3.12 ± 0.67	19.89 ± 4.47	7.81 ± 1.75
(2 × 13)F_1	0	1.91 ± 0.99	4.27 ± 0.34	1.66 ± 0.53
(2 × 13)F_1	+	12.42 ± 3.19	11.81 ± 1.98	12.57 ± 2.33

[a] Macrophages from strain 2, strain 13, or (2 × 13)F_1 guinea pigs were incubated with mitomycin C and/or purified protein derivative (PPD) for 60 minutes at 37°C, washed, and mixed with column-purified lymph node cells, as indicated. [^{3}H]Thymidine incorporation was expressed as mean cpm × 10^{-3}. [From the study of Rosenthal and Shevach (1973).]

this proliferation was totally ablated by adding anti-2 antibodies to the culture. Shevach and Rosenthal concluded that "the activation of immune lymphocytes by antigen-pulsed macrophages is dependent on the interaction of cell surface structures that are the products of the major histocompatibility complex" (1973).

Noteworthy were the studies of antigens under *Ir* gene control. Shevach and Rosenthal selected for their analysis the copolymers of L-glutamic acid and L-lysine (GL) and of L-glutamic acid and L-tyrosine (GT) previously analyzed by Benacerraf and associates (Bluestein *et al.*, 1971). The T lymphocytes from the (2 × 13)F_1 guinea pigs appropriately immunized with GT or GL were cultured with macrophages from either strain pulsed with one or the other antigen. The F_1 T cells responded only to GT pulsed on strain 13 macrophages and to DNP-GL on strain 2 macrophages, i.e., on the macrophages from the strain that responded immunologically when immunized *in vivo* (Fig. 1). Thus, although the F_1 guinea pig contained T cells capable of responding to GT or GL, these T cells did so only when presented with the antigens in macrophages bearing the appropriate *I*-region haplotype of the responder stain. A final and important experiment in this study concerned the effects of antibodies to the MHC products of strain 2 or strain 13—now known to be anti-Ia antibodies. These antibodies blocked specifically the response of the F_1 T cells to the F_1 macrophages pulsed with the antigen under *Ir*-gene control. Thus,

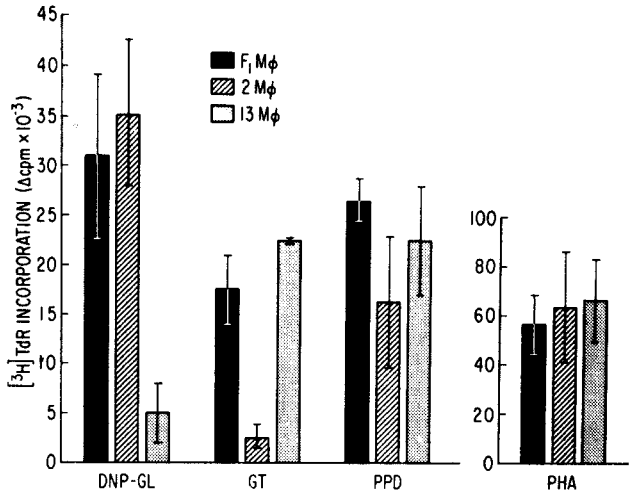

FIG. 1. The graph shows the stimulation of guinea pig (2 × 13)F_1 T cells by macrophages from each parent or from the F_1 macrophages pulsed with the antigens as indicated. Strain 2 guinea pigs respond to DNP-GL, but not to GT; strain 13 responds to GT but not to DNP-GL. Both respond to purified protein derivative (PPD). The response to phytohemagglutinin (PHA) is also shown. DNP-GL, dinitrophenyl copolymer of L-glutamic acid and L-lysine; GT, copolymer of glutamic acid and L-tyrosine. [From Shevach and Rosenthal (1973).]

anti-2 antibodies blocked the response to DNP-GL (strain 2 is a responder to GL) but not to GT (strain 13 responds to GT). The reverse is also true. The conclusion was that the anti-Ia antibodies inhibited the specific interaction between the T cell and the macrophage with its Ia product.

These series of studies, therefore, indicated that the response of T cells required the phagocyte as an *obligatory* cell and that the interaction taking place between both cells involved a product from the MHC. The antigen-presenting function of macrophages, therefore, acquired a new dimension. It clearly did not represent a simple antigen → T cell-receptor interaction but involved antigen and a specific protein, the Ia product, interacting with the T cells.

Subsequent studies confirmed the important relationship between antigen and the *I*-region products of macrophages in T cell–macrophage stimulation. A series of experiments have indicated that selective T cells proliferate upon interaction with antigen bound to the appropriate Ia-bearing macrophage. This was best shown using F_1 animals. Thomas and Shevach (1976) developed an assay system in which guinea pig T cells from a (2 × 13)F_1 guinea pig were cultured

for several days with macrophages of either parental strain pulsed with ovalbumin. At the end of the culture, the T cells were isolated and tested for the capacity to proliferate with the antigen-pulsed macrophages of each strain. The results indicated that the F_1 T cells behaved like two distinct populations, i.e., T cells from the F_1 cultured on macrophages from strain 2 proliferated only to challenge with macrophages of strain 2 but not strain 13, whereas those T cells cultured on strain 13 macrophages proliferated only when challenged with macrophages of strain 13. Paul and associates (1977) explored this issue and presented strong experiments indicating that the T cells from a $(2 \times 13)F_1$ hybrid guinea pig immune to ovalbumin were indeed composed of two independent sets. This was analyzed in two different experimental situations. The first approach was a positive selection procedure first developed by Ben-Sasson *et al.* (1975a,b): ovalbumin-immune $(2 \times 13)F_1$ T cells were placed in culture with ovalbumin-pulsed strain 2 or 13 macrophages; shortly thereafter, the lymphocytes that did not adhere to the macrophages were removed; at the end of 1 week, the T cells were isolated and tested for proliferation to macrophages pulsed with ovalbumin of either strain 2 or 13. In confirmation of the study of Thomas and Shevach, those F_1 T cells cultured initially on macrophages from strain 2 responded only to ovalbumin bound to macrophages of strain 2; the situation was also true for T cells grown initially with macrophages from strain 13. Their second approach consisted of a negative selection procedure: the T cells were cultured briefly with the antigen-pulsed macrophages from either parent and then the proliferating cells labeled by exposure to bromodeoxyuridine (and light); the remaining cells were then tested on fresh, new antigen-pulsed macrophages. The thymidine analog bromodeoxyuridine becomes incorporated into the DNA of the cell and cross-links the DNA strands when activated by light; this results in a block in proliferation. The F_1 T cells first cultured with ovalbumin in strain 2 macrophages only responded to OVA in strain 13 macrophages, i.e., the "clone" of T cells responding to ovalbumin in strain 2 macrophages had been selectively depleted. This experiment suggested that each "clone" was not represented by two cooperating sets of T cells, one recognizing Ia, the other ovalbumin; had this been the case, one or the other would have been eliminated and would not have been able to function in the assay, i.e., in the above example, the putative ovalbumin clone would have been eliminated; therefore, no proliferation would have taken place to ovalbumin bound to strain 13 macrophages.

The two clones of reactive T cells could also be identified *in vivo*

using the macrophage transfer system (Yokomuro and Rosenthal, 1979). F_1 guinea pigs were injected with DNP-ovalbumin on one parental macrophage; days later their T cells were harvested and tested for their proliferation to macrophage-bound ovalbumin; priming *in vivo* with one parental macrophage resulted in T cells that only proliferated with the same macrophages when tested in culture. Similar results to these were found in the mouse in different experimental situations, to be analyzed in the sections to follow.

The most logical interpretation of all these results with F_1 T cells is to postulate that distinct clones of T cells are selected to expand and proliferate upon interaction with antigen and a particular *I*-region product. In the normal immunization, that is, in a syngeneic situation, the clones reactive to the antigen in an allogeneic Ia, even if present, do not expand and, therefore, are not detected. The F_1 experiments also tell us that the *I*-region restrictions cannot be explained by a simple sharing of the MHC surface products of the T cell with that of the macrophage (i.e., the F_1 T cell clone selected on strain 2, for example, would still share MHC determinants with strain 13; the surface expression of MHC products does not show allelic exclusion). Finally, one should recall that, in the studies of cytolytic T cells restricted to their target by H-2K or H-2D products, similar results were found using F_1 T cells.

Analysis of the T-cell proliferative response in the mouse has yielded essentially identical results as in the guinea pig, but it also permitted a more elaborate genetic mapping because of the more extensive information on the murine *H-2* and the availability of great number of inbred strains. Schwartz, Paul, and associates examined the proliferative response of T cells to several different antigens and found a perfect correlation between the antibody responses to the antigens *in vivo* and the extent of T-cell proliferation (Schwartz and Paul, 1976; Schwartz *et al.*, 1978). The way in which they studied the genetics of the antigen-presenting accessory cell was to pulse briefly mitomycin C-treated spleen cells with antigens and then to add the live cells to the peritoneal exudate T cells. (Their accessory cell was characterized by its adherence to glass, radioresistance, and lack of B- or T-cell markers.) Using dinitrophenylated ovalbumin or the copolymer of glutamic acid.alanine, and tyrosine (GAT), they confirmed the requirement for histocompatible phagocytes in antigen presentation and mapped this requirement to the *I-A* subregion of the mouse *H-2* (Yano *et al.*, 1977, 1978). Table III reproduces results of their experiments using inbred strains of mice with recombinants at *H-2*. T cells from the B10 or B10.A proliferated upon presentation of the antigens in cells

TABLE III

CONTROL OF ANTIGEN PRESENTATION BY *I*-REGION GENES[a,b]

Spleen cells		DNP-OVA primed		GAT primed	
Strain	MHC	B10.A	B10	B10.A	B10
B10.A	*kkkkkddd*	100 (all)	15 (none)	100 (all)	19 (none)
B10	*bbbbbbbb*	9 (none)	100 (all)	0 (none)	100 (all)
B10.A (5R)	*bbbkkddd*	14 (*J, E, C, S, D*)	62 (*K, A, B*)	0 (*J, E, C, S, D*)	82 (*K, A, B*)
B10.A (4R)	*kkbbbbbb*	126 (*K, A*)	16 (*B, J, E, C, S, D*)	77 (*K, A*)	8 (*B, J, E, C, S, D*)
A.TL	*skkkkkd*	94 (*A, B, J, E, D*)	14 (none)	47 (*A, B, J, E, D*)	0 (none)
		A.TL	A.TH	A.TL	B10.A (5R)
A.TL	*skkkkkkd*	100 (all)	6 (*K, D*)	100 (all)	7 (***J, E, D***)
A.TH	*sssssssd*	0 (*K, D*)	100 (all)	ND	ND
B10.A (4R)	*kkbbbbbb*	130 (*A*)	11 (none)	85 (*A*)	10 (*B*)
B10.A (5R)	*bbbkkddd*	ND	ND	12 (*J, E, D*)	100 (all)
SJL	*ssssssss*	23 (*K*)	106 (*K, A, B, J, E, C, S*)	ND	ND

[a] This table is a summary of the series of experiments of Schwartz, Paul, and associates as reported by them (Schwartz *et al.*, 1978). DNP-OVA- GAT-primed T cells from B10.A or B10 mice were stimulated *in vitro* with the antigens bound to spleen cells from various strains of mice. The proliferative responses were measured by thymidine incorporation, and the data are expressed as percentages of the syngeneic response, i.e., the difference between antigen-pulsed cultures for the allogeneic spleen cells, divided by the difference between antigen-pulsed and nonpulsed cultures for the syngeneic spleen cells, times one hundred (Δ cpm allo/Δ cpm syn) × 100.

[b] MHC, major histocompatibility complex. Small letters denote the haplotype source of origin of the alleles at the ***K, I-A, I-B, I-J,*** *I-E, I-C, S,* and *D* regions. Capital letters in parentheses following the percentage of the syngeneic response refer to the regions of the MHC that are shared between the responding T cells and the stimulating spleen cells. ND, not determined.

from B10 or B10.A, respectively, not vice versa. Because the T cells from B10.A could not be stimulated by antigen presented in cells from B10.A (5R) but would respond to antigen in cells from B10.A (4R) or A.TL, it was possible to map the *H-2* restrictions to *I-A*. B10.A had identity with B10.A (5R) from *I-J* to *-D*, with B10.A (4R) at the *K* and I-A, and with A.TL at *I-A* to *-D*. Other combinations described in Table III confirmed this genetic mapping.

In the murine system, the important issue was raised about the absolute need for macrophages in the response to complex, multideterminant antigens not under obvious *Ir* gene control. We analyzed before that macrophage presentation of complex antigen in the guinea pig showed MHC restriction mapped at the *I* region, implying, therefore, an involvement of this genetic segment in this function. When reevaluating the response of murine lymph node T cells to a number of antigens such as DNP-ovalbumin, hemocyanin, or GLT, Rosenwasser and Rosenthal (1978a,b) found that the responses were completely abolished by depletion of phagocytes and could be reconstituted with peritoneal macrophages, in agreement with past studies. Yet two striking differences were found between the multideterminant antigens DNP-ovalbumin and hemocyanin, and the synthetic polypeptide GLT. The responses to the former two could be reconstituted with allogeneic macrophages or a macrophage culture fluid, whereas the response to GLT strictly required histocompatible macrophages. [The lack of macrophage MHC identity for the response to DNP-hemocyanin was also found in the analysis of the antibody formation *in vitro* (Katz and Unanue, 1973), to be considered in Section II,C.] This is an important point because it raises the issue of whether interactions with macrophages bearing the appropriate Ia products are essential or whether T cells immunized in a syngeneic environment can recognize allogeneic *I*-region products. It is most likely that the response to complex, multideterminant antigens is strictly MHC restricted and that the explanation for the results of Rosenthal and Rosenwasser lies with the number of contaminating phagocytes in their preparation. Their preparation of lymph node lymphocytes still contained a small, yet perhaps significant, number of phagocytes (approximately 0.1–0.5%), sufficient to give a response to PHA. It is now known that complete depletion of macrophages from the T-cell preparations impairs lectin-induced proliferation (Section II,B). We have evaluated this issue ourselves, examining the proliferative response to hemocyanin but employing a more elaborate procedure for depleting macrophages. Under conditions of complete depletion of phagocytes, the proliferative response of lymph node T cells to hemocyanin was found to be dependent strictly on live

phagocytes and to require *H-2* homology between the phagocytes and the T cells; the restrictions mapped at the left-hand side of the *H-2* (*K* to *I-B*); furthermore, the macrophage requirement could not be replaced by a macrophage-conditioned medium (Kammer and Unanue, 1980). The conclusion, therefore, is that, in the mouse as in the guinea pig, the requirement for MHC restrictions at the *I* region for macrophage–T cell interaction is absolute. Of importance is that a very small number of antigen-presenting macrophages, by themselves insufficient to promote antigen-dependent T-cell growth, can function in collaboration with macrophage-derived products. This point requires further elaboration but is inescapable from the analysis of the data described above.

The involvement of *I* region in the macrophage presentation of antigens to T cells has been explored in depth with protein antigens of well-defined structure. These results are considered in Section III,B. Noteworthy have been the studies on insulin (Barcinski and Rosenthal, 1977; Rosenthal *et al.*, 1977; Rosenwasser and Rosenthal, 1979a,b), on human fibrinopeptide B (Thomas *et al.*, 1979a,b), and on myoglobin (Richman *et al.*, 1979).

The few studies carried out in man have indicated the requirements for histocompatible macrophages matched at the HLA-D region (Rodey *et al.*, 1979; Bergholtz and Thorsby, 1978).

2. *Ia-Bearing Phagocytes*

Only a certain percentage of phagocytes bear Ia molecules on their membrane (Section III,A). These Ia-bearing cells are responsible for most macrophage–lymphocyte interactions, including the proliferation of T cells reviewed in this section. Killing the Ia-bearing set of accessory cells with anti-Ia and serum as a complement source results in an impairment of antigen-stimulated T-cell proliferation. The studies have been made on the peritoneal macrophages of the guinea pig (Yamashita and Shevach, 1977) and mouse (Farr *et al.*, 1979a; Kammer and Unanue, 1980) and on the accessory cells of the spleen (Cowing *et al.*, 1978a; Schwartz *et al.*, 1978). A recent report of Lee *et al.* (1979), claimed that Ia-negative macrophages (i.e., the population remaining after killing with anti-Ia) could interact with T cells, but only in conditions where soluble antigen was added to the culture during the entire time. A soluble mediator substituted for the macrophages. My interpretation of this result is that Lee's T-cell population contain residual macrophages—as evidenced by the high response to soluble antigen in the *absence* of macrophages—and that the experiments indicate a cooperation between the T cells and the few residual mac-

rophages with other macrophages or their products. This is a situation akin to that discussed before in the study of Rosenwasser and Rosenthal.

Limited studies have been made on antigen-induced T-cell proliferation in man as concerns the requirement of Ia-bearing macrophages. The results thus far reported indicate that removal of macrophages by killing them with anti-HLA-D antibodies or with heterologous antibodies to the *I*-region product of man abrogated antigen-induced T-cell proliferation (Rodey *et al.*, 1979; Bergholtz and Thorsby, 1978; Breard *et al.*, 1979; Raff *et al.*, 1980; Geha *et al.*, 1979).

In summary, the studies of antigen-induced T-cell proliferation have been extensive and have clearly indicated the key functional role of the macrophage in antigen presentation. In my opinion, these studies have established that (*a*) T cells will not grow in culture upon antigen stimulation in the absence of phagocytes; (*b*) phagocytes are required as an essential antigen-presenting cell; (*c*) the *I* region regulates the interaction between the T cell and the macrophage; (*d*) the phagocytes interacting with the T cell must express Ia antigens on their membranes; (*e*) the *I*-region involvement in macrophage interaction applies to all antigens, even those under no obvious Ir gene control; and (*f*) the interaction between the T cell and the appropriate macrophage selects and maintains the life of the antigen–Ia-reactive T-cell clone.

B. Interactions with T Cells in Lectin-Induced Proliferation

The response of T lymphocytes to the lectins phytohemagglutinin (PHA) and concanavalin A (Con A) requires the presence of adherent phagocytic cells. Some of the early reports examining lymphocyte populations depleted of phagocytes failed to find an impairment of the proliferative response to lectins (for example, Hersh and Harris, 1968; Waldron *et al.*, 1973). Rosenstreich and associates (1976), however, made a detailed study of the response of lymph node lymphocytes to PHA and found it to be macrophage dependent. The response was not affected by depleting macrophages by a single passage of lymphoid cells through columns containing nylon wool and glass beads, although the same cells failed to respond to the antigen DNP–ovalbumin to which the guinea pigs were immunized. A second passage of cells through a nylon-wool column resulted in a complete lack of responsiveness to PHA. Addition of purified peritoneal macrophages fully reconstituted the response. Lipsky, Ellner, and Rosenthal confirmed their results (Lipsky *et al.*, 1976; Ellner *et al.*, 1976). In their hands, passage of the lymph node cells twice through nylon wool

resulted in practically a complete depletion of phagocytes (less than one per one thousand). Lipsky *et al.* (1976), made the point that the culture conditions were critical to show the accessory cell dependence. Those situations that favored cell clustering, like high density and round-bottom culture wells, required more stringent macrophage depletion in contrast to having cells at a lower cell density in flat-bottom well dishes. A similar point had been made previously by others analyzing antibody formation *in vitro* (for example, Theis and Thorbecke, 1970). Similar accessory cell dependency of the lectin proliferative response of T cells was later found with murine cells (Habu and Raff, 1977; Ahman *et al.*, 1978b; Rosenwasser and Rosenthal, 1978a; Andersson *et al.*, 1979; Kammer and Unanue, 1980; Larsson *et al.*, 1980a; Gronvik and Andersson, 1980) and human cells (Oppenheim *et al.*, 1968; Levis and Robbins, 1970; Lohrman *et al.*, 1974; Hedfords *et al.*, 1975; Schmidtke and Hatfield, 1976; Taniguchi *et al.*, 1977; Arala Chaves *et al.*, 1978; de Vries *et al.* 1979).

In contrast to the antigen-dependent proliferation, the lectin-proliferative response was reconstituted with syngeneic or allogeneic macrophages (Lipsky *et al.*, 1976; Habu and Raff, 1977; Rosenstreich and Mizel, 1978; Kammer and Unanue, 1980) or even xenogeneic macrophages (Schmidtke and Hatfield, 1976). Interestingly, the reconstitution with phagocytes and other accessory cells was best with the population of macrophages bearing Ia. Thus, Habu and Raff (1977) found that killing the peritoneal macrophage population by exposure to anti-Ia plus fresh serum—as a complement source—markedly impaired their capacity to cooperate with the T cell stimulated by Con A. In our own studies, macrophage populations depleted of the Ia-bearing population did reconstitute the response, although less efficiently than the entire untreated cells (Kammer and Unanue, 1980). Addition of fibroblasts to the culture reconstituted the response of guinea pig T cells but only partially (Lipsky *et al.*, 1976). Habu and Raff (1977) fully reconstituted the response with mouse embryo fibroblasts but not with 3T3 cells. This finding contrasts with the results showing requirements for Ia-positive macrophages—whether the embryo cells bore Ia or contained macrophages was not studied.

The issue of the requirement for an Ia-bearing cell was analyzed in a different way by Ahman *et al.* (1978b). They confirmed the results that the response to Con A was affected by killing the entire unfractionated cells with anti-Ia (and complement) (Niederhuber *et al.*, 1976; Frelinger, 1977; Ahman *et al.*, 1978a; also Larsson *et al.*, 1980a). Such treated cells, however, could proliferate when a preparation of Ia-bearing spleen accessory cells was added to the culture. The accessory

cells were adherent to glass, lacked T-cell determinants, and contained two *I*-region determinants, one mapped to *I-A*, the other mapped from *I-B* to *I-E/C*. The accessory cell lacking Ia reconstituted the response but not as effectively, although, in their studies, a complete titration of each cell was not shown. Interestingly, the response to PHA was not found to be sensitive to treatment with anti-Ia and complement, implying that the Ia-bearing phagocytes were not an essential reconstituting cell. Direct examination of this point with regard to the PHA response has not been made.

A most interesting result with regard to the role of the macrophage in the lectin response was made by the studies of Rosenstreich *et al.* First, they found that 2-mercaptoethanol, an agent believed to replace macrophages in culture (Section II,C) would not replace the need for macrophages, a point confirmed by others (e.g., Ellner *et al.*, 1976; Rosenwasser and Rosenthal, 1978a; de Vries *et al.*, 1979; Kammer and Unanue, 1980). However, mercaptoethanol enhanced the activity of very small numbers of macrophages added to the culture (Rosenstreich and Mizel, 1978): macrophage-depleted T cells responded marginally to PHA if reconstituted with 1% macrophages, yet 1% macrophages in the presence of mercaptoethanol resulted in a very strong DNA synthesis by the T cells (about 35,000 cpm vs. 3000, respectively) (Fig. 2). Noting the lack of effect by mercaptoethanol, Rosenstreich *et al.*, carried out experiments in which the separated T cells or macrophages were pulsed with PHA briefly and then cocultured. Under both circumstances, a strong proliferative response was obtained. The macrophages had to be live and metabolically active. Thus, macrophages could "present" PHA to the lymphocytes; and, alternatively, the T cells, after binding PHA, could interact effectively with the macrophages (Rosenstreich *et al.*, 1976; Rosenstreich and Mizel, 1978).

Further analysis by Rosenstreich and associates indicated that a soluble mediator was involved in the interaction between macrophages and lymphocytes and PHA. Separating macrophages and the PHA-treated lymphocytes by a cell-impermeable membrane resulted in lymphocyte proliferation to about 60% of that found when both cells were mixed. Furthermore, a soluble molecule, named TAF or T cell-activating molecule, released from macrophage cultures replaced the physical need for macrophages. The reconstitution of the lectin-induced response by a macrophage-conditioned medium has now been found by others (for example, Rosenwasser and Rosenthal, 1978a; de Vries *et al.*, 1979; Larsson *et al.*, Kammer and Unanue, 1980; Smith, 1980). The response of T cells to Con A is believed to involve growth factors released by T cells themselves upon interaction with mac-

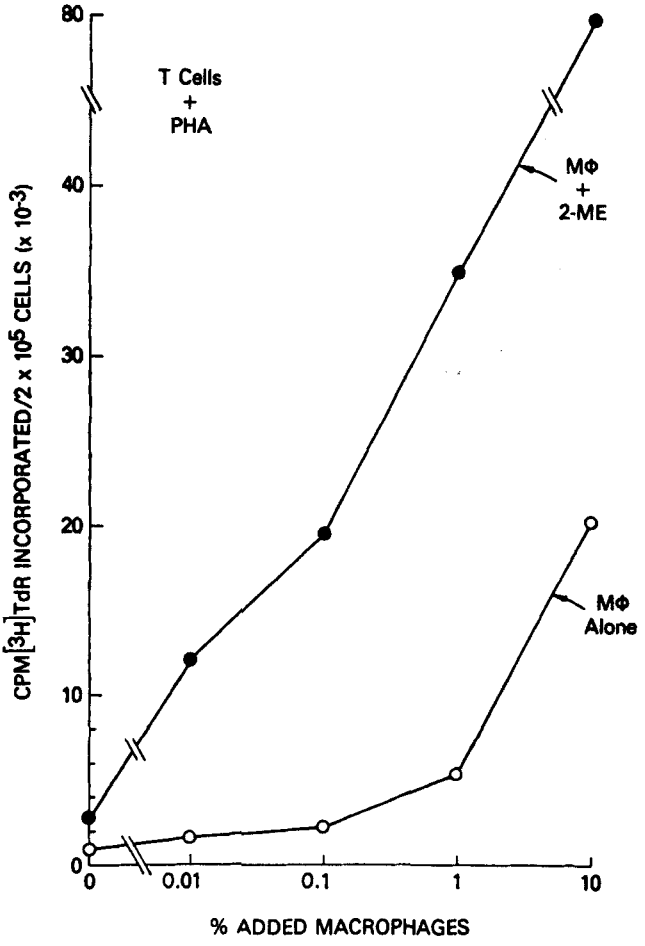

FIG. 2. The figure shows the effects of mercaptoethanol in potentiation of accessory function of macrophages. T cells were incubated with macrophages (Mϕ) and phytohemagglutinin (PHA) in the presence or the absence of 2-mercaptoethanol. [From Rosenstreich and Mizel (1978).]

rophages. The relationship between the T-cell growth factor and the macrophage products is discussed at more length in Section III,C.

Another polyclonal T-cell response that is strictly macrophage dependent is that induced by generation of aldehyde from cell-surface glycoproteins. Gentle treatment of cells with sodium metaperiodate or sequential treatment with neuraminidase and galactose oxidase induced vigorous T cell proliferation (Novogrodsky and Katchalski, 1972). The T-cell proliferation required macrophages. Actually, mac-

rophages or T cells could be treated with the chemicals and then mixed with the other untreated cells and still generate a proliferative response (Greineder and Rosenthal, 1975b). The response was not MHC restricted inasmuch as it was generated by allogeneic macrophages, but it clearly involved the surface Ia; anti-Ia antibodies directed to the allogeneic macrophages blocked the response (Greineder *et al.*, 1976). Clusters of macrophages and T cells were abundant in the stimulated cultures.

In essence, the polyclonal responses to lectins or to aldehyde generation were found to require macrophages either syngeneic or allogeneic with the T cells. An involvement of the *I* region was not found for the PHA response but was evident for the Con A response. Ia-positive macrophages were better in reconstituting the Con A response of the T cells, yet Ia-negative cells also had an effect. The involvement of the *I* region was well shown in the response to aldehydes by the inhibitory effect of antibodies. The involvement of macrophages in the lectin response involved the release of lymphostimulatory molecules (Section III,C). The nature of the involvement of the *I* region products in the T cell–macrophage interactions induced by lectins or aldehydes is not understood. The results suggest that macrophage–T cell contact induced by nonantigenic stimuli may result in interactions with the Ia antigens that generate the "signals" required for macrophage and T-cell stimulation.

C. Interactions with T and B Cells in Antibody Formation

The requirements for phagocytes in the immune response to thymus-dependent antigens have been examined extensively in a number of experimental systems. These systems vary from *in vivo* cell transfer using live macrophages with bound antigen, to cell culture assays, to approaches combining both *in vivo* and cell culture techniques. All the results indicate that macrophage-associated antigen is highly effective in promoting T-B cell collaboration; moreover, in many instances it appears essential for antigen to be bound to phagocytes for T-B cell interactions to develop. The issue of an essential role for phagocytes, however, cannot be generalized to all experimental systems.

The first series of experiments using live macrophages in a transfer system established that macrophage-bound antigen was highly efficient in inducing an antibody response (Unanue, 1972). All the antigens tested were protein antigens now known to be thymus-dependent. Thymectomized mice did not respond to transfer of an antigen like hemocyanin bound to live macrophages, indicating that

macrophage-bound antigen did not bypass the need for T cells and that both T and B cells interacted with the cell-bound immunogen (Unanue, 1970).

1. Hapten–Protein Conjugates

The interactions between T and B cells with macrophage-bound antigens were further analyzed using hapten–protein conjugates. One approach, taken by Kunin *et al.* (1972), was to immunize mice with live thioglycolate-induced macrophages previously pulsed with rabbit albumin and then to assay the spleen cells in cell cultures for the response to DNP-rabbit albumin. The presence of T cells immune to the carrier resulted in an enhanced anti-DNP response. Their results indicated an excellent generation of carrier-primed T cells by immunization with the macrophage-bound antigen. The experimental system was then manipulated using X-irradiated mice that received mixtures of thymus, bone marrow cells, and antigen-bearing macrophages. Excellent carrier-primed cells were found in spleens of X-irradiated mice injected simultaneously with thymocytes and macrophages, followed 8 days later with bone marrow cells; the reverse, however, giving bone marrow cells with macrophages first, followed 8 days later with the thymocytes, did not result in induction of carrier-specific T cells. Kunin *et al.* concluded that carrier priming developed only following T cell–macrophage interaction. A more detailed quantitative approach was followed by Klaus (1974) using the classical thymus–bone marrow Claman-type transfer system. Helper T cells were generated by transferring cortisone-resistant thymocytes into X-irradiated mice, together with soluble hemocyanin or hemocyanin-bearing live macrophages; 5 days later, the helper T-cell activity in the spleen was assayed by cotransferring them together with bone marrow cells and then measuring the anti-DNP response. The macrophage-associated hemocyanin was several thousandfold more immunogenic than soluble hemocyanin in inducing carrier-primed cells *in vivo*, in accordance with earlier studies of the antibody response to the whole molecule (Unanue and Askonas, 1968a). Askonas and Roelants (1974) extended this type of observation, noting that the number of different anti-DNP B cell clones generated *in vivo* was much larger upon immunization with a DNP-protein bound to live macrophages.

David Katz and I (1973) analyzed the response to DNP proteins using the Mishell–Dutton systems, employing spleen cells from immunized mice. We found that DNP-hemocyanin bound to macrophages was about 10,000-fold more immunogenic than a similar amount of soluble or dish-bound protein. Whether DNP-hemocyanin

was bound to macrophages directly or as part of an immune complex made no difference. One interesting result was found when adding soluble antigen together with macrophage-bound antigen to the T-B cell mixtures. In this situation, the response to macrophage-bound antigen was significantly reduced. A similar result had been previously reported in *in vivo* experiments (Spitznagel and Allison, 1971). The results imply that there are two forms of antigen that compete with each other, the macrophage-bound and the soluble antigen. Although not explored, it is possible that the soluble antigen acted by triggering suppressor T cells (Ishizaka and Adachi, 1976; Pierres and Germain, 1978).

Using hapten-conjugated proteins associated with macrophages, it was questioned whether both the haptenic and carrier protein had to be linked together in the same molecule or could be placed separately either in the same macrophages or in different ones. To do this, Askonas and Roelants (1974) transferred, *in vivo*, DNP-bovine γ-globulin-primed spleen cells with mixtures of macrophages, one bearing bovine γ-globulin and the second DNP-hemocyanin, and then assayed for the anti-DNP response. Although the control group challenged with macrophages bearing DNP-bovine γ-globulin gave excellent responses, the mixtures of macrophages was very weak. We analyzed this point ourselves by placing the hapten bound to the unrelated carrier protein (in our case, DNP-γ-globulin) and the immunizing protein (hemocyanin), both on the same macrophage (Unanue and Katz, 1973). Using radioactive proteins, we were able to estimate that the amounts of both proteins bound to the macrophage were similar to the amount of DNP-hemocyanin bound to macrophages. The results indicated that lymphocytes would respond weakly to the unlinked hapten and carrier determinants on the same macrophage, but strongly if the determinants are bound in the same molecule (Fig. 3).

2. *Depletion of Accessory Cells*

A different approach from testing the immunogenicity of macrophage-bound antigen is to examine cultures for antibody formation in the absence or the presence of accessory cell, i.e., to ask how essential the accessory cells are for the B-T cell interactions to take place. An early approach was to deplete accessory cells, usually from spleen, on the basis of their adhesion to culture dishes. Mosier's first report (1967, 1969) showing that depletion of adherent cells blunted the antibody response to SRBC, was rapidly confirmed (Roseman, 1969; Pierce and Benacerraf, 1969; Pierce, 1969; Haskill *et al.*, 1970; Hartmann *et al.*, 1970; Shortman *et al.*, 1970; Feldmann and Palmer,

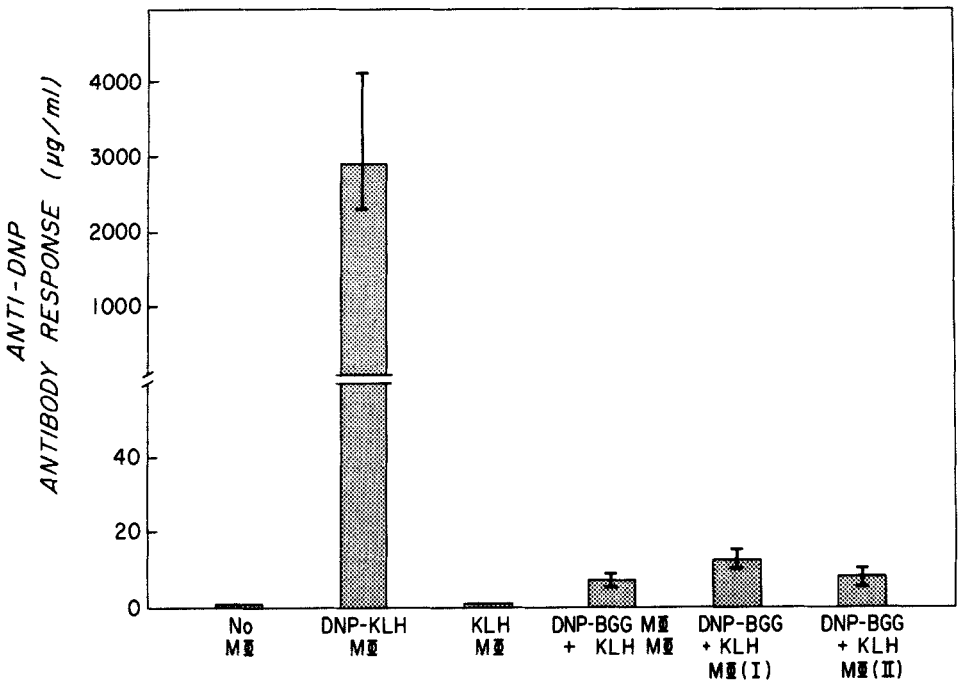

FIG. 3. This experiment shows the immunogenicity of hapten-proteins bound to macrophages. BALB/c mice received 25 × 10^6 DNP-hemocyanin-primed spleen cells with or without 12 × 10^6 macrophages. Mice were bled for anti-DNP antibody a week later. DNP-KLH MΦ: macrophages containing 3.1 μg of DNP-hemocyanin; KLH MΦ: macrophages containing 12.1 μg of hemocyanin; DNP-BGG MΦ + KLH MΦ: received simultaneously one set of macrophages containing 3.7 μg of DNP-bovine γ-globulin and another set containing 13.2 μg of hemocyanin; DNP-BGG + KLH MΦ(I): both antigens were bound to the macrophages (2.7 μg and 9.7 μg, respectively); DNP-BGG + KLH MΦ(II): as I but with 0.2 μg of bovine γ-globulin and 16.4 μg of hemocyanin. The results indicate that the optimal immune response to macrophage-bound antigen was induced by the haptenic determinant linked to the carrier molecules. [From Unanue and Katz (1973).]

1971; Shortman and Palmer, 1971; Cosenza and Leserman, 1971; Feldmann, 1972; Sjoberg *et al.*, 1972; Chen and Hirsh, 1972a,b; Moller *et al.*, 1976; Lee *et al.*, 1976; Niederhuber, 1978, 1980; Niederhuber and Allen, 1979; Wong and Hercowitz, 1979).

The response of SRBC has been the most frequently analyzed, probably because of its ease of production and analysis in culture. More recently, however, primary antibody responses in culture have been developed to protein antigens such as TNP conjugated to either synthetic polypeptides, like (TG)-AL or (HG)-AL, or to proteins like

hemocyanin. These primary responses were found to be strictly dependent on accessory cells (Hodes and Singer, 1977; Singer *et al.*, 1978; Hodes *et al.*, 1978). Likewise, the development of helper T cells in culture, to be considered below, was strictly macrophage dependent (Erb and Feldmann, 1975a,b; McDougal and Gordon, 1977). Responses to synthetic polypeptides like GAT are also macrophage dependent (Pierce *et al.*, 1974).

The extent to which the T-B cell interactions leading to antibody formation in culture are accessory cell dependent becomes specially relevant in two circumstances: one is the secondary antibody response and the second with the use of 2-mercaptoethanol (2-ME).

Pierce's study (1969) showed that, while the primary response of cultured spleen cells to SRBC was easily abrogated by depletion of accessory cells, this was not the case for a secondary response. As early as 3 days after priming, no more than 50% depletion was possible. The same spleen from a mouse immune to SRBC would still respond after depletion of adherent cells but would not respond to burro red cells—a non-cross-reactive antigen. Pierce acknowledged that his method of depletion—by adherence—still left behind up to 1% phagocytes. Reports on the anti-DNP-response using spleen cells from mice immune to DNP-hemocyanin (Katz and Unanue, 1973) or DNP-$\phi\chi$ (Bluestein and Pierce, 1973a, 1973b) also indicated significant responses following depletion of accessory cells by the adhesion method of Mosier. In contrast, other studies using more stringent methods of depletion of accessory cells indicated that the secondary responses were markedly reduced—85 to 95%—by removal of accessory cells. These removed accessory cells by dish adherence method (Lamvik, 1969; Theis and Thorbecke, 1969; Radcliffe and Axelrod, 1971), by the use of glass bead columns (Feldmann and Palmer, 1971), anti-macrophage antibodies (Feldmann and Palmer, 1971), iron carbonyl (Sjoberg *et al.*, 1971), or the Sephadex G-10 method (Ly and Mishell, 1974). The latter method has proved to be a simple, fast, and reproducible one for eliminating accessory cells.

3. *Studies with 2-Mercaptoethanol*

This reducing agent is a promoter of lymphocyte function and has been discussed as a possible substitute for macrophages in culture. Although the effects of thiols have been examined in a number of situations, some of which we have alluded to before, their actions have been particularly emphasized and discussed in the context of antibody formation *in vitro*. Early reports described that addition of 2-ME to cultures enhanced the proliferative response of lymphocytes to ligands

(Fanger *et al.*, 1970) and to allogeneic cells (Heber-Katz and Click, 1972; Bevan *et al.*, 1974); in general, 2-ME potentiated the function of all immunological cells *in vitro*, varying from antibody responses (Click *et al.*, 1972), to antigen-driven T-cell responses (Section I,A), to cytolytic T-cell responses (Cerottini *et al.*, 1974; Harris *et al.*, 1976; Igarashi *et al.*, 1977), to the growth of B lymphocyte colonies (Metcalf, 1976). Click and associates (1972) were the first to examine the effects of 2-ME on the antibody response to SRBC using the method of Mishell and Dutton. They found that addition of 2-ME, at an optimal dose of $2 \times 10^{-5} M$, resulted in a marked increase in the plaque-forming cell response of *unfractionated* spleen cells. This increase was clearly not associated with a promotion of cell viability. Because the reducing agent did not need to be added daily to the culture, Click *et al.* postulated that most likely it was affecting very early events; the half-life of the reducing agent was expected to be short. Kinetic study disclosed that the development of plaque-forming cells, in cultures with 2-ME, progressed at an exponential rate earlier and for longer periods than in cultures lacking the reducing agent.

Chen and Hirsch were the first to examine the action of 2-ME on the plaque-forming cell response of spleen cells depleted of adherent cells (1972a,b). They confirmed that depletion of adherent cells would result in marked reduction of the antibody response and that addition of peritoneal macrophages would reconstitute it. Their experiments indicated that addition of 2-ME resulted in a marked improvement of the response of the accessory cell-depleted spleens and that the 2-ME had to be present for at least 72 hours in cultures. They also found a marked improvement of cell viability by addition of the thiol; while nonadherent cells showed a poor recovery, about 3% of initial input recovered after 4 days, the addition to them of macrophages or 2-ME resulted in about 30% recovery. A logical conclusion was that at least one effect of 2-ME must be through its improvement of cell survival. Although there is no major disagreement with this point (Pierce *et al.*, 1974), the issue is that 2-ME clearly had other major effects that resulted in an increase in cell growth. The results of Click *et al.* are not contradictory to those of Chen and Hirsch inasmuch as the former used unfractionated spleen cells; in Click's experiments, it was possible to observe a marked enhancement of a response by 2-ME without a marked effect on cell viability.

The critical point in Chen and Hirsch's work is the question of whether the response of T and B cells to SRBC in the presence of 2-ME is truly macrophage independent. This issue becomes critical with regard to an essential role of a macrophage–antigen-presenting

step. Chen and Hirsch faced this question attempting to enumerate macrophages in the spleens in various experimental manipulations. Their depletion method used adherence to plastic. Cognizant of the difficulties in enumerating macrophages, they proceeded to test for their presence by counting adherent cells, using either phagocytosis as an index or by the appearance of differentiated macrophages in cultures. In their estimates, the cultures of 5×10^6 nonadherent spleen cells contained from 500 to 2000 phagocytes, and a similar number after addition of 2-ME. Another 24 hours of culture in dishes, or 48 hours—the second 24 hours with 2-ME—resulted in further depletion to the extent of 220 macrophages per culture, respectively. In all instances, the antibody response to SRBC cells with 2-ME in the culture was the same regardless of the number of residual macrophages. Hence, Chen and Hirsch concluded that they had no evidence that the reducing agent was acting by increasing macrophage development in culture and that most likely its effect was by improving culture conditions. Other groups have confirmed these observations again using the SRBC response as the test antigen. Particularly noteworthy are the reports by Opitz and associates, who, by depleting macrophages using iron carbonyl or silica, completely abrogated the SRBC response. The impaired response was fully reconstituted by 2-ME (Lemke and Opitz, 1976; Opitz *et al.*, 1977a,b).

In contrast to these results, other investigators have failed to reconstitute macrophage-depleted lymphoid cells by 2-ME. Ly and Mishell (1974), using their method of depletion by G-10, obtained inconsistent results. Hodes and Singer's (1977) studies on the primary response to TNP-proteins, in which accessory cells were depleted, also by G-10 columns, showed strict dependence on accessory cells without any effect whatsoever of 2-ME. Similar results were obtained by Erb and Feldman (1975a,b,c). The proliferative responses of T cells to antigen or lectins discussed previously were strictly macrophage dependent, and these, again, could not be replaced by 2-ME (Section II,A). The study of Rosenstreich and Mizel (1978), mentioned in Section II,B, is worth repeating in this context. Their results indicated that 2-ME synergized with macrophages to increase the response.

Two final points with regard to thiols are worth discussing before making some final conclusions on their use and significance. How are the thiols acting on the cell? Do they have a direct effect, or is it through a secondary product?

Broome and Jeng (1974) made a very thorough analysis of the effect of various thiols on the growth of normal spleen cells and various lymphoid tumor lines. Normal spleen cells and 13 of 22 tumor lines

had increased growth in cultures when various thiols were added. In contrast, other cell lines—HeLa or fibroblasts, for example—were not influenced. There is general agreement now, from their studies, the early reports referred to before, and from recent publications, that thiols are very important growth potentiators for lymphocytes. These include the normal basal proliferation of lymphocytes (Lemke and Opitz, 1976; Goodman and Weigle, 1977), the growth of T cells upon lectin stimulation (for example, Lemke and Opitz, 1976; Rosenstreich and Mizel, 1978), or of B cells in response to anti-immunoglobulin antibodies (Sidman and Unanue, 1978, 1979; Sieckmann *et al.*, 1978), or to endotoxin (Lemke and Opitz, 1976). Other lymphoid tumor lines have also been shown to require 2-ME for growth (Hewlett *et al.*, 1977, 1979).

Broome and Jeng in their study went on to test various thiols trying to establish a functional–structural relationship. Various thiols and their disulfides promoted growth, in decreasing order of activity—for example, thioglycerol, the most potent of all, 2-ME mercaptopropionic acid, and cysteamine. Several substitutions of the basic thioethane compound resulted in changes, but the relationship of these with the growth potential was not found. For example, addition of a methyl group to the primary carbon did not affect the growth activity, but that of a polar group destroyed it. For more detailed analysis of these points, their paper should be consulted.

Are the thiols acting directly on the cell? The reducing activity of the thiols was brief—a few hours (Broome and Jeng, 1973); therefore, the expectation was that, if the compounds acted directly on the lymphocyte, they should do it during the first few hours of culture. Chen and Hirsch (1972b), however, found that medium containing 10% fetal calf serum treated with 2-ME retained its activity for promoting antibody formation for at least 3 days and suggested possible secondary effects. That the thiols might have direct effects was suggested by experiments in which 2-ME was added to cells in the absence of proteins; nevertheless the optimal growth potential required the addition of serum to the media (Lemke and Opitz, 1976).

Regardless of whether thiols directly act on a cell component, one way in which they promote growth is by the generation of an active molecule from plasma (Opitz *et al.*, 1977a,b; Hewlett *et al.*, 1979; Sidman and Unanue, 1978, 1979). The initial studies of Opitz *et al.* indicated that the fetal calf serum used for culturing lymphocytes, when treated with 2-ME, generated an active molecule about the size of ovalbumin. This molecule substituted for 2-ME in the culture and supported antibody formation to SRBC. The 2-ME-treated serum pro-

tein had to be added within 24 hours of culture and kept throughout the entire period. Our studies indicated that serum treated with 2-ME and then alkylated with iodoacetamide generated a 65,000-MW molecule that migrated electrophoretically like albumin and promoted growth of B cells and T cells (Sidman and Unanue, 1978; also in Braun and Unanue, 1980). The active moiety appeared, therefore, to be closely related to albumin. The later studies of Opitz *et al.* supported this conclusion (Hewlett *et al.*, 1979; Opitz *et al.*, 1978, 1980): Using affinity chromatography with Affi-Gel Blue columns, the 2-ME-generated moiety behaved exactly like albumin; furthermore, purified bovine albumin treated with 2-ME also generated a growth-promoting activity. The biochemical basis for this effect has not been established. Last, it should be noted that incubation of macrophages with fetal calf serum did not generate the active moiety.

In essence, the thiols have been clearly shown to promote growth of all classes of lymphocytes. The issue of whether these compounds substitute for macrophages is not entirely resolved, in part, because of the difficulties in eliminating all phagocytes from heterogeneous cell populations. Taking the overall evidence from analysis of T cell–antigen interactions or the T-B cell responses, particularly to protein antigens, one tends to favor the explanation that the antigen-presenting function of macrophages cannot be replaced by thiols. The thiols increase lymphocyte activity in cultures and, therefore, may replace a growth-maintenance function of macrophages yet may not be able to bypass the essential function of antigen presentation provided by the macrophage. It is possible, however, that in the presence of an agent like 2-ME the antigen-presenting function may be done by the B cell during its handling of antigen, particularly if these B cells are in high frequency, as in primed animals. This applies particularly to the SRBC response. Some direct evidence for B-cell presentation of antigen was presented by Kammer and Unanue (1980) but awaits further studies (see below).

4. Induction of Helper Cells

Continuing the analysis of T-B cell–macrophage interactions in culture, I will now review experiments attempting to analyze and dissect the interaction. Erb, Feldman, and their associates developed a two-stage culture system for the generation of carrier-primed helper T cells (Erb and Feldman, 1975a,b,c,d; Erb *et al.*, 1976, 1979a,b). First, T cells were cultured for 4 days with a protein antigen, resulting in the production of helper cells; the function of the helper T cell was assayed in a second culture by mixing the T cells with unprimed B cells

and the appropriate hapten–protein conjugate, later measuring the anti-hapten response. The anti-hapten response—usually to DNP—was measured by a plaque-forming assay.

Carrier-specific helper T cells were generated, inducing a small to modest number of plaque-forming cells with rather large experimental variations. Nevertheless, the results in the various publications appeared to be highly consistent. The generation of helper T cells to hemocyanin or to (TG)-AL required the presence of accessory phagocytic cells in the culture. The 2-ME did not replace the need for macrophages. Macrophages pulsed with the antigen were also highly efficient in generating helper T cells. McDougal and Gordon (1977) developed a similar system. In their experiments, cortisone-resistant thymocytes developed into helper T cells upon culture with hemocyanin or fowl γ-globulin. An excellent dose-response relationship was obtained, depending on the number of macrophages added to the culture. The primed T cells also generated an important nonspecific helper effect (assayed by challenging with DNP on an unrelated carrier protein) that was most pronounced with high numbers of T cells.

Erb and Feldman made a number of observations regarding the role of the macrophage in the generation of helper T cells. Marked differences were found between the response to hemocyanin added to the T cell–macrophage cultures either in soluble form or bound to Sepharose 2B particles. Thus, macrophages syngeneic with the T cells were required for the response to soluble hemocyanin—or the synthetic polypeptide (TG)-A-L (Erb and Feldman, 1975a,b). By mixing syngeneic and allogeneic macrophages, it was found that the lack of response by allogeneic macrophages could not be attributed to a suppressor effect. Furthermore, using strains of congenic mice with intra-H-2 recombinations, it was possible to map the locus controlling the interactions to the *I-A* region of *H-2* (Erb and Feldman, 1975c). The response to hemocyanin-Sepharose also required macrophages yet could be partially replaced by 2-ME and, in contrast to the response to soluble hemocyanin, allogeneic macrophages were effective. The implications were that *I*-region restrictions did not take place with particulate antigens.

A second point explored by Erb and Feldman concerned the requirements for macrophage–T cell contact. In their first experiment (1975a), helper T cells were generated in a double-chamber culture where the macrophages were separated by a 0.2-μm pore filter. This suggested that macrophages operated by releasing a soluble molecule. Direct examination for this material was done by culturing mac-

rophages with the antigens for 4 days and then using the conditioned media to culture the T cells. Culture fluids from syngeneic, but not from allogeneic, macrophages generated antigen-specific T helper cells to soluble proteins when added at ranges from 1 to 10% (volume/volume). This "genetically restricted factor" could be removed from solution by an immunoabsorbent column containing anti-I-A antibodies, but not by one containing anti-Ig antibodies. The conclusion reached by Erb and Feldman was that the macrophage released a molecule with Ia determinants and antigen-binding specificity. A different molecule was formed in cultures of macrophages that were cultured with hemocyanin, or without, that could generate helper T cells to Sepharose–hemocyanin, without any *H-2* restrictions.

Finally, a brief comment on the differences found by Erb and Feldman between soluble and particulate-bound hemocyanin. This lack of *I*-region restriction with particulate antigens cannot be generalized because it was not found for SRBC (Kappler and Marrack, 1976; Sprent, 1978a,b,c; Niederhuber *et al.*, 1979) or for bacteria (Farr *et al.*, 1979a). One possible explanation is that the initial T-cell culture contained sufficient macrophages to serve as antigen-presenting cells, but only in cooperation with the growth factor. This bead-bound hemocyanin is very likely to interact much more efficiently with any few residual macrophages than the soluble antigen.

Important experiments to discuss are those of Ishizaka and Adachi (1976), who examined the generation of helper T cells *in vitro*. They found a major difference if the T cells were incubated with macrophage-bound antigen or with soluble antigen in the absence of macrophages. Normal spleen cells were cultured on ovalbumin bound to syngeneic peritoneal macrophages for 5 days, after which their function as helper cells was assayed by *in vivo* adoptive transfer; the T cells were inoculated with DNP-hemocyanin-primed B cells into X-irradiated mice, which were boosted with DNP-ovalbumin and then assayed for the IgG and IgE anti-DNP response. T cells cultured with macrophages developed helper T cell function. In contrast, when the lymphocytes were incubated with 10 μg of ovalbumin per milliliter in the absence of macrophages, no helper cells developed, but instead T cell suppressors that blocked the response to ovalbumin of normal mice. The results were found with both the IgG and IgE response. This is an important experiment that strongly supports that the mode of antigen presentation is a critical step in immune induction. It brings out good evidence that antigen not presented in the macrophage stimulates preferentially the suppressive pathway. The generation of

suppression *in vivo* by high doses of antigen may well be explained by a bypass of the macrophage requirement. Another experiment supporting Ishizaka's result is that of Pierres and Germain (1978), to be discussed later.

5. *MHC Restrictions*

The *I*-region-dependent regulation of macrophage–B and T cell interactions was examined in a number of systems, all of which indicated that Ia-bearing phagocytes matching at the *I* region with the T cells were required for the interaction. For example, Hodes and Singer and their associates studied extensively the primary *in vitro* responses of mixtures of T and B cells to TNP-(TG)-AL or TNP-hemocyanin (Hodes and Singer, 1977; Singer *et al.*, 1977, 1978, 1979; Hodes *et al.*, 1978, 1979). The response was strongly dependent on phagocytic accessory cells identified with the use of a cytotoxic assay as bearing both I-A and I-E/I-C determinants (Hodes *et al.*, 1978; Dickler *et al.*, 1980). The response to both proteins required that T cells and macrophages share the *I-A* region of *H-2* (Singer *et al.*, 1977, 1978).

Kappler and Marrack's laboratory, with a somewhat different approach, reached the same conclusions (Kappler and Marrack, 1976, 1978). In their system, F_1 mice from the combination (C57BL/6 × DBA/2) were inoculated *in vivo* with hemocyanin-pulsed macrophages from either of the parental strains; the T cells were subsequently isolated and cultured with B cells and macrophages from one or the other parents, together with haptenated hemocyanin; the antihapten response was measured by a plaque assay. The F_1 T cells cooperated only with the T cell–macrophage of the parental strain used for priming (Kappler and Marrack, 1976). In a subsequent study, the T cells reactive with the antigen were enriched in culture, by first placing them on a monolayer of macrophages bearing the antigen (Swierkosz *et al.*, 1978). Binding of T cells to macrophages took place if the macrophages were pulsed with the specific antigen. Furthermore, using the system of F_1 T cells primed *in vivo*, described earlier, it was possible to identify two populations of F_1 T cells, each binding to one or the other parental macrophages. The F_1 T cells isolated exhibited helper activity when tested with B cells and macrophages that had the same *K* through *I-A* region as the macrophages used for the isolation (Swierkosz *et al.*, 1979). The antigens used in the system were hemocyanin, (TG)-AL, and SRBC.

A third system of *I*-region-dependent macrophage–lymphocyte interaction is the primary SRBC response. Niederhuber's laboratory analyzed its macrophage requirement and found that macrophages

bearing Ia were essential (Niederhuber, 1978, 1980). The Ia-bearing macrophages contained antigenic determinants coded in *I-A*, *I-E/C*, and *I-J*. Niederhuber's most provocative observation concerned the effect of antibodies to the *I-J* subregion (Niederhuber *et al.*, 1979; Niederhuber and Allen, 1980). They found that a brief 30-minute treatment at 37°C of spleen macrophages with antibodies to *I-J* resulted in a complete loss of function when T-B cells were added; similar treatment with antibodies to I-A or I-E/C did not affect the macrophages.

The *in vitro* antibody response to the synthetic terpolymer of L-glutamic acid-L-alanine-L-tyrosine (GAT), an antigen under *Ir*-gene control, showed peculiar characteristics insofar as MHC restrictions that merit attention. The response to GAT was dependent on adherent accessory cells (Pierce *et al.*, 1974) and could be reconstituted with GAT pulsed to syngeneic or allogeneic macrophages (Pierce *et al.*, 1976). Furthermore, the removal of macrophages from the spleen cell suspension (of a "responder" mouse strain) resulted in the ready development of specific suppressor T cells (Pierres and Germain, 1978; Germain *et al.*, 1980) (Fig. 4). The suppressor cells were also found in unfractionated spleen cells by adding a large amount of GAT. (The suppressor T cells were assayed by their inhibition of the development of a primary anti-GAT response to syngeneic spleen cells.) This last result again depicts the balance between the development of helper and suppressor T cells, depending on the way in which antigen is introduced: suppression may be favored by antigens not presented in macrophages or presented—as in the case of an excess of soluble antigen—perhaps in the context of a "wrong" antigen-presenting cell.

Further analysis of the GAT system disclosed that, although no MHC restrictions were found insofar as macrophage presentation, these did take place in situations where the T and B cells came from mice primed *in vivo* with GAT-bearing macrophages. Thus, mice were primed *in vivo* with GAT bound to syngeneic or allogeneic macrophages, and their spleen cells were later examined in culture: response took place only with the GAT bound to the macrophages used for priming, whether syngeneic or allogeneic (Pierce *et al.*, 1976). In a different situation, the spleen cells from F_1 mice between a responder and a nonresponder strain, primed *in vivo* with "responder" or "nonresponder" strain GAT-pulsed macrophages would make antibody to the GAT pulsed with the macrophage used for priming (Pierce *et al.*, 1977). In contrast, in the T cell proliferative response to GAT, F_1 T cells would proliferate only to GAT bound to responder strain macrophages (Yano *et al.*, 1978). The same results were found by Miller

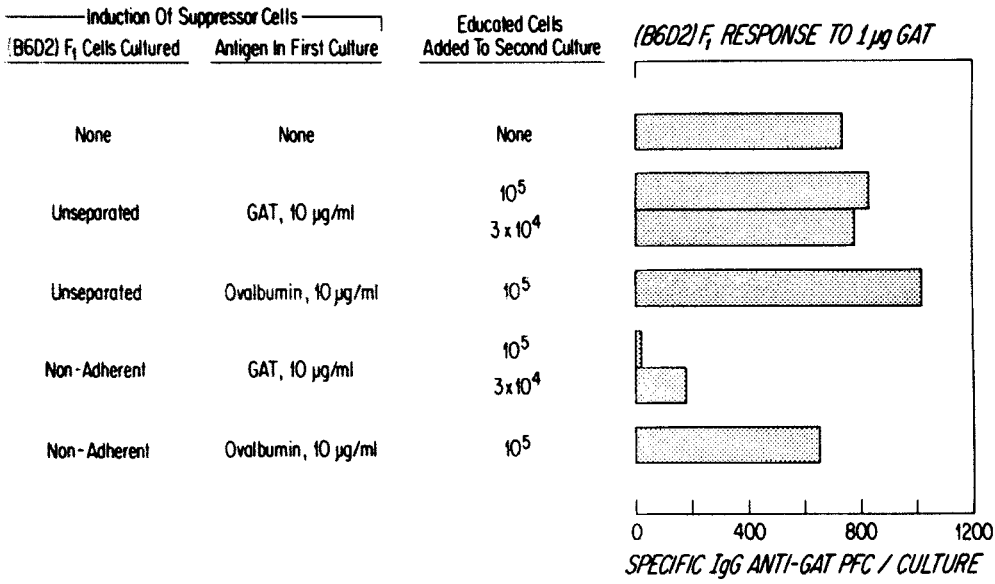

FIG. 4. This experiment shows the induction of suppressor T cells by incubation of macrophage-depleted spleen cells with copolymer L-glutamic acid-L-alanine-L-tyrosine (GAT). Unfractionated or nonadherent spleen cells were cultured for 2 days with the antigens indicated in the graph. The "educated" cells were then added to fresh cultures of syngeneic spleen cells stimulated with GAT. Cells cultured in the absence of adherent cells with 10 μg of GAT generated suppressor activity. [From Pierres and Germain (1978).]

studying delayed sensitivity (Section II,D). Also, Howie and Feldman (1978) reported collaboration to GAT only between macrophages of responder strains interacting with B cells and T helper factors. However, recent studies by Araneo and Kapp (1980), using proliferation as the assay, again found that F_1 T cells proliferated to both responder or nonresponder macrophages, which leaves the issue unresolved. The GAT system may be a unique situation, but it should be considered at a time when the molecular mechanisms underlying the recognition of antigen and Ia are better understood. Perhaps the structure of GAT is such that an association of it with a macrophage *I*-region product, if indeed taking place, results in similar configurations, regardless of *I*-region haplotype. The genetic defect of the GAT would be placed then at the level of the lymphocyte.

It should be noted that, in some studies, *I*-region-restricted interactions between the macrophages and the lymphocytes were not found (Katz and Unanue, 1973; Unanue, 1978). In these experiments, al-

logeneic macrophages presented haptenated hemocyanins effectively to *immune* spleen cells that were not fractionated or were not stringently depleted of macrophages and, therefore contained syngeneic macrophages. The possibility was raised of transfer of antigen from the allogeneic to the syngeneic macrophages (or B cells) (Unanue, 1978). Indeed, this system has been used to support a pathway of presentation of antigen from macrophages to another antigen-presenting cell not involving an MHC-restricted interaction (Section III,B). Pierce and Malek (1980) obtained evidence supporting this possibility in the *in vitro* response of mice to fowl γ-globulin. Syngeneic and allogeneic antigen-pulsed macrophages stimulated about the same response of unfractionated immune spleens. However, only syngeneic macrophages presented antigen when the spleen cells were totally depleted of macrophages by the Sephadex G-10 method of Ly and Mishell (1974). Evidence of release of immunogenic antigen was also obtained in this study.

An *in vivo* demonstration of the MHC-restricted interaction between helper T cells and antigen-presenting cells has been elegantly demonstrated by Sprent. His basic experiments consisted of injecting (CBA $\times$ BL/6)F_1 purified T cells into an X-irradiated mouse of one or the other parental strain together with SRBC. The F_1 T cells were recovered from the thoracic duct lymph and cotransferred with the antigen and parental strain B cells into X-irradiated mice, which were later examined for their plaque-forming cell number (Sprent, 1978a,b). The immune T cells in the initial host were depleted from the thoracic duct cells during the first 2 days, indicating their transient removal from the circulation, but appeared in large number by 6 days. Thus, by examining day 2 or day 6 thoracic duct cells, "negative" or "positive" selection of immune T cells could be shown. The results indicated that F_1 T cells would cooperate only with the B cells of the same parental strain used for the *in vivo* priming and supported the concept that these F_1 T helper cells comprised two distinct groups. Sprent went on to show that the selection was found only when transferring purified T cells (to the first host). Transferring spleen cells showed no preference, suggesting that antigen-presenting cells in the spleen competed with those of the host. This was shown directly by cotransferring peritoneal exudate cells of one or the other parent, together with purified T cells (Table IV) (Sprent, 1978a). In the experiment shown in Table IV, F_1 T cells transferred into BL/6 preferentially helped B cells of BL/6; this preference was not found if the peritoneal exudate cells were added to the T-cell inoculum. Sprent has recently shown that the selection process could be blocked by injection of anti-Ia antibodies (1980b). He

TABLE IV
ANTIGEN PRESENTATION *in Vivo*[a]

T-cell group	T cells	PE cells added during positive selection	B cells	Anti-SRBC PFC per spleen at 7 days in irradiated F_1	
				IgM	IgG
A	F_1 T → B6	—	CBA	1,770 (1.12)	1,580 (1.52)
			B6	17,280 (1.14)	37,050 (1.13)
B	F_1 T → B6	3×10^7 CBA PE	CBA	26,950 (1.16)	67,740 (1.13)
			B6	21,160 (1.19)	47,880 (1.16)
C	F_1 T → B6	3×10^7 B6 PE	CBA	2,150 (1.34)	8,140 (1.37)
			B6	57,330 (1.24)	114,910 (1.02)
D	F_1 T → CBA	—	CBA	19,740 (1.12)	59,400 (1.06)
			B6	1,300 (1.30)	2,160 (1.14)

[a] This table is taken from Sprent's study (1978a). (CBA × BL/6)F_1 nylon-wool-purified T cells were injected into X-irradiated BL/6 mice together with sheep red cells (SRBC). In groups B and C, CBA or B6 peritoneal exudate (PE) cells were injected together with the T cells. Thoracic duct lymphocytes were harvested 7 days later and transferred with B cells (of CBA or B6) into F_1 X-irradiated animals plus sheep red cells. The results show the plaque-forming cells (PFC) after subtraction of the background values.

has concluded that the activation of helper T cells was *H2* restricted *in vivo*, that the cells presenting antigens were enriched in spleen and in peritoneal exudates, and that they were radioresistant, belonging most likely to the phagocyte lineage (1980a).

Finally I will analyze very briefly, but speculate freely on, the controversial issue of whether the *I*-region control in the interaction of T and B lymphocytes with macrophages is at the level of the macrophage–T cell interaction exclusively or applies also to the interaction of B and T cells as initially postulated by Katz's laboratory (Katz *et al.*, 1973, 1978). There is no argument that macrophage–T cell interactions result in the development of helper T cells. The point is whether the T-cell clones reactive to an antigen in the context of an *I*-region determinant of an appropriate macrophage need also to interact with a B cell bearing the same *I*-region product. A number of experiments indicated that T cells from F_1 animals selected on macrophages from one parent will collaborate only with the B cells of the same parent (Yamashita and Shevach, 1978; Sprent, 1978a,b; Swierkosz *et al.*, 1978; Andersson *et al.*, 1980). However, other experiments suggest that the primary restrictive *I*-region interaction is only between macrophages and T cells (for example, Singer *et al.*, 1979; McDougal and Cort, 1978). An explanation for these discrepant results

awaits further studies. It may well be that at least in culture there are various pathways to activate B cells. For example, release of nonspecific helper factors from the T cell–macrophage interaction that trigger B cells without an *I*-region restrictive interaction (Section III,C).

There is no *a priori* reason why selected T cells cannot interact with the B cell in an *I*-region-restricted way. B cells take up and handle antigen in a manner analogous to macrophages (Engers and Unanue, 1973), the exception being that only the B cells with specific receptors can interact with antigens, whereas all the macrophages in a population are capable of antigen-binding. An antigen presentation role for the B cell may explain various observations: (*a*) that B cells recognize mostly conformational determinants of a globular protein (Sela, 1969); (*b*) yet T cells recognize unfolded or denatured determinants; but (*c*) hapten and carrier determinants of an antigen have to be linked together for optimal interactions. These three observations could be explained if a B cell recognizes a protein antigen in its tertiary structure, "processes" it, and then presents the "processed" or "carrier" determinants to the T cell in the context of its Ia molecule.

Direct examination of antigen presentation from B cells to T cells has been difficult to test and, in general, has not been critically examined. Technical difficulties in separating macrophages from B cells limit the experiments. Our laboratory has recently presented suggestive evidence that B cells may present hemocyanin to T cells (Kammer and Unanue, 1980). The proliferation of T cells was highly favored by the addition of macrophage-conditioned medium to the culture. More recent results using Ig as antigen support an antigen presenting function of B cells (Chestnut and Grey, 1971). It is possible that the B cell may present antigen, but only for the purpose of receiving back the T cell helper signal that it acquires by direct cell contact. However, the B cell will be limited in its capacity to reciprocate by providing a stimulus required for the activation and growth of the T cell. Thus, the stimulation of the T cell becomes the responsibility of the phagocytes, which are abundant and capable of freely binding antigen. (The inability of B cells to support T cell growth is also shown by their poor role as stimulators in the mixed leukocyte reaction.) This explanation does not exclude that a B cell, under appropriate circumstances, can also be stimulated by macrophage factors and/or non-specific T helper factors in situations that do not involve an *I*-region-restricted step (Section III,C). However, the fact that, for optimal *in vivo* responses, the hapten and carrier determinants must be linked in the same molecules (reviewed in Katz, 1977) argues for the major pathway being that of B

cell stimulation by close contact with T cells. Hence, the scenario that I envision for the three cells is that phagocytes are the primary antigen-handling cells that result in the selection, growth, and stimulation of the *I*-region-restricted T cells. The B cells are stimulated when they recognize intact antigen (either free or bound also to phagocytes) and presents it to the T cell in a process that could be modulated by soluble helper factors from either the macrophage or the T cell.

The studies described in this section permit the conclusion that: (*a*) macrophage-bound antigen is a highly effective mode of antigen presentation for T-B cell interactions; (*b*) the macrophages are required for the primary antibody response to thymic-dependent antigens, either for protein or particulate antigens; (*c*) the requirement for macrophages in secondary response or for primary responses to SRBC in the presence of a growth promoter, e.g., 2-ME, are less stringent; it is even possible that macrophages may not be essential in these situations; (*d*) in most studies, the interactions between T and B cells with macrophage-bound antigen involve the *I* region of the macrophage; that is to say, antigen presentation for T-B collaboration involves Ia-bearing macrophages and shows requirements for *I-A* homology, as in the proliferative systems described before; and (*f*) interactions of T lymphocytes with antigen in the absence of macrophages favor the development of suppressor cells. Finally, in all studies so far, the helper T cells involved in *I*-region-restricted interaction with macrophages bear the Ly1 marker and not the Ly2 antigen characteristic of the suppressor and cytolytic T cells (reviewed by Katz, 1977; and Cantor and Boyse, 1977).

D. Interactions with T Cells in Cellular Immunity Reactions

I am referring in this section to cellular immunity reactions that result in accumulation of phagocytes in foci of immune reactions and in macrophage activation for increased microbicidal and tumoricidal activity. Cellular immunity reactions constitute the basis of resistance to intracellular pathogenic microorganisms, such as the facultative intracellular bacteria, protozoa, viruses, and fungi. An important component of macrophage activation is also found in the response to tumors. Delayed-type hypersensitivity and contact sensitivity reactions are expressions of cellular immunity reactions to protein antigens or contact sensitizers deposited in the skin. These reactions involve immune T cells that, by way of various soluble mediators, call forth and activate macrophages. The activated macrophages are bone marrow-derived

phagocytes that have changed their biology, developing a heightened cytocidal activity. Such activated macrophages will be able to stop growth and/or kill bacteria, protozoa, and tumors (for papers on these issues, see MacKaness, 1962; MacKaness and Blanden, 1967; Dannenberg, 1968; North, 1969, 1978; McGregor *et al.*, 1971; Lane and Unanue, 1972; David and David, 1972; Lefford, 1975; Karnovsky and Lazdins, 1978; Cohn, 1978).

A major and apparently essential component in the stimulation of T cells to bring about macrophage accumulation and activation is an interaction of the T cell with Ia-bearing phagocytes. Several experimental systems have been used to examine this issue, some involving *in vivo* analysis, others involving culture approaches. For didactic reasons, these several approaches are now considered separately.

1. Delayed Sensitivity

The first transfer of delayed-type hypersensitivity reactions involving contact sensitizers or tuberculin were made by Landsteiner and Chase (1942) and Chase (1945) using outbred guinea pigs. They transferred peritoneal cells from outbred immune guinea pigs into nonimmune recipients, which were skin tested hours later. Successful transfer required a large number of lymphoid cells. Subsequent to these pioneering studies, lymphocyte transfers employed cells from inbred animals to avoid primarily tissue rejection. The first indication that the effective transfer of delayed reactions by lymphocytes required an important contribution by the host involving the *Ir* genes came from the studies of Green, Paul, and Benacerraf using outbred guinea pigs (Green *et al.*, 1967). They were pursuing the analysis of the genetics of the immune response to the polylysine compounds (Kantor *et al.*, 1963) and were attempting to transfer the capacity to develop delayed sensitivity from a "responder" to a "nonresponder" guinea pig. Lymphoid cells from responder guinea pigs were able to transfer the reaction to responder, but not to nonresponder, guinea pigs, suggesting to Green *et al.* "that some essential genetic controlling processing step on the antigen, specific for L-lysine sequences, must take place before sensitized cells capable of binding the antigenic determinant can be stimulated by such antigens." Subsequent studies linked the response to poly(L-lysine) compounds to the MHC of the guinea pigs (Ellman *et al.*, 1970; Martin *et al.*, 1970). Responder and nonresponder animals differed primarily at the *I* region (Benacerraf *et al.*, 1971).

In 1975, Miller and his associates reevaluated the transfer of delayed sensitivity using inbred strains of mice (Miller *et al.*, 1975). Mice were immunized with fowl γ-globulin in Freund's adjuvant; their lympho-

cytes were harvested and transferred to various inbred strains syngeneic or allogeneic with the donor of the cells. The recipient mice were later challenged in the ear with a small amount of the antigen; the accumulation of cells radiolabeled by a pulse of [^{125}I]iodobromodeoxyuridine (Vadas *et al.*, 1975) was taken as a measure of delayed sensitivity. The basic results of their experiments were that the sensitized T cells could transfer the reaction to syngeneic, but not to allogeneic, recipients; furthermore, using recipient mice from inbred strains sharing part of the *H-2*, it was found that successful transfer required identity at *I-A* subregion between the donor and recipient strains of mice. No suppressor mechanism could be ascertained for the restriction of the transfer (Vadas *et al.*, 1977).

A logical candidate for the restriction of the transfer of delayed sensitivity was the macrophage as per the results found by Rosenthal and Shevach's study of 1973 (Section II,A). Delayed sensitivity reactions were known to be very successfully induced—or the reactions elicited in the immune animal—by transferring antigen bound to live macrophages (Unanue and Feldman, 1971; Feldman and Unanue, 1971; Seeger and Oppenheim, 1972). Miller *et al.* proceeded to immunize F_1 mice (between CBA, an H-2^b strain, and BALB/c, an H-2^d) by injecting them with live macrophages from either of the parental strains pulsed with antigen (fowl γ-globulin or hemocyanin). Such mice, as expected, developed strong delayed reactions (Miller *et al.*, 1976, 1979); their lymphocytes were able to transfer the delayed reactivity only to the recipient mice of the same strain as the antigen-carrying macrophage. In other experiments, it was found that a delayed reaction could be elicited in immune mice if injected with live macrophages bearing antigen, but only if the macrophages were syngeneic or semiallogeneic. Overall, these experiments, therefore, pointed to the antigen-presenting event as the one responsible for restricting the transfer of the reaction.

Other experiments used an antigen under *Ir*-gene control, i.e., an antigen that results in responsive or unresponsive states among inbred strains, like the copolymer of glutamic acid–alanine–tyrosine (GAT). It was found that GAT-immune cells from an F_1 between a responder and a nonresponder would transfer the delayed sensitivity state only to mice from a responder strain (Miller *et al.*, 1977, 1979). Furthermore, F_1 recipients could be sensitized by macrophages pulsed with GAT from a responder strain but not with macrophages from the nonresponder (Miller *et al.*, 1979). Finally, cultured bone marrow cells pulsed with antigen were able to induce a delayed sensitivity state when transferred into normal recipients. Killing the Ia-bearing mac-

rophages by treatment with anti-Ia (plus serum as complement) did not sensitize (Motram and Miller, 1980).

2. *Contact Sensitivity*

The Langerhans cells of the skin are further reviewed in Section III, A. In this section, we consider this phagocytic cell as a strong candidate for presentation of antigens introduced by way of the epidermis. Langerhans cells are found throughout the epidermis, mainly in the upper layers of the stratum malpighii. They appear in hematoxylin and eosin stains as cells with a clear cytoplasm containing a peculiar organelle—the Birbeck granule—consisting of a rod-shaped structure contained in a membrane-limited vesicle. Langerhans cells can be distinguished by their content of ATPase and their uptake of gold salts (reviewed by Breathnach, 1975; and by Silberberg-Sinakin *et al.*, 1978).

Interest was aroused in the finding that, in man, guinea pigs, and mice, Langerhans cells contained Fc and C3 receptors (Stingl *et al.*, 1977) and bore Ia antigens (Rowden *et al.*, 1977, 1978; Klarskog *et al.*, 1977; Stingl *et al.*, 1978a,b,c; Forsum *et al.*, 1979b; Tamaki *et al.*, 1979). Langerhans cells also took up a variety of soluble and particulate antigens and thus had characteristics with the cells of the mononuclear phagocyte system (Shelley and Juhlin, 1977; Silberberg-Sinakin *et al.*, 1978). In contrast to classical mononuclear phagocytes, the Langerhans cells contained few lysosomes and showed little pinocytic activity. The phagocyte, in turn, lacked the typical Birbeck granule (Birbeck *et al.*, 1961).

Stingl and associates made a major observation by isolating the keratinocytes rich in Langerhans cells and establishing in culture that they could present antigen in systems involving T cell proliferation (Stingl *et al.*, 1978a,b, 1980). They isolated epidermal cells and selected by density gradient those bearing Fc receptors and capable of binding opsonized red cells. This Langerhans cell-rich fraction was added with antigen (ovalbumin) to cultures of immune T cells from syngeneic or allogeneic strains of guinea pigs. A strong proliferative response was obtained by ovalbumin presented in Langerhans cells, but only in syngeneic combinations. Killing the Langerhans cell with anti-Ia resulted in a complete loss of the response. Langerhans cells were also found to be good stimulators in the mixed leukocyte reaction; also, T cells from guinea pigs immune by skin sensitization with picryl chloride were found to proliferate in culture upon interactions with trinitrophenyl conjugated to syngeneic macrophages (Thomas *et al.*, 1977a) or to isolated Langerhans cells (Stingl *et al.*, 1978a, 1980).

Thus, overall, Langerhans cells were behaving as the conventional Ia-bearing macrophage, insofar as their capacity to take up and present antigen.

The finding of a phagocyte-like cell in the epidermis bearing Ia and capable of antigen binding and presentation raises the issue of whether this is the cell responsible for immunization to antigens that contact and bind to the skin (Silberberg *et al.*, 1976). There is evidence that the Langerhans cell increases in the skin after application of a contact sensitizer and migrates into the dermal lymphatics and into the draining lymph nodes (Silberberg *et al.*, 1975; Silberberg-Sinakin and Thorbecke, 1980). These events may be essential steps in transporting and providing the immunogen to the lymphoid elements. Experiments of Macher and Chase (1969) provided evidence that the skin was directly responsible for the presentation of antigen. They painted the guinea pig ears with a contact sensitizer and evaluated the effects of excising the ear on the later development of contact sensitivity. Ear excisions 24 hours after painting, when 80% of the sensitizer had escaped via the circulation, completely abolished the development of delayed sensitivity. This indicated that the antigen that was rapidly removed was not immunogenic. However, removal of the skin site at later times showed progressively less effect until the third and fourth days, at which times were was no effect, i.e., sensitization had taken place. They concluded that the skin depot was responsible for sensitization; to quote, "these results support the concept of interaction between allergen and host tissue at the site as being essential for transformation of the simple chemical into the sensitizing antigen" (Macher and Chase, 1969). Very convincing evidence that this is by way of the Langerhans cell was provided by Toews, Bergstresser, and Streilen (Toews *et al.*, 1980). They sensitized mice with DNFB on areas of the skin varying in their content of Langerhans cells and later measured the delayed reactions by ear swelling after application of the sensitizer. Painting of the tail skin with a content of 100 Langerhans cells per square millimeter resulted in very weak reactivity in contrast to painting on the skin of the abdomen, a site with a high content of Langerhans cells (700/mm^2). Toews *et al.* went on to show that Langerhans cells disappeared after ultraviolet irradiation of the skin and that painting the irradiated skin resulted in no sensitization. As a control, they showed that mice sensitized on areas not directly exposed to ultraviolet light were able to develop a delayed sensitivity state. Very interestingly, not only was the painting on tail skin or ultraviolet-irradiated skin ineffective, but it apparently resulted in a tolerant state; i.e., mice painted with DNFB on tail or on ultraviolet-irradiated

skin were later painted for a second time on normal abdominal skin and subsequently challenged; the first exposure resulted in specific abrogation of sensitization. The data, therefore, clearly supported a cardinal effect of the skin most likely via the Langerhans cells in inducing a local state of immunity.

3. *Lymphokine Secretion*

Cellular immunity reactions are believed to be mediated by a number of mediators released by T cells in the course of antigen stimulation. The secretion of macrophage inhibitory factor by guinea pig or murine T cells challenged by antigen in culture required the presence of macrophages (Wahl *et al.*, 1975a,b; Nelson and Leu, 1975; Ohishi and Onoue, 1975; Landolfo *et al.*, 1977) and was inhibited by anti-Ia antibodies (Ben-Sasson *et al.*, 1974). The same macrophage requirement applied to the secretion of macrophage chemotactic factor induced by antigen (Wahl *et al.*, 1975a), osteoclast-activating factor induced by PHA (Horton *et al.*, 1974), and mitogenic factor induced by human T cells (Larsson, 1978; Breard *et al.*, 1979). The secretion of macrophage-activating factor by murine T cells required Ia-positive macrophages (Farr *et al.*, 1979b). Once secreted, the various lymphocyte mediators acted on macrophages regardless of their strain of origin or Ia content (Fidler, 1975; Riisgaard *et al.*, 1978; Farr *et al.*, 1979b). As in most interactions in culture between macrophages and lymphoid cells, their amount and state of macrophage activation was critical. Lymphokine secretion was suppressed, for example, using activated macrophages from tumor-bearing mice (Varesio *et al.*, 1979). The nature of the suppressive function of the activated macrophages may be multiple (Section III,C).

4. *Antibacterial Immunity and Macrophage Activation*

The only studies of induction and development of cellular immunity to facultative bacteria have used *Listeria monocytogenes*, an organism that induces a severe infection in mice. Resistance to *Listeria* depends on the recruitment and activation of phagocytes resulting from T cell stimulation (MacKaness and Blanden, 1967). I will not review here the process of macrophage recruitment and activation but will focus exclusively on the events leading to T cell stimulation. The first indication of the involvement of the MHC in anti-*Listeria* immunity came from the studies of Zinkernagel and associates (Zinkernagel, 1974; Zinkernagel *et al.*, 1977). The experimental setup consisted of measuring *Listeria* growth in tissues of recipients of *Listeria*-immune T cells; i.e., T cells were harvested from immune mice and injected intravenously

into normal mice, followed a few hours later with live *Listeria* organisms; viable bacteria were estimated by colony counts a day later. It was found that the *H-2* complex restricted the successful transfer of antibacterial immunity; in order for bacterial counts to be reduced by 100- to 1000-fold, it was necessary that the recipient and donors of the T cells match at the *I-A* region. Limited experiments also suggested the presence of two *H-2* reactive T cells in F_1 recipients. The interpretation of these studies was the same as that given to Miller's, i.e., that the development of T cell reactivity required an initial interaction with host accessory cells regulated by the *I* region.

In our laboratory, we investigated the interactions between *Listeria*-immune T cells and macrophages that resulted in either macrophage or T cell stimulation (Farr *et al.*, 1977, 1979a,b; Ziegler and Unanue, 1979; Beller *et al.*, 1980). *Listeria*-immune T cells were placed in culture, together with peritoneal macrophages and heat-killed *Listeria* organisms. After 24 hours, the culture supernatants were harvested and assayed for a number of mediators, while the macrophages were tested for tumoricidal function. After 24 hours of interaction with T cells, the macrophages were highly cytocidal and killed ^{51}Cr-labeled P815 tumor cells. In order for the macrophages to develop cytocidal function, it was necessary that there be *I*-region homology between T cells and macrophages and that the macrophages bear Ia antigens. By mixing immune *Listeria* T cells from one strain with macrophages from various congenic lines, it was possible to map the *H-2* restriction to *I-A* subregions with a minor involvement in *I-E/C*. At the same time that macrophages developed cytolytic activity, secretion of mediators took place. Among them we found a macrophage-activating factor and a 15,000 MW mitogen identical to the lymphocyte-activating factor (LAF) made by the macrophage (Section III,C). Again, the secretion of all mediators involved a first interaction regulated by the *I* region. Identical results were obtained assaying for T-cell proliferation. Using a functional assay that measures the direct binding of T cells to the macrophages, it was possible to ascertain that T cells would interact with macrophage-bound *Listeria* only if the macrophages showed surface Ia and genetically shared the *I-A* region (Ziegler and Unanue, 1979 (Section III,B).

In summary, the studies in cellular immunity have indicated that (*a*) macrophage-bound antigen can transfer—or elicit—a delayed-sensitivity state and that these reactions involve the *I* region of the macrophage; (*b*) an antigen-handling step restricts the cellular transfer of delayed reactions and cellular immunity to infection, and this restriction maps to the *I* region; (*c*) skin phagocytes are most likely the

cells responsible for elicitation of contact sensitivity by presenting and carrying the sensitizer to the T cells, again involving an *I*-region function; (*d*) in culture, the production of mediators and the activation of the macrophage requires recognition of antigen bound to a phagocyte. Such recognition has the same characteristics shown for the proliferative response. Finally, the T cell involved in the cellular immunity reactions described above bears the Ly1 antigen and thus belongs to the helper subset.

E. Interactions with B Cells and Polyclonal Stimuli

The critical issue here is whether polyclonal stimuli, most of which are regarded as thymic-independent antigens, will act on B cells without the need of macrophages or do so only with the intervention of the phagocytes. The macrophage dependency of B cell proliferation and differentiation induced by polyclonal stimuli in cell cultures has not been an easy problem to tackle and, furthermore, has not been pursued as thoroughly as the T-cell responses to antigen. Discrepant results are frequent, even within a single laboratory, most likely because of the difficulties in counting and eliminating phagocytes. The problem of separating phagocytes from B cells has not been adequately resolved, and most methods have some problems: for example, the iron-carbonyl method, while removing macrophages, also eliminates some B cells; overall, whether polyclonal stimuli act directly on the B cell or by way of the macrophage still requires critical evaluation.

Some polyclonal stimuli, most notably *Escherichia coli* lipopolysaccharide (LPS), induce B cell proliferation and differentiation after macrophage depletion and thus appear to be macrophage independent (reviewed in Coutinho and Moller, 1975; Persson *et al.*, 1978). Some of the responses involving LPS, however, do involve the macrophage. The LPS stimulates the release of powerful lymphostimulatory molecules from macrophages, and these molecules definitely have an effect in B- and T-cell responses in culture (see Section III,C). In a system extensively analyzed by Hoffman and colleagues (1979), it was found that spleen cells responded with an antibody response to TNP coupled to autologous red cells, but only in the presence of LPS, confirming earlier reports (Schmidtke and Dixon, 1972b). Such a response, however, required macrophages. The macrophage requirement could be replaced by medium conditioned by macrophages treated with LPS (Hoffman *et al.*, 1979). Therefore, although a powerful molecule like LPS apparently can act on B lymphocytes directly, its effects on macrophages result in responses involving a multiplicity of cells.

DNP-levan (Desaymard and Feldman, 1975) and lanatoside C

(Smith and Hammarstrom, 1977; Hammarstrom *et al.*, 1978) are antigens that have been shown not to require macrophages. Other stimulants, such as the polyanion dextran sulfate require accessory cells (Diamanstein *et al.*, 1973; Persson *et al.*, 1977; Hammarstrom *et al.*, 1978; Smith and Hammarstrom, 1978). There are discrepant results with regard to polymerized flagellin used so extensively by the Australian workers. While many studies showed no impairment of the antibody response in culture (Shortman *et al.*, 1970; Feldman and Palmer, 1971; Shortman and Palmer, 1971), a recent report claims a marked reduction by elimination of phagocytes using the iron-carbonyl method (Lee *et al.*, 1976).

TNP-derivatized Ficoll, a polysucrose compound, induces differentiation of spleen cells to antibody formation in cultures. Although the first report claimed that this response was macrophage independent (Mosier *et al.*, 1974), subsequent studies agreed that macrophages were required (Lee *et al.*, 1976; Chused *et al.*, 1976; Nordin, 1978; Kirkland *et al.*, 1980; Boswell *et al.*, 1980). The antibody response to TNP-polyacrylamide beads was also found to be macrophage dependent in both the mouse (Duclos *et al.*, 1979) and in man (Delfraissy *et al.*, 1977).

The response of human peripheral blood lymphocytes to pokeweed mitogen has been extensively studied. Pokeweed mitogen induced strong B-cell differentiation that required the presence of T cells (Keightley *et al.*, 1976). The response also required a critical number of monocytes (Rosenberg and Lipsky, 1979; Knapp and Baumgarten, 1978; Gmelig-Meyling and Waldmann, 1980); macrophages also inhibited the response. The nature of the inhibition is unexplained but did not appear to be mediated by prostaglandins (Gmeling-Meyling and Waldmann, 1980). The response of human B cells to a water-soluble antigen from *Nocardia asteroides* was not affected by monocyte depletion (Bona *et al.*, 1979; Gmelig-Meyling and Waldmann, 1980).

How the macrophage operates in the response to the macrophage-dependent polyclonal stimuli is not well understood, although there are some experiments indicating that they do this by releasing a growth-promoting component. The antibody response to dextran sulfate developed in macrophage-depleted cultures in the presence of macrophage-conditioned medium (Persson *et al.*, 1977, 1978; Smith and Hammerstrom, 1978). A similar degree of reconstitution could be obtained by using 2-mercaptoethanol (2-ME). The macrophage culture fluids consisted of 24-hour culture of unstimulated macrophages. There is yet no biochemical characterization of the active principle. The experiments of Persson suggest that the B cells required two interactions, one with dextran sulfate, the second with the growth-

promoting molecule from the macrophage-conditioned medium; whether this is the case has not been proved yet nor critically examined. Studying the TNP-Ficoll response, Nordin (1978) found a partial reconstitution of macrophage-depleted spleen cells by either 2-ME or macrophage-conditioned medium.

The interaction of the B cells with the polyclonal stimuli associated with the phagocytes has been the subject of recent studies. Boswell *et al.* (1980), found that the antibody response to TNP-Ficoll was macrophage dependent; spleen cells depleted of adherent or latex-ingesting cells (using the fluorescence-activated cell sorter and fluoresceinated latex beads) did not develop an anti-TNP antibody response. The spleen-adherent cells were pulsed with TNP-Ficoll, then washed to eliminate the unbound compound, and added to Sephadex G-10-fractionated spleen cells; the spleen B cells went on to develop an excellent anti-DNP response. The spleen accessory cells had to be live; but unfortunately, no definite information was given on their uptake of the TNP-Ficoll. An interesting observation on Boswell's study was the response of pulsing the nonadherent spleen cells with the antigen; such cells could act as antigen-presenting cells when added to cultures of spleen cells but not when added to cultures of spleen cells depleted of accessory cells. The conclusion was that the B cells in the nonadherent population were binding antigen and eventually passing it to the accessory cells, which were then responsible for the presentation.

Kirkland *et al.* (1980), have now confirmed most of the points made in Boswell's study, including the last one on the possible B cell transfer of antigenic material; they also reported that treatment with anti-IgM antibodies stopped the putative antigen presentation supporting the contention that B cells were responsible, by way of membrane Ig, as the TNP-receptor. It is obvious that the cellular basis of TNP-Ficoll response is of interest and requires further analysis.

Weigle's laboratory, examining the polyclonal response of B cells to Fc fragment of Ig, have recently found a novel pathway of macrophage–lymphocyte cooperation. They found that Fc fragments of human Ig strongly stimulated proliferation of murine B cells (Berman and Weigle, 1977), as well as their differentiation to antibody formation (Morgan *et al.*, 1980). The Fc fragments could be derived from all classes of Ig except IgE. The proliferative response to the Fc fragments was dependent on adherent phagocytic cells (Morgan and Weigle, 1979a), whereas the differentiation required both phagocytes and T cells (Morgan and Weigle, 1980). The evidence so far obtained indicated that a portion of the Fc fragment was cleaved following its interaction with macrophages; this Fc subfragment was the one re-

sponsible for inducing B-cell proliferation (Morgan and Weigle, 1979b). The experiments consisted of culturing briefly Fc fragment with spleen-adherent cells, after which the culture fluid was tested and fractionated on Sephadex chromatography. The active moiety eluted with an apparent molecular weight of 15,000. It could be removed by an immunoabsorbent containing antibodies to the Fc fragment and subsequently eluted and isolated. The isolated subfragment could stimulate B cell proliferation in the absence of phagocytes. Insofar as the differentiation of B cells to antibody-forming cells, the results indicated that, following interaction of the B cells with the Fc subfragment, there was a stimulation to differentiation involving the T cells (Morgan and Weigle, 1980). These experiments of Weigle and associates, therefore, suggest a cellular amplification reaction by immune complexes involving the macrophage and T cells that are somehow brought into the reaction.

The conclusions from the studies reported herewith are that (*a*) macrophages are definitely involved in some response to many of the conventional polyclonal stimuli, but the molecular and cellular events still await further analysis; (*b*) involvement of the macrophage may be by way of secreted active molecules; (*c*) Fc fragments of Ig act as a polyclonal stimulant only after a processing of the fragment by the macrophages.

F. Other Interactions of the Macrophage: Mixed Leukocyte Reaction and Anti-Tumor Immunity

The *mixed leukocyte reaction* involves the interaction of T cells (responding cells) with *I*-region determinants of allogeneic cells (the stimulator cells). Phagocytes are considered to be the major stimulatory cells in the mixed leukocyte reactions in man (Rode and Gordon, 1974), guinea pigs (Greineder and Rosenthal, 1975a), mouse (Talmage and Hemmingsen, 1975; Davidson, 1977; Shirrmacher *et al.*, 1975; Minami *et al.*, 1980), and rat (Oehler and Herberman, 1977).

Greineder and Rosenthal did a detailed study of the cellular nature of the stimulatory cell and found excellent stimulation with highly purified populations of peritoneal and alveolar macrophages as well as spleen cells. This stimulation was blocked by anti-Ia antibodies (Greineder *et al.*, 1976). The depletion of phagocytes from the spleen cell suspension resulted in a loss of stimulation, making them conclude that B cells, although bearing Ia antigens, were poor in stimulating and/or triggering the T cells. There are several reports in the literature claiming stimulation by B cells, but these did not eliminate the phago-

cytes (a review on most papers on this subject can be found in Davidson, 1977).

Two recent studies using murine spleen cells as stimulators confirmed the observations of Greineder and Rosenthal and added new information. Ahmann *et al.* (1979), found that purified T cells did not respond to allogeneic B cells, but only to a phagocyte-rich adherent population of cells expressing Ia determinants encoded in *I-A* and *I-E/C*. The main subregion of *I* stimulating in their combinations was *I-A* but also to lesser extent *I-B* to *I-C*. Minami *et al.* (1980) confirmed the lack of stimulation by B cells and pointed to the macrophage as the major stimulatory cell. The degree of proliferation correlated perfectly with the number of phagocytic cells.

Steinman and Witmer (1978) have reported strong stimulatory activity by "dendritic" cells isolated from the spleen (Section III,A) and very weak activity from peritoneal macrophages, The lack of stimulation by peritoneal macrophages in their experiments is puzzling. In our hands, we found excellent stimulatory activity. Of interest is that the stimulatory activity of the peritoneal macrophage decayed after 24 hours of culture, correlating with the loss of Ia biosynthesis (Section III,A); this loss of Ia was reversed if the macrophages were given a phagocytic stimulus that resulted in stimulation of T cells (Beller and Unanue, 1980b).

The immune response to tumors, like that to protein antigens and microorganisms, is highly complex, involving interactions among various lymphocytes, particularly T cell subsets. Several effector cells participate in the anti-tumor response: natural killer cells, cytolytic T cells, and activated macrophages. Phagocytes may participate in the anti-tumor response by (*a*) presenting tumor antigens, thereby inducing cellular immunity; (*b*) regulating the level of natural cytotoxicity; and (*c*) participating as effector cells. Activated macrophages are found during development of an anti-tumor response *in vivo*. Such macrophage activation resembles that found during antibacterial immunity and is caused by lymphokines released by immune T cells. The effector function of the activated macrophage will not be reviewed.

Overall, the role of macrophages in presenting tumor antigens for the development of effector T cells has not been critically examined. Whether T cells are generated upon direct contact with the tumor or indirectly by tumor antigens shed into the medium and captured by the phagocytes is not known. Several attempts were made, with some success, to generate tumor-immune lymphocytes by incubating them with tumor (Schechter *et al.*, 1976; Kedar *et al.*, 1977), but the cellular

interactions involved in the reactions were not studied; suppressor cells were also generated in some cases (Small and Trainin, 1976). Treves and associates generated effector T lymphocytes by incubating the lymphocytes for 2–4 days with thioglycolate-induced peritoneal macrophages pulsed with tumor extracts (Treves *et al.*, 1976; Treves, 1978). Syngeneic macrophages were more effective than allogeneic macrophages; fibroblasts were not capable. The effector cells were active *in vitro*, by reducing the number of tumor cells synthesizing DNA, most likely reflecting the presence of cytolytic T cells, and also *in vivo*, by conferring protection. The Treves studies indicated that macrophages could present tumor antigens and sensitize lymphocytes, but they did not address the issue of whether this was the way in which normal tumor antigens were presented to T cells. The expectation is that, at least for the generation of the helper T cells that secrete lymphokines, a presentation of tumor antigens by the macrophage may be essential (Section II,D).

I will now review briefly the macrophage in the induction of cytolytic T cells to tumors, as well as to virally infected cells and modified autologous cells. The induction of cytolytic T cells involves a number of regulatory interactions with various subsets of T cells and with the participation of various growth and differentiation factors (reviewed by Wagner *et al.*, 1980); a large series of studies agreed on the requirement for accessory cells (for example, Wagner *et al.*, 1972; Koren and Hodes, 1977; Woodward and Daynes, 1979; Woodward *et al.*, 1979; Yamashita and Hamaoka, 1979; Taniyama and Holden, 1979; Friedman *et al.*, 1979; Pettinelli *et al.*, 1980). The exact role of the macrophages, whether presenting antigens or supplying stimulatory factors, is not known. Some reports indicated that the generation of primary cytolytic T cells to allogeneic cells or tumors took place with both syngeneic or allogeneic spleen adherent cells, but that, in either case, the Ia-bearing macrophages were essential (Pettinelli *et al.*, 1980; Yamashita and Hamaoka, 1979). In experiments reported by Woodward and associates, the T cells obtained from lymph nodes draining the tumor implant developed into effector cytolytic T cells in the presence of Ia-positive accessory cells but without the need for addition of tumor antigens, suggesting that the function of the macrophage was to promote growth and/or differentiation independent of antigen presentation (Woodward and Daynes, 1979; Woodward *et al.*, 1979). One report examining the secondary cytolytic T-cell response to syngeneic tumors required syngeneic macrophages (Taniyama and Holden, 1979). Finally, macrophages activated by cellular immune reactions, such as by infection with bacillus Calmette-Guérin (BCG)

were found to inhibit development of cytolytic T cells by mechanisms not yet known (Klimpel and Henney, 1978; Klimpel *et al.*, 1979).

The phagocytes are important regulators of natural killer (NK) cells. NK cells are believed to be involved in the innate resistance to tumors. Although their exact lineage is in dispute, the NK cells have a series of distinctive properties including surface markers. The relationship between NK cells and macrophages can be gathered from three major observations: (*a*) that the increase of NK cell activity *in vivo* brought about by interferon inducers is impaired by the injection of agents that are toxic for macrophages; (*b*) that, in culture, the increase in NK activity produced by incubation of spleen cells with interferon inducers is ablated if macrophages are depleted; (*c*) that macrophage culture fluids contain a molecule that induces NK activity *in vivo* and *in vitro*. It is likely that this molecule is interferon, a molecule known to be secreted by macrophages and found in the culture fluids that induce NK activity. Some details on these points are now given.

NK activity *in vivo* increased by severalfold by administration of interferon or interferon inducers (Schultz *et al.*, 1977; Gidlund *et al.*, 1978; Djeu *et al.*, 1979a). That the action of the interferon inducers might be by way of the macrophage was suspected by the effects of silica or carrageenin, agents known to be toxic for phagocytes. These agents administered 4 hours before poly(I : C) prevented the increase in NK activity in the spleen, usually observed after 18 hours (Djeu *et al.*, 1979b). The effects of carrageenin on spleen NK activity was also reported by Cudcowicz and Hochman (1979).

Since poly(I : C) also increases NK activity on cultured spleen cells, it became possible to test more definitively the involvement of the macrophage (Djeu *et al.*, 1979b). Thus, removal of macrophages from the spleen cells, or treatment of spleen cells with silica or carrageenin, impaired the generation of NK activity induced by poly(I : C), as well as the production of interferon. Furthermore, a conditioned medium from macrophages treated with poly(I : C)—containing interferon—induced NK activity in spleen cells. Other experiments established that the generation of NK activity by interferon did not require macrophages (Djeu *et al.*, 1979b).

Essentially similar results were obtained by Tracey (1979). Infection with BCG resulted in a pronounced activation of the macrophages and also in an increase in NK cells (Wolfe *et al.*, 1976; Tracey *et al.*, 1977). Injection of silica prior to infection stopped the increase in NK activity. Furthermore, intraperitoneal injection of macrophages from BCG-infected mice—but not from normal mice—or a conditioned medium from such activated macrophages resulted in the generation of NK

activity. The active principle in the macrophage-conditioned medium was not known but was presumed to be interferon. In a recent study, Tracey and Adkinson (1980) found that the BCG-activated macrophages secreted prostaglandins (Section III,C) that could inhibit NK activity. Indomethacin, which stops prostaglandin synthesis, increased the NK-inducing activity of the activated macrophages, both *in vivo* and *in vitro*.

G. Interactions Not Mediated by Antigens

Mononuclear phagocytes may be involved in the antigen-independent differentiation of lymphocytes and also of granulocytes. Their role in such processes, exclusively studied in culture, may be related to the production and secretion of growth-promoting and -differentiating molecules, as well as to the presence of surface-bound Ia molecules.

In Section III,C, we mention that macrophages secrete colony-stimulating factor that results in the development of granulocyte/macrophage colonies in culture (reviewed by Kurland and Moore, 1977). Colonies of B cells will develop in soft agar (Metcalf *et al.*, 1975) from B lymphocytes harvested from the spleen. The growth of these B cell colonies in soft agar is stimulated by a feeder layer of macrophages or by molecules secreted by macrophages (Kurland *et al.*, 1976; Kincade *et al.*, 1978), as well as inhibited by macrophage-derived prostaglandins (Kurland *et al.*, 1976). Growth of B cells around macrophages has also been reported in liquid cultures (Garland and Owen, 1978).

A most important function of phagocytes may be in the intrathymic maturation of T cells. The maturation of the T cell in the environment of the thymus gland has been the subject of intense study for many years. How such maturation takes place is not known, but speculations have centered on the role of thymic "hormones" and thymic "epithelial" cells. The thymic gland is made up of thymocytes at various maturational stages and a stroma highly rich in reticular—or epithelial—cells (Raviola, 1975). Among the stromal cells are star-shaped or oval cells, with thin cytoplasmic-dendritic prolongations, particularly in the cortex. These stellate dendritic cells have Ia antigens as detected by immunofluorescence of frozen sections of thymic tissue (Rouse *et al.*, 1979; Janossy *et al.*, 1980). Some of the stromal cells studied by electron microscopy have macrophage characteristics, and a few contain the Birbeck granule, the cytoplasmic marker of the special phagocyte of the skin, the Langerhans cells (Sections II,A and

III,A) (Hoefsmit *et al.*, 1980). Typical macrophages were abundant in the medulla and in the corticomedullary areas.

The cells found by tissue immunofluorescence bearing Ia are indeed related to the phagocytes. Thus, David Beller isolated, from suspensions of murine thymus cells, two distinct types of phagocytic cells: one, represented by 85% of the cells, had typical macrophage morphology, bore Fc receptors, and took up latex particles; the remaining 15% had dendritic appearance, also showed Fc receptors, but were poorly phagocytic (Beller and Unanue, 1980a). Both populations had a high content of cells with surface-bound Ia antigens. The dendritic cells are probably the counterpart of the Langerhans-type phagocytes. Excellent antigen presentation to immune T cells was found by a mixture of these two cells (Beller and Unanue, 1980a). Stromal cells have been isolated from the thymus after delicate handling so as not to grossly disrupt their architecture. Small clusters of very large reticular-type cells with extensive cytoplasmic extensions surrounded by thymocytes could be identified. Those large "nurse cells" contained Ia antigens (Wekerle and Ketelsen, 1980; Wekerle *et al.*, 1980). The nature of the cells in the cluster and its relation to phagocytes is not clear.

An effect of thymic phagocytes in thymocyte differentiation has now been documented in culture. Generally speaking, attempts to reproduce the entire sequence of thymic maturation in culture have not been satisfactory. Some differentiation limited to the expression of thymic surface proteins by immature prothymocytes was found using soluble products obtained from the thymus gland (Komura and Boyse, 1973). However, results using thymic epithelial cultures have, on the whole, not given positive results. Cultures of thymic epithelia have been poorly characterized as to their culture types and frequently are highly contaminated by fibroblasts and macrophages (Loor, 1979; Beller and Unanue, 1978). An early study of Mosier and Pierce (1972) found that thymocytes lost more Thy.1 antigen—an expression of maturation—when cultured on "epithelial" cells or on adherent cells from the spleen. Part of the results were explained by a selective growth of mature thymocytes rather than by a true maturational process. In our studies (Beller and Unanue, 1977, 1978), thymocytes were separated into mature and immature sets by density using sedimentation in gradients of bovine albumin (Konda *et al.*, 1973). Immature thymocytes were characterized by their low content of *H-2*, the presence of TL antigens, and, functionally, by not responding in the mixed leukocyte reaction or to PHA. Immature thymocytes cultured on a layer of thymic macrophages adhered very avidly to the macrophages

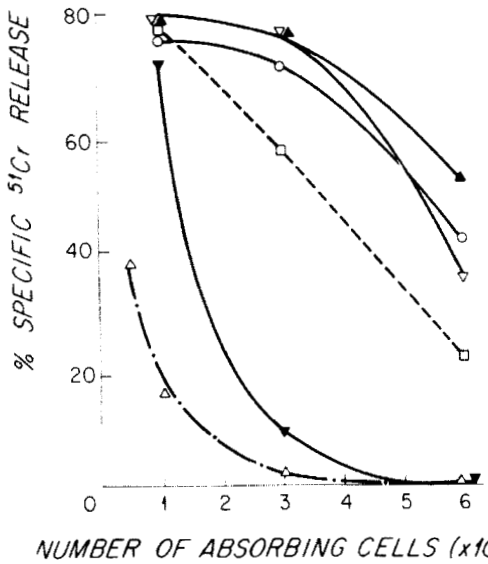

FIG. 5. This figure shows the H-2 maturational changes found in thymocytes cultured on thymic macrophages. Fractions rich in immature thymocytes were cultured without added cells (○), on fibroblasts (▲) or on thymic macrophages (▼) for 48 hours, after which the amounts of H-2 antigen in the cell were measured by their capacity to absorb a standard anti-H-2 serum. The serum was titrated by its capacity to lyse ^{51}Cr-labeled cells. The immature thymocytes showed helper content of H-2D after their culture on macrophages. The content of H-2D on mature thymocytes (△) and on mixtures of mature and immature ones (□) is shown for comparison. [From a study by Beller and Unanue (1978).]

(Section III,B) and dramatically changed within a period of 48–72 hours. Maturation was evidenced by changes in the amounts of surface *H-2*, which increased severalfold to the level shown in mature thymocytes, as measured by quantitative absorption, by a loss of sensitivity to killing by anti-TL antibodies, and by a capacity to respond in the mixed leukocyte reaction (Fig. 5). The increase in H-2 antigens was found in thymocytes treated with mitomycin C and therefore not synthesizing DNA, hence ruling out the possibility that the previous results were accounted by a selective growth of a few mature cells contaminating the fractionated population. The maturational changes seen by macrophages could be reproduced by a conditioned medium containing a 40,000 MW material without interferon activity (Beller and Unanue, 1977) and also quite distinct from the 15,000 MW thymocyte mitogen secreted by macrophages (Section III,C). Curiously, thymic maturation was not accomplished by cultures of purified thymic "epithelial" cells devoid of macrophages (Beller and Unanue,

1978). Changes in reactivity of guinea pig thymocytes to Con A were reported after incubation with macrophages (Van den Tweel and Walker, 1977), but the nature of the change, either selection of cells or maturation, was not studied.

In summary, the thymus gland contains a large number of cells with Ia antigens, some of which clearly belong to the phagocyte series. The reasons for the high concentration of Ia-bearing cells, their nature, and their role are not known and require evaluation. Ia-bearing stromal cells are found in the thymus from early fetal life (Jenkinson *et al.*, 1980); Ia-bearing macrophages were reported from birth (Lu *et al.*, 1980). If Ia antigens are involved in antigen-independent maturational events, then it is likely that these cells may well be the substrate for such events (Section III,B). Clearly, part of the differentiative process of immature thymocytes can be reproduced in culture using phagocytes or a differentiation factor derived from them. The extent to which the T cells are functional after maturation on thymic macrophages has not been fully examined. This is a point of particular importance, particularly as it concerns the MHC involvement.

H. Macrophages in Suppressive Phenomena

There are three basic situations to analyze where macrophages are involved in suppressive-type effects: (*a*) in the generation of suppressor T cells; (*b*) in a direct suppressive effect on lymphocytes in culture; and (*c*) as a mediator or carrier of T-cell suppressor factors.

While presentation of antigen by way of the macrophages is the essential way to elicit helper T cells, the interaction of antigen with T cells in the absence of macrophages resulted in generation of suppressor T cells (Ishizaka and Adachi, 1976; Feldman and Kontiainen, 1976; Pierres and Germain, 1978). This is an important point, analyzed in Section II,C, which, at face value, implies that the induction of T suppressors may take place by direct interaction with antigen without any other cellular mediation. Sherr *et al.* (1980) reported that suppressor T cells to fowl γ-globulin were induced *in vivo* by transfer of Ia-positive adherent spleen cells containing the antigen, a palmitoyl derivative of fowl γ-globulin. Mice that received as little as 0.1 μg did not respond upon immunization with TNP-fowl γ-globulin (plus adjuvant); and moreover, their spleen cells injected into normal mice suppressed the specific response. The responsible cell was adherent and bore I-A determinants, not I-J determinants. Interestingly, immunization with the antigen coupled to any cell type, including red cells, but at a 100-fold higher dose, induced an unresponsive state without any detectable suppressors. This is an interesting study worth pursuing. How the Ia-positive macrophages with the antigen enter into the suppressor pathway requires more analysis. Sherr speculated

that perhaps Ia-positive macrophages may induce helpers, which, in turn, regulate the generation of suppressor cells such as those postulated in the studies of Eardley *et al.* (1978).

Macrophages in cultures, as was indicated in the preceding sections, presented antigen but inhibited lymphocyte functions, particularly at high numbers or if activated by cellular immune reactions. The inhibitory effect of macrophages was explained to a great extent by their release of prostaglandins, oxygen intermediates, and perhaps other factors (discussed in Section III,C). Interestingly, the release of the inhibitors was increased by situations that stimulated the macrophage, such as phagocytosis of the antigen and interaction with T cells, or its products, or both. A whole loop of interactions could be followed starting with a T cell induced by antigen presented in macrophages to secrete lymphokines; these, in turn, activated macrophages, which then released prostaglandins; the prostaglandins inhibited proliferative as well as T-cell secretion of mediators. It would appear that the system regulates itself by these multiple interactions.

Finally, macrophages may regulate the action of suppressor factors. One of the best-analyzed systems was that worked by Pierce and associates: suppressor T cells (Ly2 bearing) secreted a protein that, upon oxidation by a small metabolite from the macrophage, converted into a highly active inhibitor (Section III,C). In a different system, it was shown that suppressor factors released by T cells would bind to macrophages, resulting in the inhibition of the transfer of contact sensitivity (Asherson and Zembala, 1974; Zembala and Asherson, 1974; Ptak *et al.*, 1977, 1978a,b; Kojima *et al.*, 1979). As worked out by Ptak *et al.* (1978a), cells immune to trinitrobenzene sulfonic acid transferred the contact-sensitivity state if injected into normal mice. A brief 1-hour incubation of these cells with a suppressor factor (induced by incubation of tolerant T cells with the antigen) abolished their capacity to transfer the reaction. The point is that the suppressive factor required the macrophages (Kojima *et al.*, 1979) that would bind and remove the activity (Ptak *et al.*, 1978a). The macrophages with the bound factor inhibited the immune T cells. Ptak *et al.* (1978b) have brought out preliminary evidence that the macrophage might cause the suppression by releasing a nonspecific inhibitor. As envisioned by Asherson, Ptak, and Gershon, the macrophage would act, therefore, as a conduit of suppressor signals.

III. Basis for Macrophage Regulation

The role of macrophages in immune induction can be explained by two distinct, yet interrelated, functions: handling and presentation of antigen to lymphocytes, and secretion of lymphostimulatory mole-

cules. Key molecules in the antigen-handling process are the *I*-region products. Section III,A considers some general properties of phagocytes. It also reviews other accessory antigen-presenting cells. Section III,B reviews antigen handling, and Section III,C reviews the secretory function of macrophages.

A. Properties of Macrophages and Other Accessory Cells

The classical mononuclear phagocytes are responsible for the interactions described in the preceding section. In most studies, these cells were isolated from the peritoneal cavity. Cultured bone marrow, liver, lung, and spleen macrophages have been used. In this section, we first review some general characteristics of the phagocytes and then consider the expression and synthesis of the Ia proteins.

1. The Mononuclear Phagocyte and the Ia Antigens

Mononuclear phagocytes develop from self-renewing stem cells found in bone marrow and, to lesser extent, in lymphoid organs. The cell line is represented by cells in various stages of differentiation, from the early monocyte, to the mature, well-differentiated macrophage found in the various tissues. Maturation in the line is characterized by a progressive development of lysosomes, increase in Fc and C3 receptors, and in various functions such as phagocytosis, pinocytosis, and adhesiveness to culture dishes. Mononuclear phagocytes are affected by the local environment and by systemic stimuli such as bacterial products and lymphocyte mediators. These local and systemic factors contribute to the functional heterogeneity of phagocytes harvested from various tissues, under different conditions.

An issue still unresolved is the migration of phagocytes into tissues and the factors that control it in steady-state and in pathological conditions. There is little doubt that the bone marrow contains a large number of cells that give rise to mature phagocytes (Volkman and Gowans, 1965a,b; Cline and Moore, 1972; Cline and Summer, 1972; van Furth and Cohn, 1968; van Furth and Diesselhoff-den Dulk, 1970). Thus, destruction of dividing cells by systemic irradiation resulted in a loss of inflammatory exudates, but these exudates developed if the irradiated animal was infused with bone marrow cells (Volkman and Gowans, 1968a,b; Volkman, 1970). [Also, macrophages were found to develop in cultures of bone marrow cells grown with appropriate growth factors (reviewed by Stewart, 1980).] While kinetic analyses supported the sequence of bone marrow precursor → monocytes → inflamed tissue (van Furth *et al.*, 1973; Volkman and Collins, 1974), not yet clear is the extent to which tissues renew their phagocyte population in the normal steady state; that is to say, whether the mac-

rophages in tissues—all or part of them—derive from bone marrow via blood monocytes or from local cells. Most *in vivo* studies using radiolabeled thymidine indicated a small (2–5%) but still significant proportion of dividing cells in exudates (Volkman and Gowans, 1968a; van Furth and Cohn, 1968; Volkman and Collins, 1968). Furthermore, one study failed to trace the flow of monocytes into peritoneal exudates in *normal* conditions (Volkman, 1976), implying that in steady-state conditions macrophages were renewed by local precursors. The issue of self-renewal in tissue and the control of monocyte–macrophage populations is important not only because it may relate to the response to inflammation, but it may be crucial as it pertains to the immunogenic function of macrophages.

As is evident from the previous sections, phagocytes are essential cells, mediating the interactions of lymphocytes with antigen. Within the phagocyte population, however, there is a definite heterogeneity in function. One major reason for this heterogeneity is the difference in the expression of Ia within the macrophage population. As was analyzed in the preceding section, it is the presence of the Ia proteins that gives the phagocyte the capacity to interact with T lymphocytes. Early studies identified Ia antigens in some, but not all, macrophages using immunofluorescence (Unanue *et al.*, 1974) or cytotoxicity (Hammerling *et al.*, 1975). There is now general consensus following more extensive analysis that the mononuclear phagocytes in all tissues can be grouped into two sets, one bearing and the other lacking Ia molecules (for example, R. H. Schwartz *et al.*, 1976; Yamashita and Shevach, 1977; Dorf and Unanue, 1978; Cowing *et al.*, 1978a,b; Lee *et al.*, 1979; Beller *et al.*, 1980). The methods for detecting Ia in macrophages included immunofluorescence or cytotoxicity using anti-Ia antibodies. Immunofluorescence studies required careful consideration of the nonspecific binding by the antibody to the macrophage Fc receptor. Nevertheless, the nonspecific binding was totally minimized using strong alloantibodies (Cowing *et al.*, 1978a) and, particularly, monoclonal anti-Ia antibodies (Beller *et al.*, 1980), both of which gave excellent and very specific localization to the surface Ia. Figure 6 is an example from our studies using the monoclonal anti-I-A^k raised in the Herzenberg's laboratory (Oi *et al.*, 1978). Ia-bearing macrophages were also identified using cytotoxicity, in particular with strong cytolytic serum as the source of the complement (Hammerling *et al.*, 1975; Cowing *et al.*, 1978; Dorf and Unanue, 1978). Macrophages bearing Ia antigens were also recognized using fluorescent antibodies in the fluorescence-activated cell sorter (R. H. Schwartz *et al.*, 1976; Dickler *et al.*, 1980).

The presence of Ia in only a fraction of macrophages has raised several questions, not all of which are currently answered. What is the

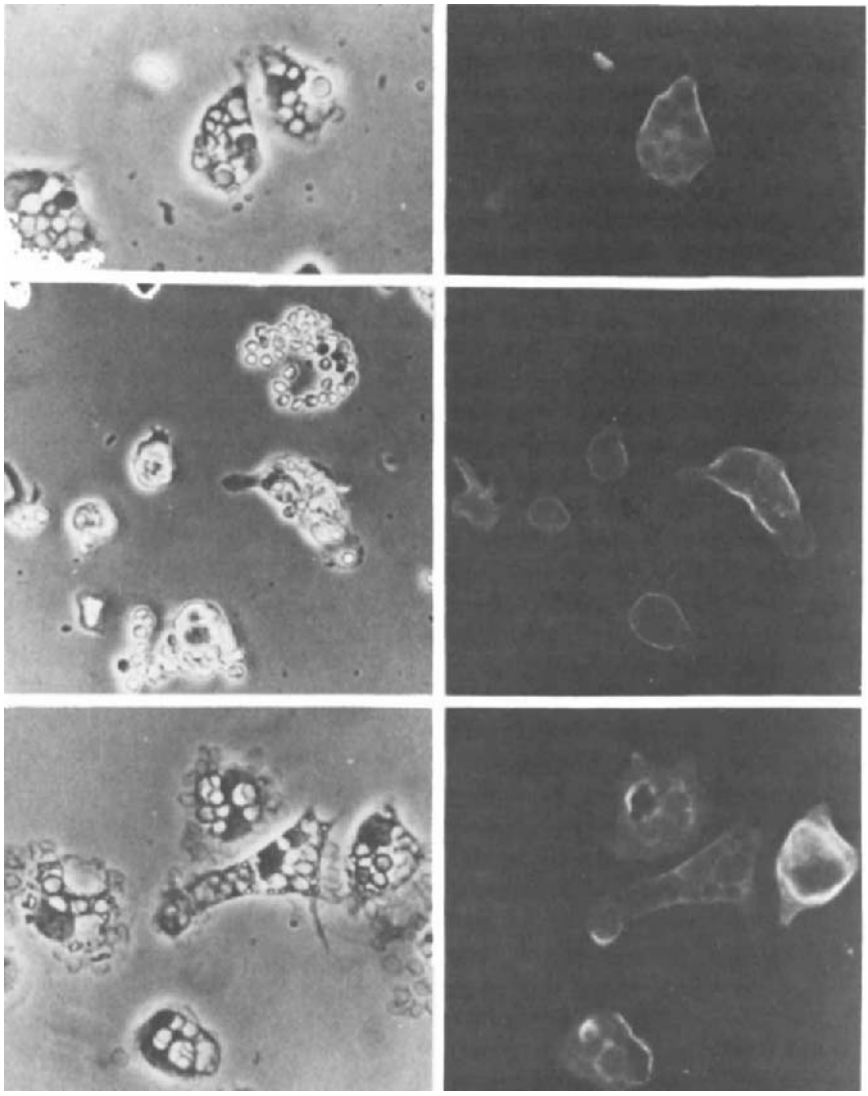

FIG. 6. Immunofluorescence of macrophages using anti-I-A antibodies. In the upper and lower panels, peritoneal macrophages were incubated at 37°C with red cells opsonized with IgG antibodies, then fixed and stained with anti-I-A. Both Ia-positive and Ia-negative macrophages phagocytized the opsonized red cells. In the middle panel, the macrophages were incubated at room temperature. Binding of red cells took place, without phagocytosis, to both Ia-positive and Ia-negative macrophages. [From Beller *et al.* (1980).]

relationship between Ia-positive and Ia-negative macrophages? Are the two cells related? Do the two cells have distinct functions? Are there regulatory mechanisms controlling the percentage of Ia-positive and Ia-negative in various tissues?

Cytological examinations disclosed that both macrophages, i.e., for ease of terminology, Ia-positive and Ia-negative, had about the same general properties. Both sets of macrophages were morphologically identical, adhered to plastic surfaces, and took up particles (Yamashita and Shevach, 1977; Cowing *et al.*, 1978a; Beller *et al.*, 1980). Both had Fc and C3 receptors, although most Ia-positive showed less when quantitated using appropriately opsonized red cells (Beller *et al.*, 1980).

a. Tissue Distribution. The percentage of Ia-positive and Ia-negative macrophages varied among the different tissues and exudates of mice; but within a given tissue or exudate, the distribution was highly consistent (Table V). It is important, however, in studying distribution of Ia-positive macrophages that consideration be given to the fact that environmental stimulation changed the percentage (see below). This may account for the occasional reference to high numbers of Ia-positive macrophages, for example, in ther peritoneum (Unanue *et al.*, 1974; Hammerling *et al.*, 1975).

A brief comment on the distribution of macrophages in the various tissues follows. The *peritoneal cavity* was frequently examined because of the ease for sampling and obtaining a pure population of phagocytes. Cultured peritoneal macrophages were practically all classical phagocytes, synthesized Ia (see below), and, as noted in Section II, were highly active in antigen-presenting function. The peritoneal macrophages were harvested from normal animals or from animals injected with mineral oil, peptone, or thioglycolate broth; the former was used mainly for studies in the guinea pig, and the last two were used particularly in the mouse (Table V). The percentage of Ia-bearing macrophages in the inflammatory exudates was about the same as in the resident population (Yamashita and Shevach, 1977; Beller *et al.*, 1980). Overall results using T cell proliferation as the readout to measure macrophage–T cell interactions showed comparable antigen-presenting function by the various exudates. In general, however, the more activated the macrophage, the more critical was the number in culture because of their inhibitory effects. Thus, in the mouse, resident macrophages (i.e., from noninduced peritoneal exudates) or macrophages from peptone, thioglycolate, or oil exudates had good antigen-presenting function, but the thioglycolate-induced macrophages were more inhibitory. Other culture assays that explored at the same time the antigen-presenting role and a second macrophage function, such as secretion or activation for tumoricidal activity, did

TABLE V

TISSUE DISTRIBUTION AND FUNCTION OF Ia-POSITIVE MACROPHAGES[a]

Source	Stimuli	Ia-positive (%)	Antigen-induced T cell proliferation	Secretion of LAF[b]	Activation for cytotoxicity
Peritoneum	None (resident)	10–20	++	+++	1±
	Peptone	10	++	++	++
	Thioglycolate	5–10	+	—	++
	Oil	5–10	++	NS	
	Listeria infection	50–75	++	++	++++
Spleen	None	50	++	++	NS
	Listeria infection	>75	NS	NS	NS
Skin	None	90	++	NS	NS
	Contact reactions	> In absolute numbers	NS	NS	NS
Liver	None	25–30	++	NS	NS
Thymus	None	75	++	++	NS
Lung	None	10	++	++	++
		80 (guinea pig)	++	NS	NS
Bone marrow	L-Conditioned medium	30	++	++	++
Blood	None	75 (man)	++	NS	NS

[a] References are given in text. All studies were done in the mouse except as indicated.

[b] LAF, lymphocyte activity factor; NS, not significant.

show considerable variation depending on the type of exudate macrophage. The secretion of lymphostimulatory molecules resulting from macrophage–T cell interactions (Section III,C) took place best with resident macrophages, to a lesser extent with peptone-induced macrophages, and very poorly with thioglycolate-induced macrophages; in contrast, the inflammatory macrophages in the same culture developed tumor cytotoxicity, while the resident macrophages were practically inactive (Farr *et al.*, 1979a,b; Unanue *et al.*, 1980).

Whether or not there is heterogeneity within the Ia$^+$ population insofar as antigen-presenting function is an important point that has not been critically addressed. A study by Lee *et al.* (1979), suggested that this might be the case. They separated macrophages on the basis of size, using velocity sedimentation in albumin gradients, and found some fractions that were better than others, despite the presence of Ia-bearing cells in all. Detailed cytological studies were not reported, nor was the issue of nonspecific suppression analyzed. Thus, the reasons for this heterogeneity remained unexplained.

The *spleen Ia-bearing macrophages* were first studied by Cowing and associates (1978a,b). They isolated a population of adherent phagocytic cells capable of taking up latex beads and with a high representation of Ia using immunofluorescence or cytotoxicity. The figures in Cowing's studies amounted to about 50% of the phagocytes. Figures about the same range were found in other studies (Beller *et al.*, 1980; Lu *et al.*, 1980). The spleen macrophages synthesized Ia in culture and expressed determinants both in *I-A* and in *I-E/C* region (Cowing *et al.*, 1978a). Dickler *et al.* (1980), isolated the population of Ia-bearing phagocytes using the fluorescence-activated cell sorter and found excellent antigen-presenting function. Other accessory cells found in spleen besides the mononuclear phagocyte include the interdigitating cells, the Langerhans cells, and the dendritic-type isolated by Steinman and Cohn (1973). These will be discussed in Section III,A,2.

The *skin Ia-bearing Langerhans cell* was discussed in Section II,D and will also be considered in the next section.

The *liver Kuppfer cells* were isolated and tested for percentage of Ia- and antigen-presenting function (Richman *et al.*, 1979, 1980; Forsum *et al.*, 1979a; Lipsky and Rogoff, 1980; Rogoff and Lipsky, 1979, 1980). Berzofsky's laboratory isolated the Kuppfer cells from the mouse by treatment of liver slices with collagenase (Richman *et al.*, 1979). The cells were typical phagocytes, 50% of which bore Ia. The Ia-positive cells contained molecules coded in the *I-A* and *I-E/C* subregions. Isolated Kuppfer cells were essential for the proliferative response of purified T cells to sperm whale myoglobin (Richman *et al.*, 1969). Lipsky's laboratory isolated the Kuppfer cells from guinea pig liver

using collagenase and trypsin (Rogoff and Lipsky, 1980; Lipsky and Rogoff, 1979, 1980). They found that about 27% bore Ia. These liver macrophages were essential for the proliferative response to lectins and antigens and showed typical MHC restrictions, but were less active when compared to peritoneal macrophages. Both groups commented on the biological significance of an antigen-presenting function by the Kuppfer cell. Clearly, the Kuppfer cells have the potential to interact with lymphocytes, but evidence *in vivo* suggests that antigens that flow through the liver lose their immune potential. It is not clear how both phenomena can be related.

There are two current studies on Ia in *alveolar macrophages.* Our laboratory found about 10% Ia-positive cells in the macrophages from nonstimulated murine lungs (Weinberg, 1980; Weinberg and Unanue, 1981), while Uhr's laboratory, working in the guinea pig, found about 80% (Toews and Lipscomb, 1980; Lipscomb *et al.*, 1981). Alveolar Ia-positive macrophages presented antigens and were as active as peritoneal macrophages. Curiously, in our own studies, we found that alveolar macrophages lacked the capacity to bind *Listeria monocytogenes* and, therefore, presented poorly these bacteria to immune T cells in functional assays. Murine alveolar macrophages also lacked C3 receptor (Alblas and van Furth, 1979). Both C3 receptor and the structures that bind to *Listeria* are trypsin sensitive, and it may be that the normal proteases of the lung fluid are affecting the macrophage surface and modifying its function. Opsonization of *Listeria* allowed for its binding to the trypsin-resistant Fc receptor of the macrophage with the development of antigen-presenting function (Weinberg, 1980; Weinberg and Unanue, 1981).

Thymic macrophages were found to contain a very high percentage of Ia-positive cells (Beller and Unanue, 1978, 1980; Lu *et al.*, 1980) (Section II,G).

Monocytes were used as antigen-presenting cells mostly in human studies. As referred to in Section II, their antigen-presenting function resided in the Ia-bearing population. The percentage of monocytes bearing Ia varied from 50 to 75 (Albrechtsen, 1977). A recent study of Raff and associates (1980) employed a monoclonal antibody that discriminated two populations of glass-adherent monocytes, each containing Ia determinants; of great interest is that clearly functional dichotomy was found after testing the two populations. Elimination by cytolysis, with complement, of macrophages bearing the 120,000-dalton determinant (termed Mac-120 antigen) markedly diminished the antigen-induced proliferation but not the capacity to induce an alloreactive mixed leukocyte reaction. The Mac-120-positive cells were highly active in stimulating basal proliferation of T cells. Cytological and biological examination of both subsets have not yet

been reported, except with regard to their similar uptake of latex particles. Mac-120 was found in 40% of the monocytes.

Macrophages have been cultured from bone marrow in the presence of medium containing growth-promoting molecules. The cells isolated from the marrow were pure macrophages bearing Fc and C3 receptors and having phagocytic properties. Bone marrow-grown macrophages were tested in various antigen-presenting systems and found to be highly effective (Stern *et al.*, 1979; Lee and Wong, 1980; Erb *et al.*, 1980b; Mottram and Miller, 1980; Calamai and Unanue, 1980). These included induction of helper T cells (Stern *et al.*, 1979); presentation of tuberculin, hemocyanin, and flagellin for T-cell proliferation (Lee and Wong, 1980); presentation of *Listeria monocytogenes* assaying T-cell proliferation, macrophage secretion of lymphostimulatory molecules, and macrophage activation for cytocidal activity (Calamai and Unanue, 1980); and macrophage induction of delayed sensitivity *in vivo* (Mottram and Miller, 1980). The antigen-presenting function has been definitely ascribed to the Ia-bearing macrophage population—about 30%—isolated by cell-sorting experiments (Erb *et al.*, 1980b,c).

b. Regulation of Ia in Macrophages and of Macrophage Ia Population. Three recent important observations indicate that the biosynthesis of Ia by macrophages takes place during a brief period of time, that the activity or stimulation of the macrophage may regulate the expression of membrane Ia, and that the relative percentage of Ia-positive and Ia-negative macrophages in tissues is under regulation. These are results that are mostly coming from studies with David I. Beller and other members of our laboratory (Beller *et al.*, 1980; M. G. Scher *et al.*, 1980; Beller and Unanue, 1980, 1981).

The time that Ia-bearing macrophages expressed Ia was brief and limited, under basal conditions, to a few hours. Fluorescent antibody studies of peritoneal macrophages in culture indicated progressive loss of Ia so that, by 24–72 hours, most of the Ia-bearing cells were no longer detectable (Beller and Unanue, 1980a). Biosynthetic studies indicated strong synthesis of Ia by the macrophages during the first day but then a progressive loss with time. This loss of Ia surface expression and biosynthesis could not be accounted for by loss of macrophages from the culture dish. This progressive loss of the capacity to synthesize Ia explains the early results of B. D. Schwartz *et al.* (1976), who reported a negligible amount of Ia made by cultured macrophages. In their experiments, biosynthesis was studied only after 3 days of culture. Also noteworthy are the studies of Cowing *et al.* (1978b), who found a loss of Ia-positive spleen macrophages after 1 week of culture but could not differentiate between selective loss of cells and lack of expression.

The loss of Ia biosynthesis in Beller's experiments was selective.

Total protein synthesis was not impaired at the time that Ia was not being synthesized. Furthermore, a different membrane protein, the H-2K protein, was synthesized continuously in culture (Fig. 7).

In vivo studies disclosed a loss of Ia by the macrophages. These experiments were done by examining X-irradiated mice (Beller and Unanue, 1981). Such mice showed a marked loss of Ia-bearing macrophages time after irradiation with kinetics very similar to that found in culture. To ascertain truly that the loss of Ia represented selective loss by the macrophage—not the disappearance of a subset of macrophages—cell transfers were done. C57BL/6 mice were irradiated, then transplanted intraperitoneally with macrophages from (C57BL/6 × A)F_1 mice; at times thereafter, the peritoneal cavity was examined, and the transplanted macrophages were identified immunocytochemically by examining for surface-bound H-2K^k and I-A^k molecules (corresponding to the haplotype of the A strain). Indeed, the transplanted macrophages could be easily identified by the presence of H-2K, yet the I-A^k molecule had completely disappeared with time after examination. Because the percentage of Ia-positive macrophages of the donor macrophages was very high, the results strongly indicated a selective loss of this protein from the recovered macrophages. The implication of this finding as regards the effects of X-irradiation in immunity is discussed below.

When examining the loss of Ia in culture, the question was asked whether the biosynthesis of Ia was affected by the stimulation of the macrophage (Beller and Unanue, 1980b). Although nonstimulated macrophages lost Ia during the first 48 hours of culture, the uptake of latex beads, dead *Listeria monocytogenes* organisms, opsonized red cells, or soluble antigen–antibody complexes resulted in continuous biosynthesis. After phagocytosis, there was progressive expression of Ia to the surface of the macrophage as well as incorporation of labeled amino acids into the newly synthesized Ia protein. Interestingly, the biosynthesis of Ia stimulated by particle uptake was taking place only in the set of macrophages that originally expressed surface Ia. Elimination of such Ia-positive macrophages by antibody (with complement) resulted in no expression of Ia after particle phagocytosis by the remaining Ia-negative macrophages.

A different series of observations indicated an *in vivo* control of the Ia-positive and Ia-negative macrophage populations. Beller *et al.* studied the peritoneal exudate for possible changes in Ia-positive macrophages under different inflammatory conditions (Beller *et al.*, 1980). Mice were inoculated with a number of inflammatory molecules—mineral oil, peptone, thioglycolate broth, endotoxin—and were also infected with *Listeria monocytogenes* organisms. While the first group of materials produced an increase in macrophages, this increase con-

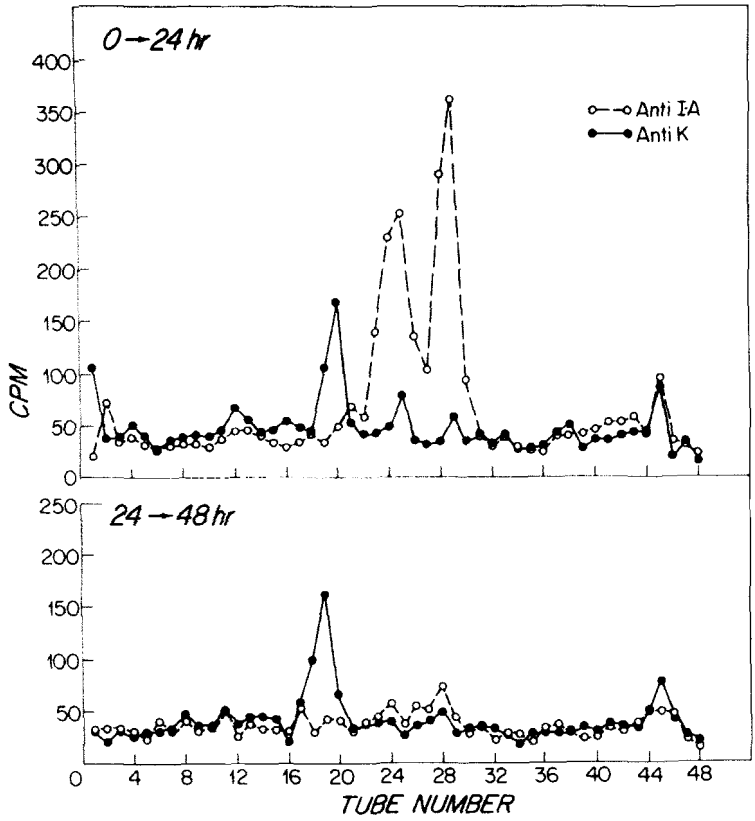

FIG. 7. Macrophages were cultured from zero to 24 hours or from 24 to 48 hours after planting with [^{3}H]leucine, after which the cell-bound I-A or -K products were isolated and precipitated with specific monoclonal antibodies and examined by polyacrylamide gel electrophoresis. During the first 24 hours, the macrophage synthesizes both K and I-A proteins; after 24 hours, the synthesis of I-A is markedly reduced. (From Beller and Unanue, 1981.)

sisted predominantly of Ia-negative macrophages. The absolute number of Ia-negative macrophages was actually increased by about 10-fold 3 days after the intraperitoneal injection. In contrast, infection with live *Listeria* resulted in a complete shift in the macrophage population to 50–90% Ia-positive macrophages. Similar results were obtained after infection with *Mycobacterium tuberculosis* (Beller *et al.*, 1980) or with *Trypanosoma cruzi* (Behbehani *et al.*, 1981). The marked increase in Ia-positive macrophages following infection resulted from T cell stimulation. An early indication that this was the case came from analysis of immune mice. Mice immune to hemocyanin or *Listeria* were challenged intraperitoneally with either hemocyanin or *dead Listeria* organisms, and the percentage of Ia-

positive macrophages was studied days later. Either of the two antigens did not induce a noticeable change in the distribution of Ia-positive macrophages in unimmunized mice. However, a *secondary* boost resulted in a marked increase in Ia-positive macrophages, but only following specific challenge, i.e., hemocyanin-primed mice did not respond to dead *Listeria* but only to hemocyanin. Further analysis disclosed that T cells transferred the capacity to develop an exudate rich in Ia-positive macrophages. T cells were harvested from immune mice and transplanted intraperitoneally together with the specific antigen: 3 days later, there was a marked increase in Ia-positive macrophages in the peritoneal exudate, but not in the spleen.

T cells regulated the appearance of Ia-positive macrophages by way of a soluble mediator (Scher *et al.*, 1980). Peritoneal exudate cells from *Listeria*-immune mice composed of macrophages and T cells were cultured with dead *Listeria* organisms; the conditioned medium from such cultures, when injected intraperitoneally, induced the appearance of Ia-positive macrophages. The production of the mediator—termed macrophage Ia-recruiting factor or by the acronym MIRF—was dependent on the interaction in culture of Ia-positive macrophages and T cells, together with the specific antigen. While the production of MIRF, like all mediators, required the involvement of the *I* region, its action was unrestricted by the *H-2* (Fig. 8). Preliminary studies indicate that MIRF is a nondialyzable protein, stable to 56°C and to repeated freeze-thawing (Scher *et al.*, 1980).

How are the T cells—or MIRF—regulating the appearance of Ia-positive macrophages? We have now found, in studies not yet published, that the action of T cells in recruiting Ia-positive macrophages is radiosensitive. The experiments in X-irradiated mice are informative inasmuch as they show one possible mode of action of the various phlogogenic stimuli in regulating macrophage populations. Normal and irradiated mice were injected with T cells and antigen, or mineral oil, and the peritoneal exudates were examined a few days later. The X-irradiated mice, in contrast to the normal mice, did not develop either exudates rich in Ia-positive macrophages in response to T cells, or exudates rich in Ia-negative macrophages, in response to the mineral oil. Such exudates were found if the X-irradiated mouse was reconstituted with bone marrow stem cells. The experiments indicated that the two responses, to immune or nonimmune stimuli, operated on the bone marrow precursors, but not at the level of the mature tissue macrophages (Scher *et al.*, 1981).

More recent studies have tested the response in culture of Ia-negative macrophages to lymphocyte mediators (Steinman *et al.*, 1980; Steeg *et al.*, 1980). After several days of incubation with the lymphokines, a high number of the Ia-negative macrophages went on to

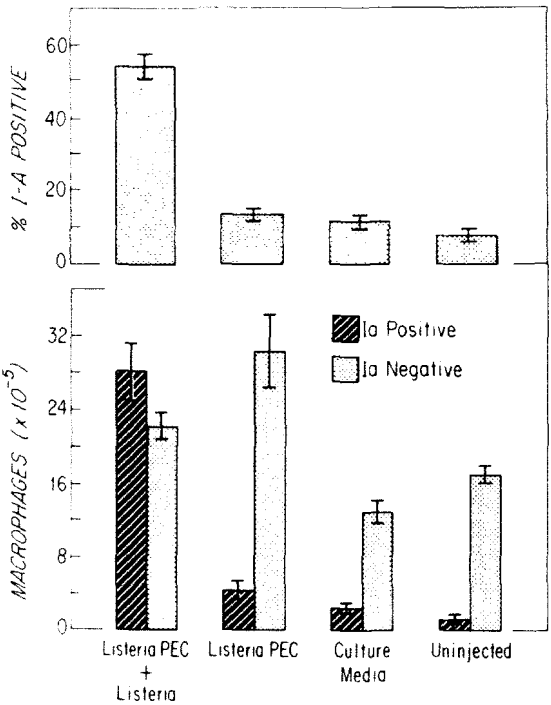

FIG. 8. Recruitment of Ia-positive macrophages by a soluble mediator. Mice were injected intraperitoneally with (*a*) a culture fluid from mixtures of macrophages and *Listeria*-immune T cells with heat-killed *Listeria monocytogenes* organisms (*Listeria* PEC + *Listeria*; (*b*) a culture fluid from the same cells but not challenged with *Listeria* (*Listeria* PEC); and (*c*) regular medium. The percentage of Ia-positive macrophages and the absolute numbers of Ia-positive and Ia-negative in the peritoneum were investigated 12 hours later. The active factor that recruits Ia-positive macrophages is secreted *in vitro* only by challenge of immune T cells with macrophage-bound antigen. PEC, peritoneal exudate cell. [From Scher *et al.* (1980).]

synthesize and express Ia, indicating that most macrophages have the potential to make these modulatory proteins. In agreement with the *in vivo* studies of Scher *et al.* (1981), the expression of Ia by the cultured phagocytes depended highly on their state of maturation. Most monocytes and exudate macrophages readily expressed Ia but only a small minority of the resident macrophages (Beller and Unanue, unpublished observations).

c. Ontogeny. The control of the Ia-positive and Ia-negative macrophage subgroups is evident in studies on their ontogeny in the mouse. Up to the second week to the fourth week of life, the macrophage population is made up mostly of Ia-negative cells (Lu *et al.*, 1979; Nadler *et al.*, 1980). Lu's studies examined the peritoneal and spleen macrophages for their uptake and presentation of *Listeria*

monocytogenes. The macrophages took up the bacteria but were unable to stimulate proliferation of immune T cells from an adult. The macrophages from the neonate were phagocytic, could be activated by lymphokines, and secreted lymphostimulatory molecules when stimulated with endotoxin. Their lack of antigen-presenting function was best attributable, as would be expected, to their low content of Ia-positive macrophages. No suppressive effects were noted when the macrophages from the neonate were mixed with adult spleen cells. In Nadler's study, the immunoincompetence of the spleen accessory cells was studied in the primary antibody response to haptenated hemocyanin, and, as in Lu's experiments, it correlated with the absence of Ia-positive cells. These results may explain previous studies indicating a deficient function of adherent cells from neonates in the primary antibody response to SRBC (Landahl, 1976; Nakano *et al.*, 1978); to be noted is that an initial study by Fidler *et al.* (1972) had failed to see a defect in the adherent cells. It is of interest that Argyris in 1968 reported that injection of macrophages from adult mice into newborns resulted in the development of an antibody response to SRBC. This observation was confirmed (Hardy *et al.*, 1973; Blaese and Lawrence, 1977).

Another interesting point concerning the ontogeny of Ia-positive cells is their appearance in the thymus. In the thymus, Ia-positive macrophages with effective antigen-presenting function were found since birth, in contrast to their absence in the spleen (Lu *et al.*, 1980). In a different study, Jenkinson *et al.* (1980) reported finding thymic stromal cells bearing Ia antigens since day 14 of gestation. We have speculated that the Ia-positive accessory cells found in the neonate since very early life function not to present antigen, but rather to modulate the antigen-independent differentiation of T cells (Lu *et al.*, 1980) (Section II,G).

Recent studies may explain the defect in Ia expression by the macrophages of the neonate (Snyder *et al.*, 1981). Neonates injected intraperitoneally with MIRF did not respond with exudates rich in Ia-positive macrophages. Furthermore, a cell was identified in the neonatal spleen that, when injected into adults, interfered with the development of exudates rich in Ia-positive macrophages induced by T cells. This "suppressor" cell did not bear surface markers of T or B cells, was actively proliferating, and poorly adherent. That the macrophage from the neonate has the potential to express Ia was evident in the cell culture systems where Ia could be induced in them by a T cell-conditioned medium. Thus, it appears that the lack of Ia expression in the neonate is caused by an active suppression induced by dividing hematopoietic cell and not be an intrinsic defect of the phagocyte.

The lack of Ia-positive macrophages in the secondary lymphoid tissues and in exudates appears, at face value, to place the neonate at a disadvantage during a critical time of life. It may be, however, that this lack of Ia-positive macrophages is what regulates the lack of response to self antigens during this important developmental stage, and it ensures the establishment of self-tolerance.

d. Summary of Ia Macrophage Subsets. The more recent data indicate that macrophages synthesize Ia molecules for a brief period of time, after which they lose the capacity to express this important regulatory molecule. The loss of Ia by tissue macrophages implies that a certain amount of the Ia-negative macrophages were cells that initially contained Ia. However, it is not resolved whether *all* Ia-negative macrophages derive from Ia-positive cells. Indeed, the Ia-negative macrophages from fresh exudates induced by nonimmune stimuli were young bone marrow-derived cells that did not contain Ia from the time that they left the marrow; whether these cells contained Ia during their intramarrow life is not known. It may well be that the phagocyte is a single cell lineage in which Ia is expressed at only a certain stage, which can be regulated by external stimuli. It may also be that the line splits at some point, one branch never expressing Ia, while the other is capable of making the protein at least during a period of time. The recent experiments cited before, in which a majority of monocytes and early macrophages express Ia following culture with lymphocyte mediators, support the former statement.

The fact that Ia is synthesized briefly establishes that the maintenance of Ia-positive macrophages in tissues requires a continuous supply of new cells from their precursors. Indeed, the X-irradiated mouse showed, together with the loss of stem cells, the loss of Ia from its radioresistant tissue macrophages (Beller and Unanue, 1980b). Although macrophages harvested from irradiated mice presented antigen (Schmidtke and Dixon, 1972a), one would predict that macrophages harvested from mice several days after irradiation would function poorly as antigen-presenting cells in an MHC-restricted system. Indeed, macrophages from X-irradiated mice presented antigen poorly in an early experiment done by Galilly and Feldman (1967), perhaps explained by the loss of Ia.

In essence, the macrophage Ia phenotype is regulated by (*a*) the activity of the macrophage; (*b*) immune and nonimmune stimuli; (*c*) unknown tissue conditions that result in varying ratios; and finally (*d*) ontogenetic events. This regulation of Ia expression underlines the importance of controlling the synthesis of such an important regulatory protein in a multifaceted cell like the macrophage.

2. *Accessory Cells besides the Macrophages*

A number of cells have been identified in lymphoid organs with some characteristics in common with classical phagocytes, but also with some differences. We now review their characteristics, role as accessory cells, and possible relationship with the classical phagocyte.

The Langerhans cells of the skin were discussed in Section II,D. These are cells with typical morphological appearance: a dendritic appearance in the skin, a clear cytoplasm, a few lysosomes, and a typical organelle, the Birbeck granule. Langerhans cells bear Fc and C3 receptors and present antigen in a typical MHC-restricted fashion. Cells resembling Langerhans cells have also been described in the secondary lymphoid organs, in the thymus, and in the afferent lymph. Thus, histological analysis of lymphoid organs identified in the thymus-dependent areas stellate cells, called interdigitating reticular cells, in intimate contact with the surrounding lymphocytes (Veerman, 1974; Van Ewick *et al.*, 1974; Hoefsmit *et al.*, 1980; Veerman and Van Ewick, 1965). These cells had morphological features of the Langerhans cells, and many contained Birbeck granules. Interdigitating cells increased in number after antigenic stimulation (Silberberg-Sinakin *et al.*, 1976; Kamperdijk *et al.*, 1978). The studies of Silberg and Thorbecke, mentioned in Section II,D, indicated a rapid increase in cells in dermal lymphatics and in nodes draining the sites of contact reactions. Such cells were classified, on the basis of Birbeck granules, as Langerhans but were indistinguishable with the interdigitating cells. Other studies indicated that the interdigitating cells of the lymph nodes derived by way of the afferent lymphatics inasmuch as the cells disappeared after the ligation of the afferent vessels (Kamperfijk and Hoefsmit, 1978). To this effect, it is noteworthy that Langerhans cells were found to originate from bone marrow precursors in experiments using transplanted bone marrow cells (Katz *et al.*, 1979; Frelinger *et al.*, 1979). A histological study identified stellate Ia-bearing cells in the thymus-dependent areas of lymph nodes; these cells were compatible in morphology with the interdigitating cells (Hoffman-Feizer *et al.*, 1976) (Section II,G). In the thymus, cells with morphological features of interdigitating cells were identified, some of which contained Birbeck granules (Hoefsmit *et al.*, 1980). Cells with dendritic morphology bearing Ia were identified in frozen section and actually isolated (Section II,G).

Cells with characteristics akin to classical phagocytes and Langerhans cells were found in lymph from afferent lymphatics of rabbits (Kelly *et al.*, 1978), pigs (Drexhage *et al.*, 1980), and man

(Sokolowski *et al.*, 1978; Spry *et al.*, 1980). These were medium-size mononuclear cells, some having C3 and Fc receptor, and a few having Birbeck granules. The cells emitted long cytoplasmic projections; thus, the term "veiled cell" given to them. Veiled cells were particularly abundant in the lymph after skin irritation. In man, the large mononuclear cells with veiled cell characteristics contained Ia molecules (Spry *et al.*, 1980).

The Langerhans-interdigitating-veiled cell should be compared to a fourth cell with dendritic appearance, the dendritic cells isolated by Steinman and Cohn (Steinman and Cohn, 1973, 1974; Steinman *et al.*, 1974, 1975). These investigators isolated from the spleen a glass-adherent cell with dendritic extensions and scanty cytoplasm containing few organelles. The dendritic cells did not exhibit Fc and C3 receptors but had a high content of Ia antigens (Steinman *et al.*, 1979). They were isolated after initial adherence to culture, after which they spontaneously detached. In culture, the dendritic cells were found to be strong promoters of the mixed lymphocyte reaction (Steinman and Witmer, 1978) and also stimulated growth of autologous lymphocytes, the so-called syngeneic mixed lymphocyte reaction (Nussenzweig and Steinman, 1980). The dendritic cell isolated by Steinman is also bone marrow derived and tends to cluster with lymphocytes in a manner similar to that shown for the veiled cells.

A comparison of the various cells discussed above is shown in Table VI, which, in great part, I derived from a recent and very excellent review by Thorbecke (Thorbecke *et al.*, 1980). To me, the evidence is strong that the interdigitating cell, the Langerhans cell, and the veiled cell are the same: their characteristic morphology, the presence of Fc and C3 receptor, and the Ia content argue for this. It is my opinion, too, that the dendritic cell of Steinman is most likely part of this lineage: its morphological resemblance and presence of Ia also is in favor. However, the lack of Fc and C3 receptors argues against it. Also, no Birbeck granules have been reported in the cells isolated by Steinman. Although these arguments are powerful, they are not conclusive. Fc and C3 receptors are dynamic membrane structures that may fluctuate in their expression as a cell develops, matures, or is activated. The lack of Birbeck granules must be taken with caution: the amounts of this organelle among skin dendritic cells vary considerably, and some even lack them (see Thorbecke *et al.*, 1980, page 33). Some species, particularly the mouse, appear to have few Birbeck granules.

It may well be, therefore, that all the four cells discussed above belong to the same lineage. The definite answer awaits until such a time as detailed culture analysis and surface phenotype studies be-

TABLE VI

COMPARISON OF VARIOUS TYPES OF CELLS[a]

Property	Macrophages	Langerhans cells	IDC[b]	Veiled cells	Thymus dendritic cell	Spleen dendritic cell (Steinman)	Germinal center dendritic cell
Surface markers							
Fc receptor	++	+	NK	±	++	—	?
C3 receptor	++	+	NK	±	—	—	++
Ia	++	++	+ (?)	+	++	++	NK
Glass adherence	++	++	NK	±	+	+	NK
Latex phagocytosis	++	±	NK	±	—	—	NK
Lysosomes	++	±	+	NK	NK	±	±
Birbeck granule	—	±	Some	Some	Some	—	—
Pinocytic vesicles	+++	++	++	++	NK	+	±
Antigen presentation	++	++	NK	NK	NK	NK	NK
Stimulator: MLC	++	++	NK	NK	NK	++	NK
Origin	Tissue and bone marrow precursors	Bone marrow	NK	NK	NK	Bone marrow	NK

[a] References are included in the text.

[b] IDC, interdigitating cells; NK, not known.

come available. The relationship of the Langerhans cell—and others—with the classical phagocyte also needs evaluation. The issue is whether both sets of cells represent two completely distinct lineages, two lines that split from a common precursor, states in the differentiation of the phagocyte, or adaptation of the classical phagocyte to a particular tissue microenvironment. Although the phagocytes have shown a great degree of plasticity in responding to stimuli, no direct evidence has been brought up that macrophages in culture develop characteristics of Langerhans cells. Therefore, the first two possibilities are the most likely.

Critical comparison of the function of the two cells should inform us of what may be functions in common or particular to each type. So far, Langerhans cells and the typical mononuclear phagocyte presented antigen to the same extent and were both active as stimulators in the mixed leukocyte reaction. Both cells most likely cooperate in bringing about a dynamic response to antigen. The macrophage, because of its antigen-handling properties, should be the major cell involved in the response to bacteria, virus, protozoa, and tumors, as well as proteins, whereas the Langerhans group of phagocytes may be more involved in the response to soluble sensitizers that attach to the skin. The Langerhans group of cells may represent a line of phagocytic cells that selectively homes to certain specialized tissues and is endowed with less inflammatory properties—hence its presence in skin and in the thymus. The macrophages, on the other hand, have the added function of more actively responding to various inflammatory agents.

Finally, a comment on the antigen-trapping mechanism of the germinal center seems pertinent. Not many studies have been made in the past few years, so its mechanisms and role remain to be elucidated. Antigen trapping to germinal center requires antibody and, particularly, complement (Papamichael *et al.*, 1975) and may be associated with dendritic-type cells studied ultrastructurally by Nossal's laboratory (see our past review, Unanue, 1972; and Nossal and Ada, 1978). The dendritic cells of the follicles have *not* been shown to contain Birbeck granules and appear different from the interdigitating cells (Hoefsmit *et al.*, 1980); their precise identification and role require their isolation (Klaus *et al.*, 1980).

B. Antigen Presentation

In Section II we reviewed the many examples of antigen presentation via the macrophage and the essential role it played in bringing about the stimulation of the lymphocyte, particularly the T cell. It became obvious that the development of all T-cell functions required

an interaction with macrophage-bound antigen regulated by the *I* region. No stimulation of T cells took place by direct interaction with antigen. In general, the regulation of the macrophage–T cell interaction by the MHC was akin to that shown for T-B cell collaboration or for the interactions between cytolytic T cells and their target cells.

1. Ir Gene Function and Determinant Selection

The genetic basis of the MHC "restriction" has been the subject of extensive and fascinating studies, still in progress, that have examined the cellular basis of *Ir* genes, the role of the nonlymphoid cell "environment" prior to the entrance of antigen, the involvement of the thymus and extrathymic cells in regulating the interactions, etc. Hypotheses have been put forward to explain the restrictions, one postulating that the T cell bears two receptors, one for the antigen and a second for the MHC product; another postulating a single T cell receptor that recognizes an antigen–MHC complex. No definitive answers are yet available and will not be, in my opinion, until the immunogenic moiety from the macrophage and the T cell receptors are isolated. I will limit this section to a consideration of important pieces of information that associate the macrophage to *Ir* gene function. Extensive reviews of immune response genes have appeared (Katz, 1977; Benacerraf and Germain, 1978; Zinkernagel, 1978; Berzofsky, 1980).

A number of important points came out from the studies reviewed in Section II. The involvement of the *I* region in the T cell–macrophage interactions applied not only to antigens under *Ir* gene control—as defined by states of responsiveness or nonresponsiveness—but to all proteins, even complex ones with multiple antigenic determinants. Analyzing antigens under *Ir*-gene control, it became evident in several studies that the macrophage was a key cell responsible for the responder or nonresponder status. This was shown with synthetic polypeptides where *Ir* gene status could be easily identified. The first study by Shevach and Rosenthal (1973), indicating responses of T cells only to antigens bound to macrophages from responders, was confirmed, for example, using another synthetic polypeptide like (TG)-AL (Erb and Feldman, 1975b; Hodes *et al.*, 1978; Kappler and Marrack, 1978) or natural proteins, to be discussed below.

Let us first discuss the selection of T cell antigen-reactive cells by the MHC environment in which they mature and then continue with the analysis of the antigen-presenting defect in "nonresponder" macrophages. We can start by questioning why there is such limited, if any, T cell reactivity to antigens bound to allogeneic macrophages. The lack of T cell reactivity to antigen-allogeneic Ia may mean that (*a*)

such cells are never generated during the normal non-antigen-driven differentiation of the T cell in its syngeneic thymus milieu; or (*b*) that the T cells did develop and joined the pool of mature T cells but are not found in the usual assays simply because they are a very minor component, overshadowed by the growth of the syngeneic reactive T cells. (It is indeed clear that an antigen-reactive cell, if found in the secondary lymphoid tissues, can be selected for and expanded during the handling of the antigen by macrophages. These were the studies showing that F_1 T cells consisted of two antigen-reactive sets of cells, each of which could be selected *in vivo* or *in vitro* by one or the other parental macrophage.) There are experiments in the literature in support of both explanations.

A role of the MHC of the thymus in generating and selecting the various antigen-reactive T cells was theorized by Jerne in 1971. His hypothesis has served as the basis for a number of experimental studies transplanting bone marrow cells into X-irradiated mice of different haplotypes, thus allowing maturation of stem cells to lymphocytes in different H-2-bearing hosts. Simply stated, the Jerne hypothesis envisions lymphocytes carrying germ line genes that encode for the receptors of the MHC of the species. The diversity of receptors to foreign antigens takes place as a result of mutations in T lymphocytes upon reaction with the thymic accessory cells bearing the self-MHC product. Thus, a lymphocyte would express two sets of receptors, one for the MHC product, the other to the foreign antigen (Von Boehmer *et al.*, 1978). Data particularly on cytolytic T cells but also with helper T cells support the fundamental premise of the hypothesis to a greater or lesser extent. Without entering into detail, the general consensus is that the host MHC environment, be it in the thymus and/or in the extrathymic milieu, has an influence, at least in the phenotypic expression of antigen-reactive T cells (reviewed in Berzofsky, 1980). The phagocytes, by virtue of their Ia content, their capacity to present antigen, their widespread tissue distribution, and their migratory patterns, may well be a major cell, not only handling and presenting antigen, but also regulating the non-antigen-driven maturation or "selection" of T cells (Section II,G).

Experiments examining helper cells support the statement that a T cell reactivity, not previously apparent, can be generated if the T cell develops in the tissues of a host bearing the appropriate MHC haplotype. Kappler and Marrack (1978), for example, produced bone marrow chimeras by transplanting bone marrow cells from one mouse strain (i.e., parent *a*) into lethally X-irradiated F_1 mice (from the same parent strain *a* and a different one, i.e., $a \times b$) and, weeks later, studied

the reactivity of the T cells. The T cells from such chimeras cooperated especially well with B cells and macrophages of either parental H-2 type (*a* or *b*) in bringing about a response to SRBC or hemocyanin. This basic result was confirmed by others (Singer *et al.*, 1979; Erb *et al.*, 1979, 1980a,b). However, T cells from chimeras made of bone marrow from an F_1 into a parent cooperated only with B cells and macrophages of the parental host (this result was also supported by other studies, i.e., Sprent, 1978c; Katz *et al.*, 1978). Thus, in their situation, the potential antigen reactivity of T cells could be expanded or decreased by their differentiation in an appropriate host. This same experimentation also applied to antigens under *Ir*-gene control showing state of responsiveness or unresponsiveness. For example, chimeras were made by transplanting bone marrow stem cells from a "nonresponder" mouse into X-irradiated recipients from an F_1 cross between a responder and a nonresponder; the T cells were subsequently examined. Kappler and Marrack (1978) found that such T cells provided helper cells to (TG)-AL if now tested in cultures with B cells and macrophages from responder, but not from nonresponder, mice [the basic conclusions were also confirmed by Hodes *et al.* (1979)]. The studies of Erb *et al.* (1980a,b), used the *in vitro* system described in Section II,C and confirmed the previous results using hemocyanin, adding experiments with insulin, an antigen under strict MHC control.

A different approach was taken by Thomas and Shevach (1977). They explored the possibility that mature T cells reactive to antigen in allogeneic macrophages could be found if appropriately selected for. They examined a system of *in vitro* generation of hapten-specific T cells. The system consisted of three sequential cultures: (*a*) T cells from strain 13 guinea pigs were cultured with strain 2 macrophages for 3 days, then exposed to bromodeoxyuridine and light to inactivate the anti-strain 2 alloreactive T cells; (*b*) such treated cells were then incubated for 5 days with strain 2 macrophages that contained surface TNP groups; after which (*c*) the T cells were cultured with fresh TNP-macrophages from strain 2 and assayed for proliferation. The strain 13 T cells still reacted with strain 2 macrophages (not derivatized) about 80% less than the T cells not treated with the drug. Significantly, the T cells proliferated vigorously to the TNP-macrophages of strain 2, and this reaction was inhibited by antibodies to the Ia antigens of strain 2 (Thomas *et al.*, 1977b). Not killing the antigen-alloreactive T-cell clone did not allow for the demonstration of the TNP-specific clone. These results, if confirmed, imply that the cells recognizing antigen in the context of allogeneic Ia are present but require to expand in order to become operative. Another situation where T cells react with anti-

gen in an allogeneic macrophage was discussed before for the GAT polymer. In essence, the experimental evidence suggests that the interactions of T cells with the MHC products expressed by the individual is important in the expression of different antigen-reactive T cells. Some experiments suggest that the selection of T cells is a developmental event that takes place prior to contact with antigen, but others have shown it following antigen exposure of mature T cells. Overall, the chimera experiments are difficult and may contain complicating features, and whether the selection of T cells is more apparent than real requires further studies. The way in which the MHC "environment" regulates antigen selection prior to antigen still remains unexplained.

How does one explain the nonresponder status of the macrophage? Alan Rosenthal's laboratory provided evidence that macrophages, through their handling of antigen, would select the appropriate determinant for the T cell. This concept of "determinant selection" became evident during the analysis of the response to insulin by guinea pig T cells (Rosenthal *et al.*, 1977; Barcinski and Rosenthal, 1977; Rosenthal, 1978). *Ir* genes controlled the immune response to the two chains of insulin: T cells from strain 13 proliferated upon challenge with oxidized B chain of pork insulin, but T cells from strain 2 did not. In contrast, T cells from strain 2 proliferated upon interaction with A-chain determinants. With this information at hand, Rosenthal and associates examined the T cell response of F_1 guinea pigs to insulin or its chains presented on macrophages from either strain 2 or strain 13. T cells from guinea pigs immunized with pork insulin proliferated upon interaction with insulin on either macrophage. Using cross-reactive insulin or isolated chains, it was possible to show that the F_1 T cells proliferated to the B chain only when insulin was presented on strain 13 macrophages and to a sequence of three amino acids of the A chain when presented to strain 2 macrophages. Furthermore, using manipulations with bromodeoxyuridine and light, it was possible to identify two distinct sets of cells, the selection of which depended on the macrophage. Similar selection was found in *in vivo* experiments using F_1 guinea pigs transplanted with strain 2 or strain 13 macrophages (Yokomuro and Rosenthal, 1979). Rosenthal concluded that "a selected amino acid sequence and/or conformation within the antigen itself is seen by the T cell receptor and that generation or display of such antigenic determinants is a function of immune response genes operating at the level of the antigen-presenting cell." Perhaps the *Ir* genes "are, or regulate the activity of, families of enzymes which modify or metabolize polypeptide antigens" (Rosenthal, 1978).

The determinant selection phenomenon was found for several proteins. Thomas' laboratory reported it for the response to human fibrinopeptide B, a 14 amino acid fragment derived from the B β chain of fibrinogen (Thomas *et al.*, 1979a,b; Thomas and Wilmer, 1980). Macrophages from immune strain 2 guinea pigs, but not from strain 13, presented the antigen or synthetic analogs that contained 10 critical amino acids (positions 5 through 14). The terminal arginine was essential, and its absence resulted in a lack of immunogenicity. A further illustration of this phenomenon involving the *I-C* region in the mouse was shown for myoglobin (Richman *et al.*, 1980).

A final point to analyze is the relationship between *Ir* genes and the Ia antigens. Are they the same? The conclusion, based on the genetics of *Ir* genes and of Ia antigens, is that this is the case. Strong experiments to support this claim are those showing *Ir* gene complementation between the *I-A* and *I-E/C* regions for the response to the peptide poly(Glu-Lys-Phe), GLPhe. The response to this peptide required two complementing *Ir* genes, one mapped at the *I-A* to *I-B* subregion, the other to the *I-E/C* subregion. The responder haplotypes were $I\text{-}A^{b}$ and $I\text{-}E/C^{d}$ (i.e., $H2^{b}$ and $H2^{d}$ mice did not respond, but their F_1 offspring or appropriate intra-H2 recombinants mounted a strong immunity (Dorf *et al.*, 1975; Dorf and Benacerraf, 1975). In order to obtain T cell proliferation, the two gene products had to be expressed in the same antigen-presenting cells, which had to come from a responder or from the recombinants bearing the appropriate crossovers at *I-A* and *I-E/C* (Schwartz *et al.*, 1979). In subsequent studies, Jones *et al.* (1978) and Cook *et al.* (1979) brought structural and serological evidence of two complementing gene products in the same strain combinations coding for the I-E/C antigens. One gene product mapped at *I-A* and coded for the β chain, and the second mapped at *I-E/C* and coded for the α chain. The finding of a hybrid molecule provides a biochemical explanation to the complementation studies using GLPhe and also brings out a molecular mechanism for generating multiple *I*-region products on the cell surface.

2. *Correlation with Antigen Handling*

Several approaches have been taken to study the relationship between antigen handling and the immunogenic moiety recognized by lymphocytes. These include (*a*) correlating metabolism of labeled antigen with immunogenicity; (*b*) attempting to modulate the macrophage presentation of antigen with antibodies to Ia or to the antigen, or by treatment with proteases: (*c*) analyzing macrophage-released products; and (*d*) studying the binding of lymphocytes to mac-

rophages in the presence or absence of antigen. These last studies will be described in Section III,B,3.

Metabolism of Labeled Antigens—Use of Antibodies and Trypsin. The two major attempts so far to correlate the handling of a radioactive antigen with immunogenicity were carried out in my laboratory and in Alan Rosenthal's. The results of many of the experiments were remarkably similar, yet there were important differences, too, that indicate two pathways of antigen presentation.

In our first experiments initiated in the laboratory of Dr. B. A. Askonas, macrophages were pulsed with ^{125}I-labeled hemocyanin; at various times thereafter, the macrophages were transferred live into syngeneic recipients and assayed for their capacity to induce an antibody response. Macrophages were also cultured, and the amounts of ^{125}I-labeled antigen remaining in the cell or released into the medium was determined at various times. Three main results were obtained:

1. Most protein antigens were bound initially to the cell surface, then interiorized in vesicles and rapidly catabolized; the bulk of the catabolized antigen was found in the form of ^{125}I bound to amino acids; however, a small percentage of antigen invariably escaped catabolism (approximately 20%) and remained cell associated or was released (Unanue and Askonas, 1968a,b; Cruchaud and Unanue, 1971; Schmidtke and Unanue, 1971a; Calderon and Unanue, 1974). The antigen that escaped rapid catabolism was identified as (*a*) a released soluble product; (*b*) a small component on the macrophage surface, solubilized by trypsin; (*c*) an internal pool of antigen that was slowly degraded.

2. Culturing macrophages for several periods of time established that there was no correlation between the amount of antigen degraded and its immunogenicity; for example, with hemocyanins, the immunogenicity of the macrophage-bound antigen was relatively stable for a few days, while the bulk of the protein was catabolized (Unanue and Askonas, 1968b); with albumins, the life of the immunogenic moiety was shorter, lasting about a day.

3. Incubating the macrophages with antibody or trypsin prior to *in vivo* cell transfer markedly reduced their immunogenicity (Unanue and Cerottini, 1970); these results were confirmed by testing for antibody formation *in vitro*. The immunogenicity of macrophage-bound hemocyanin did not decay after 28 hours of culture and was sensitive to trypsin treatment of macrophages pulsed with antigen 24 hours *earlier* (Unanue, 1978). It is noteworthy that, in this latter case, the presentation of antigen to T and B cells did *not* require genetic iden-

tity at *H-2* (Unanue, 1978). The lack of MHC involvement was explained by a transfer of the macrophage-associated antigen to an antigen-presenting cell syngeneic with the T cell (Section II,C).

The major point in the catabolism studies was that antigen from phagocytes was available for some periods of time, albeit in decreasing amounts. The antigen molecules were found secreted and on the macrophage surface. The antigens secreted from macrophages included all those tested so far: hemocyanin (Unanue and Askonas, 1968b; also, Askonas and Jaroskova, 1970), albumin (Schmidtke and Unanue, 1971a), iodinated SRBC (Cruchaud and Unanue, 1971; also, Cruchaud *et al.*, 1975), and products from *Listeria monocytogenes* (Unanue, 1980). Calderon did a detailed analysis of this released antigen trying to determine its source, pathway of release, and immunogenicity (Calderon and Unanue, 1974). The released hemocyanin derived from active, live macrophages, not from dead cells, and was represented by 3–7% of the protein initially bound to the macrophages. The released product was not affected by trypsin treatment of the macrophage indicating that it was not derived from any hemocyanin on the cell surface; furthermore, it was of heterogeneous size, indicating changes in the molecule, although a substantial amount was still reactive with antibody. The antigen was secreted mainly during the first 2 hours following uptake, but a small amount was slowly released during later times of culture. [Using γ-globulins, the released antigen was much smaller than the native molecule (Cruchaud and Unanue, 1971; Cruchaud *et al.*, 1975).] The hemocyanin released from macrophages was as immunogenic as the native molecule (our unpublished studies). Products from *Listeria* were also found to be released by macrophages and to be immunogenic without any H-2 restrictions (Unanue, 1980). The released antigen, therefore, appeared to derive from the intracellular compartment and probably represented a few molecules in phagosomes that reverted to the surface and were exocytosed.

The surface-bound molecules, released by trypsin (Unanue *et al.*, 1969; Unanue and Cerottini, 1970) and by EDTA (Unanue and Cerottini, 1970; Askonas and Jaroskova, 1970), included a few molecules that remained membrane bound after the internalization of the bulk of antigen molecules. The amount of protein that persisted on the surface was small, no more than 1%, and this amount decayed with time. Why a small number of molecules was kept on the surface is not known. The surface-bound molecules, at least with hemocyanin, retained their basic native structure: the protein was visualized by electron microscopy in unpublished studies with J. Rosenblith and M. Karnovsky

(mentioned in Unanue, 1975); also, antibody reacted with the trypsin-released hemocyanin (Unanue and Cerottini, 1970). Surface-bound molecules were also found after the binding and internalization of particulate antigens (Cruchaud and Unanue, 1970; Cruchaud *et al.*, 1975). These molecules might have derived from either the action of ectoenzymes on the antigen prior to its interiorization or following the endocytosis of the antigen by reversal to the surface of endocytic vesicles containing the antigen. Limited experiments were done in which macrophages were trypsinized twice, first to remove the bulk of the initial membrane-bound antigen, followed by a latent period to allow for "reexpression," then by a second trypsinization to determine whether new labeled protein could be released. The results indicated that the bulk of the surface-bound molecules was found early after the initial period of antigen binding and that very little was reexpressed.

The studies from Rosenthal's laboratory are important inasmuch as they focused on the relationship between the antigen handling and antigen presentation in a strictly MHC-dependent system, that of the proliferation of guinea pig T cells to syngeneic macrophage-bound antigen. Radioiodinated DNP-guinea pig albumin was rapidly catabolized, but approximately 20% remained cell associated for at least 3 days of culture (Ellner and Rosenthal, 1975). Some of the cell-bound protein was surface bound and releasable by trypsin. Also, a small amount of protein was secreted into the culture supernatant. These results are, in essence, identical to the ones described above. The major differences between the two studies concerned the functional effects of treating the antigen-pulsed macrophages with trypsin or antibodies. First, the immunogenicity of macrophage-bound albumin was lost progressively with time. By 24 hours, there was a 40% drop, and this increased further to 80% by the next day. It is noteworthy that the function of macrophages not pulsed until 48 hours of culture also dropped somewhat (by 40%). This can be explained now by the loss of Ia biosynthesis (Section III,A).

Treatment of the macrophages pulsed with DNP-albumin with trypsin removed cell surface molecules so that antibodies to DNP-guinea pig albumin would no longer bind to macrophages. Notably, the immunogenicity was *not* affected by the treatment after 24 or 48 hours of pulsing with antigen. Trypsinization affected the immunogenicity only if applied immediately after a fresh pulse with antigen at 4°C, at a time when the bulk of the molecules had not been interiorized. This result also applied to the protein PPD (Waldron *et al.*, 1974). Furthermore, exposure of the macrophages to antibody did not have an effect on their immunological function. Ellner and Rosenthal (1975) concluded

that "a trypsin-resistant, antibody-inaccessible component of macrophage-associated antigen, presumably at an intracellular site, is of major relevance in macrophage-dependent T cell recognition." Further analysis assaying for lymphokine production instead of T-cell proliferation and examining for clusters of T cells with macrophages (next section) confirmed that antibodies were not inhibitory (Ellner *et al.*, 1977).

Other attempts to block the responses of T cell–macrophage interactions with antibodies also failed. Werdelin and Shevach (1979) did not block the binding of T cells to macrophages pulsed with DNP-albumin, using a functional assay. Ben-Sasson *et al.* (1977) found no effect assaying morphologically the clustering of T cells to macrophages. In our system, antibodies had no effect on macrophage-bound *Listeria monocytogenes* (Farr *et al.*, 1979a).

The point can be made that T cells recognize "sequential," linear amino acid sequences of the protein, not antigen in its native configuration, whereas B cells recognize conformational determinants (Sela, 1969). Therefore, antibodies would not be expected to block a T-cell reaction with antigen. Loblay *et al.* (1980) prepared monoclonal antibodies to the antigenic determinant of the A chain of insulin (amino acids 8 to 10), which is the sequence recognized by T cells. These antibodies did not inhibit the T cell proliferative response to macrophage-bound insulin. Chestnut *et al.* (1980) prepared antibodies to denatured determinants of albumin and also failed to inhibit T-cell proliferation.

Another major attempt to localize the macrophage-bound immunogen was carried out by Shevach and Thomas. They developed an assay system in which immune guinea pig T cells were generated upon culture with trinitrophenylated live macrophages (TNP-macrophages). The TNP groups were bound to most surface proteins of the macrophages, including the transplantation antigens (Forman *et al.*, 1977). Subsequently, the T cells were assayed by their proliferative response to fresh TNP-macrophages (Thomas and Shevach, 1978a,b). Their main findings were as follows:

1. TNP-macrophages were immunogenic if added to the primed T cells immediately after conjugation of TNP or 24 hours later ("aged" macrophages, to use their term).
2. Addition of anti-TNP antibodies to fresh macrophages blocked the response by about 75%.
3. The TNP presentation by "aged" macrophages (i.e., 24 hours after pulsing with TNP) was not affected by anti-TNP antibodies.

Thus, the immunogen was available early, but not late. Shevach (1980) went on to report that the presentation of antigen by aged TNP-macrophages could be inhibited by anti-TNP antibodies if the cells were derivatized again after 24 hours: (*a*) TNP-macrophages → 24-hour culture → anti-TNP antibody: T cell proliferation; while in (*b*) TNP-macrophages → 24-hour culture → TNP → anti-TNP antibody: no T cell proliferation. Thomas (1978), on the other hand, examined the effects of glutaraldehyde fixation on this system. He noted that very light fixation affected the mixed leukocyte reaction and the response to PPD bound to macrophages but had a small effect on the response to TNP-macrophages. This finding allowed him to manipulate the system. Presentation by TNP-macrophages was not inhibited by antibodies after 24 hours of culture, but was blocked if the macrophages were lightly fixed with glutaraldehyde immediately after TNP conjugation, and then cultured, i.e., TNP macrophages → 24 hours → antibody: no blocking; TNP-macrophages → glutaraldehyde → 24 hours → antibody: T cell response is blocked. Furthermore, macrophages that were cultured 24 hours, then derivatized, were immunogenic if fixed in glutaraldehyde, whereas TNP-macrophages cultured for 24 hours, then fixed, lost all their function (macrophages → 24 hours → TNP → glutaraldehyde: immunogenic; TNP-macrophages → 24 hours → glutaraldehyde: not immunogenic).

Shevach interpreted his results to mean that a few molecules of TNP were available on the membrane but, being so few, could not be cross-linked by antibody and were not modulated; hence, the need to add fresh antigen. Thomas interpreted his experiments to mean that during culture TNP became inaccessible but was expressed upon contact with T cells. Glutaraldehyde "locked" the antigen in place and, therefore, affected the aged TNP-macrophage but not the fresh TNP cell. In other experiments, Thomas and Shevach found that anti-Ia antibody, even for a short period of 1 hour before or after derivatizing the macrophages, blocked very effectively; they argued, therefore, that TNP must be associated with Ia antigens.

One can argue that TNP conjugation of the macrophage is a drastic manipulation that complicates the issue inasmuch as it brings into operation many surface proteins of the macrophage. It is obvious that haptenation does not simplify an already complex system, yet some of Thomas and Shevach's results are striking and do suggest the early availability of a pool of surface antigen for a period of time; the experiments of Shevach with antibody are provocative and so are those of Thomas with fixation and must be further explored with better defined antigens.

Kirk Ziegler and I have a series of yet unpublished experiments using *Listeria monocytogenes* that address the issue of whether the antigen recognized by T cells required a step of internalization and digestion. The system consisted of a brief, 15–30 minute interaction of *Listeria*-immune T cells with macrophages; this resulted in the firm adherence of the T cells with concomitant loss of functional cells from the nonadherent population (Section III). Radiolabeled *Listeria* was added to macrophages and followed cytologically and biochemically as it bound to the macrophage membranes and was then internalized and catabolized. The two major findings were (*a*) that the T cells did not bind to the macrophages until about 30 minutes after the interiorization of bacteria; and (*b*) that *following* this lag period, macrophages fixed lightly in paraformaldehyde could interact with the T cell. The implications are that the bacteria had to be taken in to be digested, after which some of the immunogenic products reverted to the cell surface. Of interest is that iodinated *Listeria* peptides could be released by trypsin, yet this treatment did not affect immunogenicity.

A summary of the main findings and my interpretation of them are given in Table VII, taken from a previous discussion (Unanue, 1978). I conclude that antigen is available from the macrophage in two major forms: (*a*) "unrestricted" by an MHC product and thus free to interact with any cells; and (*b*) linked—at least functionally—with an *I*-region product.

The "unrestricted" antigen is represented by a small number of molecules that are secreted and by a few that remain membrane bound and easily releasable by trypsin, both of which retain, at least in part, their native structure and can be recognized by specific antibodies. The population of "unrestricted" molecules are found with every antigen so far tested. The macrophage, therefore, serves as a source—or "reservoir"—of this antigen for finite periods that vary from antigen to antigen. With hemocyanin, for example, it was long to the point where its immunogenicity *in vivo* could be shown, up to 2–3 weeks (Unanue and Askonas, 1968). Indeed, this available antigen is an *unavoidable* complication in every system that examines antigen presentation and MHC restrictions. All the indications are that the free "unrestricted" antigen can enter into the Ia-restricted pathway by having another antigen-presenting cell interact with it. This is surmised by observations that allogeneic macrophages can present the antigen to T cells (that contain syngeneic macrophages) or to T and B cells that are in themselves *I*-region restricted. Ellner *et al.* (1977) and Pierce and Maleck (1980) (Section II,C) have presented clear evidence in support of this conclusion. Conceivably, as discussed in Section II,C, the B cell in T-B cell mixtures might interact also with this macrophage-

TABLE VII
TWO FORMS OF ANTIGEN PRESENTATION

	I-region restricted	MHC-independent[a]
Assay	Antigen-pulsed macrophages are added to purified T cells	Antigen-pulsed macrophages are added to unfractionated T cells or to mixtures of T and B cells (with macrophages)
Effects of antibody	Usually does not block the interaction	Effective in blocking interaction
Effects of trypsin	Not sensitive	Removes membrane-bound molecules important in the interaction
Effects of anti-Ia antibodies	Blocks the interaction	Not tested
Presentation by allogeneic macrophages	Present poorly	Can present
Presentation by Ia-bearing macrophages	Strictly required	Probably not required
Localization in macrophages	Not known. Intracellular?	Membrane-bound and soluble secreted molecules
Major role	MHC-restrictive interactions with T cells	Source of antigen available for B cells and/or Ia-bearing macrophages

[a] MHC, major histocompatibility complex.

associated antigen and, in turn, present it to T cells in an MHC-regulated interaction (Unanue, 1978).

The antigenic moiety involved in the Ia-restricted interaction has not been characterized: its chemical forms, its location in the macrophage, its association with Ia need to be fully explored. In favor of a processing step for the antigen are the observations that T cells recognize small sequences of amino acids, and that proteins that are not digestible do not stimulate T-cell reactivity. The lack of accessibility of the antigen to proteases (Ellner and Rosenthal, 1975); the requirement for an intracellular handling stage, as suggested by Ziegler's experiment; the lack of inhibition by antibodies to the T cell determinant in the case of the experiments of Thomas and Shevach, Loblay *et al.*, and Chestnut *et al.*, all argue for an intracellular depot. Yet it is clear that the T cell specifically contacts, and adheres tenaciously in a specific way to, macrophages containing antigen. The next section discusses this specific interaction in detail. Rosenthal has argued that the T cell

may first contact macrophages in an antigen-independent manner and that this contact then triggers the flow of specific antigenic molecules from an intracellular site to the membrane, making them available to the T-cell antigen receptor. This argument is echoed in Thomas's experiments with glutaraldehyde described above. Against it, at face value, is Shevach's experiment blocking "aged" macrophages with anti-TNP antibodies or the experiments of Ziegler showing that paraformaldehyde-fixed macrophages are immunogenic. Both situations may not be contradictory: it may well be that the flow of immunogenic material from an intracellular site to the membrane is highly dynamic and that there is a continuous recycling; this recycling could be regulated by a membrane interaction. Depending on how much immunogen is at a given time on the outside—which may vary from antigen to antigen—the T cells will bind to it with different degrees of avidity. Once the T cell binds to it, the process of recycling may augment, making available more immunogen. In this situation, it may be difficult for antibodies to block this close cell-to-cell contact interaction, particularly if the determinants are sparse. Along the lines discussed above, in unpublished studies reproduced in Fig. 9, I have found that the intracellular vesicles which contain antigen also have Ia antigens.

In all the studies on antigen presentation, one puzzling and unexplained fact still remains, and that is the ease of suppression of the interaction by anti-Ia antibodies in contrast to the lack of effects of antibodies to the antigen. One wonders if Ia may have two distinct functions in the macrophage, one to regulate the antigen-handling and antigen-presentation step, and the other to modulate the contact be-

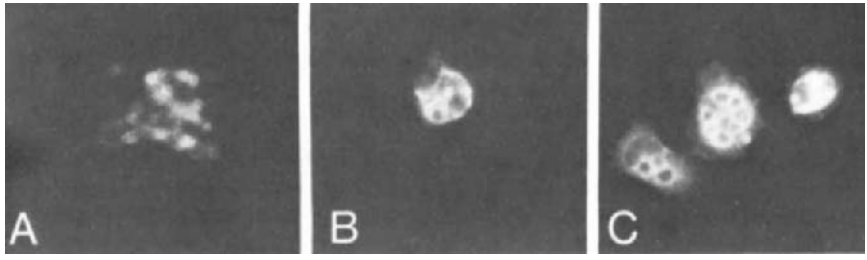

FIG. 9. Macrophages were incubated with heat-killed *Listeria monocytogenes* (panel A) or with opsonized red cells (panels B and C), after which the cells were fixed in acetone and stained with a monoclonal antibody anti-I-A (clone 10-2.16). The endocytic residues with the phagocytized particles are very rich in I-A molecules. Macrophages that have not phagocytized show few very weak punctate fluorescence. [Unpublished studies of E. R. Unanue (1979).]

tween the T cell and the macrophage. To this effect, two observations discussed in Section II showed T cell–macrophage interactions in the response to polyclonal stimuli, involving the macrophage Ia, but not restricted by the *I*-region haplotype—these were the interactions of T cells with macrophages in the response to Con A and to aldehyde. Particularly, the response to aldehydes was thoroughly examined and shown to be blocked with the specific anti-Ia antibody (Greineder *et al.*, 1976) (Section II,B). The results imply a surface-to-surface effect where allogenic Ia modulates the interaction. Ia proteins, therefore, may be versatile proteins capable of influencing the key and specific antigen-handling event at the same time that they can also interact with surface structures of the T cells in a way not involving antigen.

The association of antigen with Ia has been thought to be the immunogenic complex, although this has not been easy to demonstrate directly by biochemical techniques. Perhaps Ia is a protein that can bind sequences of amino acids and form a complex to which the T-cell receptor(s) binds with high affinity. Determinant selection may then involve the specific attachment of a piece of the antigen, resulting from lysosomal digestion to Ia—the degree of binding will vary depending on both the antigen and the structure of the Ia protein, which eventually translates into responder or nonresponder status. Or, perhaps, as Rosenthal speculated, genes code for an enzyme that directs the cleavage of the proteins at the same time that the Ia antigens function also as cellular interaction molecules.

Two experiments have been published in support of a soluble antigen–Ia complex. Erb and Feldmann's studies, described in Section II,C, indicated that a soluble material released from 4-day cultures of macrophages with antigen was effective in inducing helper T cells in an *I*-region-restricted way. The material was specifically removed by its passage through an anti-Ia column. Puri and Lonai (1980) recently reported on a similar phenomenon. Their first approach was to study the binding of ^{125}I-labeled (TG)-A-L to T cell suspensions. The binding, using autoradiography, took place if the T cells were first incubated for 2 hours with a 48-hour conditioned medium from macrophages. Furthermore, the binding required the presence of adherent cells in the T cell suspensions. A culture of T cells (containing adherent cells) with labeled antigen for 40 minutes contained a radiolabeled material that would rapidly bind to T cells treated with the macrophage-conditioned medium. The "processed" antigen did not bind to T cells bearing a different *I* region and was removed from solution by an anti-Ia immunoabsorbent. In a second series of experiments, the processed antigen was immunogenic *in vivo* and could, if

highly radioactive, kill the specific T cells (i.e., T cells incubated with "processed" antigen for 40 minutes would no longer show carrier function in a T-B cell collaboration protocol). [Some evidence of Ia released from macrophages and attaching to T cells was also brought up by Lee and Paraskevas (1979).] The Erb–Feldmann and Puri–Lonai observations are at an early stage in development but imply the release of an immunogenic antigen-Ia-bearing product as one pathway of antigen presentation. The results are of obvious importance and need to be further explained and characterized as well as integrated with other phenomena showing direct T cell-macrophage associations.

3. *Lymphocyte-to-Macrophage Interactions*

One of the critical issues relating to the mechanisms of lymphocytes interacting with antigen bound to macrophages is that of cell-to-cell contact. The early anatomical studies of macrophage–lymphocyte association in lymph nodes and spleen were reviewed previously (Unanue, 1972). Few histological studies have appeared in the past few years, and these, in general, have contributed little beyond a descriptive analysis of frequent and intimate associations between phagocytes and lymphocytes in the deep cortex and medulla of stimulated nodes. The early reports on macrophage–lymphocyte association in antigen and lectin-driven cultures were also reviewed. These indicated the frequent finding of multicellular aggregates between phagocytes and lymphocytes (for example, Mosier, 1969; Salvin and Nishio, 1969; Pierce and Benacerraf, 1969; Schechter and MacFarland, 1970; Seeger and Oppenheim, 1970; Salvin *et al.*, 1971). Several recent reports, particularly from five laboratories, have analyzed the issue of cell contact using cell cultures. These reports have indicated the specific and intimate anatomical and functional association between antigen-committed T cells and antigen-containing Ia-positive phagocytes.

The general approach taken was to produce a monolayer of macrophages, to which immune lymphocytes were added, in the presence or the absence of antigen. The interaction was quantitated by counting either the percentage of macrophages binding lymphocytes or the number of lymphocytes per 100 macrophages; or, functionally, by testing in a bioassay the removal of the antigen-specific lymphocytes from the cell suspension.

The studies of Lipsky and Rosenthal (1973, 1975b) using the guinea pig confirmed the observations that *thymocytes* would avidly bind to macrophages in the absence of antigens (Siegel, 1970a,b). About 90% of the macrophages in a preparation would bind the thymocytes; 5% of them went on to ingest the thymocytes. Serum had no effect on the

interaction. Thymocytes attached to cultured macrophages, even better to macrophages from oil-induced exudates, but not to neutrophils or fibroblasts. Lipsky and Rosenthal considered this binding an example of *antigen-independent* lymphocyte–macrophage interaction. The binding required live, active macrophages, took place optimally at 37°C, and by way of a trypsin-sensitive structure of the macrophage (Siegel, 1970b; Lipsky and Rosenthal, 1975b; Lopez *et al.*, 1977). The binding was species specific (Siegel, 1970a, 1970b; Lopez *et al.*, 1974), but not strain specific (Lopez *et al.*, 1977). In contrast to thymocytes, the degree of antigen-independent binding of mature T cells obtained from lymph nodes, although present, was much less pronounced (three- to fivefold less). The antigen-independent binding of either thymocytes or T cells took place *briefly* following culture, reaching maximal values after 1 hour, and disappeared progressively with time, the cells leaving their substrate (Lipsky and Rosenthal, 1973, 1975b; Lopez *et al.*, 1977). The thymocytes that dissociated from the macrophages lost their capacity to bind again (Lopez *et al.*, 1977).

In our own experience using mouse cells, this binding of thymocytes was very marked both in terms of number of thymocytes adhering to the macrophage and percentage of macrophages binding thymocytes (Beller and Unanue, 1978). Interestingly, thymocytes would also bind to macrophages isolated from the thymus (Beller and Unanue, 1978). I am not convinced, however, that the molecular basis for antigen-independent binding of thymocytes to macrophages is the same as that of mature T cells. It is possible that the much greater binding of immature thymocytes is because of a unique surface structure or one common with the mature T cell but at a much higher concentration.

The *antigen-dependent* binding has been tested most frequently by adding immune T lymphocytes to the monolayers of antigen-pulsed macrophages and quantitating the binding morphologically (Fig. 10). Depending on the culture conditions, maximal binding took place between 4 and 8 hours after the initiation of the culture and was maintained for a few days, decaying slowly; the binding was clearly antigen-specific (Werdelin *et al.*, 1974; Lipsky and Rosenthal, 1975a; Lipscomb *et al.*, 1977; Ben-Sasson *et al.*, 1977, 1978; Braendstrup and Werdelin, 1977). The lymphocyte that remained attached to the macrophages exhibited morphological signs of transformation and synthesized DNA (Lipsky and Rosenthal, 1975b; Braendstrup *et al.*, 1976; Ben-Sasson *et al.*, 1978). The lymphocyte that dissociated from the macrophages could bind *de novo* to fresh antigen-pulsed macrophages, suggesting that their release from the macrophage might be because of the loss of immunogenic antigen (Ben-Sasson *et al.*, 1978).

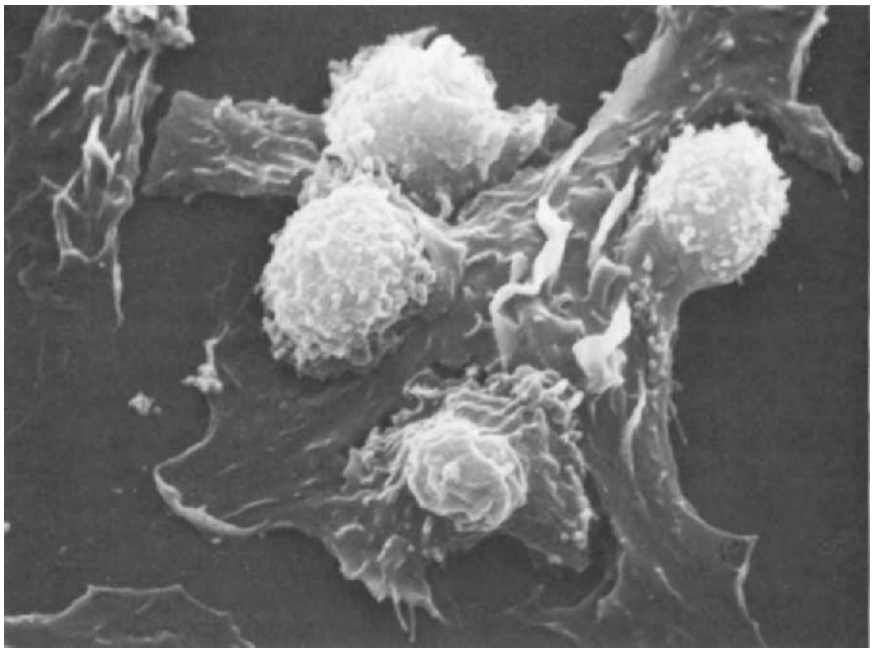

FIG. 10. Clustering of immune T cells to macrophages. *Listeria monocytogenes*-immune T cells were planted onto macrophages previously pulsed with *Listeria* for 1 hour, and then the loosely adherent cells were removed. Note the attachment of four lymphocytes to the macrophage. The attachment required phagocytosis of *Listeria* and Ia molecules on the macrophage surface. (Experiment of K. Ziegler, R. S. Cotran, and E. R. Unanue.)

It was clear that the binding of T cells to antigen-pulsed macrophages showed the same MHC restrictions as found in functional assays. Immune T cells from strain 2 guinea pigs preferentially clustered on antigen-pulsed macrophages of strain 2 rather than on macrophages of strain 13 (Lipsky and Rosenthal, 1975a; Braendstrup *et al.*, 1979; Lyon *et al.*, 1979). Anti-Ia inhibited cluster formation (Braendstrup *et al.*, 1979; Lyon *et al.*, 1979). In their study, Lyon *et al.* (1979) inhibited the clustering of T cells from $(2 \times 13)F_1$ guinea pigs to macrophages of strain 2 pulsed with DNP-GL (an antigen to which strain 2 responds, but not strain 13) by addition of an anti-strain-2-Ia, but not by an anti-strain-13-Ia, suggesting that the macrophages were the target. Functional assays established that this was indeed the case (Ziegler and Unanue, 1979).

Not all antigen-pulsed macrophages bind T cells; the number, although not studied in detail, was about 20–40% (Werdelin *et al.*, 1974;

Ben-Sasson *et al.*, 1974). This could well be explained by the different content of Ia-bearing macrophages inasmuch as the T cells bind preferentially to them. This point was studied in our laboratory by K. Ziegler, who, after allowing the lymphocytes to layer and cluster on the macrophage, fixed the preparation and stained for the Ia antigens of the macrophage by fluorescence. Clearly, the antigen-specific binding was taking place on Ia-bearing macrophages and could be enhanced severalfold using macrophage monolayers with a high content of Ia-positive macrophages.

Detailed morphological and cinematographic studies of the macrophage–lymphocyte clustering were made by Werdelin's laboratory using guinea pig cells (Nielsen *et al.*, 1974; Braendstrup and Werdelin, 1977; Petri *et al.*, 1978, 1979). They found that the lymphocyte–macrophage clusters were formed by a phagocyte to which a "central" lymphocyte was attached to by long areas of intimate membrane-to-membrane contact; interestingly, various other lymphocytes surrounded and attached to the central lymphocyte by way of long uropods (Fig. 11). The "peripheral" lymphocytes interacted sometimes briefly with the central lymphocytes, left, and reattached to other macrophages. Stopping cell motility by drugs, as expected, reduced cluster formation (Braendstrup *et al.*, 1977). The antigen specificity and kinetics of binding of central and peripheral lymphocytes was quite different. The central T lymphocyte bound optimally by 4 hours of culture, whereas the interaction of the peripheral lymphocytes took several hours longer. More important, the binding of the central lymphocyte to the macrophage was antigen specific, whereas the binding of the peripheral lymphocyte was not. Thus, a suspension of lymphocytes could be specifically depleted of their capacity to cluster on antigen-pulsed macrophages by a first culture on macrophage monolayers; i.e., after 4 hours, the cells that were not adherent did not form clusters on a fresh antigen-pulsed macrophage monolayer (Werdelin and Braendstrup, 1979). The peripheral lymphocyte, on the other hand, from immune or nonimmune, syngeneic, or allogeneic guinea pigs, associated and clustered on the cultures of macrophages with a central lymphocyte (Braendstrup *et al.*, 1979). Peripheral lymphocytes were represented by both T and B cells (Petri *et al.*, 1978). The interpretation was that the binding of the peripheral lymphocytes could be an example of lymphocyte–lymphocyte collaboration.

Particularly significant results that addressed the issue of T cell recognition of antigen were obtained using the binding of T cells to macrophages as a means to deplete T cells in a functional T cell–

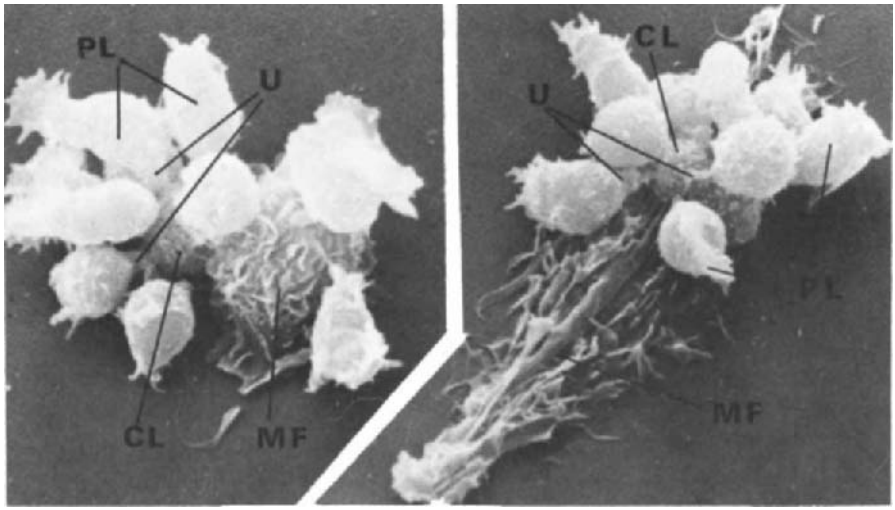

FIG. 11. Clustering of peripheral (PL) and central (CL) lymphocytes to macrophages (MF). In this study from Werdelin's laboratory, guinea pig purified protein derivative (PPD)-primed T lymphocytes were incubated with PPD-pulsed macrophages. The peripheral lymphocytes attached to the central lymphocyte by slender uropods (U). [From Nielsen *et al.* (1974).]

macrophage assay (Swierkosz *et al.*, 1978, 1979; Werdelin and Shevach, 1979; Werdelin *et al.*, 1979; Ziegler and Unanue, 1979; Werdelin, 1980).

The experiments reported by Werdelin and Shevach used guinea pig T cells immune to ovalbumin added to monolayers for 4 hours, after which the nonadherent cells were recovered and again added to a fresh macrophage monolayer and tested for DNA synthesis. The optimal removal of the antigen-specific T cells—74–98%—occurred after three such cycles of binding. In the mouse, we technically modified the system and obtained excellent depletion using *Listeria monocytogenes* as the antigen, after one or two 1-hour cycles of binding (Ziegler and Unanue, 1979). One important manipulation was to lightly centrifuge the immune T cells onto the macrophage monolayer. Werdelin's studies assayed T-cell function by DNA synthesis; Ziegler's did it by DNA synthesis and also by measuring mediator production or macrophage activation.

The main results were that T cells interacted only with antigen molecules associated with the appropriate Ia-bearing macrophages. In contrast, T cells did not bind to soluble antigen. Werdelin *et al.* (1979), were unable to show competition for the T-cell binding to mac-

rophages by an excess of soluble antigen, and neither did Ben-Sasson *et al.* (1977), who also did not observe binding to insolubilized antigen. We did not find binding of immune T cells to *Listeria* attached by poly-L-lysine to a culture dish.

Using F_1 mice, it was possible to find a specific interaction of two clones of T cells, each binding to the antigen in the context of one of the parental macrophages. The experiments of Swierkosz *et al.* (1978, 1979), assayed for helper T cells to SRBC (Section II,C). The F_1 T cells, isolated directly from the macrophage monolayer, were highly enriched in helper cells when tested for an antibody response in a mixture of B cell and macrophages from either parent. Similar results assaying for depletion of T cells rather than enrichment were obtained by Werdelin *et al.* (1979) and Ziegler and Unanue (1979).

We used the F_1 mouse with its two clones of T cells to explore the blocking effect of the anti-Ia antibodies. T cells from (BL6 × A)F_1 mice were bound specifically to the *Listeria*-pulsed macrophages from A or BL/6 and then tested functionally on each parental macrophage. Each reactive clone would be depleted by the specific macrophages. The depletion of the A-reactive clone did not take place when specific anti-Ia antibodies were added during the binding reaction. In contrast to the effects of antibodies to Ia, antibodies to the antigen did not result in abrogation of T cell–macrophage binding (Ellner *et al.*, 1977; Ben-Sasson *et al.*, 1977; Werdelin and Shevach, 1979).

In contrast to the many studies described above showing the specific binding of T cells to macrophages bearing Ia and antigen, there are few reports on the binding of B cells to macrophages. One of our early studies with Schmidtke showed binding of B cells to macrophage monolayers (in the absence of antigen) in a process competed by excess Ig (Schmidtke and Unanue, 1971b). Lipsky and Rosenthal (1975b) also observed antigen-independent binding of guinea pig B cells to macrophages but found no inhibition by excess immunoglobulin. Petri *et al.* (1978) observed binding of B cells to the central lymphocytes bound to macrophages (see above). O'Toole and Wortis (1980) reported antigen-independent binding of B cells to macrophages, particularly of the B cells bearing the Lyb3 marker. The binding of B cells was limited to the first 2 hours of culture if T cells were also present, but persisted in the absence of T cells.

C. Secretory Function of Macrophages

The mononuclear phagocytes can decisively affect their surrounding environment through the release of a number of biologically active products. These products influence the function of lymphocytes, con-

nective tissue cells, and extracellular proteins. Table VIII summarizes the major secretory products grouped into four major categories. The secretory products include enzymes that affect connective tissue and extracellular proteins—collagenase, elastase, plasminogen activator; a number of molecules critical for "defense" processes—complement proteins, interferon; molecules that affect cells—growth factors such as the lymphostimulatory molecules; and a variety of small molecular weight active compounds—prostaglandins, thymidine, oxygen intermediates. Several reviews of secretory processes have been published (Unanue, 1976; Unanue *et al.*, 1976a; Page *et al.*, 1978; Gordon, 1980). Many of the products secreted by macrophages are molecules destined exclusively for export, plasminogen activator for example, whereas others are intracellular products that are partially released under appropriate stimuli, such as the lysosomal enzymes. Some molecules are secreted continuously, but others only in response to external stimuli.

TABLE VIII
SECRETORY PRODUCTS OF MACROPHAGES

Products That Affect Extracellular and Connective Tissue Proteins	
Lysosomal enzymes (1)[a]	Various other neutral proteases (5, 6)
Plasminogen activator (2)	Esterases (7)
Collagenase (3)	Fibronectin (8)
Elastase (4)	Procoaglulant (9)
Products Involved in Defense Processes	
Complement proteins (C1, C2, C3, C4, factor B) (10)	Interferon (12)
Lysozyme (11)	
Biologically Active Proteins	
Lymphostimuatory molecules (LAF) (this review)	Mesenchymal growth factor (14)
Colony-stimulating factor (13)	Angiogenesis factor (15)
Small Molecular Weight Compounds	
Prostaglandins (this review)	Thymidine (this review)
Cyclic nucleotides (16)	Oxygen-derived products (this review)

[a] Numbers in parentheses indicate references. This is a partial list that includes the main molecules studied so far. References are not complete and refer to either key studies or review papers. More extensive review of the various secretory products can be found in Unanue, 1976; Page *et al.*, 1978; and Gordon, 1980. Key to numbers: (1) Davies *et al.*, 1974; (2) Unkeless *et al.*, 1974; (3) Werb and Gordon, 1975a; (4) Werb and Gordon, 1975b; (5) Hauser and Voes, 1978; (6) Gordon, 1980; (7) Wiener and Levanon, 1968; (8) Alitalo *et al.*, 1980; (9) Edward and Rickles, 1980; (10) Colten, 1976; (11) Gordon *et al.*, 1974; (12) Smith and Wagner, 1967; (13) Chervenick and LoBuglio, 1972; (14) Leibovich and Ross, 1976; (15) Polverini *et al.*, 1977; (16) Gemsa *et al.*, 1975.

The secretory function of the macrophages is important in the relationship of the macrophage with the lymphocyte for three reasons.

1. Some of the molecules secreted by the phagocytes are powerful stimulants of T and B cells and, moreover, are released following macrophage–lymphocyte interactions regulated by the MHC of the species (Unanue *et al.*, 1976b; Farr *et al.*, 1977, 1979a). Thus, the lymphostimulatory molecules may explain part of the molecular events taking place between the phagocytes and the lymphocytes, each cell regulating the other.
2. The macrophage–lymphocyte interaction, or products derived thereof, may also modify the secretion of many of the molecules listed in Table VIII.
3. The negative side of the macrophage, its inhibition of lymphocyte function can be explained to a great extent by the release of small biologically active products.

1. Secretion of Lymphostimulatory Molecules

It is now well established that phagocytes release proteins that decisively control the function of T and B lymphocytes. The biosynthesis and secretion of lymphostimulatory molecules may, therefore, be considered one of the important molecular events that follow macrophage–lymphocyte interaction.

a. Lymphocyte-Activating Factor. One of the earliest demonstrations of an active soluble material released by the phagocytes came from the studies of the human mixed leukocyte reaction (Bach *et al.*, 1970). No reaction developed between responder and stimulatory lymphocytes depleted of adherent cells (Alter and Bach, 1970). However, a culture medium conditioned by adherent, monocyte-rich cells promoted the development of the allogeneic interaction. That macrophage-conditioned medium might also affect *in vitro* antibody formation was suggested by experiments indicating that such medium could substitute for the macrophage (Hoffman and Dutton, 1971). (The nature of the product in this study was never elucidated. The fact that it was absorbed by the red cells, which were then strongly immunogenic, suggests that it was antibody rather than a true macrophage product.)

The first clear demonstration of a mitogenic activity secreted by macrophages came from the studies of I. Gery, B. Waksman, and associates at Yale (Gery *et al.*, 1972; Gery and Waksman, 1972). In their study, the adherent cells from human peripheral blood released, after 24 hours of culture, a "factor" that promoted the response of murine

thymocytes to PHA. Addition of the factor without PHA also promoted proliferation, but to a lesser extent. The factor was termed lymphocyte-activating factor and became known by the acronym LAF. We are defining LAF, therefore, as an activity in macrophage cultures that, added to thymocytes together with or in the absence of PHA, increases, after 3–4 days of culture, their uptake of tritiated thymidine by severalfold. Gery *et al.* found that LAF was best secreted following stimulation of the human monocytes or murine adherent spleen cells with endotoxin. LAF had a weak mitogenic effect on spleen cells but was active on lymph node lymphocytes, particularly in cultures of high cell density. Gery *et al.* concluded their study by speculating that LAF might well explain part of the helper role of macrophages in immune induction.

These observations were rapidly confirmed and extended. LAF is now considered the most important, perhaps the only, lymphostimulatory molecule released by phagocytes with a wide range of biological effects that extend from the original effects described by Gery, to promotion of T cell secretion of mediators, to stimulation of B cell differentiation.

After Gery's observations, our laboratory reported some important observations of particular relevance for our understanding of the biological effects of LAF. First, it became apparent that macrophage-conditioned medium was a mixture of both stimulatory and inhibitory molecules, the latter being represented by dialyzable low-molecular-weight molecules frequently at concentrations that masked the stimulatory effects. This "inhibitory" factor became more evident in high-density cultures of macrophages (for example, at approximately 5 to 10×10^6 per milliliter in a round dish 35 mm in diameter). Dialyzing the inhibitor resulted in the clear demonstration of a "mitogenic protein" that turned out to be identical to LAF (Calderon and Unanue, 1975; see also Hoessli *et al.*, 1977). The inhibitors in many instances were represented by thymidine molecules, in others by prostaglandins [another study stressing the dual effect of macrophage-conditioned medium, in this case on hematopoietic cell growth, is that of Kurland *et al.* (1977), discussed below]. Of particular importance are the observations that LAF secretion was under strict regulation and that the key regulating event involved the interactions of macrophages with antigen and with immune T cells (Unanue *et al.*, 1976b; Farr *et al.*, 1977, 1979a; Beller *et al.*, 1978; Ziegler and Unanue, 1979). These studies involving the regulation of LAF secretion are now described in detail.

b. Regulation of LAF Secretion. Under basal conditions, peritoneal

macrophages from unstimulated mice secreted a small and variable amount of thymocyte mitogenic activity. Addition of various stimulants, such as an antigen–antibody complex, a bacterium like *Listeria monocytogenes*, latex beads, or an adjuvant like beryllium sulfate, increased secretion approximately from 2- to 20-fold (Calderon *et al.*, 1975). The increase in secretion, however, depended very critically on the life history of the macrophage: although peptone-induced macrophages were also triggered to release LAF, the macrophages from thioglycolate-injected mice were poor secretors (Calderon *et al.*, 1975; Unanue *et al.*, 1976b; Unanue and Kiely, 1977). Human monocytes also increased their basal secretion of LAF after interaction with latex spheres and antigen–antibody complexes (Blyden and Handschumacher, 1977). It should be noted that the most potent stimulant to secretion—second only to T cells and antigen—was endotoxin. Endotoxin strongly stimulated most macrophages (Meltzer and Oppenheim, 1977), spleen macrophages (Gery *et al.*, 1972), and human monocytes (Gery *et al.*, 1972; Blyden and Handschumacher, 1977; Lachman *et al.*, 1977). The basal or stimulated secretion of LAF usually was optimal during the first 24–48 hours of culture (Fig. 12).

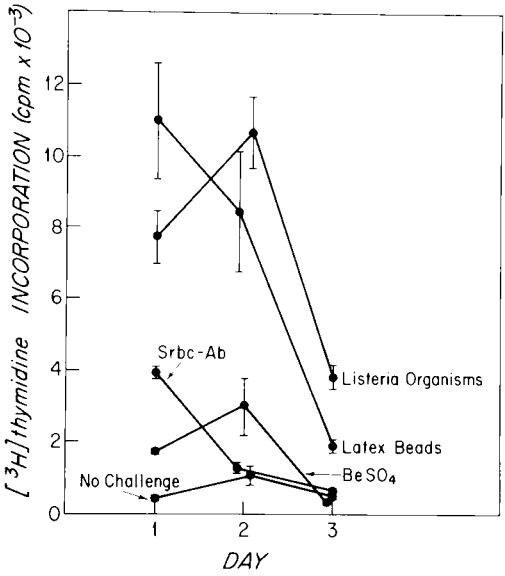

FIG. 12. This figure shows the increase in thymocyte mitogenic (lymphocyte-activating factor) activity secreted from macrophages after exposure to various materials. Culture fluids were tested on their effects on thymocyte proliferation at 25% volume per volume. [From Unanue *et al.* (1976).]

The release of the thymocyte mitogen was regulated by immune T cells upon interaction with macrophage-bound antigen. In our studies, peripheral macrophages from *Listeria*-immune or peptone-injected mice were cultured with heat-killed *Listeria* organisms, without, or together with, *Listeria*-immune T cells. The basal level of secretion by macrophages of LAF was low, but the uptake of *Listeria* increased secretion to a small extent. The addition of immune T cells produced a remarkable increase in secretion (immune T cells plus *Listeria* did not result in any production of mitogen). The secretion of thymocyte mitogenic LAF activity by T cell-macrophage interaction was found to (*a*) require intimate contact between the macrophage and the T cell—their separation by a filter did not result in secretion (Farr *et al.*, 1977); (*b*) be antigen specific; and (*c*) be under control of the *I* region of the MHC (Farr *et al.*, 1977, 1979a; Beller *et al.*, 1978; Ziegler and Unanue, 1980). Thus, the release of the mitogen involved an initial recognition of antigen that followed all the parameters discussed in the preceding sections. Indeed, the secretion of LAF required not only the presence of Ia-bearing phagocytes, but that macrophages and T cells share the *I-A* region of *H-2*. The last experiment was done by culturing macrophages from congenic strains of mice together with immune T cells and *Listeria* organisms for 24 hours, after which the culture fluid was assayed for thymocyte mitogenic activity. A representative result is shown in Table IX. It should be stressed that the secretion of LAF was best induced after cell contact, as described above; addition to macrophages of lymphokine-containing medium resulted in relatively weak stimulation of LAF secretion. The release of LAF following T cell–macrophage interaction was also found with an antigen like hemocyanin (Unanue *et al.*, 1976b) or with antigens in fetal calf serum (Farr *et al.*, 1977, 1979a). Thioglycolate macrophages were also poor secretors of LAF following macrophage-T cell interaction.

The macrophage line P388D1 (Mizel *et al.*, 1978a,b) was found to release LAF. This release was increased by addition of PHA and T cells, even xenogenic, requiring also cell contact; in this unphysiological situation, there was no MHC involvement of the secretory process (Mizel *et al.*, 1978b). The P388D1 line also increased the secretion of LAF following exposure to phorbol myristate acetate (Mizel *et al.*, 1978c) and endotoxin (Mizel *et al.*, 1978b).

There are other important characteristics of LAF secretion that bear on the question of why the very activated macrophages were poor secretors of this protein. The basal or particle-induced secretion of LAF showed a superinduction phenomenon (Unanue and Kiely, 1977). Superinduction refers to the paradoxical increase in the production of a

TABLE IX

ABILITY OF B10.A *Listeria*-IMMUNE T CELLS TO INTERACT WITH MACROPHAGES SHARING DIFFERENT REGIONS OF THE H2 GENE COMPLEX[a,b]

Macrophage strain	H-2 haplotype	H-2 formulas							H-2 homology with T Cell	Mitogenic activity	
		K	*I-A*	*I-B*	*I-J*	*I-C*	*S*	*D*		No antigen	With *Listeria*
B10.A	*a*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	All	1,164 ± 120	11,395 ± 1,069
B10	*a*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	None	1,259 ± 209	1,348 ± 137
B10.A (4R)	*h*4	*k*	*k*	*b*	*b*	*b*	*b*	*b*	*K* + *I-A*	689 ± 46	11,163 ± 1,434
B10.A (5R)	*i*5	*b*	*b*	*b*	*k*	*d*	*d*	*d*	*I-J, I-C, S, D*	501 ± 7	1,190 ± 405
B10.A (15R)	*h*15	*k*	*k*	*k*	*k*	*d*	*d*	*b*	*K, I-A, I-B I-J, I-C, S*	1,745 ± 238	13,188 ± 1,313
B10.A	*a*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	All	1,164 ± 120	11,195 ± 1,069
B10.G	*q*	*q*	*q*	*q*	*q*	*q*	*q*	*q*	None	1,693 ± 68	1,050 ± 20
B10.A (6R)	*y*2	*q*	*q*	*q*	*q*	*q*	*q*	*d*	*D*	1,687 ± 269	1,587 + 113
B10.AQR	*y*1	*q*	*k*	*k*	*k*	*d*	*d*	*d*	*I-A, I-B, I-J, I-C, S, D*	1,035 ± 68	12,297 ± 925

[a] From Farr *et al.* (1979a).

[b] The experiment involved culturing B10.A *Listeria*-immune T cells with macrophages in the presence or the absence of 10^6 *Listeria* for 24 hours and then assaying the culture fluid for its content of mitogen. Mitogenic activity generated in cultures of T cells and *Listeria* antigen was 1068 ± 23. Background proliferation of thymocytes was 530 ± 10. Previous experiments have established that the lack of response in the combinations where macrophages and T cells did not share *I-A* could not be attributed to a suppressor mechanism.

cell product following inhibition of RNA or protein synthesis (McAuslan, 1963). It was observed, for example, for the secretion of tyrosine aminotransferase (EC 2.6.1.5) by liver cells (Steinberg *et al.*, 1975) or for the release of interferon by fibroblasts (Vilcek and Ng, 1971). One interpretation of superinduction is that the inhibitors of protein synthesis stop the production of a control protein that regulates the synthesis and/or metabolism of the product. In our studies, we found that freshly harvested macrophages did not contain any preformed LAF (freeze-thawed extracts had no thymocyte mitogenic activity); however, a few minutes after planting, LAF could be extracted from the microsomal fraction, reaching peak levels by 1–2 hours and then steadily declining so to disappear altogether by 24 hours. The loss of internal LAF did not correlate with its appearance in the culture fluid. In other words, contact with the dish stimulated the appearance of cell LAF, but not its secretion. The inhibitors of protein synthesis had two contrasting effects, depending on the time at which they were added to the culture. Cycloheximide added to cells immediately after planting stopped all production of internal LAF; however, the addition of cycloheximide after 1 hour of culture when LAF had already been made resulted in a marked *increase* in the secretion of LAF; in this case, the internal LAF did not decline and remained in the cell for long periods of culture (Fig. 13). We postulated that LAF secretion involved an internal control protein that regulated the expression and/or degradation of the cellular LAF; shutting off the secretion of the putative control proteins allowed for the persistance and maintenance of LAF secretion. The fact that highly activated macrophages secreted less LAF argued for an increased content of this inhibitor. In this regard, thioglycolate macrophages treated with cycloheximide 1 hour after planting released the mitogen.

c. Biochemistry of LAF. Lymphocyte-activating factor is a protein with a molecular weight of about 13,000–16,000 (Gery and Handschumacher, 1974; Calderon *et al.*, 1975; Blyden and Handschumacher, 1977; Lachman *et al.*, 1977; Economu and Shin, 1978; Mizel, 1979). The protein is sensitive to protease and papain digestion but is relatively resistant to trypsin (Calderon *et al.*, 1976; Blyden and Handschumacher, 1977). It is not inhibited by diisopropyl fluorophosphate (Calderon *et al.*, 1975) nor by phenylmethane sulfonylfluoride (Blyden and Handschumacher, 1977), indicating that it is not a serine protease. LAF does not bind to columns of Con A–Sepharose (Mizel, 1979), nor does it contain antigen products encoded in the *H-2* of the species (Calderon *et al.*, 1975). LAF shows charge heterogeneity with isoelectric points ranging from 4.5 to 6.5. Economu and Shin's

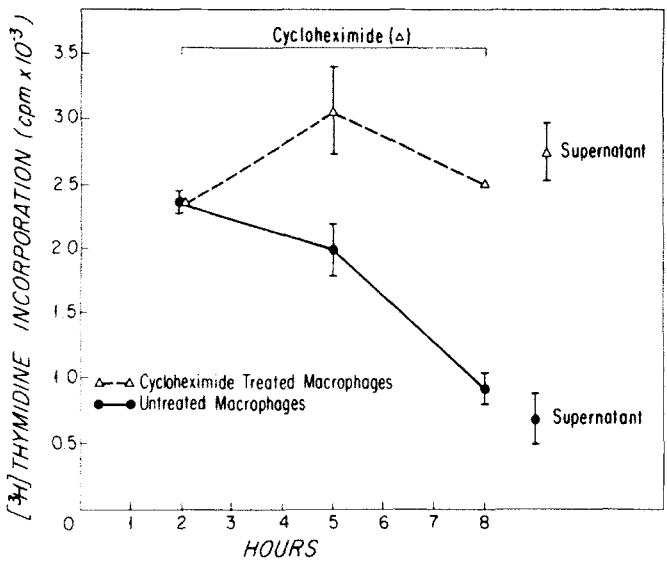

FIG. 13. Superinduction of the release of thymocyte mitogenic protein (lymphocyte-activating factor). The figure depicts the amounts of activity extracted from untreated macrophages or macrophages treated with 10 μg of cycloheximide per milliliter starting 2 hours after planting and terminating at 6 hours. The amount of cell-bound mitogen in untreated macrophages decreases with time, and little is released into the supernatant (after 8 hours of culture). After cycloheximide, mitogenic activity persists longer and is released. [From Unanue and Kiely (1977).]

(1978) excellent study indicated that the charge heterogeneity may result from some proteolytic changes during the isolation. In their studies, analysis of LAF, after a brief period of culture and with enzyme inhibitors in the processing steps, resulted in more homogeneous preparations with an isoelectric point of 4.8. Other reports indicated isoelectric points of 6 to 8 (Lachman *et al.*, 1977) and 5.0 to 5.4 (Mizel, 1979). Economu and Shin's study indicated that LAF had a Stokes' radius of 20 Å, a sedimentation coefficient (SE_{20w}) of 2.0, a buoyant density of 1.30 gm/cm^3, and a calculated molecular weight of 16,400–19,600. There are reports of size heterogeneity of LAF-type molecules having apparent molecular weights of 30,000, sometimes 80,000, besides the 15,000 product. The reasons for this size heterogeneity have not been adequately resolved. In fact, whether all these molecular weight products are the same as the major 15,000 MW protein is far from clear. There is some indication that some—but not all—of the size heterogeneity may be caused by protein–protein aggregates (Togawa *et al.*, 1979); thus, some of the isolated 15,000 MW product added to serum rechromatographed in the 50,000–70,000 size

range. In our studies, we have found similar effects, indicating that LAF may easily complex to serum proteins. To be noted is that the intracellular LAF of peritoneal macrophages was highly homogeneous in size, approximately 15,000 MW (Unanue and Kiely, 1977), whereas that found in the macrophage P388 line showed marked size heterogeneity (Mizel and Rosenstreich, 1979).

Lymphocyte-activating factor has been purified extensively by combinations of size and gel chromatography (Lachman *et al.*, 1977; Blyden and Handschumacher, 1977; Economu and Shin, 1978; Mizel, 1979). Not enough material has been isolated for detailed amino acid analysis.

d. Mode of Action. In this section, I will discuss the mitogenic effect of LAF on thymocytes and will also examine the relationship between LAF and other growth-promoting molecules released by cultured lymphoid cells.

Various growth-promoting molecules have been found not only in mixed cultures of macrophages and lymphocytes, but in cultures of purified macrophages. The biological studies of LAF analyzed above disclosed some size and charge heterogeneity that is not entirely explained. Therefore, I will focus the analysis of the action of LAF to situations where the 15,000 MW product has been the major component.

There is no question that the main target cell of the mitogenic effect of LAF is the thymocyte. Directly assaying proliferative activity—without the addition of PHA—established that T cells obtained from spleens or lymph nodes responded poorly, if at all, whereas thymocytes were stimulated by the mitogen, maintaining their level of DNA synthesis (Gery *et al.*, 1972; Calderon *et al.*, 1975; Beller and Unanue, 1979). The thymocytes also responded to LAF by an augmented response to PHA; lymph node T cells also responded to both stimuli (LAF alone or LAF plus PHA), but very much less than the thymocytes (Gery *et al.*, 1972). Within the thymocytes, is there a difference in response? Mature and immature subsets of thymocytes—separated in albumin gradients—responded to LAF; the mature subset, which normally proliferated to PHA, showed a higher DNA synthesis upon culture with LAF (Gery *et al.*, 1972; Beller and Unanue, 1979). Thymocytes, separated on the basis of their agglutinability to peanut agglutinin, were stimulated to LAF, but the "peanut-agglutinin-negative" subset exhibited a greater response (Oppenheim *et al.*, 1980). Attempts to identify a LAF receptor by determining whether it could be removed from solution by thymocytes have so far failed (Beller and Unanue, unpublished observation).

Experiments of David Beller indicated a relationship between the

spontaneous growth of the thymocyte and its response to LAF (Beller and Unanue, 1979). Many of the thymocytes freshly harvested were in cell cycle and responded to the continuous presence of LAF in culture by maintaining their proliferative activity for several days. In the absence of the growth factor, there was a rapid decline in their DNA synthesis. Addition of LAF at a time when spontaneous growth had stopped (by 24 or 48 hours of culture) did not reactivate the cells to go into DNA synthesis. However, such resting thymocytes were stimulated if pulsed briefly with the lectins Con A or wheat-germ agglutinin, followed by removal of the lectins and then addition of LAF. It should be noted that wheat-germ agglutinin is a nonmitogenic lectin and, moreover, is highly inhibitory when added to cultures. However, a brief, 2-hour interaction with the thymocytes was effective in inducing a first "signal" to the cell. Reversing the two stimuli, i.e., LAF first, followed by the lectins, was ineffective. Our results, therefore, indicated that the direct mitogenic action of LAF required a cell in an "activated" state that was inherent in the population of spontaneously cycling thymocytes. In the resting thymocyte, the activated state had to be induced by an initial interaction with a nonmitogenic ligand. Precedent for two growth-promoting molecules in the regulation of cell growth could be found in studies on fibroblasts. In these studies, one growth-promoting molecule had to take the cell to the state where it was able to "receive" the second factor (for example, Pledger *et al.*, 1977; Vogel *et al.*, 1978). Our opinion is that LAF may be an important endogenous regulator of thymocyte proliferation *in situ*. To this effect, we have found a definite basal level of secretion by macrophages directly isolated by the thymus. Thymic macrophages, therefore, by virtue of their content of Ia and their secretion of LAF and differentiation molecules, are a likely candidate for exercising a powerful role in the intrathymic maturation of T cells (Section II,G).

How LAF interacts with antigen or Con A to bring about proliferation of *mature* T cells and the relationship between LAF and other growth-promoting molecules found in lectin-stimulated lymphoid cells, is the subject of intense study at the present time. Cultures of unfractionated lymphocytes with Con A contained a growth-promoting molecule for T cells (Chen and DiSabato, 1976). The T-cell growth factor (TCGF) or "costimulator" has been studied by several groups, especially Smith and Gillis (Gillis and Smith, 1977; Gillis *et al.*, 1978; Smith, 1980) and by Paetkau and associates (Paetkau *et al.*, 1976; Mills *et al.*, 1976; Paetkau, 1980). The TCGF was shown to be a molecule required to maintain proliferation of Con A-activated T cells as well as lines of cytolytic T cells; TCGF appears to be different from LAF,

although similarities are abundant. Biologically, TCGF did not promote DNA synthesis of thymocytes unless Con A was added (Mills *et al.*, 1976; Smith, 1980), whereas LAF had a direct mitogenic action. In contrast, LAF did not stimulate proliferation of cytolytic T cells *selected* to grow in TCGF (Oppenheim *et al.*, 1980); nor did macrophage-conditioned medium stimulate the growth of Con A-activated blast cells (Larsson *et al.*, 1980). In the mouse, TCGF showed a molecular weight of 30,000 (Paetkau *et al.*, 1976; Smith, 1980), higher than that of LAF.

Evidence obtained from several laboratories indicates that the stimulation by Con A of immune T cells is highly complex, involving the participation of macrophages, producing a factor, and T cells of the Lyl subclass, producing TCGF. The evidence is that the addition of Con A to cultures of T cells (with macrophages) resulted in the production of TCGF (measured by its effect in promoting growth either of clones of cytolytic T cells or of Con A blasts). This production of TCGF correlated with the mitogenic response of the T cells. Removal of adherent cells ablated the Con A response, as we discussed in Section II,B but also resulted in the lack of production of TCGF (Smith *et al.*, 1980; Smith, 1980; Larsson *et al.*, 1980; Paetkau, 1980; Gronvik and Andersson, 1980). More important, addition of a macrophage factor, together with Con A, generated TCGF (Larsson *et al.*, 1980; Smith *et al.*, 1980), and with it, the recovery of the proliferative activity. Thus, Con A induced proliferation of mature T cells (*a*) in the presence of macrophages; (*b*) in the absence of macrophages if a macrophage factor was added; (*c*) in the absence of macrophages if TCGF, a T-cell product, was added; furthermore, (*d*) production of TCGF by Con A required macrophages or a factor derived from them. The nature of the macrophage factor that stimulates growth of Con A, or production of TCGF, is not altogether clear, although the assumption has been made that it is LAF. The first "factor" reported by Rosenstreich and Mizel (1978), produced by guinea pig macrophages, had a molecular weight of about 30,000; that studied by Larsson *et al.* (1980), produced by a macrophage cell line, was close to 25,000; the factor studied by Paetkau (1980) and by Smith *et al.* (1980) had the size characteristic of LAF. Thus, LAF may indeed be responsible for TCGF production, but perhaps other macrophage products may also do it.

Is the effect of LAF on Con A-induced T-cell proliferation mediated entirely by the production of TCGF? Smith *et al.* (1980), suggested that this was the case, based, in part, on the correlation between TCGF production and growth. They also brought evidence that dexamethasone affected the release of TCGF, but not its mitogenic effect

on mature T cells. Also, the drug inhibited the mitogenic effect of LAF (plus Con A) on thymocytes, but not the growth induced by TCGF; thus, the assumption was made that LAF acted only by inducing TCGF production. I find this interpretation to be on weak grounds; it is based mainly on the single experiment described above. The LAF and TCGF molecules clearly acted on different T-cell populations. While LAF had a direct mitogenic effect on thymocytes, TCGF required the action of Con A. Thus, the former was acting, as discussed before, on cells already in cycle, including immature cells, whereas the latter was acting on cells that required Con A stimulation. Thymocytes have different sensitivities to dexamethasone, and this may explain Smith's results.

Regardless of the final explanation, the point is clear: there is great cooperation between two major mediators, which in their production and mode of action are highly interrelated. A comparison of both has been summarized in a recent Letter to the Editor in the *Journal of Immunology* (Aarden *et al.*, 1979).

Some of the effects of macrophage-conditioned medium on *antigen*-driven T-cell responses were evaluated in the preceding sections and will only briefly be restated here. In Section II,A, we discussed the studies of Rosenwasser and Rosenthal, which indicated that a macrophage factor, most likely LAF (Rosenwasser *et al.*, 1980), allowed for the proliferative response of T cells to hemocyanin. This response required a small number of antigen-presenting cells (Kammer and Unanue, 1980) and, therefore, implies an amplification of a T-cell response by the macrophage product that still requires, in my opinion, antigen presentation. This is the same way in which I interpret the effect of the nonspecific factor studied by Erb and Feldman (Section II,C) in the response to particulate antigens and the activity reported by Lee *et al.* (1976) (Section II,A).

e. Effects on B Cells. In culture, macrophage-conditioned medium exerts effects on the antigen-driven B cell differentiation to antibody-forming cells. All the indications are that one of the molecules responsible may be similar, if not identical, to LAF. Experiments on the action of macrophage culture fluids on antibody formation in culture are now summarized.

Schrader reported (1973) that spleen cells from athymic mice developed an antibody response if cultured with peritoneal macrophages or their conditioned medium. Detailed studies were not done of the phenomenon, nor of the activity of the macrophage culture fluid. The activity was generated only by peptone-activated macrophages, particularly if cultured with medium containing fetal calf serum, and was sensitive to trypsinization.

Our laboratory examined the effects of macrophage-conditioned medium using a hapten-carrier system, with spleen cells from mice immune to fluorescein-bound hemocyanin (Calderon *et al.*, 1975; Unanue *et al.*, 1976b). Macrophage-conditioned medium induced a definite, albeit modest, differentiation of spleen cells in the absence of antigen. Challenging with fluorescein on a heterologous carrier (rabbit IgG) in the presence of the macrophage-conditioned medium also induced a mild response, not much over that induced by the conditioned medium without the antigen. The response to a challenge with the homologous antigen was marked and depended very critically on the immunological status of the primed mice donating the spleen cells. Cells from mice primed several months earlier, which responded with a modest antibody response, increased their activity by as much as 100-fold when macrophage-conditioned medium was added, in a dose-related response. Spleen cells from mice primed recently were also stimulated by macrophage-conditioned medium but only at low concentrations; at high concentration, a suppressive effect was induced. Two other experiments indicated that the non-antigen-driven differentiation did not require T cells; and nude mice, in confirmation of Schrader's experiment, responded to SRBC in the presence of macrophage-conditioned medium.

Wood's laboratory examined the response of SRBC of either murine spleen cells depleted of T cells (by killing them with anti-Thy.1 and serum as a complement source) or of spleen cells from athymic nude mice (Wood and Gaul, 1974; Wood *et al.*, 1976; Wood and Cameron, 1975, 1976). The macrophage-conditioned medium was obtained from human monocytes. The experiments indicated a substantial response of the B cells, in the presumed absence of T cells. The degree of reconstitution was about 50% when compared to that produced by T cells. A similar degree of reconstitution was obtained by medium conditioned by a mixed leukocyte reaction. The macrophage culture fluid also increased to a small extent the non-antigen-dependent "basal," plaque-forming cells and clearly augmented the response of whole spleen (i.e., with T cells).

The system used by Hoffman to study the effect of macrophage-conditioned medium was the response to TNP bound to autologous red cells (Schmidtke and Dixon, 1972b). The addition of endotoxin with the antigen resulted in antibody response to TNP that was macrophage dependent (Hoffman *et al.*, 1976a,b); this was taken as an example of a thymus-independent effect modulated by the polyclonal effect of the endotoxin on macrophages. A major point was that spleen cells responded to TNP-mouse red cells in the presence of conditioned medium from macrophages cultured with LPS (Hoffman *et al.*, 1979).

Because the response was found in C3H/He mice that are not responsive to LPS, the logical argument was made that the effect was only mediated by the macrophage products. The response observed by Hoffman was dose-dependent and was claimed not to be impaired by anti-Thy.1 treatment of the spleen cells, although the data did show some effect of the treatment. Addition of T cells with the macrophage-conditioned medium increased the response to a noticeable extent. Thus, as in Calderon and Wood's experiments, a clear potentiation of an antibody response was also taking place as a result of a macrophage product, plus the T cell "stimulus."

What are the conditions resulting in the production of the B cell stimulatory molecule(s)? What is their relationship with LAF? How do they act?

A major stimulus for the production of the stimulatory molecules was the endotoxin activation of the monocytes as found for the case of human cells (Wood and Gaul, 1974; Wood and Cameron, 1976). Human monocytes, unstimulated, or after phagocytosis of antigen–antibody complexes or latex did not release the B cell-activating factor (Wood and Cameron, 1976). We compared the release of B-cell stimulatory molecules and the thymocyte mitogen by the mouse peritoneal macrophages and found about the same results in most cases. As reported for LAF, increased B-cell stimulatory activity was found after uptake of macrophages of *Listeria monocytogenes*, latex beads, or antigen–antibody complexes, or following T cell–antigen interaction (Unanue *et al.*, 1976b). Also, as with LAF, highly activated macrophages released less material in basal conditions or after phagocytosis.

The two studies examining the B-cell stimulatory activity indicated that a substantial part of it separated in Sephadex columns with an apparent size of about 15,000 MW (Calderon *et al.*, 1975; Wood *et al.*, 1976; Wood, 1979a). In our studies, LAF and the B-cell stimulatory molecules usually chromatographed in the same position (Calderon *et al.*, 1976), but this was not the case in Wood's studies using human material (Wood *et al.*, 1976). His last report (1976) did conclude that similar size material contained both thymocyte mitogenicity as well as B-cell stimulatory activity. Although it may well be that the LAF molecule contains the B-cell activity, it has not been conclusively proved. We have also found some B-cell stimulatory molecules in the range of 110,000 and 35,000 that may or may not relate to LAF (Unanue, 1978).

Concerning the mode of action of the B cell-activating molecule, there is general agreement on the following points.

1. It induces to a small extent some differentiation to antibody secretion in the absence of antigen; whether this target cell is the imma-

ture, small B cell or B cells in already an activated state has not been critically examined and deserves consideration.

2. It increases the response of B cells in the presence of T cells in a situation where there is clear synergy between the macrophage-derived molecule and the helper function of T cells.

3. To some extent, it stimulates B cells in the apparent absence of T cells. The few studies that have critically compared the response of B cells to antigen and macrophage-conditioned media in the presence or absence of T cells have shown much better responses in the former situation, i.e., with T cells (Calderon *et al.*, 1975; Hoffman *et al.*, 1979). Unquestionably, the macrophage product exerted a good effect on T cell-deprived spleen cells, particularly noticeable with the SRBC response. Certainly with hapten-carrier we could not state that the macrophage product "substituted" for T cells.

The important issue of how the macrophage products act on B cells, if at all, and its synergy with T cell helper activity still requires analysis. Hoffman's studies indicated an effect on the expression of Ia antigens by bone marrow cells incubated for just 2–5 hours with the macrophage-conditioned medium. This was taken to mean that the macrophage product, which in his case was not characterized, might influence B-cell maturation. It is also possible that the macrophage products in cultures of B cells with antigen were acting as maturation molecules on T-cell precursors remaining in the culture. This has not been critically ruled out. Regardless, a striking feature is the interaction between the macrophage product and the T cell that results in heightened B-cell differentiation. Early studies of Farrar and associates (1977) had shown a number of "activities" in a mixed leukocyte supernatant that, in part, "replaced" T cells in the anti-sheep response *in vitro*. These activities were resolved by gel filtration in molecules of apparent sizes of 15,000 and 40,000 MW that *synergized* when added together. The 15,000 MW material was shown to have thymocyte mitogenic activity compatible with LAF (Koopman *et al.*, 1977). Indeed, in support of this cooperativity are recent studies where the macrophage product, together with a T cell-replacing factor (induced by Con A-activated T cells), induced strong B-cell responses in culture (Hoffman and Watson, 1979; Hoffman, 1980).

Finally, there are few studies on the effects of macrophage-conditioned medium on the B-cell responses in man. One analysis indicated that macrophage-conditioned medium potentiated the response to pokeweed mitogen (Dimitri and Fauci, 1978).

f. Summary: Other LAF Activities. There is no question that macrophages secrete powerful lymphostimulatory molecules that exert effects on T and B cells and that the major principle is the 15,000 MW

LAF. Still to be resolved are two questions: (*a*) whether all the molecules of larger size found with some biological activities represent aggregates of LAF; and (*b*) whether all the activities of the 15,000 MW protein can be attributed to a single protein or to a family of them. The action of LAF, if indeed a single molecule, is extensive as we just analyzed.

Two other activities have been associated with a 15,000 MW molecule. Endogeneous pyrogen has been partially purified and found to be a 15,000 MW protein (Dinarello *et al.*, 1977; Dinarello and Wolff, 1977). This purified material was found to potentiate the antigen-driven T cell response in the system developed by Rosenwasser and Rosenthal (Rosenwasser *et al.*, 1980). Another preparation of rabbit endogeneous pyrogen (from peritoneal exudates stimulated with endotoxin) was shown to contain thymocyte mitogenic activity; furthermore, a purified rabbit LAF had pyrogenicity (Murphy *et al.*, 1980). The results, therefore, strongly indicate that the LAF molecule may be the major leukocyte protein that mediates fever.

A human monocyte product has been identified, again of low molecular weight, that when added to cultured synovial cells markedly increased their secretion of collagenase and prostaglandins. This secretion could possibly be involved in the connective tissue changes associated with the inflammatory response of the joints (Dayer *et al.*, 1977a,b). Obviously, the role of LAF and LAF-like products in the inflammatory response involving macrophages needs more analysis, but these two studies strongly indicate the protean role that this special molecule(s) may play.

Last, it is worthy of note that macrophage-conditioned medium may contain other lymphostimulatory molecules aside from LAF. A 40,000 MW protein has been identified in macrophage-conditioned medium, distinct from LAF, that induced marked maturational changes in early thymocytes (Beller and Unanue, 1977) (Section II,G).

2. *Modulation of the Macrophage Secretory Process by the Lymphocyte*

The addition of T cells to macrophages with antigen or a polyclonal stimulus, or the addition of T cell-derived products—a lymphokine-containing culture fluid—may change the pattern of secretion of the macrophage. We discussed before our own studies showing that the secretion of lymphostimulatory molecules by the macrophages was regulated by the T cell when it responded to antigen under an MHC-regulated interaction. Addition of lymphocytes with antigens also stimulated the macrophage to secrete the C2 protein, a key early com-

ponent in the complement cascade (Littman and Ruddy, 1977). In this study, an eightfold increase in hemolytically active C2 was found by human peripheral blood leukocytes stimulated with SK-SD; addition of a lymphokine to the monocytes resulted in less stimulation, about fivefold.

Lymphokines stimulated macrophages to secrete lysosomal enzymes (Pantalone and Page, 1975), interferon (Neumann and Sorg, 1977), collagenase (Wahl *et al.*, 1975a), and plasminogen activator (Vassali and Reich, 1977; Klimetzek and Sorg, 1976, 1977; Nogueira, *et al.*, 1977; Greineder, *et al.*, 1979). The study of Vassali and Reich (1977) is one of several that documented in detail the stimulation of secretion by a lymphokine-rich medium. They demonstrated a dose-response relationship between the amount of lymphokine added to the culture and the secretion of plasminogen activator: the unstimulated macrophage did not release the enzyme until after the addition of the conditioned medium, as little as 0.3% volume per volume. Thioglycolate-stimulated macrophages secreted the enzyme but increased their rate of synthesis and secretion about 10-fold after addition of lymphokine. In these studies, it was possible to document that most macrophages secreted the plasminogen activator. Macrophages also released a procoagulant activity following interaction with lymphocytes stimulated by endotoxin (Edwards *et al.*, 1979; Edwards and Rickles, 1980; Levy and Edgington, 1980); in a comparable situation, macrophages also released a molecule that stimulated lymphocytes to secrete colony-stimulating factor (Apte *et al.*, 1980).

3. *Molecules Involved in Suppressive Effects*

Macrophages release a number of compounds that are directly responsible for inhibitory effects. These compounds include prostaglandins, oxygen intermediates, thymidine, and some macromolecular inhibitors. The suppressive role of macrophages depends on their number and degree of activation, their response to a stimulus, and on the nature of the target cell. This will become evident as we analyze the various observations.

a. Prostaglandins. Prostaglandins are important metabolites derived from the cyclooxygenation of arachidonic acid following its release from phospholipids by phospholipases (Fig. 14). Prostaglandins are believed to be important mediators of inflammation, inducing vasodilatation and edema. There is very good evidence that prostaglandins mediate many of the inhibitory effects produced by macrophages in cell cultures. It is likely that these highly active metabolites may mediate effects *in vivo* under appropriate circumstances. Prostaglandins

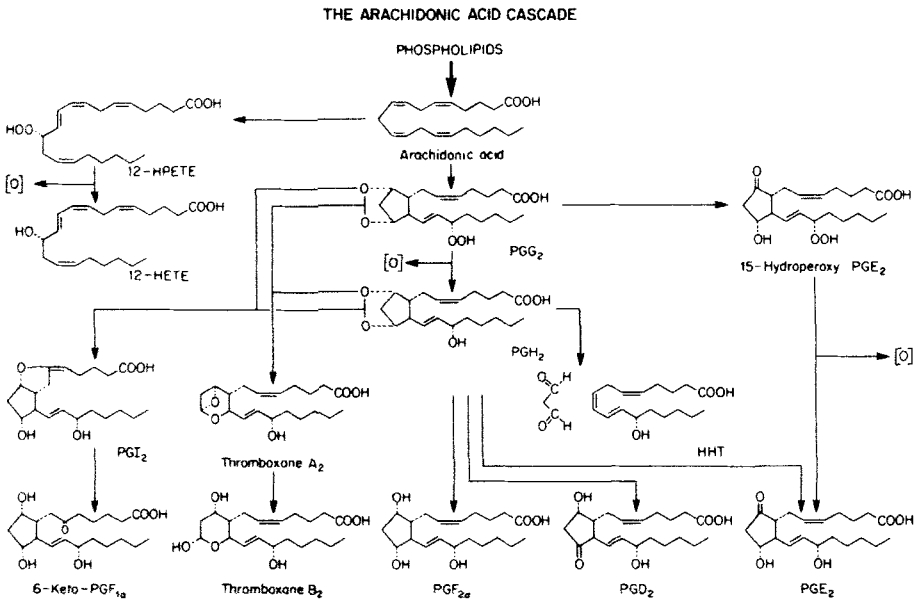

FIG. 14. The prostaglandin cascade taken from a diagram by courtesy of Dr. Philip Davies.

increase cellular cyclic AMP, which may be responsible for many of the modulatory effects on lymphocytes. The interest in the role of prostaglandins in macrophage-lymphocyte interactions came about from several observations. First, macrophages definitely synthesize prostaglandins, and the synthesis increases quite dramatically after their stimulation. Second, prostaglandins of the E series, added to cultures of lymphocytes, inhibit a number of functions. Third, the inhibitory effects of macrophages in macrophage–lymphocyte interactions can be reversed by the addition of drugs that inhibit the cyclooxygenase pathway, such as indomethacin.

Several studies indicated that prostaglandins were synthesized and released by cultured macrophages (Gordon *et al.*, 1976; Goodwin *et al.*, 1977; Humes *et al.*, 1977; Gemsa *et al.*, 1978; Kurland and Bockman, 1978; Brune *et al.*, 1978; Davies *et al.*, 1980). The prostaglandins were measured by radioimmunoassays or directly analyzed biochemically in cultures of macrophages pulsed with [^{3}H]arachidonic acid. The major biosynthetic products were PGE_2 and the 6-keto prostaglandin $F_{1\alpha}$, the stable product of prostaglandin I_2 (Hume *et al.*, 1977; Bonney *et al.*, 1978; Davies *et al.*, 1980). Small amounts of

$PGE_{2\alpha}$ and thromboxane B_2 were found in one study (Brune *et al.*, 1978). The synthesis and release of prostaglandins took place continuously, but increased markedly after stimulation of the macrophage. For example, the studies of Humes *et al.* (1977), showed that the addition to resident macrophages of zymosan, antigen–antibody complexes, or phorbol myristate acetate resulted in a marked increase in synthesis—up to 60-fold—by 30 minutes and continuing for at least a day of culture. Phagocytosis of latex did not have an effect. Essentially similar results were obtained in other studies (Gemsa *et al.*, 1978; Bonney *et al.*, 1979; Scott *et al.*, 1980). Increases in prostaglandins were reported following addition of endotoxin to murine and human phagocytes (Kurland and Bockman, 1972), colony-stimulating factor to murine macrophages (Kurland *et al.*, 1978), and lymphokines to guinea pig macrophages (Gordon *et al.*, 1976). Human monocytes increased their secretion by severalfold after addition of the Fc fragment of IgG, soluble aggregated human IgG, or Con A, but not by phagocytosis of latex particles (Paswell *et al.*, 1979).

The life history of the macrophage has an important effect on the synthesis of prostaglandins. In the studies of Hume *et al.* (1977), summarized by Davies (Davies *et al.*, 1980), normal, resident murine peritoneal macrophages and peritoneal macrophages, following injection of thioglycolate broth or *Corynebacterium parvum*, released equal amounts under basal conditions; yet, while the unstimulated macrophages responded to zymosan with a 20- to 30-fold increase in synthesis, the response of the stimulated macrophages was small, about 5-fold. Four papers reported an increase in basal stimulation of activated macrophages *in vivo*, one from tumor-bearing mice (Pelus and Bockman, 1979), two from mice that received *C. parvum* (Farzad *et al.*, 1977; Grimm *et al.*, 1978), and one from mice infected with BCG (Tracey and Adkinson, 1980); these last studies are of interest in view of the inhibitory effects of *C. parvum*- or BCG-stimulated macrophages in cultures (Scott, 1972; Klimpel and Henney, 1978). The issue of basal secretion by activated macrophages is, on the whole, not completely settled, with some discrepant results; the study of Farzad studied the synthesis for a brief period in comparison to Davies' study, and the study of Grimm was made from whole spleen and did not characterize critically the adherent cells responsible for biosynthesis. Further analyses of macrophage populations are warranted. In most reports, the addition of indomethacin inhibited the synthesis of prostaglandins.

Addition of prostaglandins inhibited various functions of lympho-

cytes in culture. Not all the studies, however, used prostaglandins at physiological concentrations. A list of the inhibitory effects is shown in Table X. Noteworthy is the study of Goodwin *et al.* (1977). They found that stimulation of human peripheral blood lymphocytes with lectins resulted in the production of prostaglandins in amounts similar to those inducing a significant suppression of the proliferative response when directly added to the culture. Addition to the culture of indomethacin stopped prostaglandin biosynthesis and also resulted in an increase in proliferation. Indomethacin had no effect on the action of suppressor T cells induced by the lectin. These investigators went on to show that patients with Hodgkins' disease contained adherent cells (monocytes?) that produced large amounts of prostaglandins; the poor responsiveness of these patients' cells to lectins resulted in marked improvement following addition of indomethacin.

The addition of indomethacin has been found to improve the response of lymphocytes in a number of situations, reducing to a great extent the inhibitory effects of excess numbers of macrophages (Webb and Jamieson, 1976; Webb and Osheroff, 1976; Webb and Nowowiejski, 1977; Demenkoff *et al.*, 1980; Metzger *et al.*, 1980).

It is noteworthy that not all the effects of prostaglandins are inhibitory. Some secretory functions, in fact, are mediated by them, such as the secretion of osteoclast-activating factor by T cells stimulated by lectins (Yoneda and Mundy, 1979) and collagenase by endotoxin-stimulated macrophages (Wahl *et al.*, 1977).

There are some studies that are worth discussing in relation to the

TABLE X
INHIBITORY EFFECTS OF PROSTAGLANDINS

Mitogen-induced proliferation (1–6)[a]
Anti-red cell responses (7, 8)
Lymphokine production (9, 10)
Killing by cytolytic T cells (11)
Mixed leukocyte reaction (12)
Antibody-dependent cell cytotoxicity (13)
Hematopoietic colony formation (14–17)
Natural killer cell generation and activity (13, 18)

[a] Numbers in parentheses indicate the following references: (1) Bourne *et al.*, 1974; (2) Goodwin *et al.*, 1977; (3) Webb and Jamieson, 1976; (4) De Rupertis *et al.*, 1974; (5) Berenbaum *et al.*, 1976; (6) Smith *et al.*, 1971; (7) Webb and Nowowiejski, 1977; (8) Plescia *et al.*, 1975; (9) Gordon *et al.*, 1976; (10) Lomnitzer *et al.*, 1976; (11) Henney *et al.*, 1972; (12) Webb and Osheroff, 1976; (13) Droller *et al.*, 1978; (14) Kurland *et al.*, 1977; (15) Kurland *et al.*, 1978; (16) Williams 1979; (17) Pelus *et al.*, 1979; (18) Tracey and Adkinson, 1980.

regulation of prostaglandin synthesis by macrophages and its significance. The study of Goodwin *et al.* (1977), mentioned above, showed the critical relationship between the accessory function of the monocyte, on the one hand, and its suppressive effect on the other: addition of indomethacin improved the proliferative response to lectins, yet the removal of the monocyte resulted in a drop in the response. The message is clear, therefore, that the number and state of activation of the macrophage becomes critical in closed cell culture systems where the active metabolites accumulate.

The study of Gordon *et al.* (1976) called attention to a possible feedback loop involving the T cells and the macrophages. Indeed, lymphokines stimulated prostaglandin secretion, but these, in turn, stopped the biosynthesis of lymphokines produced by antigen in T cell–macrophage mixtures. A whole loop of macrophage–T cells → lymphokines → macrophages → prostaglandins → lymphocytes can be discerned. A similar kind of situation was reported by Kurland and associates: a critical number of macrophages by way of a growth-promoting molecule stimulated formation of bone marrow-derived hematopoietic colonies; yet an excess amount of macrophages suppressed colony formation through the release of prostaglandins. Colony-stimulating factor in itself enhanced prostaglandin secretion by the macrophage (Kurland *et al.*, 1977, 1978).

Finally, some studies have addressed the issue of synthesis by macrophages of arachidonic acid products produced by the lipooxygenase pathway. Macrophages have been shown to secrete 12-hydroxyeicosatetranoic acids (Rigaud *et al.*, 1979; Scott *et al.*, 1980) and the leukotrienes (slow reactive substance of anaphylaxis) (reviewed by Stenson and Parker, 1980).

b. Oxygen-Derived Products. A recent study of Metzger *et al.* (1980) is of importance in that it adds oxygen metabolites to the list of inhibitors of lymphocyte function in culture, together with the prostaglandins. Leukocytes, neutrophils, and macrophages, particularly if stimulated with various particles and agents, can release a number of highly reactive oxygen intermediates, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and possibly hydroxyl radical ($OH\cdot$) and oxygen in its excited state, singlet oxygen ($^1O_2$). These very active radicals are believed to be of particular importance for the microbicidal action of the cell and can also damage cells. Good evidence has been presented for a role of H_2O_2 in the tumoricidal effect of activated macrophages (Nathan *et al.*, 1978). Release of oxygen intermediates best takes place following phagocytosis by macrophages, particularly if previously activated by a cellular immune reaction (for a review of

these studies, see the papers of Nathan, 1980; Johnston, Jr. *et al.*, 1980; Klebanoff, 1975, 1980; Karnovsky *et al.*, 1975; Babior, 1978). In their study, Metzger *et al.* confirmed that indomethacin added to cultures of Con A-activated T cells stopped the suppression produced by phagocytes, except when these were in high numbers; they also added to their cultures catalase, an enzyme that rapidly converts H_2O_2 to H_2 (plus O_2). Addition of catalase resulted in an improvement in DNA synthesis, particularly in cultures strongly inhibited by an excess of macrophages; moreover, addition of indomethacin and catalase had a significant syngergistic effect. The indications from their results is that part of the inhibitory effect of macrophages in culture could be due to H_2O_2. This is an important observation that needs to be extended, but which points out the existence of oxygen products as powerful inhibitors. Clearly, situations that result in stimulation of the phagocytes and thus increase their release of prostaglandins or H_2O_2 result in adverse effects in cultures.

c. Thymidine. Thymidine is released continuously from the cultured macrophages. Depending upon the concentration of macrophages, the amount of thymidine that accumulates in the medium can be significant. We calculated that a 24-hour culture of 10^7 macrophages in 1 ml of medium planted in a culture plate of 35 mm diameter contained thymidine at a concentration of 4×10^{-6} *M* (Stadecker *et al.*, 1977). The thymidine in the macrophage-conditioned medium may compete for radiolabeled thymidine used for pulsing cells growing in such medium; the thymidine may also directly block DNA synthesis (Stadecker *et al.*, 1977) by preventing the conversion of cytidilate to deoxycytidilate (Morris and Fischer, 1963).

A number of reports indicated the presence of an inhibitor of DNA synthesis in macrophage-conditioned medium (Nelson, 1973; Waldman and Gottlieb, 1973; Calderon *et al.*, 1974; Calderon and Unanue, 1975; Opitz *et al.*, 1975a). Our studies indicated that a dialyzable molecule was clearly masking the presence of lymphostimulatory molecules in macrophage-conditioned medium (Calderon and Unanue, 1975). Opitz and associates showed that the inhibitor was dialyzable and affecting only the incorporation of ^{3}H-labeled thymidine by the lymphocyte, not its growth. The inhibitor was behaving, in a limited number of biochemical analyses, as thymidine (Opitz *et al.*, 1975b; Kasahara and Shioiri-Nakano, 1976). In our laboratory we found a dialyzable inhibitor that truly stopped DNA synthesis of the tumor cell line EL-4 (Calderon *et al.*, 1974). Biochemical characterization of the inhibitor also revealed a molecule identical to

thymidine (Stadecker *et al.*, 1977). The observations that the EL-4 tumor line was truly inhibited in its growth was explained by an unusual sensitivity of this cell to thymidine. Most tumor lines or growing lymphocytes were blocked by concentrations of thymidine ranging from 10^{-3} to 10^{-4}, while EL-4 was blocked at 10^{-6} *M*. This is a clear example of the importance of the target cell in determining the effects of macrophage-secretory products.

The release of thymidine by the macrophage is quite unique. The possibility was raised by Opitz *et al.* (1975b) that the molecule could derive from the degradation of nuclear material phagocytized by macrophages. The bulk of the released thymidine, however, was synthesized by the macrophage, as was evident from biochemical studies using [^{14}C]formate (Stadecker *et al.*, 1977). The release of thymidine was explained by the absence of thymidine kinase, the enzyme that phosphorylates thymidine and rescues it from being released (Stadecker and Unanue, 1979). We found that macrophages lacked thymidine kinase and that addition to them of the growth-promoting molecule from L cell-conditioned medium resulted in *de novo* appearance of the kinase and the expected drop in thymidine release. Previous studies in fibroblasts had shown that mutant lines lacking thymidine kinase released thymidine into the culture (Chan *et al.*, 1974).

d. Macromolecular Inhibitor. One of the most interesting inhibitor molecules produced in culture is the soluble immune response suppressor (SIRS) identified in C. Pierce's laboratory (Tadakuma and Pierce, 1976, 1978; Tadakuma *et al.*, 1976). SIRS is a molecule found in culture of Con A-activated T cells that suppresses immune responses *in vitro*. When added to a culture of spleen cells, it abruptly stopped the plaque-forming cell response at day 4. The molecule was made by Ly2-positive T cells but required macrophages for the suppressive effect. Recent studies showed that the T cell product isolated from Con A-stimulated T cells was in a precursor, inactive form that could be converted into the active moiety upon direct incubation with macrophages. The macrophages did not require synthesis of new protein, nor was the generation of the active inhibitor blocked by indomethacin. Addition of catalase, however, stopped the production by the macrophage. To this effect, addition of H_2O_2 to the precursor molecule in the absence of macrophages resulted in generation of the active molecule. The inhibitory effects of SIRS was rapid, probably during G_2 or mitosis, a unique situation for a cell cycle inhibitor, and was reversed by 2-mercaptoethanol. Both the precursor and the active product had a molecular weight of approximately 55,000 (Pierce and Aune,

1981). Thus, all the indications are that an Ly2-positive suppressor T cell releases a molecule that, upon oxidation by H_2O_2 released by macrophages (probably only upon activation), converts into a strong and rapid inhibitor of cell proliferation.

Cooperativity of two cells by way of their released products has also been found between malignant plasma cells and macrophages. Plasmocytomas are known to inhibit normal Ig production. In an attempt to investigate the basis for this suppressor, it was found that cultured plasma cells released a large-molecular-weight protein that, upon interaction with macrophages, resulted in the release of a small-molecular-weight product of about 6000 MW. The product inhibited antibody formation *in vitro* in a noncytotoxic reaction and did not affect lymphocyte proliferation (Kennard and Zolla-Pazner, 1980).

Macromolecular "toxins" have been identified in high-density cultures of macrophages. Whether all are derived from macrophages, and their mode of action on lymphocytes, is not clear. A discussion of these groups of molecules can be found in a study of Chen *et al.*, who, in their own work, found that high-density cultures of macrophages released a 110,000 MW protein that inhibited DNA synthesis and antibody formation *in vitro* (Chen *et al.*, 1977).

IV. Conclusions

Historically, the participation of the macrophage in immunobiology was first studied in the context of the resistance to intracellular pathogenic bacteria. The analysis of the activated macrophage started with Koch's early description of the tuberculous granuloma and has continued up to the present time. We accept the notion that resistance to many infectious agents requires the mobilization of bone marrow-derived phagocytes to infectious foci, where they are activated, acquiring potent cytocidal functions; this process of macrophage recruitment and activation is brought about by soluble mediators secreted by antigen-stimulated T cells. Our information on the role of the macrophage in regulating immune induction is more recent, dating from the past 20 years. The intensive work of this period has demonstrated that the phagocytes exert a fine control on the early events that lead to antigen stimulation of T and B cells. Thus, the phagocytes participate at two very distinct stages in the immune process: first, as a cell regulating the extent to which lymphocytes are stimulated; second, as an effector cells responding to the signals from stimulated lymphocytes. Both processes are obviously deeply interrelated. It is indeed fascinating to note how the specific cells of the immune system—the

lymphocytes—and the nonspecific element—the macrophage—are so deeply dependent upon each other to the point of symbiosis.

The immunological cellular network is disturbed by the entrance of antigen, an event that sets in motion a complex number of cellular interactions. The phagocytes play a central role at this stage essentially because of the inability of the specifically reactive components of the immune system to respond to soluble antigen molecules. The T cells, or at least the helper subset, respond primarily to antigen that has undergone a handling process in which the products of the *I* region of the MHC play a critical and essential modulatory role (Sections II and III,B). The macrophages thus come into action at an early stage of the immune process by virtue of their capacity to take up and handle antigen, to express Ia, and to secrete lymphostimulatory molecules. It is noteworthy that two of these key functions, Ia expression and secretion of active factors, are relatively brief events (Sections III,B and III,C) in the life of the macrophage and that both are controlled by antigen and T cells in positive and negative ways. Thus, we reviewed how phagocytes displaying Ia antigen are able, after their uptake of antigen, to establish physical and functional interactions with T cells, the result of which is a burst of synthesis of lymphostimulatory molecules and Ia antigens, on the part of the macrophage, and of secretion of mediators and DNA synthesis on the part of the T cell. In closed cell cultures, the interactions are shut off when the macrophage, as it becomes stimulated, synthesizes inhibitory molecules (Section III,C). This negative control function appears likely to apply to *in vivo* situations. It is also likely from some of the evidence brought out in Sections II,H and III,C that suppressor cells may intervene in the T cell–macrophage interaction, even using macrophages as an intermediary. Other reciprocal control mechanisms that are now emerging are those regulating the percentage and number of Ia-bearing macrophages in tissues. This is particularly evident in the response to various inflammatory stimuli and in the ontogeny of the macrophage in the mouse (Section III,C). The ontogeny studies are indicating the need to limit the number of Ia-bearing phagocytes in the various tissues at a very critical stage of development. Overall, it appears that the macrophage antigen-presenting function is controlled internally as well as by external signals.

A major advancement in our understanding of the role of the macrophage came from the discovery of its participation in *I*-region-regulated events (Section II,A). The essential role of the *I*-region products in controlling all macrophage–T cell interactions is now an accepted fact. The antigen-presenting function of the mac-

rophage extends beyond a simple focusing of antigen to a more sophisticated and elaborate process. Such antigen-presenting function is essential in promoting growth of the selective T cells that recognize antigen in the context of the proper *I*-region molecules of the macrophage. The importance of the antigen-presenting function can be surmised from the many experiments mentioned in Section II. It becomes especially pointed in the case of the response to peptide antigens under *Ir*-gene control. The *Ir* gene effects indicate a very specific interaction between a fragment of antigen and the various *Ir* gene products, most likely the Ia antigens. The selective interaction between *I*-region products and antigen imparts specificity to the antigen-handling step. It is, in my view, a device by which the system ensures that there is no reaction against soluble or tissue self-molecules, but only against those antigens whose intrinsic properties allow them to bind to phagocytes (i.e., microorganisms, for example).

The macrophage regulates the interaction with antigen, in part, by selecting the key antigenic determinants, a point clearly brought out in the experiments using the insulin molecule in the guinea pig (Section III,B). Determinant selection appears to take place at the same time that antigen molecules are degraded, an apparent paradoxical situation. The macrophage destroys most of the antigen but conserves a few key determinants for the lymphocytes to interact. The molecular and biochemical events in determinant selection and antigen presentation are not entirely known and represent key areas of future research, together with the issues of why and how the helper T cells interact with macrophage-bound antigen, the molecular nature of the T cell receptor, and the development of the various T cell subsets. With regard to the latter point, there are indications that the macrophages in tissues may also play a role in non-antigen-driven maturational events (Section III,B).

Several cells aside from lymphocytes bear Ia antigens, the key modulatory proteins in antigen-driven and non-antigen-driven events. The question that comes to mind is whether all such cells are capable of antigen presentation or other forms of immune modulation. We discussed the Langerhans cell and a number of related dendritic cells, abundant in skin and lymphoid tissues, some of which are capable of expressing Ia and presenting antigen (Section III,A). The developmental and functional relationship between the Langerhans cells and the classical phagocyte require more analysis, yet the distinctive features of the Langerhans cell indicates the presence of a novel accessory cell performing in conjunction with the phagocytes. Aside from the Langerhans cells, stromal cells in the thymus have been found to bear Ia and, more recently, so were human endothelial cells in culture

(Hirschberg *et al.*, 1980). Will all these present antigen? Or are they involved in non-antigen-driven events? Aschoff coined the term reticuloendothelial system to encompass a number of connective tissue cells all having the common feature of taking up vital dyes and particles to a major or lesser extent. The term lost its use when each cell type—fibroblasts, macrophages, endothelial cells, etc.—was identified into their proper lineage. Perhaps the time has come to reintegrate these cells into a system with a unifying function, that of regulation of cellular interactions by the Ia antigens.

The developments in macrophage biology over the past few years were not predicted at the time of my 1972 review in this Series. It is safe to assume that the discoveries in the next few years will prove equally surprising, with the study of the macrophage continuing to provide unique, illuminating insights into the complexities of immune cellular interactions.

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T-Cell Growth Factor and the Culture of Cloned Functional T Cells

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I. Introduction

Twenty years have passed since the discovery that plant lectins initiated blastic transformation and mitosis in small resting lymphocytes (Nowell, 1960). As a result of this discovery and the development of assays for specific antigen-induced lymphocyte triggering, the ensuing decade brought forth a veritable explosion of information relative to the cellular basis of immunity. Together with the single-cell assay for antibody-forming cells promoted by Jerne and Nordin (1963), lectin- or antigen-initiated T-lymphocyte proliferation assays gradually led to the appreciation that several distinct cell types were involved in immune responses. As a result, the attention of immunologists has fo-

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cused during the past decade toward the dissection of the mechanisms of communication among the principal cells involved in the immune response.

Soon after the realization that lymphocytes were capable of self-renewal, soluble mitogenic factors were discovered in the media of cultured lymphocytes (Kasakura and Lowenstein, 1965; Gordon and MacLean, 1965). Initially, it was thought that the mitogenic activities were by-products of the antigen- or lectin-induced cellular proliferative response, and functioned simply to augment a cellular activation process that was essentially ligand-driven. As experimental evidence accumulated, however, definitive proof of this hypothesis became elusive. Macrophages, as well as T cells, were found to be essential for T-cell proliferative responses to antigens or lectins (Oppenheim *et al.*, 1968; Seeger and Oppenheim, 1970; Rosenstreich *et al.*, 1976). The awareness that macrophages played an obligatory role in immunocyte proliferation and differentiation, thus abolished the notion that ligand–lymphocyte binding was the sole requirement for the initiation of blastic transformation and mitosis.

The identification of the cellular requirements for immune responses relied progressively upon sophisticated techniques of cellular identification and separation. Significant and most timely advances were the discovery of functionally distinct T-cell surface markers, the development of the fluorescence-activated cell sorter, and the advent of monoclonal antibodies. Gradually, as a result of these technical improvements, it became apparent that different functional T-cell subsets cooperated to generate a fully competent T-cell and B-cell immune response. All of these techniques, however, depended upon the assumption that the reagents identified and allowed separation of functionally distinct and homogeneous cell populations. Thus, despite intricate efforts to ensure homogeneity of separated cell populations, it always remained possible that the results obtained from functional experiments were derived from small numbers of remaining contaminating cells rather than the positively selected cells in question.

This deficiency of experimental design has now been overcome owing to the discovery that T lymphocytes can be maintained, apparently indefinitely, in continuous exponential proliferative culture (Morgan *et al.*, 1976; Ruscetti *et al.*, 1977). Moreover, under the proper conditions, subsequent studies revealed that it is possible to derive and maintain monoclonal functional T-cell lines (Gillis and Smith, 1977; Baker *et al.*, 1979; Von Boehmer *et al.*, 1979; Schreier *et al.*, 1980; Engers *et al.*, 1980; Sredni and Schwartz, 1980). The active moiety responsible for this advance was subsequently found to be a

soluble lymphokine of T-cell origin, termed T-cell growth factor (TCGF) (Gillis *et al.*, 1978b). Thus, lymphokines suddenly assumed a central importance to those interested in the regulation of the immune response; it is now apparent that the lectin/antigen-initiated proliferation of T-cells originally described by Nowell, is actually mediated by an intricate interaction of both macrophage- and T-cell-derived soluble factors. The insight gained by this realization has for the first time integrated immunologists who have approached immunocyte regulation from seemingly disparate disciplines. Although the molecular basis for the functional interaction of the soluble factors and the various cells involved in competent immune responses are now at the threshold of discovery, it may be beneficial at this time to review the established facts, and to explore the assumed facts based on the information presently available.

At the outset it is helpful to present a working model of T-cell activation. The model to be described is a condensation of information derived from several groups of investigators working independently, who have almost simultaneously arrived at strikingly similar conclusions (Smith *et al.*, 1979b; Coutinho *et al.*, 1979; Bonnard *et al.*, 1979; Larsson *et al.*, 1980a,b; Gronvik and Andersson, 1980; Paetkau *et al.*, 1980; Ruscetti and Gallo, 1981). As displayed in Fig. 1, the T-cell proliferative response results from the interaction of at least three distinct cell types. When antigen or lectin is introduced to a mixed population of these cells, all the cells that bind the ligand through specific binding sites become "activated." This results in at least three responses: (*a*) release of lymphocyte-activating factor (LAF) from monocytes and macrophages; (*b*) under the influence of LAF, the release of TCGF by a specific T-cell subset; and (*c*) binding of TCGF by separate T-cell subsets resulting in the proliferative response. The importance of this concept has been the understanding that functional T-cell clonal expansion, although ligand initiated, is under a strict and obligatory regulatory control by soluble immunocyte products that have features characteristic of hormones. Thus, the clonal expansion and differentiation of functional T-cell immune responses can no longer be viewed simply as an externally driven phenomenon; rather there exists a unique signaling apparatus designed to transmit external foreign stimuli to internal hormone-like control.

It is apparent from this model that, because the ultimate realization of T-cell proliferation depends upon an intricate communication network between at least three functionally distinct cells, those signals that determine activate participation of each of the cells must assume a central importance. While there exists some circumstantial evidence to

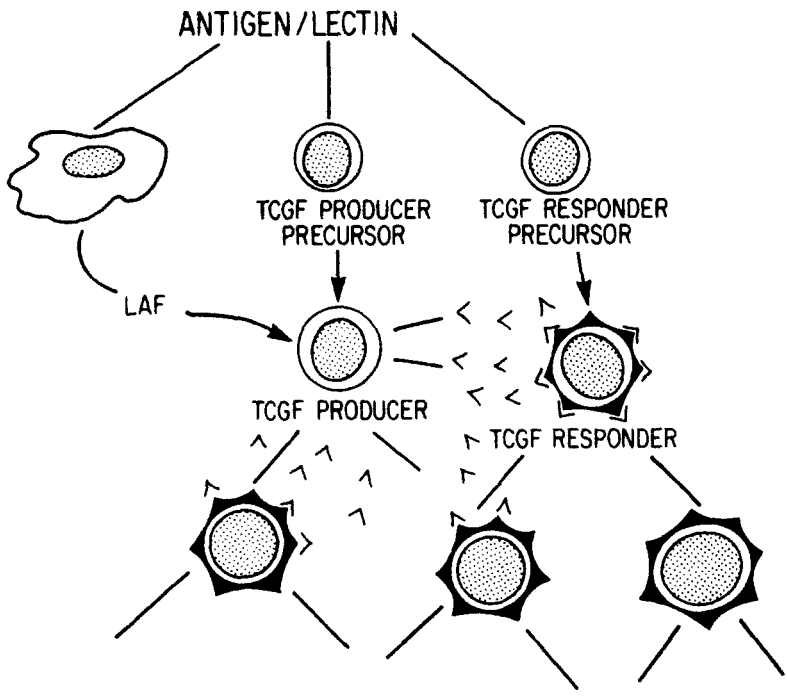

FIG. 1. A model for T-cell activation. LAF, lymphocyte-activating factor; TCGF, T-cell growth factor.

indicate that activating signals may vary for different cells, many more data are required before it will be possible to ascribe with certainty the exact reaction sequences. It is evident, however, that lectin or antigen are obligatory to initiate the transformation of resting cells to an activated, receptive state. In addition, major histocompatibility complex (MHC) gene products perform an essential role in the activation process. Although these early triggering signals appear to initiate the reaction and dictate the specifity of the ultimate immune response, the nonspecific soluble factors of macrophage and T-cell origin actually mediate the antigen-directed clonal expansion of effector cells. Since more experimental data are required before the dissection of the functional role of activating signals can be realized, and because such a dissection will necessarily depend upon monoclonal functional cellular reagents, this review will concentrate on the events that occur subsequent to ligand-initiated MHC-modulated cellular activation, and which actually mediate the proliferative clonal expansion of activated T cells.

II. Lymphocyte-Activating Factor

A. General Considerations

Although it was initially assumed that the mitogenic factors found to be present in leukocyte cultures were lymphocyte products, Bach *et al.* (1970) reported that adherent cells appeared to release soluble factors that had mitogenic properties for lymphocytes. This report was subsequently confirmed and extended by Gery *et al.* (1972), who coined the term "lymphocyte-activating factor" (LAF) for macrophage-derived mitogenic factors. There seems to be no doubt that LAF is macrophage-derived: adherent cells originating from human (Bach *et al.*, 1970), mouse (Gery *et al.*, 1972), guinea pig (Rosenstreich *et al.*, 1976), monkey (Blyden and Handschumacher, 1977), and dog (Ulrich, 1977) cells have been found to produce LAF. Additional evidence was reported by Lachman *et al.* (1977b), who found that murine tumor cell lines having macrophage characteristics released LAF activity. These workers also demonstrated that human acute monoblastic and myelomonocytic leukemia cells could be induced to release LAF activity (Lachman *et al.*, 1978). In contrast, attempts to identify LAF release by lymphoid cell lines or freshly isolated malignant and normal lymphoid cells have been unsuccessful.

While LAF appears to be spontaneously released by macrophages soon after they are placed *in vitro* and allowed to attach to the surface of a culture vessel (Unanue and Kiely, 1977), various stimuli have been reported markedly to increase LAF release. LAF inducers fall into two major groups: those that enhance LAF release in the absence of other cells, and those that require T cells (Oppenheim *et al.*, 1979). Among the direct LAF inducers are bacterial lipopolysaccharide (LPS), phagocytic stimuli such as latex particles, phorbol myristic acetate, pyran copolymer, gram-negative and gram-positive bacteria, yeast, and mycobacterial peptidoglycolipids. It is noteworthy that this list contains many of the agents that have been used experimentally and clinically as immunological adjuvants.

Lipopolysaccharide is the prototype LAF inducer that is most commonly employed experimentally. Human monocytes have been found to be exquisitely sensitive to LPS, such that as little as 10 pg/ml can stimulate LAF release, with maximum release observed between 1 and 10 ng/ml (Wood and Cameron, 1978). In this regard, therefore, it is probable that many, if not all, *in vitro* immunological studies performed over the years employed reagents contaminated with LPS in sufficient quantities to induce maximal LAF release from macro-

phages. The effects of the LAF release on the results of the experiments, and thus the experimental conclusions drawn, can only be inferred at this time: future experiments where LPS is rigorously excluded and LAF carefully monitored, will be necessary to delineate the relative importance of LAF to the interpretations of experimental results.

Detailed studies by Rosenstreich *et al.* (1976), have shown that T cells are required for the induction of LAF release by macrophages in the presence of T-cell lectins or soluble protein antigens. Cell separation experiments have indicated that activated T cells (most probably of the $Lyt1^+2^-3^-$ subset) stimulate macrophages to release LAF, and the resultant product is indistinguishable from the LAF released after LPS stimulation. It is unclear whether direct T cell–macrophage contact is required for this type of stimulation or whether a T-cell-derived lymphokine is responsible for the macrophage stimulation. A recent report suggested that T-cell-derived macrophage-specific colony-stimulating activity or macrophage growth factor is the lymphocyte product that fulfills this role (Moore *et al.*, 1980). Further experiments are necessary, however, to ascertain the exact sequence of signals passing between macrophages and T cells. Suffice it to say at this point that it is quite possible that a cyclical interaction may exist between activated T cells and macrophages, the products of one cell type amplifying the release of products of the other cell type and vice versa.

B. The LAF Assay

One of the major impediments to the dissection of the role and mechanism of action of LAF has been the absence of an assay that specifically detects LAF and no other mitogenic factor. LAF is traditionally assayed by its mitogenic effect on murine thymocytes (Oppenheim *et al.*, 1979). It is important to characterize the sample to be assayed as LAF, for the thymocyte assay will also detect lymphocyte-derived mitogenic proteins, such as TCGF. Additionally, if the samples to be assayed contain T-cell lectins or antigens, the *in situ* production of mitogenic factors by the target thymocytes may be superimposed upon any mitogenic effect of LAF. The assay is conducted by culturing thymocytes (which contain less than 1% macrophages, thus serving as a good target cell population for a macrophage product) at high cell densities (usually 10 to 15×10^6 cells/ml) in the presence of a suboptimal concentration of a T-cell lectin and varying concentrations of LAF (Oppenheim *et al.*, 1979). After 72–96 hours of culture, thymocytes exhibit a marked proliferative response that is dependent on LAF concentration. Displayed in Fig. 2 are the

results from a typical assay. Several observations are noteworthy when the kinetics of the thymocyte response (Fig. 2A) are examined. Thymocytes incorporate considerable amounts of [^{3}H]thymidine (Tdr) when initially placed into culture, whether or not LAF or lectin are

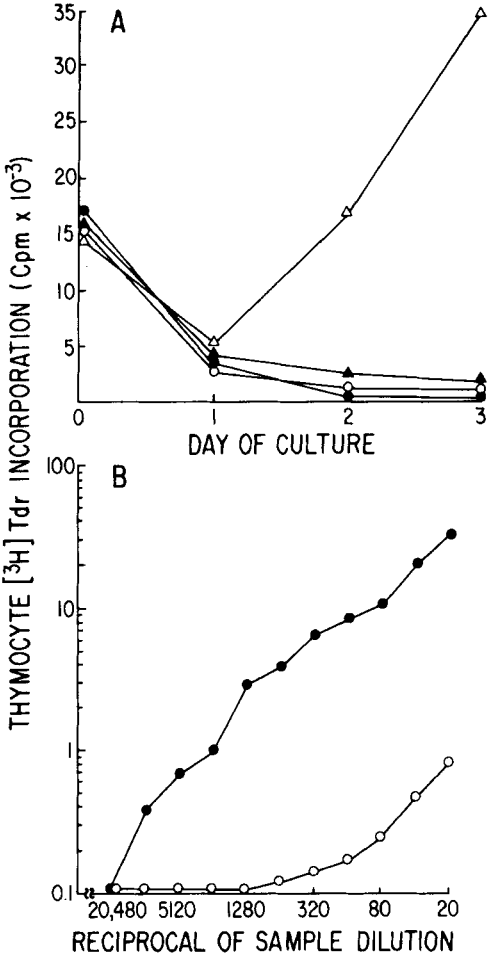

FIG. 2. The lymphocyte-activating factor (LAF) thymocyte assay. (A) The kinetics of LAF activity. C3H/HeJ thymocytes were cultured (1×10^7 cells/ml) in the presence of medium (●); phytohemagglutinin (PHA) (▲); LAF derived from lipopolysaccharide-stimulated human mononuclear cells at a maximal stimulatory concentration (1 : 20 dilution, see Fig. 2B), (○); PHA plus LAF (△). At the times indicated, [^{3}H]Tdr was added for 4 hours prior to the cessation of the culture. (B) The LAF concentration-dependent effect on thymocyte [^{3}H]Tdr incorporation determined 72 hours after the initiation of culture in the absence (○) and the presence (●) of PHA (1 μg/ml). [From Smith *et al.* (1980d).]

present. This proliferative activity, however, declines regardless of the presence of lectin and/or LAF, and [^{3}H]Tdr incorporation progressively decreases as the culture period is extended. By the second day of culture, cells exposed to both LAF and lectin exhibit a significant increase in [^{3}H]Tdr incorporation, which is of even greater magnitude by the third day. Concomitant with the increase in [^{3}H]Tdr incorporation is the appearance of blastoid cells, their proportion increasing as the culture period is extended. In contrast to the marked proliferative response observed when both LAF and lectin are present, cells exposed to LAF or lectin alone exhibit only minimal blast transformation and [^{3}H]Tdr incorporation.

The concentration dependency of the LAF effect after 72 hours of culture is displayed in Fig. 2B, where thymocyte [^{3}H]Tdr incorporation is plotted as a function of the reciprocal of the LAF sample dilution (the kinetic data shown in Fig. 2A were derived from the highest LAF concentration utilized, a 1 : 20 dilution). In the presence of PHA, as the concentration of LAF is increased thymocyte [^{3}H]Tdr incorporation increases. The marked difference in LAF-induced [^{3}H]Tdr incorporated in the presence and the absence of PHA deserves comment, for there is also a LAF concentration-dependent increase in [^{3}H]Tdr in the absence of PHA. This modest difference in the amount of [^{3}H]Tdr incorporated solely in the presence of LAF (800 cpm at the highest LAF concentration) compared to the medium control (100 cpm) has led some investigators to ascribe direct mitogenic activity to LAF (Rosenstreich and Mizel, 1978). It is also quite possible, however, that LAF, or perhaps other constituents in the LAF preparations, may simply retard the decline of proliferative activity present at the initiation of the culture (Fig. 2A).

A final point with regard to the traditional LAF assay is important. Although the experiment depicted in Fig. 2 displays the concentration-dependent effect of LAF, most investigators have assayed only one concentration (usually a 1 : 4 dilution) and compared the difference in [^{3}H]Tdr incorporation affected by one LAF preparation to that of another. If one compares the dilutions (or titers) of the LAF samples that mediate comparable amounts of thymocyte proliferation, the assay can yield more useful information as to the quantity of LAF present in a given sample.

C. Biochemical Characteristics of LAF

Human LAF, derived from LPS-stimulated peripheral blood monocytes, has been examined by several groups (Blyden and Handschumacher, 1977; Lachman *et al.*, 1977a,b; Wood, 1979). There is

general agreement that human LAF elutes from molecular gel chromatography in a position corresponding to that of a globular protein of 13,000–15,000 molecular weight. Although species of higher molecular weight have also been identified, most investigators feel that these arise as a consequence of noncovalent interactions between LAF and contaminating molecules: such higher molecular weight forms are commonly observed when LAF is produced in the presence of serum and are not present in preparations derived in the absence of serum.

When LAF recovered from gel filtration was examined by isoelectric focusing (IEF) (Lachman *et al.*, 1977a), most of the activity centered in a relatively broad band at pH 6.5–7.0. Minor peaks of activity were also identified in the pH range of 5.0 to 6.0. The heterogeneity found on IEF has not been further delineated, and thus it is unclear whether LAF activity is present in several molecular species or in one protein that has undergone posttranslational modification or degradation. It should be noted, however, that Lachman *et al.* (1977a) demonstrated that the major band of LAF isolated from IEF in the pH range 6.8 to 7.2 migrated on polyacrylamide gel electrophoresis (PAGE) with a uniform size.

Like human-derived LAF, analytical biochemical characterization of murine LAF by gel filtration has resulted in the active material migrating in a position corresponding to a globular protein of 13,000–15,000 daltons (Calderon *et al.*, 1975; Lachman *et al.*, 1977a; Economou and Shin, 1978; Mizel *et al.*, 1978; Lachman and Metzgar, 1980). Again, higher molecular weight species have been identified by these methods, but most workers have concentrated on the lower molecular weight form. Analysis of active fractions obtained from gel filtration by DEAE chromatography has yielded a heterogeneous elution pattern over a broad range of salt concentrations (between 25 and 90 m*M* NaCl) (Mizel *et al.*, 1978), thus suggesting that although the activity is fairly homogeneous in size, there is considerable heterogeneity in charge. Similar results have been obtained from LAF derived from LPS-activated peritoneal macrophages, as well as LPS- or T-cell-activated $P388D_1$ tumor cells. In fact there have been no discernible differences noted between LAF derived from these sources (Rosenstreich and Mizel, 1978).

Economou and Shin (1977) have examined LAF derived from serum-free cultures of murine peritoneal exudate cells by isoelectric focusing and have reported the activity to be heterodisperse, but present in two broad major peaks centered at pH 4.8 and 5.3. In contrast, Mizel (1980) reported that $P388D_1$-derived LAF focused in one dis-

tinct peak at pH 5.7. Suffice it to conclude at this point that murine-derived LAF focuses at a considerably lower pH than does human-derived LAF. In this regard there are some parallels between LAF and TCGF derived from mouse and human cells (see the biochemical characteristics of TCGF, Section III,C).

While studies on the physicochemical nature of human LAF have revealed the activity to be relatively resistant to some proteases, both papain and chymotrypsin were found to decrease LAF activity (Wood, 1979), thus suggesting that the activity can be ascribed to a protein. Blyden and Handschumacher (1977) found that human LAF partially purified by gel filtration and ion exchange chromatography was unaffected by treatment with sodium periodate and iodoacetate, thus suggesting that, if carbohydrate or sulfhydryl residues are present in LAF, they are not essential for activity. Although the activity is resistant to low pH (0.18 *N* HCl at 23°C for 24 hours), alkalinization (pH 9.0 for 24 hours) results in inactivation. The activity is remarkably resistant to high temperatures: LAF partially purified by gel filtration and ion exchange chromatography loses activity only gradually at 100°C (Wood, 1979).

Physicochemical characterization of murine LAF has yielded many similarities to human LAF. Calderon *et al.* (1975), found LAF derived from murine peritoneal exudate cells to be resistant to trypsin, ribonuclease, carboxypeptidase A, papain, and neuraminidase. Activity of LAF, however, was reduced 41% by treatment with Pronase and totally destroyed by chymotrypsin and pepsin. Mizel (1980) also found LAF derived from phorbol myristic acetate-stimulated P388D_1 cells to be destroyed by Pronase, but not by trypsin, chymotrypsin, and papain. In the presence of 8 *M* urea, LAF activity was completely destroyed by papain: Mizel has interpreted this finding as being consistent with a reversible denaturation by urea that allows previously unexposed papain-sensitive sites to become accessible to proteolytic attack. Other data reported by Mizel (1980) suggested that a carbohydrate component either is not present or is not essential for biological activity: P388D_1-derived LAF was not inactivated by neuraminidase or sodium periodate, nor did it bind to concanavalin A(Con A)–Sepharose. Additionally, since LAF activity was not inactivated by reduction and alkylation, the active conformation of LAF does not appear to depend upon disulfide linkages.

D. Functional Characteristics of LAF

There has been considerable confusion as to the functional mechanism of LAF action, owing in part to the use of either unprocessed or only partially processed conditioned media for biological experiments.

LAF was originally defined as a macrophage-derived soluble factor that promoted thymocyte proliferation (Oppenheim *et al.*, 1979). As mentioned previously, some investigators attributed a direct mitogenic function to LAF owing to the fact that LAF-containing conditioned media were shown to promote a modest increase in thymocyte [^{3}H]Tdr incorporation in the absence of other exogenous additives. This interpretation necessarily assumes that other entities, either present in the LAF-containing conditioned media or the fetal calf serum, do not provide low levels of antigenic stimulation required for the reception of an activating signal provided by LAF. Upon examination of the published data it is clear that LAF causes a marked concentration-dependent enhancement of lectin-initiated thymocyte [^{3}H]Tdr incorporation (e.g., see Fig. 2). As a result of these data, most recent reports have redefined the operational definition of LAF as a macrophage-derived soluble factor that enhances lectin-initiated thymocyte proliferation. It is important to note, however, that the effects of LAF are not confined to thymocytes or lectin-initiated T-cell responses. In fact, the first report of the effects of LAF dealt with its macrophage replacing-activity in mixed lymphocyte cultures of human peripheral blood lymphocytes (Bach *et al.*, 1970). Subsequent studies revealed that LAF-containing conditioned media would enhance the generation of cytotoxic T cells (Farrar *et al.*, 1980) as well as the generation of antibody-forming cells (Wood and Cameron, 1976; Koopman *et al.*, 1978; Hoffman and Watson, 1979). The mechanism whereby LAF mediates these seemingly diverse biological activities, however, has remained ill-defined.

Soon after it was realized that lectin-stimulated mononuclear cells released an activity that maintained continuous T-cell proliferation, it was questioned whether LAF was the moiety responsible for the T-cell growth. It was found, however, that partially purified human or murine LAF that clearly enhanced lectin-initiated thymocyte proliferation did not support continuous T-cell growth (Oppenheim *et al.*, 1980). The most likely explanation, which could account for the thymocyte mitogenic effect of LAF and the lack of a growth-promoting effect of LAF on T cells, was the LAF-mediated enhancement of a T-cell released, and T-cell-specific, mitogen. Several groups of investigators have provided evidence that supports this hypothesis (Smith *et al.*, 1979a; Larsson *et al.*, 1980b; Smith *et al.*, 1980b; Shaw *et al.*, 1980). Partially purified human or murine LAF promotes TCGF release by thymocytes and adherent cell-depleted splenocytes. In the absence of adherent cells or LAF, T-cell enriched populations release little or no TCGF upon lectin or antigen stimulation.

Although these data support the idea that LAF is mitogenic for T

cells because it promotes the production of the T-cell-specific mitogen, TCGF, direct evidence in support of this concept was lacking. Additionally, since T-cell-enriched populations were utilized in the aforementioned experiments, it was impossible to rule out the possibility that the LAF effect was mediated via residual macrophages or intricate interactions among various T-cell subsets. To directly approach the LAF mechanism of action, we examined cloned murine T-lymphoma cells that released TCGF upon lectin stimulation. When these cells were cultured in a lymphokine-free medium, partially purified LAF promoted a LAF concentration-dependent increase in TCGF release (Smith *et al.*, 1980c) (Fig. 3). Thus, it appears that LAF is mitogenic for T cells because it facilitates TCGF production. Additional experimental approaches have supported this model. Glucocorticoids, which cause a concentration-dependent inhibition of TCGF production, mediate a parallel suppression of the mitogenic effect of LAF on lectin-initiated T-cell proliferation (Smith *et al.*, 1980b). Additionally, LAF has no mitogenic effect on splenocytes from athymic nude mice, which are incapable of TCGF production (Smith *et al.*,

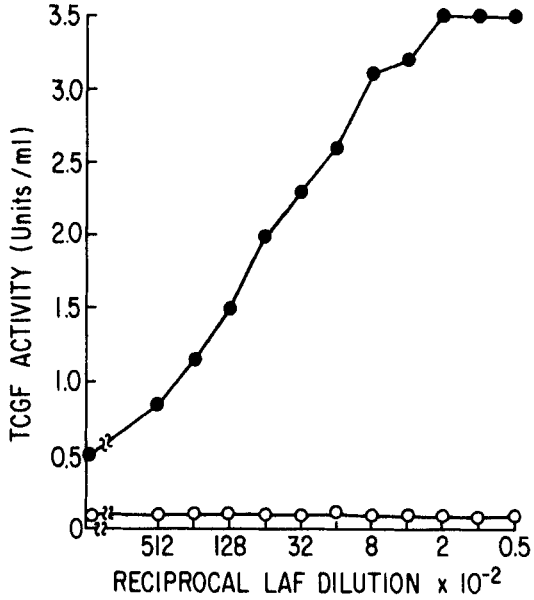

FIG. 3. Lymphocyte-activating factor (LAF)-concentration-dependent production of T-cell growth factor (TCGF) activity from WEHI-7 clone 49 (●), and clone 8 (○). The LAF preparation was partially purified by Sephadex G-75 chromatography followed by isoelectric focusing. The LAF used for the experiment displayed was derived from the isoelectric focusing gradient, pH 6.85. [From Smith *et al.* (1980c).]

1980b). The implications of these results are that LAF serves as an early amplifier of T-cell proliferation by promoting the production of a second, T-cell-derived, soluble factor that actually mediates T-cell proliferation.

III. T-Cell Growth Factor

A. General Considerations

Beginning with the original reports by Gordon and MacLean (1965) and Kasakura and Lowenstein (1965) of the presence of mitogenic factors in mixed leukocyte cultures, many mitogenic factors thought to be of T-cell origin were described. For the most part, only unfractionated supernatants were assayed for biological activity, and the mitogenic factors were named according to the assay used to detect the activities. Thus, there are reports of mitogenic factors designated thymocyte mitogenic factor (TMF), thymocyte-activating factor (TAF), killer helper factor (KHF), thymocyte-stimulating factor (TSF), lymphocyte mitogenic factor (LMF), and others (for review, see Smith *et al.*, 1979a). Because different lymphoid sites from different species (e.g., human, mouse, rat, and guinea pig) were utilized as cellular targets for assays by investigators, it is difficult to ascertain whether an activity described by one group of investigators is similar or divergent from an activity described by others. Additionally, because all the assays employed mixed populations of target cells, it was never clear whether the activity detected was due to the presence of LAF or to a separate mitogenic factor of T-cell origin.

With the discovery that one can select and maintain the continuous proliferation of T cells (Morgan *et al.*, 1976; Ruscetti *et al.*, 1977), it was possible to construct a specific, quantitative assay for the mitogenic factor responsible for T-cell growth (Gillis, *et al.*, 1978b). Thus, for the first time experiments clearly demonstrated the existence of an activity separable from LAF that functioned as a mitogen for T cells (Smith *et al.*, 1979a). As a result of these observations, at the Second International Lymphokine Workshop, which was held at Ermatingen, Switzerland in May, 1979, a group of investigators unofficially agreed to use common terms for putative macrophage-derived mitogenic factors and lymphocyte-derived mitogenic factors. The aims of this group of scientists were to list the known criteria of these two groups of factors so that investigators could establish to which group a particular activity belonged, and to provide for a common nomenclature to facilitate communication. A term with no meaning in the

English language was purposely chosen so that there would be no confusion with existing terms. Interleukin 1 was chosen for macrophage-derived mitogenic factors, and interleukin 2 was chosen for putative lymphocyte-derived mitogenic factors. It should be understood that the terms interleukin 1 and interleukin 2 designate many possible hormone-like factors. These terms should not be misconstrued as names for a particular entity, but rather as classes of biologically active molecules, a viewpoint stressed by Watson and Mochizuki (1980).

Because it can be ascribed to a single protein on the basis of size and charge, and because it can be defined by a specific activity (i.e., the maintenance of continuous T-cell proliferation), TCGF appears to be the first entity that deserves a separate designation within the interleukin 2 class. The name T-cell growth factor necessarily implies both the cellular specificity and the only known function for this moiety. It is hoped that future studies will delineate other "factors" as single proteins with similar target cell specificities, so that we may continue to dissect the interleukins and elucidate their functional importance for the immune response. In retrospect it is impossible to decide whether the biological properties of mitogenic factors described prior to the development of the TCGF assay were due to TCGF, or to other T-cell and macrophage-derived mitogenic factors, since reported biochemical characteristics of the activities are either nonexistent or too scanty to make meaningful comparisons. For these reasons, this review will concentrate on the biological and biochemical characteristics of the activity that is readily detectable in the TCGF assay and is responsible for the maintenance of continuous T-cell growth.

B. The TCGF Assay

The TCGF assay is simply a lymphocyte proliferation assay whereby the TCGF-dependent proliferation of cloned cytolytic T cells is monitored by [^{3}H]Tdr incorporation. There are three important characteristics of this assay that distinguish it from traditional assays of lectin- or antigen-initiated lymphocyte proliferation: (*a*) the target cells are cloned cytolytic T cells; (*b*) [^{3}H]Tdr incorporation is quantified after only 24 hours of culture; and (*c*) the sample to be assayed for TCGF activity is added to the culture in $\log_2$ dilutions.

The use of cloned cytolytic T-lymphocyte lines (CTLL) as the target cells for the assay is an absolute requirement for the unambiguous interpretation of results. Detailed studies have shown that TCGF is the sole proliferative stimulus for CTLL: TCGF-dependent CTLL do not proliferate in response to lectin, antigen, or LAF in the absence of other cells; TCGF purified by molecular gel chromatography and

isoelectric focusing, which migrates as a single molecular entity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), mediates the proliferation of TCGF-dependent CTLL (Meir and Gallo, 1980; Smith *et al.*, 1980a; Robb and Smith, 1981). The addition of lectin or LAF to highly purified TCGF does not enhance its effect. Therefore, when TCGF-containing conditioned media are assayed using these target cells, one can be confident that any proliferative effect that occurs is due to TCGF. This is not true if other lymphoid cell populations are utilized as target cells, since the presence of antigen, lectin, and/or LAF in the conditioned media may stimulate the *in situ* production of TCGF from potential TCGF-producer cells present in the target cell population.

Since the target cells are harvested from TCGF-dependent growth prior to assay, all the cells are in the replicative cell cycle. Thus, when CTLL are placed into culture with limiting concentrations of TCGF, one measures their *continued* TCGF-dependent proliferation. In the absence of TCGF, cells cease to proliferate and lyse within 12–18 hours. If the [^{3}H]Tdr pulse is delayed until after 20 hours, cells cultured in the absence of TCGF incorporate minimal amounts of [^{3}H]Tdr (usually less than 100 cpm), whereas, if TCGF is present in excess, the cells proliferate over 24 hours and incorporate large amounts of [^{3}H]Tdr (usually 15,000–20,000 cpm). The obvious advantages of such an assay are that only 1 day is required for completion, and there is a marked difference between maximum and minimum TCGF effect. In contrast to murine CTLL, murine helper T-cell clones (Schreier *et al.*, 1980) and human T-cell lines (Ruscetti *et al.*, 1980) still incorporate appreciable amounts of [^{3}H]Tdr after 24 hours of culture in the absence of TCGF, and the assay must be extended for 48–72 hours.

The magnitude of continued CTLL proliferation is dependent upon the concentration of TCGF present in the assay sample. Thus, the dose response curve for TCGF-stimulated CTLL [^{3}H]Tdr incorporation is symmetrically sigmoid when the response is plotted against the logarithm of the TCGF dose (Smith, 1980b). Because the [^{3}H]Tdr incorporation of CTLL is dependent upon the concentration of TCGF, it is possible to compare one sample with another. A representative assay is depicted in Fig. 4A. It is obvious from this plot that the sample depicted by the open symbols contains more TCGF activity than the sample depicted by the filled symbols: the former could be diluted 1 : 512 before losing activity whereas the latter lost activity at a dilution of 1 : 64. The sigmoid shape of the dose-response curve indicates a Poisson distribution pattern, and thus the data are best quantified

using probit analysis, which is commonly employed to quantify interferon (Jordon, 1972). The transformation of the data for probit analysis is accomplished by assigning a value of 100% to the counts per minute of [^{3}H]Tdr incorporated at the highest dose of TCGF. All other data points are then calculated as a percentage of the maximum. Data points that fall within two standard deviations about the mean (50% point) then fall upon a straight line. The data from the two TCGF samples depicted in Fig. 4A have been plotted by probit analysis as shown in Fig. 4B. The TCGF activity is quantified by noting the *X* axis intercept where the *Y* axis is at 50% of the maximum. Thus, the *titer* of TCGF in the sample depicted by the open symbols is 1 : 93, whereas for the other sample the titer is 1 : 16. Thus the TCGF concentration is

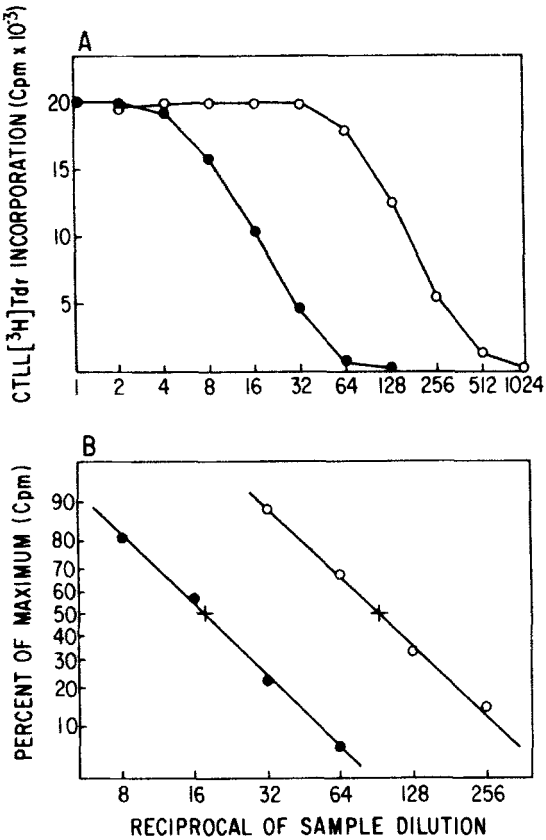

FIG. 4. T-cell growth factor (TCGF) assay. (A) Cytolytic T-lymphocyte lines (CTLL) [^{3}H]Tdr incorporation as a function of the TCGF sample dilution. (B) Probit analysis. [From Smith (1980b).]

5.6-fold greater in one sample compared to the other. For ease of discussion one can arbitrarily define a unit of TCGF activity. In our laboratory we have defined a unit as the quantity of TCGF that has a 50% titer of 1 : 10. To standardize the results derived from successive assays, it is convenient to employ a laboratory standard that contains 1 unit of TCGF activity per milliliter. Thus, when unknown samples are quantified by comparison to the standard, any variations from one assay to another will be canceled. In practice, we have found the assay to be remarkably reproducible: the reciprocal titer of our laboratory standard in 120 successive assays was 10 ± 0.5 (mean ± SEM).

If performed as described, the assay is specific for TCGF, rapid, quantitative, and reproducible. Because the assay requires very few CTLL cells (4×10^3 cells/well), it is possible to assay several hundred samples on a daily basis with relative ease. The quantitative aspects of the assay cannot receive enough emphasis; the lack of quantitative assays for other lymphokines has greatly impeded progress directed toward the definition of the biological and biochemical characteristics of the active moieties under study.

C. TCGF Production: The Cellular Origin of TCGF

Mature T cells are required for TCGF production: although freshly isolated cells do not appear to contain intracellular TCGF, nor do they release TCGF spontaneously, various T-cell stimuli including lectins (e.g., PHA, Con A, and pokeweed mitogen) and antigens (alloantigens, tumor antigens, soluble proteins) induce the release of TCGF, provided mature thymic-derived cells are present. Thymocytes release only 1–2% of TCGF as compared to splenocytes or cortisol-resistant thymocytes, and lymphoid cells from athymic nude mice do not release detectable TCGF activity upon lectin stimulation (Gillis *et al.*, 1978b, 1979).

Through the use of anti-Lyt sera, it has been shown that the phenotype of the murine T-cell required for TCGF production is $Lyt1^+2^-3^-$: lysis of splenocytes with anti-$Lyt1^+$ sera completely abrogates TCGF production whereas lysis with anti-$Lyt2^+$ sera has no effect (Wagner and Rollinghoff, 1978). These data suggest that the classic T-helper cell, which functions to promote the generation of antibody-forming cells, is the source of TCGF activity. Perhaps the most definitive demonstration of this concept are the results of Schreier *et al.*, (1980), who have shown that cloned, erythrocyte antigen-specific T-helper cell lines release TCGF in an antigen-specific and H-2 restricted fashion. Because antigen-specific TCGF release could only be induced in the presence of I-A compatible adherent cells, it cannot yet

be concluded unequivocally that normal Lyt1$^+$ T cells are the source of TCGF. It also remains to be determined whether all Lyt1$^+$ T cells are capable of cooperating with adherent cells to promote TCGF elaboration or whether this is a function of only a fraction of Lyt1$^+$ cells. It is established, however, that Lyt1$^-$2$^+$3$^+$ cells do not release TCGF by protocols employing histocompatible adherent cells and either specific antigen or lectin (Schreier *et al.*, 1980).

In contrast to the ambiguity of the normal cellular source of TCGF, studies with malignant cell lines strongly suggest a T-cell origin. As detailed in the section on LAF activity, cloned murine lymphoma cells that express T-cell markers release TCGF and respond to LAF by enhanced TCGF production in the same manner as normal, T-cell-enriched populations (Smith *et al.*, 1980c). Others have found that murine T-lymphoma cell lines can be induced to release TCGF (Shimizu *et al.*, 1980; J. J. Farrar *et al.*, 1980; Gillis *et al.*, 1980a), while others (Schrader *et al.*, 1980; Harwell *et al.*, 1980; Pfizenmaier *et al.*, 1980) have reported that T-cell hybrid cell lines release TCGF upon lectin stimulation. In contrast to these positive findings with T-cell lines, cell lines with B-cell or macrophage characteristics have not been found to release TCGF activity (Gillis *et al.*, 1980a).

Three signals are involved in the elicitation of TCGF release from TCGF producer cells: lectin or antigen cell membrane binding, *Ir* gene products, and LAF (Smith *et al.*, 1980b; Smith, 1980a; Schreier *et al.*, 1980). The relative importance of these signals and their sequence of application to the T cell have yet to be delineated. Future experiments, which employ cloned T cells, defined culture medium, and purified antigens and lymphokines, will doubtless provide the answers. These experiments will be important, not only because they will provide for our understanding of the process of T-cell activation, but also because such an understanding will yield insight into possible defects of TCGF production that may have clinical relevance. Similarly, studies of the molecular mechanisms of TCGF production are necessary to understand the limits of TCGF-driven T-cell clonal expansion. Results to date suggest that after activation of both murine (Wagner and Rollinghoff, 1978) and human (Ruscetti *et al.*, 1980) T cells, transcription and translation precede the appearance of TCGF in the culture medium. The kinetics of TCGF appearance are consistent with these data: TCGF first appears in measurable quantities within 4–6 hours after stimulation, and peak levels are attained after 12–24 hours (Gillis *et al.*, 1978b). By analogy to other polypeptide hormone systems, one might anticipate any one of a number of sequences involved in the synthesis and release of TCGF.

D. Biochemical Characteristics of TCGF

The biochemical characteristics of human, rat, and mouse TCGF have been reported. Of the three, TCGF of human origin has been the most extensively characterized and therefore will serve as the focus for discussion. TCGF derived from lectin and alloantigen-stimulated peripheral blood mononuclear cells and tonsilar cells elutes from molecular gel chromatography in a position corresponding to that of a globular protein of 15,000–20,000 molecular weight (Gillis *et al.*, 1980b; Meir and Gallo, 1980; Robb and Smith, 1981). When active material isolated by gel filtration was subjected to isoelectric focusing, initial reports suggested that the active material was heterogeneous with respect to charge. Meir and Gallo (1980) found that the activity focused diffusely between pH 5.0 and 7.5 with the peak at pH 6.8 when a gradient of pH 3.0 to 10.0 was applied to a 5 to 60% glycerol density gradient as support. Similarly, we found the activity to focus diffusely with the peak activity centered at pH 6.0–6.5 using a linear pH gradient ranging from pH 3.0 to 10 and a flat-bed apparatus with Sephadex G-75 as support (Gillis *et al.*, 1980b). Subsequently, we found that TCGF derived from human tonsils focused in three major discrete peaks when applied to polyacrylamide gels and subjected to a narrower pH gradient (Fig. 5A) (Robb and Smith, 1981). The approximate p*I* values obtained from several experiments were 8.2 (peak a), 7.9 (peak b), and 7.6 (peak c). Similar results were obtained with a 10-fold greater sample size using a flat-bed apparatus and Sephadex G-75 as support; in both cases total recovery ranged from 40% to 70%. Essentially, the same profile was obtained with material focused in gels containing 4 *M* or 6 *M* urea, which suggested that the peaks did not arise as a consequence of noncovalent interactions between TCGF and contaminating molecules.

Glycosylation with a variable sialic acid content was one explanation that could account for the heterogeneity of the material. To test this possibility, tonsil-derived TCGF isolated by gel filtration was incubated with and without neuraminidase prior to IEF. As depicted in Fig. 5B, the three peaks of TCGF activity present in the untreated sample were replaced by a single peak after neuraminidase treatment. This peak consistently corresponded to the position of the high p*I* (8.2), peak a, of the untreated sample. Assays of TCGF following enzyme treatment revealed no detectable loss of activity, thus suggesting that the material had actually shifted to a single, higher p*I* value after removal of negatively charged *N*-acetylneuraminic acid. This was confirmed by isolation of peaks b and c, treatment with neuraminidase, and reelectrophoresis (Robb and Smith, 1981).

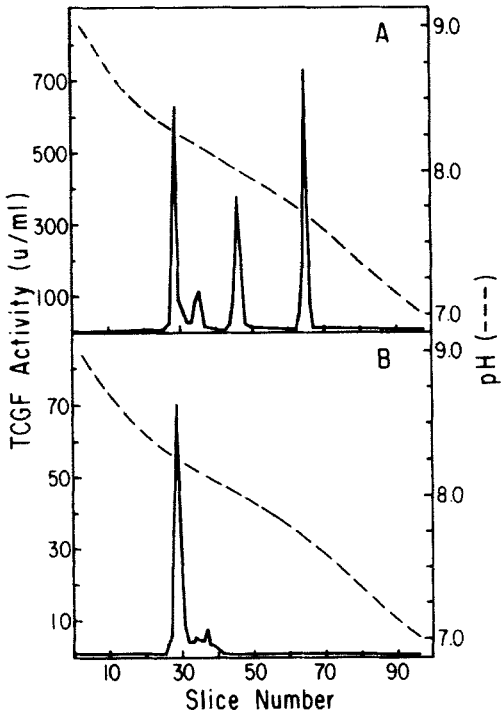

FIG. 5. T-cell growth factor (TCGF) activity isolated from isoelectric focusing gels (Pharmolyte pH 6.5–9.0). (A) Sephadex G-100 pool of human tonsil-derived TCGF (4000 units of TCGF loaded). (B) Sephadex G-100 pool of human tonsil-derived TCGF after incubation with neuraminidase (120 units of TCGF loaded). [From Robb and Smith (1981).]

The heterogeneity of TCGF derived from normal tonsil cells was also demonstrable on SDS-PAGE. As shown in Fig. 6A, TCGF activity isolated by gel chromatography separated into two peaks after SDS-PAGE with nominal molecular weights of 16,000 and 14,500. Essentially the same results were obtained with and without 2-mercaptoethanol in the sample buffer. This apparent heterogeneity in molecular size also appeared to be related to variable glycosylation and sialylation, since incubation with glycosidases and neuraminidase converted all the activity to a single peak (Fig. 6B).

In contrast to the heterogeneity of TCGF derived from normal human cells on IEF and SDS-PAGE, TCGF derived from a human T-leukemia cell line (JURKAT) (Kaplan *et al.*, 1976) focused as a single discrete peak corresponding to the p*I* of the asialo-form of tonsil-derived TCGF (p*I* 8.2) (Robb and Smith, 1981). Also, on SDS-PAGE

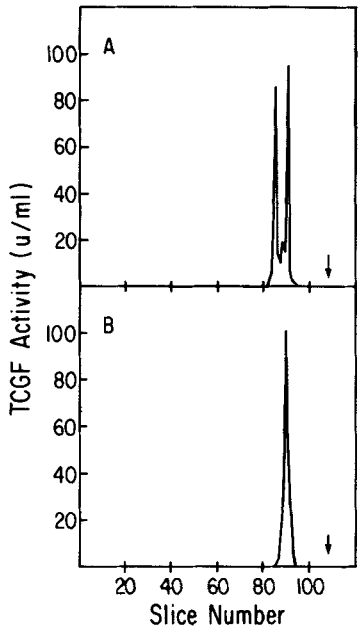

FIG. 6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human tonsil-derived TCGF. (A) Sephadex G-100 pool. (B) Sephadex G-100 pool after incubation with a mixture of glycosidases and neuraminidase (*Diplococcus pneumoniae*). The arrows indicate the position of the tracking dye. [From Robb and Smith (1981).]

the JURKAT-derived TCGF migrated as a single protein with a nominal molecular size corresponding to asialo tonsil-derived TCGF (M_r 14,600). Treatment with neuraminidase and glycosidases did not alter its apparent p*I* or size. The results obtained with the tonsil and JURKAT-derived material are consistent with a single but variably glycosylated and sialylated protein entity as the moiety responsible for TCGF activity. While minor differences in size or amino acid sequence, particularly in neutral residues, could have gone undetected, these results are the first indication that a single protein is responsible for T-cell growth.

The observation that TCGF derived from normal cells exists in both sialylated and asialo forms may be of physiological and pharmacological importance. The rapid removal of circulating desialylated glycoproteins is well documented (Neufeld and Ashwell, 1979), and is mediated by a carbohydrate recognition system present only in hepatocytes. The initial step in clearance involves specific binding to a plasma membrane receptor that recognizes glycoproteins having terminal

galactose or glucose residues on their oligosaccharide chains. Since desialylated TCGF bound to *Ricinus communis* agglutinin I affinity columns and could be specifically eluted with galactose (Robb and Smith, 1981), it appears that desialylated TCGF could be susceptible to rapid, physiological, hepatic clearance. Thus, like erythropoietin, one might anticipate that desialylated TCGF might have *in vitro* biological activity but no *in vivo* biological activity (Goldwasser *et al.*, 1974). From the potential immunotherapeutic standpoint, the removal of *all* of the carbohydrate component may improve the *in vivo* half-life of the molecule (as has been demonstrated for interferon) (Bose and Hickman, 1977), and thus prolong biological activity, since the carbohydrate component does not appear to be required for the mediation of T-cell proliferation. In this regard, JURKAT-derived TCGF, which can be produced in large quantities and is homogeneous in size and charge, may be especially useful. Additionally, looking toward the future, TCGF derived from recombinant DNA techniques (which would be nonglycosylated), should be active *in vivo* if the carbohydrate component only functions to provide for a hepatic clearance mechanism rather than a T-cell growth effect.

Rat- and murine-derived TCGF, although not as extensively characterized as the human material, demonstrate some parallels but also some differences from human TCGF. Rat-derived TCGF elutes from gel filtration with a nominal molecular size of 15,000–20,000 M_r whereas murine TCGF elutes in a position corresponding to a globular protein of 30,000–35,000 M_r (Watson *et al.*, 1979; Gillis *et al.*, 1980b). On ion exchange chromatography, rat and murine TCGF, like human TCGF, appear heterogeneous: rat-derived material elutes in a fashion similar to human TCGF with 0.05 *M* phosphate buffer, whereas murine TCGF requires 0.1–0.2 *M* salt for elution, and both elute in a relatively broad peak. Additionally, we found murine TCGF to be heterodisperse on isoelectric focusing with two peaks of activity centered at pH 4.3 and 4.9, while rat-derived TCGF focused in one diffuse peak centered at pH 5.5 utilizing a broad linear pH gradient (Watson *et al.*, 1979; Gillis *et al.*, 1980b). Using a narrower pH gradient, however, McKenzie and Culp (1981) found rat-derived TCGF to focus in two distinct peaks at pH 5.7 and 6.1. Thus it appears that, like human TCGF, murine- and rat-derived TCGF may be variably sialylated, which could account for the multiple peaks of activity that focus in the acidic pH range.

Physicochemical characterization of human TCGF has revealed that treatment with DNase or RNase had a negligible effect on activity, whereas trypsin abolished all activity (Meir and Gallo, 1980). Since

human LAF is insensitive to trypsin degradation, this may be one criterion to identify LAF in conditioned media that may also contain TCGF. Additionally, human LAF focuses at a considerably lower pH (6.5–7.0) than does human TCGF even though both activities coelute on gel filtration. Treatment of human TCGF with the serine-specific protease inhibitor phenylmethylsulfonyl fluoride or with sulfhydryl alkylator *N*-ethylmaleimide had no effect on the biological activity, suggesting that the mechanism of TCGF action does not involve proteolysis or a strategically located cysteine (Meir and Gallo, 1980).

E. Functional Characteristics of TCGF

TCGF functions to provide the stimulus necessary for T-cell proliferation, while an initial signal supplied by lectin/antigen-cell membrane binding is obligatory for the activation of T cells to a TCGF-responsive state. As is the case for the activation of TCGF-producer cells, MHC gene influences and LAF may also be involved in the activation of TCGF-responder cells; however, definitive data in support of their involvement in the activation process has not yet been presented. It is clear, on the other hand, that, once the activation signals have been received, lectin/antigen and LAF are no longer necessary, and TCGF responsive cells will proliferate indefinitely solely in the presence of asialo TCGF that migrates as a single moiety after SDS-PAGE (Robb and Smith, 1981). Since this TCGF preparation was devoid of detectable LAF and lectin, and since the addition of LAF or lectin do not enhance its growth-promoting effect, these data are consistent with the concept that TCGF provides the mitogenic stimulus to the cell rather than antigen or lectin. It follows that antigens or lectins are mitogenic for T cells because they cause T-cells both to release and to become responsive to the T-cell-specific mitogen, TCGF.

The acquisition of the TCGF-responsive state appears to be mediated by the appearance of TCGF-specific membrane binding sites on appropriately activated cells (Smith *et al.*, 1979b; Coutinho *et al.*, 1979; Bonnard *et al.*, 1979). Freshly isolated T cells will neither bind nor proliferate in response to TCGF, whereas T cells that have had prior exposure to antigen or lectin will absorb TCGF and proliferate, apparently indefinitely, as long as TCGF is present. Because activated T cells absorb TCGF in a time-, temperature-, and cell concentration-dependent manner, the depletion of TCGF by proliferating T cells accounts for the finite nature of short-term, lectin/antigen-initiated T-cell proliferation, and for the apparent infinite proliferative capacity of T-cell lines when they are supplemented with TCGF. It follows that once T cells become activated, the initial cells and all the subsequent

daughter cells continue to express TCGF-specific responsivity. In fact, this unique feature of T-cells allowed for the creation of continuous long-term T-cell lines.

Responsiveness to TCGF appears to be a functional change that is specific for cells of the T-cell lineage. Small resting B cells, B-cell blasts, fibroblasts, and fibrosarcomas do not absorb TCGF, nor is there any discernible effect of TCGF on these cell populations (Smith *et al.*, 1979b). If a mixed population of cells are placed into culture in the presence of antigen/lectin and TCGF, only T-cell growth results (Morgan *et al.*, 1976; Ruscetti *et al.*, 1977). Thus, the appearance of TCGF binding sites on T cells, but not on cells of other lineages, determines the mitogenic specificity of T-cell lectins such as Con A or PHA: although B cells and macrophages bind these lectins, only T cells proliferate, since only T cells become responsive to TCGF.

Cells responsive to TCGF have been found among both mature thymic-derived T-cell and immature T-cell populations. These observations have necessitated a redefinition of the concepts of T-cell maturity and immunocompetence. For example, Wagner *et al.* (1979) showed that the majority of cortical, immature thymocytes, as detected by $Lyt1^+2^+3^+$ surface antigens and receptors for peanut agglutinin, proliferated in response to TCGF and differentiated into both alloantigen-specific and H-2 restricted cytolytic effector cells. Additionally, we have found that lymphoid cells from athymic nude mice proliferate in response to TCGF in the presence of lectin and are also capable of differentiating into alloantigen-specific cytolytic T cells provided TCGF is supplied exogenously along with alloantigen (Gillis *et al.*, 1979). Although athymic mice appear to have fewer precursor cells than do normal mice (Hönig and Bevan, 1980), these results suggest that, although the thymus appears to be required for the maturation of potential TCGF producer cells, TCGF-responder precursor cells may mature independently of the thymus. Formally, the definitions of maturity and immunocompetence relied upon the demonstration, either *in vitro* or *in vivo*, of a proliferative response to antigen followed by differentiation to a measurable effector function. Thus, because antigen-initiated proliferation is obligatory prior to immunocyte differentiation, cell populations may have been defined as immunoincompetent and immature, not because they necessarily lacked the capability of a proliferative response, but rather because they could have lacked the components required to elaborate TCGF, the actual mediator of the proliferative response (Smith, 1981).

T cells from all three functional T-cell subsets (i.e., cytolytic, helper, and suppressor) have now been shown to be capable of proliferating in

response to TCGF (Baker *et al.*, 1979; Schreier *et al.*, 1980; Fresno *et al.*, 1980; MacDonald *et al.*, 1980). Of these three classes, only helper T cells have been found to both produce and respond to TCGF (Schreier *et al.*, 1980). Since TCGF is obligatory for the clonal expansion of functional T cells, the T-helper cell necessarily assumes a pivotal role in the regulation of the immune response as the only cell capable of releasing TCGF. One might anticipate, therefore, that regulatory influences that either suppress or enhance immune responses, may focus on the functional capabilities of the T-helper cell.

One final point that deserves emphasis with regard to TCGF function is the observation that T-cell proliferation is TCGF concentration-dependent. This fact should already be evident, since the concentration-dependent stimulation of CTLL proliferation is the basis for quantitation in the TCGF assay. From the practical standpoint this means that the extent of T-cell clonal expansion, and thus the saturation density of TCGF-dependent T-cell lines, is dictated by the concentration of TCGF present in the culture medium (Smith, 1980b). From the physiological standpoint, one may anticipate that the local and systemic concentration of TCGF will ultimately determine the magnitude of both the T-cell and B-cell immune reponse.

IV. The Initiation, Clonal Derivation, and Maintenance of Functional T-Cell Lines

A. General Considerations

The initial strategy used by ourselves (Gillis and Smith, 1977; Gillis *et al.*, 1978a; Baker *et al.*, 1979) and others (Nabholz *et al.*, 1978; Rosenberg *et al.*, 1978; Von Boehmer *et al.*, 1979) to create functional cytolytic T-cell lines was to select for antigen-specific cells by repetitive *in vivo* sensitization followed by repetitive *in vitro* exposure to specific antigen. By such a process, the *in vivo* sensitizations expand antigen-specific clones, which are then selected for *in vitro*. By virtue of the fact that antigen nonreactive cells eventually die after a secondary or tertiary *in vitro* sensitization, one can selectively enrich for antigen-specific cells prior to initiation of long-term culture with TCGF-containing conditioned media.

As one might expect, with no prior selection for cytolytic cells, the antigen-reactive cells harvested after the *in vitro* selection period are functionally heterogeneous, as so nicely demonstrated by Glasebrook and Fitch (1980): one can detect cells with T-helper characteristics (i.e., TCGF-producer cells that release TCGF upon stimulation with specific antigen and histocompatible adherent cells) as well as cyto-

lytic T cells. The relative frequency of functional T-cell subsets after short-term *in vitro* sensitization has yet to be determined; therefore, it is probable that selection of cells for a particular function prior to antigen-specific selection may skew the functional expression of the cells toward the desired subset.

After the cells are placed into continuous culture with TCGF, it is important to determine the saturation density of the cells. As discussed previously, this will depend upon the concentration of TCGF added to the cultures. We have found that a concentration of 1 unit/ml (i.e., a 50% TCGF titer of 1 : 10) will support the growth of murine T cells to a saturation density of 2 to 5 × 10^5 cells/ml. If the cells are allowed to deplete the medium of TCGF, irreversible damage occurs rapidly and the cells will die (Gillis *et al.*, 1978b; Kurnick *et al.*, 1979). A representative experiment depicting the TCGF-dependent growth and consumption of TCGF by CTLL is shown in Fig. 7. As the cell concentration increased over the culture period, the TCGF concentration decreased. Replacement of TCGF after saturation density occurred on day 7 resulted in a further increase in cell concentration; however, TCGF was then depleted rapidly, resulting in cell death by day 9.

We and others (Engers *et al.*, 1980) have experienced a most troublesome phenomenon after long-term culture is initiated if conditioned media that contain lectin as well as TCGF are utilized. After 3–4 weeks of culture, the cells may become refractory to the growth-stimulating properties of the media, the doubling time may lengthen, and often all the cells may die. There are several possible reasons for this phenomenon, some of which may be physiological, and others may be *in vitro* artifacts. It is clear that the presence of lectin in the con-

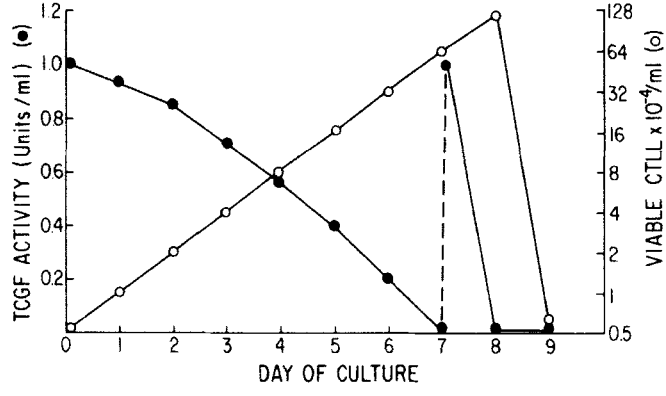

FIG. 7. T-cell growth factor (TCGF) activity present in cultures of cytolytic T-lymphocyte lines (CTLL) as a function of cell concentration. [From Smith (1980b).]

ditioned media may become toxic to the cells. We found that PHA, Con A, and wheat germ agglutinin all exerted a concentration-dependent inhibition of TCGF activity with one-half maximal suppression occurring at approximately 1 μg/ml. In contrast, pokeweed mitogen caused only a 20% inhibition at the highest concentration tested (a 1 : 10 dilution). In addition to lectin, there are other known inhibitors of T-cell proliferation present in TCGF-containing conditioned media (e.g., interferon and E prostaglandins). Therefore, by the use of crude conditioned media, the cells are necessarily exposed to growth-promoting substances and growth-inhibiting substances, and it is quite possible that eventually the negative influences overcome the positive influences.

Another possibility that has yet to be explored fully is the loss of critical cells upon passage, which may be initially present in limiting numbers. Likely candidates are macrophages, TCGF-producer cells, and possibly dendritic cells. Macrophages most probably play a dual role during prolonged culture: the removal of inhibitory substances, and the contribution of positive signals (e.g., LAF and Ia antigens). Empirically it has been determined that if T cells stop proliferating, the addition of irradiated adherent cells to the cultures will restore the growth rate of the T cells. Obviously, additional studies are necessary, utilizing cloned T-cells, defined lymphokine-free culture medium, and pure lymphokines to determine optimum growth conditions.

It is equally possible that it may be physiological for the cells to become unresponsive to the growth factor after a predetermined number of replicative cycles. "Down regulation" of TCGF receptors may occur due to continued exposure to TCGF or other polypeptide hormones, and thus the loss of TCGF receptors may be a physiological control mechanism favoring the return to the resting state after several weeks of culture. It has also been observed that cell lines often have chromosomal abnormalities after several months of culture (Engers *et al.*, 1980). This has led some workers to speculate that T-cell lines cultured for long periods are highly selected, abnormal cells, and are not really reflective of their original progenitors. Despite these phenomena, it is clear that under the proper conditions T cells from all three functionally distinct subsets can be cultured for extended periods and clonally derived without losing their antigen-specific function present at the initiation of continuous TCGF-dependent cell growth.

B. The Clonal Derivation of T-Cell Lines

It is obvious that cells must be truly monoclonal, or the asexual progeny of a single cell, if studies of the immunological function of

such cells are to be meaningful. Sredni *et al.* (1980, 1981) have pointed out that if one attempts to clone after the initial *in vivo* and *in vitro* sensitization, the plating efficiency is low (1.5×10^{-5}) whereas if one clones after the initiation of TCGF-dependent growth, plating efficiencies approach 100%. Whether the initial low plating efficiency is a reflection of the absolute numbers of antigen-reactive cells present in the population, or whether only small numbers of cells are initially clonable, has yet to be explored.

When clones are derived from soft agar or methyl cellulose it is difficult to determine whether a colony resulted from a single cell unless the cloning procedure is repeated. If clones are derived by limiting dilution in suspension cultures, however, it is possible to predict the probability, based on Poisson statistics, of the occurrence of cofertile wells. At cell concentrations distributed at 0.3 cell/well or below, the probability that more than one cell was distributed to a well is less than 0.05 (Baker *et al.*, 1979). The most convincing characteristic of a monoclonal cell line is the demonstration that the immunological activity and specificity is identical in all subclones. We have found that monoclonal cytolytic cell lines yield remarkably similar functional activities upon subcloning (Baker *et al.*, 1979) (Fig. 8), and Schreier *et al.* (1980), as well as Sredni and Schwartz (1980), have observed similar

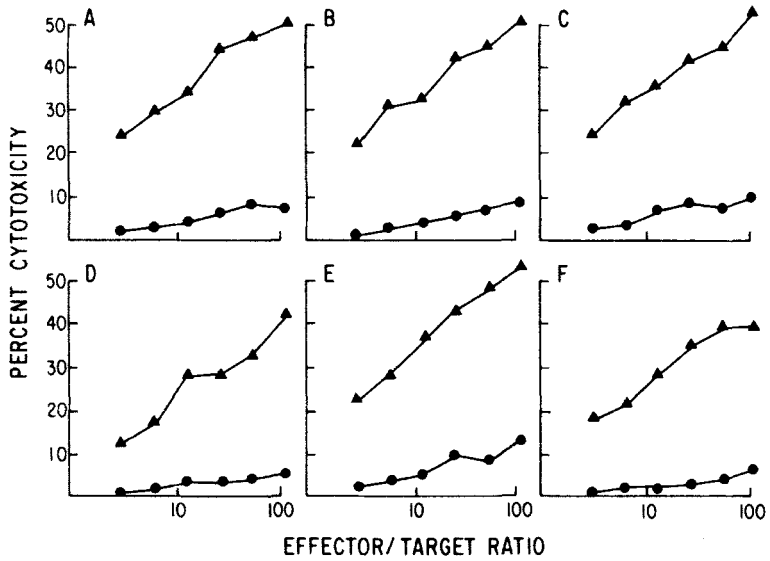

FIG. 8. Cytotoxicity of cytolytic T-lymphocyte lines (CTLL)-2-clone 15 and subclones against FBL-3 [Hn (▲), and P815 (●)] 6 weeks after insolation of the subclones; (A) clone 15; (B) subclone 15-1; (C) subclone 15-2; (D) subclone 15-3; (E) subclone 15-4; (F) subclone 15-5. [From Baker *et al.* (1979).]

exquisite specificity and activity of subclones derived from a single helper T-cell clone.

Alternative methods that favor the argument that a cell line is truly monoclonal can be derived experimentally by examining multiple markers in addition to immunological function. For example, one could derive two different cytolytic T-cell lines directed against different antigens in two strains of mice differing by *H-2* and *Thy-1* alleles. Additionally, if one cytolytic line was derived from a male and the other from a female, chromosome markers would be available. If one then mixed the cell lines prior to cloning, one could examine the clones for homogeneous expression of these multiple markers.

Although we and others have found cytolytic T-cell clones to remain remarkably stable with regard to their cytolytic specificity and efficiency for as long as a year, over prolonged culture periods both of these functions may deteriorate. Schreier *et al.* (1980) have found that the loss of immunological function and specificity may be due to the use of crude conditioned medium as a source of TCGF and to the use of fetal calf serum. When cytolytic T-cell clones were switched from such conditions to growth in serum-free, defined medium and partially purified TCGF, cytolytic specificity and efficiency returned.

C. Cytolytic T-Cell Lines

There has been much interest in the *in vivo* function of cytolytic T-cell lines grown *in vitro*. In addition to the obvious goal of the potential therapeutic utility of tumor antigen-specific cytolytic T cells, the *in vivo* transfer of different functional subsets of T cells may help us to understand the relative importance of each cell type in allograft rejection. As a first step toward such experiments, Lotz *et al.* (1980) have studied the tissue distribution of radiolabeled TCGF-dependent T cells after *in vivo* transfer to mice and humans. They have reported similar results for both species: cells initially appeared to localize within the lungs (4 hours); thereafter, at 24 hours and 48 hours they were found almost exclusively in the liver and spleen. Although these investigators did not determine whether the cells were removed from the circulation and destroyed, their results will serve as a base line for future experiments of a similar nature.

We and others have examined the effect of tumor-specific cytolytic T-cell clones administered therapeutically to tumor-bearing mice. Our initial results are promising, in that cloned tumor specific cytolytic T cells administered 3 days after a lethal intravenous inoculation of Friend leukemia virus-induced leukemia cells resulted in a significant prolongation of survival or the CTLL-treated group (Smith, 1980b). Mills *et al.* (1980) have reported similar results using T-cell popula-

tions that had been generated *in vitro* by means of exogenous TCGF supplementation of mixed tumor–lymphocyte cultures. Obviously, many more experiments are necessary, but the preliminary data suggest that T cells that are cytolytic for tumor cells *in vitro* may function to retard tumor growth *in vivo*.

D. Helper T-Cell Lines

Schreier and co-workers have extensively studied the *in vitro* and *in vivo* function of cloned T-helper cell lines. In addition to the aforementioned capacity to release TCGF, these workers have shown that T-helper cells cooperate with histocompatible adherent cells in the presence of specific antigen to cause the release of factors that enhance the generation of antibody-forming cells, the promotion of erythroid progenitor formation, and both macrophage- and granulocyte-specific colony formation (Schreier and Iscove, 1980). Although the exact cellular origin (i.e., T cell or adherent cell) of each of these factors cannot yet be assigned with certainty, it is tempting to speculate that T-helper cells are the source of this family of hematopoietic-promoting factors and that they are derived from a common precursor.

With regard to the mechanism of T-cell help in the generation of antibody-forming cells, Melchers, in collaboration with Schreier (1981) and others (Melchers *et al.*, 1980), has reported the antigen-specific, H-2 restricted release of factors from cultures of cloned helper T cells and adherent cells that stimulate B cells to replicate and mature to immunoglobulin secretion. They have observed different effects on small resting B cells and B-cell blasts. When added to B-cell blasts, the factors act as B-cell mitogens, stimulating activated B cells to proliferate in the absence of specific antigen and irrespective of the *H*-2 haplotype of the B cells. Small resting B cells on the other hand, are not stimulated to proliferate by the same preparations of factors, even in the presence of specific antigen. They do, however, undergo polyclonal blast transformation and maturation to secretion of IgM and IgG. Although the number of factors present in such supernatants has yet to be defined, it is possible that at least one of these activities is due to macrophage-derived LAF. Wood (1979) and others (Koopman *et al.*, 1978; Hoffman and Watson, 1979) have shown that LAF markedly potentiates the generation of antibody-forming cells from T-depleted normal splenocytes and athymic nude mouse splenocytes. Other than a direct effect of LAF on B cells, the only alternative route for a LAF effect in such circumstances is through the activation of residual T-helper cells. In addition, it is quite possible, as suggested by Mel-

chers *et al.* (1980), that the mitogenic effect of their supernatants for activated B-cell blasts is mediated by a specific B-cell growth factor (BCGF) that operates in a nonspecific and H-2 unrestricted fashion (as does TCGF) to initiate B-cell proliferation. If this were indeed the case, then one would predict that it may be possible to create antigen-specific, BCGF-dependent B-cell lines.

Tees and Schreier (1980) have examined the function of cloned T-helper cell lines after *in vivo* transfer. They have shown that T-helper cells, if injected along with specific antigen into nude mice, persist for as long as 3 months. Of interest is the fact that T-helper cells recovered from such specifically reconstituted nude mice stimulated the generation of even greater numbers of antibody-forming cells than expected in a primary response of normal mice, suggesting that normally active suppressive mechanisms may be absent from nude mice. Additionally, a switch from IgM to IgG secretion was not observed in the clonally reconstituted mice. Thus, this experimental model may be useful for studying the function and regulation of specific helper T cells, especially to discriminate between useful and misleading *in vitro* phenomena.

Clones of T-helper cells have also recently been found to mediate delayed-type hypersensitivity (DTH) when injected into histocompatible hosts along with specific antigen (Bianchi *et al.*, 1981). Of interest was the fact that DTH responses of the same kinetics and magnitude were observed when cloned T-helper cells were inoculated into athymic mice and normal mice, thus indicating that host T cells were not essential for the response and that the cloned T-helper cells were the DTH-effector cells. Histologically, the DTH response mediated by cloned helper T cells in athymic and normal mice was indistinguishable from other Jones–Mote-type DTH responses. Because the nature of the cellular infiltration strongly suggested the release of chemotactic factors, it appears that cloned helper T cells may be responsible for the elicitation of these moieties as well.

Sredni and Schwartz (1980) have reported convincing data suggesting that a single T-helper clone is capable of responding to two separate stimuli, a specific alloantigen and a soluble antigen in conjunction with self-MHC products. Thus, these data support the hypothesis (Janeway *et al.*, 1980) that T cells bear at least two types of antigen receptors—one for allogeneic MHC antigens, and one for nominal antigen. Obviously, further studies of the antigen receptors of T-cell clones at the molecular level should provide the highly sought after information on the nature of the T-cell antigen receptor and the nature of the signals required to activate T cells.

E. Suppressor T-Cell Lines

Data regarding TCGF-dependent suppressor T-cell lines are preliminary, however; Fresno *et al.* (1980) reported that they had generated a sheep erythrocyte-specific suppressor T-cell line. Antigen-activated $Lyt1^{-}2^{+}3^{+}$ cells were selected by fluorescence-activated cell sorting prior to initiation of TCGF-dependent growth. After clonal selection a cell line was identified that exerted antigen-specific suppression of the generation of antibody-forming cells. Through internal cellular radiolabeling procedures, it was found that the cells released a 70,000 M_r polypeptide that dissociated upon proteolysis into two major polypeptides of 45,000 and 24,000 M_r. The 70,000 polypeptide specifically bound to sheep erythrocyte glycophorin and suppressed the effect of antigen-specific T-helper cells when added to cultures in nanogram amounts. These data indicate, therefore, that suppressor T cells, like cytolytic and helper T cells, have the capability of proliferating in response to TCGF and thus should serve as fertile reagents for the elucidation of the molecular mechanism of T-cell suppression.

F. Neoplastic T Cells and TCGF

Once the striking TCGF dependency of functional T-cell lines derived from normal tissues was appreciated, malignant lymphoid cell lines were investigated for evidence of TCGF dependency. We have examined over 50 human leukemia and lymphoma cell lines (15 terminal deoxyribonucleotidyltransferase-positive T-cell, 20 B-cell, 8 non-T–non-B-cell, and 8 non-lymphoid myeloid-macrophage) for constitutive TCGF production and found all to be negative for detectable TCGF release (Ruscetti *et al.*, 1981). Similar results were reported upon testing murine cell lines of lymphocytic and macrophage origin (Gillis *et al.*, 1980a). Furthermore, we have found no evidence for TCGF absorption by established human malignant cell lines using protocols that easily detect TCGF absorption by TCGF-dependent T-cell lines derived from normal tissue. Finally, we have been unable to detect any effects by supplementing malignant T-cell lines with lectin-free, partially purified TCGF. Taken together, these results indicate that TCGF plays no role in the proliferation of established cell lines derived from neoplastic tissue. Rather, these data suggest that the existing cell lines that we have examined were established owing to the selection of cells that either do not require TCGF or depend upon some other growth factor for which we have no means of detection. In contrast to these results, a lymphoid cell line that expresses T-cell markers derived from a lymphoma of a gibbon ape was recently found

to release TCGF spontaneously (Rabin *et al.*, 1918). The TCGF activity released by this cell line supports the growth of human, monkey, and murine TCGF-dependent T-cell lines. Further studies are necessary to ascertain whether this cell line replicates in response to its own growth factor; however, it is of interest that this cell line appears to be unique with regard to TCGF release.

Although the exploration of a possible TCGF-dependency of human T-cell leukemias and lymphomas has just begun, the initial findings are interesting. TCGF-dependent T-cell lines have been established from 12 of 14 patients with cutaneous T-cell lymphoma (CTCL), and 8 of 11 patients selected as having acute lymphocytic leukemia (ALL) of T-cell origin (Poiesz *et al.*, 1980). Several observations point toward a malignant cell origin of these cell lines. All the lines were established using lectin-free, partially purified TCGF that was ineffective in initiating T-cell growth from lymphocytes isolated from normal volunteers in the absence of an activating signal supplied by lectin or antigen. Two cell lines originally dependent upon TCGF became independent of TCGF. These cell lines have been found to be constitutive low-level TCGF producers (Ruscetti *et al.*, 1981). Morphologically the cell lines derived from CTCL patients are markedly different from those derived either from ALL patients or normals. The cultured CTCL cell lines contain many giant multinucleated cells in addition to mono- and binucleated cells. Ultrastructural analysis revealed the presence of highly convoluted nuclei in three of the CTCL lines, which were very similar to the cells of the primary tumors of these patients and are considered to be diagnostic for CTCL. Finally, the TCGF-dependent cells isolated from one CTCL patient had the same chromosomal aberrations that were found in freshly isolated tumor cells from this patient. Obviously, further work is necessary before concluding that TCGF plays any role in the pathogenesis of malignant lymphopoietic diseases, however, this area may provide for new therapeutic approaches to these diseases. Future studies of TCGF-specific binding sites on malignant cells, the search for spontaneous TCGF production by the cells, and the examination of *in vivo* TCGF levels in patients would appear to be worthwhile areas of pursuit.

V. The Functional Implications of TCGF

The ability to maintain antigen-specific functional T cells in continuous proliferative culture has provided the cellular reagents necessary for our initial studies of the hormone-like factors that were respon-

sible for this technological advance. Thus, for the first time we have had the capacity to dissect T-cell immune responses from a functional standpoint. Only by utilizing cloned functional TCGF responsive T-cell lines, defined media, and highly purified TCGF and LAF preparations, was it possible to understand the critical roles played by LAF and TCGF in the T-cell immune response. The key to the significance of the LAF–TCGF amplification system for the ultimate realization of clonally expanded T-effector cells rests with the fact that these soluble factors act in series to provide a hormone-like concentration-dependent expansion of antigen-reactive T cells. The LAF effect on TCGF production, although not obligatory for the release of TCGF, greatly expands its cellular output. TCGF, in contrast, appears to be essential for T-cell mitosis: in situations where TCGF production does not occur (for example as is the case with the athymic mouse), there results a total absence of a competent T-cell immune response. The fact that the effects of both LAF and TCGF are concentration-dependent indicates that minor alterations in LAF production or action could have profound effects on TCGF production. It follows that physiological regulatory control mechanisms would have the most dramatic effects on the immune response if their influences were mediated at the levels of production and action of these essential hormones.

Since it is now possible to dissect the T-cell proliferative response into four functionally distinct components (i.e., LAF production, LAF action, TCGF production, and TCGF action), and since we can now assay each of these functions separately, for the first time we can begin to explore the site and mode of action of physiological, pharmacological, and pathological effects on T-cell proliferation. Our own initial efforts in this regard have centered on the mechanism of action of glucocorticoid hormones, since glucocorticoids are known to suppress profoundly ligand-initiated T-cell proliferation *in vitro* (Nowell, 1961) and are immunosuppressive when administered in pharmacologic doses *in vivo*. Through a series of experiments we found that the inhibitory effect of glucocorticoids on T-cell proliferation was primarily mediated by a glucocorticoid-specific, concentration-dependent suppression of TCGF production. The mechanism whereby glucocorticoids mediate the suppression of TCGF is twofold: an inhibition of LAF production, and an inhibition of LAF activity (Smith, 1980a). Thus glucocorticoids exert a concentration-dependent inhibition of LAF release and simultaneously suppress the TCGF-producer cell response to LAF, resulting in a complete abrogation of TCGF release. From a functional stand point, therefore, glucocorticoids produce the same

defect afflicting the athymic mouse—an inability to respond to antigenic stimuli by the elaboration of TCGF.

Because glucocorticoids are physiological hormones, these data suggest that there may be an intimate functional interaction between the classical endocrine system and the lymphocytotrophic hormones. Thus, our concepts of immunocyte regulation, once thought to be intricately controlled by a self-contained network of immunocyte products, necessarily must now be viewed as part of a broader neuroendocrine–immunocyte interaction. A corollary of this concept is that physiological, pharmacological, and pathological agents may modulate the immune response at any one of several points in the endocrine–lymphocyte hormone system, resulting in either immunosuppression or immunoenhancement. An example is the effect that bacterial products may have in augmenting both macrophage and lymphocyte function. Lipopolysaccharide is one of the most potent agents known to induce LAF release. It is quite possible that the well-known adjuvant effects of LPS may be mediated, at least in part, by LAF-facilitated TCGF release. Since the clonal expansion of both T-helper cells and T-cytolytic cells is TCGF-concentration-dependent, the ultimate effect of LPS would be the LPS-LAF-TCGF-dependent promotion of both the humoral and cellular immune response.

Because LAF and TCGF both appear to interact with their target cells by means of specific membrane binding sites, it can be anticipated that alterations of the active sites of these hormone-like factors, or their receptors, may lead to alterations in immunocyte function. Larsson (1980) has already provided some insight into the mechanism of action of one immunosuppressive drug (cyclosporin A), which appears to impede T-cell proliferation by interfering with the appearance of TCGF receptors on antigen-activated T cells. By analogy to other polypeptide hormone systems, we may expect to discover LAF–TCGF receptor agonists and antagonists that not only may be of therapeutic importance, but also may underlie clinical symptomatology manifested by either hyperimmune reactivity or immunodeficiency. Such examples already exist in the neuroendocrinological field: agonistic autoantibodies to the thyrotropic hormone receptor may underlie the etiology of hyperthyroidism, and antagonistic autoantibodies to the acetylcholine receptor appear to be responsible for myasthenia gravis.

Studies designed to explore the potential therapeutic utility of LAF and TCGF are embryonic at this time; however, recent experiments offer the promise that TCGF replacement therapy may be beneficial for immunodeficiency disease states manifested by defective TCGF

production. Wagner *et al.* (1980) have shown that TCGF administration to athymic nude mice along with alloantigen results in the appearance of alloantigen-specific cytolytic T cells. Similarly, Kindred and Corley (1977) found that treatment of athymic mice with Con A-conditioned media led to the appearance of alloantibody, provided the mice were also immunized with alloantigen. Although it is impossible to ascertain whether these results were due to an effect of TCGF, a report by Stotter *et al.* (1980) suggests that TCGF administration to athymic mice leads to the generation of antigen-specific T-helper cells. Obviously, many more experiments are necessary, and before results can be attributed to a TCGF effect, homogeneous TCGF uncontaminated by other proteins must be administered. At this time, however, it is encouraging that positive results have been obtained in the initial experiments.

VI. Conclusion

The ability to maintain cloned functional T cells in continuous proliferative culture, combined with the ability to assay separately at least two of the hormone-like lymphokines that allowed for this technological advance, has provided us for the first time with the capacity to dissect T-cell immune responses from a functional standpoint. The accumulated experimental data indicate that a T-cell proliferative response to antigen/lectin is actually mediated by a soluble protein termed T-cell growth factor (TCGF). The realization that lectins or antigens are mitogenic for T cells because they cause both the release and the acquisition of responsiveness to TCGF has thus opened many new approaches to the study of the regulation of T cells. Detailed studies have indicated that the production of TCGF is augmented by macrophage-derived lymphocyte-activating factor (LAF). Since the effects of LAF are concentration dependent, and since T-cell clonal expansion is TCGF concentration dependent, the magnitude of the resultant T-cell immune response is necessarily dictated by the available quantities of these lymphokines. The implications of these findings are that minor alterations in the production or action of LAF and TCGF may result in major changes in T-cell proliferation that may have physiological and pathological relevance. Thus, through the use of cloned populations of functional T cells, future studies directed toward the molecular mechanisms of T-cell function and lymphocytotrophic hormone action should assure a more complete understanding of the T-cell immune response.

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Formation of B Lymphocytes in Fetal and Adult Life

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I. Introduction

A considerable amount has been written about development of the potential to mount highly diverse antibody responses (Sigal and Klinman, 1978). This question of the generation of diversity can only grow more interesting in light of recent advances in our understanding of molecular events in antibody gene expression. It is also important, however, to consider the overall biology of cells that utilize these genes, and this review will focus on the problem of B-lymphocyte emergence in embryonic and adult life.

At the earliest time in development and in experimental situations when cells of the humoral immune system can be detected, these are infrequent with respect to surrounding nonlymphoid cells. There is probably also a cascade effect such that the least differentiated members of the series are present in the smallest numbers. In the last few years several limiting dilution (cloning) procedures have been devel-

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oped that considerably facilitate the unambiguous detection of such cells. Procedures will also be discussed that may not only allow the differentiative potential of B-cell precursors to be assessed but provide some insight into the inductive signals and regulatory mechanisms involved. Of equal importance is the utilization of appropriate cell surface markers to enumerate and manipulate early B-lineage cells. Until very recently, it was considered by many that lymphoid and hematopoietic stem cells were "undifferentiated" and thus would lack distinctive markers. It is now clear that these precursors must be highly specialized and antigens recently identified by several laboratories may be useful in resolving categories and stages of differentiation.

Another subject of interest is the apparent functional heterogeneity of B lymphocytes, which suggests the possibility of specialized cell sets. That is, it has been considered likely that particular categories of B cells with similar receptors for antigen may be uniquely able to respond when the antigen is presented in different ways. Our understanding of this question is incomplete, and any conclusions should be considered speculative. However, this is an intriguing aspect of B-cell diversity whose origin might be traced back to very early differentiation steps.

Finally, an important objective of this review will be to identify some areas of agreement about early B-lineage events that are not adequately supported by direct evidence. The extraordinary progress of experimental hematologists in resolving the precursors of erythroid and myeloid cells has created a false sense of understanding about the nature and fate of stem cells relevant to the humoral immune system. An inspection of the literature will reveal a number of timely and critical questions about these processes.

II. Some Definitions

This particular branch of immunology profits from the collective wisdom of individuals schooled in experimental hematology, embryology, immunodeficiency diseases, and molecular biology. Partly because of this, the commonly employed nomenclature is often confusing, if not ambiguous. Terms such as stem cell, differentiation, maturation, induction, precursor, progenitor, pre-B, and B cell all may have quite different meanings to different individuals. It is unlikely that any precision will soon be achieved in this area because very complex and continuously evolving concepts are involved. For the present and for the purposes of this review, such terms will have to be defined to suit the context in which they are used.

Stem cells have been commonly defined as cells with extensive self-renewal capacity, which have multiple differentiation options and are unresponsive to some of the specific stimuli that regulate their more differentiative progeny. This description had practical value in distinguishing multipotential cells in the hematopoietic system from committed progenitor cells of erythroid, myeloid, and other lineages (Metcalf and Moore, 1971; Till and McCulloch, 1980). However, it has been found that under specialized culture conditions, committed granulocyte precursors, T cells, B cells, mast cells, and macrophages proliferate indefinitely (Dexter *et al.*, 1980; Gillis *et al.*, 1978; Melchers *et al.*, 1975a; Tertian *et al.*, 1981; Ralph, 1980). These studies were done with normal, nonmalignant cells, and serially propagated memory B cells have also been shown to have extensive, but finite, self-renewal capacity (Askonas and Williamson, 1972). Some vagueness is also given to the term stem cell by the possibility that certain multipotential cells, capable of giving rise to erythroid, neutrophil, macrophage, eosinophil, and megakaryocytic cells in culture (Johnson and Metcalf, 1977; Metcalf *et al.*, 1979), may not be involved in lymphoid differentiation (see below). For purposes of this review, adjectives will be used to restrict and, it is hoped, clarify the use of this term; i.e., multipotential stem cells, lymphoid stem cells, myeloid stem cells, B stem cells, etc.

In the most restricted sense, the term differentiation refers only to events that occur within the nucleus of a cell to restrict options available to subsequent generations. Holtzer and colleagues (1972) have advanced the theory that rearrangements of chromosome structure occur only during special or "quantal" cell cycles whereby a new genetic program is conferred on daughter cells. Such differentiation steps are now known to take place within the B lineage during the initial selection of antibody-variable region genes and when certain switches in production of Ig isotypes are made (Brack *et al.*, 1978; Seidman and Leder, 1978; Rabbits *et al.*, 1980; and Davis *et al.*, 1980). However, for most of the dramatic changes that occur in cells during development and function of the humoral immune system, it is not clear whether these are true differentiation events or rather the expression of a precommitted genetic program in response to appropriate stimuli. Through habit and convenience, various steps will be referred to in this communication as differentiation events when they might be less ambiguously called maturation. Similarly, an embryologist might use the term induction to describe a critical cellular interaction that has irrevocable consequences for the cells involved. The word will be used here more loosely to refer to the initiation of protein synthesis and/or surface marker expression on cells resulting from external stim-

ulation (e.g., induction of Ig synthesis and display in normal B-cell precursors or established cell lines). It is unlikely that all these changes would be permanent.

Roitt *et al.* (1969) first coined the term "B" lymphocyte for the effector cells of the humoral immune system. It is doubly appropriate because it could represent either bursa of Fabricius or bone marrow-derived cells. In practice and in this review this will be used to refer to cells with readily detectable surface immunoglobulin (sIg) that did not acquire it through passive means. However, it should be cautioned that distinctions between cells that have small amounts of sIg and those that have none are dependent on the assay techniques that are used. Newly formed lymphocytes in marrow acquire increasing amounts of sIg in a process that spans 2 days (Osmond and Nossal, 1974a,b), and it is improbable that absolute numbers of B cells determined by autoradiography, for example, would always precisely agree with estimates made using a fluorescence-activated cell sorter (FACS) with a different antibody preparation. This is one area in which use of monoclonal anti-Ig antibodies (Kincade *et al.*, 1981b) might lead to a new level of standardization. It happens that appearance of sIg corresponds closely with the ability of murine cells to be stimulated by mitogens in semisolid agar cultures (Kincade *et al.*, 1980a), and for convenience the term clonable cell will in some instances be used interchangeably with B cell.

Lafleur and colleagues (1972a,b) introduced the term "pre-B" to describe large cells that required a period of residence in irradiated recipients before being able to mount specific immune responses. Some confusion arises from the fact that another group refers to cells responding under similar circumstances, but with different response kinetics, as "preprogenitors" (Nossal *et al.*, 1977). In both cases the responding cells were described as being sIg^+. Others have taken up the term pre-B to denote cells with detectable cytoplasmic, but not surface, Ig, since there is a variety of circumstantial evidence to suggest that they are the immediate precursors of sIg^+ cells (Cooper, 1981). We refer to any cell capable of giving rise to functional B cells as a precursor. This includes pluripotent stem cells, committed precursor cells whose only option is to generate B cells, and the type of cells that already are synthesizing subunits of Ig. It has not been shown that all cIg^+ cells eventually become B cells, and it is not certain that all B cells derive from cells with those phenotypic characteristics. For this reason, the term pre-B might for now be reserved for use with cells that are demonstrably close to becoming B cells.

One of the more problematic words that it is necessary to use is

"mature." The timely appearance of B cells and their precursors in various tissues of developing mice is documented in the following sections, and it will be shown that those emerging during fetal and neonatal life may differ considerably from those present in adult tissues. These are "immature" in the sense that they may lack cell surface receptors and characteristics that typify adult cells. However, these may have reached the most developed stage necessary for them to function, and there is no information available on their survival and contribution to B-cell populations later in life. In some cases the acquisition of markers by cells of the B lineage seems to relate more to the chronological age of the animal than to the extent of differentiation that has occurred (Kincade *et al.*, 1980b). The task is to distinguish between changes that take place coincident with development of the animal and those that correspond to the progression of B-lineage cells through various compartments. Pains will be taken to avoid equating and directly comparing, for example, cells from neonates, which lack sIgD, with those that are recently formed in adult marrow.

In order to deduce a probable sequence of events in embryonic life, it is often necessary to make measurements at various stages of gestation. Timed pregnancies are commonly estimated from the finding of vaginal plugs after overnight matings. However, there is considerable variability in the degree of development between and even within litters. Some laboratories consider the finding of plugs as day 0 but since matings usually occur at night, others consider this day 0.5, and still others consider this day 1 of gestation. It is obvious that a great deal of confusion can arise, for example, as to the precise stage when stem cells colonize an organ rudiment and when B cells and their precursors emerge. The problem is particularly acute for early embryos where a few hours can make a considerable difference and one suspects there is strain-dependent variability as well. For these reasons somite counts or other independent assessments of developmental stage should be reported (Gruneberg, 1943). It is unfortunate that this information is seldom included in immunology papers.

III. Expression of Immunoglobulin Genes

The most important, best understood, and perhaps most unique function of cells of the immune system is to synthesize immunoglobulin (Warner, 1974). Substantial progress has been recently made in understanding the molecular events associated with Ig gene rearrangement, transcription, and translation. It appears that complex mechanisms have evolved to utilize this genetic information maxi-

mally. Indeed there have been so many new and interesting findings that this subject alone would form the basis for a timely review. However, a summary of only the most salient observations will suffice as a background for the discussion that follows.

Immunoglobulin molecules are constructed from three sets of unlinked genes that encode the heavy chains, κ light chains, and λ light chains (Gottlieb, 1980). Each of these is in turn made from minigenes that are separated from each other in embryonic and somatic cells by a considerable stretch of DNA. This possibility was hypothesized by Dreyer and Bennett in 1965 and demonstrated experimentally by Hozumi and Tonegawa in 1976. It is now known that genes for light chains include those encoding the variable regions, five *J* (joining) segments, and those corresponding to the constant or Fc portions of the molecule. For the heavy chain, the genes include in addition to the variable region genes, the four *J* genes, and the constant region genes an additional set of genes called *D* (for diversity). At some point in the B lineage a critical differentiation event occurs such that V_H-D-J_H- and C_H genes are brought closer together into a transcriptional unit (Early *et al.*, 1979, 1980a; Davis *et al.*, 1980). A similar rearrangement occurs on one of the chromosomes for light chains bringing the V_L-J_L-C_L genes in proximity to each other (Sakano *et al.*, 1979; Brack *et al.*, 1978; Max *et al.*, 1979). Transcription produces a precursor RNA that is processed to remove introns while still within the nucleus (Perry *et al.*, 1980). Analysis of fetal liver hydrid cell lines suggests that the heavy-chain differentiation step occurs first (Maki *et al.*, 1980). This also is consistent with the finding that the earliest Ig-synthesizing cells make only the μ chains of IgM (Burrows *et al.*, 1979). However, the precise stage in embryogenesis when this first occurs, the nature of inductive stimuli that might bring it about, and its relationship to the initial commitment of stem cells to the B lineage are unknown.

Only one of a pair of chromosomes is involved in active synthesis of Ig molecules, and this process has been called allele or haplotype exclusion (Herzenberg *et al.*, 1976). It would seem that this could be explained in terms of selective rearrangement of genes of a single allele with concomitant blockage of a similar event on the homologous chromosome. However, it is now clear that in many cases rearrangements take place on both chromosomes (Perry *et al.*, 1980; Cory *et al.*, 1980; Hurwitz *et al.*, 1980). Many of these rearrangements are totally nonproductive (abortive), and others might result in production of incomplete messenger RNA species that can even be detected in nonlymphoid cells (Kemp *et al.*, 1980).

Complete messenger for Ig heavy chains carries information for two

alternative forms of the protein. One has a specialized hydrophobic tail that makes it an appropriate cell surface receptor molecule, and the other has a hydrophilic Fc portion and is destined to be secreted Ig (Alt *et al.*, 1980; Rogers *et al.*, 1980; Early *et al.*, 1980b; Kehry *et al.*, 1980). RNA processing results in messengers of the two kinds, and the ratio of these changes as a function of the progression of B cells toward plasma cells (Sibley *et al.*, 1980, 1981).

All cells of the B lineage derive from cells that first synthesize IgM (Kincade *et al.*, 1970; Lawton *et al.*, 1972). This conclusion derives from the observation that B-cell emergence can be aborted by treatment with anti-μ antibodies at an early stage of development. Further analysis revealed that there is a critical period just after the initial display of sIgM when recently formed B cells are hypersensitive to contacts with sIg receptors (Raff *et al.*, 1975; Kearney *et al.*, 1978; Burrows *et al.*, 1978; Sidman and Unanue, 1975). The anti-Ig antibodies bind to surface IgM molecules, cause them to be lost or internalized, and unlike the case with adult type B cells or when immature B-cell Ig is enzymically removed, it does not reappear. Cross-linking of sIg molecules is required, and analogies have been drawn between this phenomenon and neonatal type (clonal abortion) tolerance (Raff *et al.*, 1975; Nossal and Pike, 1975; Bruyns *et al.*, 1976; Teale and Mandel, 1980).

The possibility has been considered of a relatively high probability of abortive rearrangements occurring in the immunoglobulin genes of both chromosomes of a differentiating B-cell clone (Perry *et al.*, 1980). It is also conceivable that a given set of light-chain genes that were selected might not encode a protein that would adequately fold with the preselected heavy-chain molecule. Presumably, in either of these cases, the cells would never produce functional Ig and would be short-lived. The daily rate of B-lymphocyte production in bone marrow (and T cells in the thymus) is high (Osmond, 1979), but the fraction of these that survive outside the marrow is not known.

For the B cells that succeed in acquiring sIgM, there are options for further utilization of their Ig genes. A majority of peripheral B cells in adult animals bear both IgM and IgD (Abney *et al.*, 1978), and these molecules have equivalent antigen-binding sites (variable regions) (Fu *et al.*, 1974). The δ constant region gene is located in the DNA adjacent to that for μ, and it has been suggested that B cells that simultaneously produce both isotypes might do so by means of alternate splicing of a single transcript (Liu *et al.*, 1980).

The permanent switching of a given B-cell clone to the synthesis of another Ig isotype may occur according to a mechanism proposed by

Honjo and Kataoka (1978; and Kataoka *et al.*, 1980). The probable gene order in the cluster of heavy-chain constant region genes is μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ε, α in the mouse, and sequential deletions of intervening sequences may occur in expressing all possible Ig classes (Cory *et al.*, 1980; Davis *et al.*, 1980; Rabbits *et al.*, 1980; Hurwitz *et al.*, 1980; Nishida *et al.*, 1981). That is, actively secreting cells producing IgA may be incapable of making IgM, IgG, etc., because these genes were excised during the switching process.

It was long considered possible that individual B cells undergoing immune responses could switch from the production of one isotype to another (Nossal *et al.*, 1964). However, the actual demonstration of intraclonal switches has been relatively recent (Pierce and Klinman, 1975; Gearhart *et al.*, 1975; Andersson *et al.*, 1978; Teale *et al.*, 1981). It has been suggested that some elements of the immune response might induce a particular pathway of Ig class expression. On the other hand, there are examples of Ig isotype diversification that seem to be independent of obvious antigenic stimulation (Lawton and Cooper, 1974). It remains to be seen whether class switches occur in B cells through some stochastic process only to be selected by appropriate conditions or, alternatively, whether this true differentiation event can be directed by particular inductive stimuli.

Many of the conclusions summarized in this section of the review derived from studies facilitated by the use of cloned tumor or hybridoma cell lines. For example, a cell line (70Z/3) that was established in this laboratory is providing some insight into mechanisms of Ig biosynthesis and cell surface display. The heavy- and light-chain (κ) genes are both rearranged in this cell line, and it continuously produces μ chains (Paige *et al.*, 1979; Maki *et al.*, 1980). When lipopolysaccharide (LPS) is present in the cultures during the G_1/S phase of the cell cycle, κ-chain messengers of different sizes are produced and assembled IgM appears on the cell surface (Sakaguchi *et al.*, 1980; Perry and Kelly, 1979). Virtually all of the cells become sIg^+ within 24 hours, but these convert to their original state within 48 hours of removal of the LPS. In contrast, when the mitogen dextran sulfate is added to the cultures instead of LPS, only μ chains appear on the cells and light chains are not detectable within the cytoplasm (Paige *et al.*, 1981b). This induction of surface μ display does not seem to require protein synthesis, whereas expression of complete molecules following LPS stimulation does (Ralph, 1979). Even though active secretion of μ chains or IgM is not detectable with this line, approximately 30% of the intracytoplasmic μ chains are not of the hydrophobic (membrane insertion) form (Sibley *et al.*, 1981).

As interesting as studies of myeloma and B-lymphoma cell lines may be, there are many questions that can only be addressed with normal cells. For example, it seems unlikely that a full understanding of the relative contributions of germ line genes, combinatorial associations between minigenes, and somatic mutations to the overall diversity of antibody specificities could be gained from studies of cell lines alone. The following sections will consider methods now available for enumerating and manipulating B cells and their precursors.

IV. Cloning Assays for Functional B Cells

Much of the progress that has been made in recent years in our understanding of early events in B-lymphocyte differentiation has followed the development of new ways of identifying relatively rare cells. That is, when B cells or their precursors first appear during ontogeny or during regeneration of irradiated animals, they are diluted among nonlymphoid hematopoietic cells. Passing reference has already been made to the use of cytoplasmic immunoglobulin as a marker for pre-B cells, and various cell surface antigens are discussed in detail below. However, assays that require an active, immune-type response are uniquely important for studies of lymphocyte development. It should be stressed that probably none of the available assays provide an unbiased assessment of all functional B-cell populations. This was most dramatically demonstrated in experiments of Howard and co-workers (1979) where the physical characteristics of virgin B cells were compared using two different assays. Practically all of the response in an adoptive transfer system resulted from relatively infrequent large cells, whereas typical small B cells contributed most of the response in cell culture assays. On the other hand, much of B-cell diversity is currently defined in terms of differential responsiveness in various assays, and it is possible that this reflects physiologically important heterogeneity. Immune responses that take place in bulk cultures or after adoptive transfer of large numbers of cells mimic normal immune responses in that only a proportion of potentially reactive B cells participate and their function is highly influenced by regulatory T cells, accessory cells, feedback antibody, and idiotypic networks. Limiting dilution techniques make it possible to overcome these problems and obtain at least relative estimates of the frequencies of particular B-lineage cells. Three of the most commonly used approaches are summarized here.

Perhaps the broadest spectrum of B-lineage cells is detected by the Klinman splenic focus technique (Klinman, 1972; Klinman *et al.*,

1976; Sigal and Klinman, 1978). Mice are usually preimmunized with a carrier protein and then some weeks later used as irradiated recipients of the cells to be assayed. Approximately 5% of the injected B cells are present in the recipient spleen when it is removed 16 hours later. The spleen is then cut into fragments that are placed in individual microcultures with a hapten–carrier conjugate and antibody is subsequently measured in the culture supernatants. The assay depends on suppressor T-cell activity declining more rapidly than helper T cells after priming and the ability of accessory and primed helper cells to function after irradiation. For some time this procedure was used only to measure T-cell-dependent immune responses, but recently it has been shown that T-independent responses can be obtained with particular antigens (Fung and Kohler, 1980). Sedimentation velocity separations indicate that cells of all sizes are detected in this assay and a conspicuous population of large Ia^- cells that may be easily tolerized in adult spleen is detected at longer culture intervals (J. L. Press *et al.*, 1977; Teale *et al.*, 1978a,b). It was estimated that about 80% of the DNP-specific B cells that lodge in the irradiated spleens can be stimulated to antibody secretion with DNP-hemocyanin in the fragment cultures (Klinman *et al.*, 1976). This method also reveals B cells in neonatal spleen, sIg^- cells in adult spleen, and cells that appear in fetal liver by 14 days of gestation (Teale *et al.*, 1979; Teale and Mandel, 1980). All of these presumably immature cell types are distinguishable from the more typical adult B cells by their susceptibility to tolerization and their tendency to form IgM only clones of antibody-secreting cells. It thus appears that some type of B-cell precursors are capable of maturing during the initial period of splenic fragment culture (a lag of 24 hours is usual between the time when cells are injected and the time when antigen is added to the cultures). However, the incidence of functional precursors dramatically increased when the donor fetal tissue was first held in organ culture (Teale and Mandel, 1980). The nature of sIg^- precursors that give rise to antigen-responsive clones under these circumstances requires further characterization.

Lefkovits (1972) first devised assays where B cells are diluted in cultures containing antigen and constant numbers of accessory and filler cells. The incidence of cultures containing antibody-secreting cells is calculated at each dilution, and the Poisson equation is used to estimate the frequency of functional B cells. This basic technique has been adapted by many laboratories to detect primary and memory B cells stimulated with T-cell-independent and T-cell-dependent antigens. In one such study, one out of two NIP-specific purified B cells

produced antibody to NIP-polymerized flagellin (Nossal and Pike, 1976). Mitogens can also be used in this way to estimate the frequency of cells that respond to polyclonal stimulation (Quintans and Lefkovits, 1974; Andersson *et al.*, 1977a). Using thymus filler cells and a reverse plaque assay, Andersson and colleagues (1977a) found that one out of three small splenic B cells could be driven to immunoglobulin secretion with LPS. When an appropriate fetal calf serum is used in the culture medium, and especially when dextran sulfate is added to the cultures, this system also permits the maturation of B-cell precursors (Melchers, 1977a). The method has been used also to assess the diversity of antibody specificities among mitogen-responsive B-cell populations (Andersson *et al.*, 1977b; Eichmann *et al.*, 1977).

Metcalf discovered that addition of 2-mercaptoethanol (2-ME) to culture medium permitted murine B cells to form colonies in semisolid agar cultures (Metcalf *et al.*, 1975). It was later learned that this clonal proliferation was dependent on the presence of mitogens that are native to laboratory grade agar (Kincade *et al.*, 1976). With most hematopoietic or lymphoid cell suspensions and when 10^5 or fewer cells are placed in 1-ml cultures, all but the responding B cells lyse and disappear within a few days. In order to achieve a linear relationship between numbers of cells cultured and numbers of resulting colonies, it is also necessary to add one of several potentiators to the cultures (Metcalf, 1976; Kurland *et al.*, 1977). Sheep red blood cells (SRBC), lipopolysaccharide (LPS), and adherent layers of peritoneal exudate macrophages have been used for this purpose, and the relative advantages of each are discussed in detail elsewhere (Kincade, 1981a). The usual fraction of B cells that form colonies (divide 5 or more times) in the presence of one potentiator is 2–4%, and up to 13% clone in the presence of all three (Kurland *et al.*, 1977). A much larger number of B cells (perhaps as many as 60% in some circumstances) proliferate sufficiently only to form small aggregates (Metcalf, 1977). With LPS-potentiated cultures, the cloning efficiencies of sIg^+ cells in newborn spleen, adult bone marrow, spleen, lymph nodes, and peripheral blood are approximately equivalent (Kincade *et al.*, 1978b). In contrast, if SRBC are used alone in semisolid cultures, certain immature B cells in marrow clone poorly and a conspicuous population of abnormal B cells in autoimmune NZB mice are not detected at all (Lala *et al.*, 1979; Kincade *et al.*, 1979).

The characteristics of cells that can be cloned in semisolid agar are now reasonably well known. In immature tissues such as newborn spleen or liver and spleen of late-gestation embryos, all are $Lyb\text{-}2^+$, IgM^+, Ia^+, and $Qa\text{-}2^+$. They resist pretreatment with anti-Ig antibodies

but, like immature B cells, do not proliferate in the continuous presence of anti-μ antibodies. Subsets of these can be defined on the basis of expression and function of sIgD. That is, there are clonable B cells that lack IgD, and two qualitatively distinct types of $sIgD^+$ cells can be distinguished by their sensitivity to anti-δ antibodies added to the cultures. Clonable cells are as heterogeneous as all other B cells with respect to physical properties, and the only defined population that thus far has been found totally to lack colony-forming cells are in partially immunodeficient CBA/N mice (Kincade *et al.*, 1977, 1978b, 1980a,b; Metcalf *et al.*, 1976).

One particularly useful aspect of the semisolid agar cloning technique is the fact that it distinguishes between sIg^+ B cells and their immediate precursors. Clonable B cells emerge simultaneously with sIg^+ cells in fetal liver and spleen and are diminished in parallel with B cells either by suppressive regimens of anti-μ antibodies administered from birth, or by adherence to anti-Ig coated plastic petri dishes (Johnson *et al.*, 1976; Kincade *et al.*, 1980a, 1981a). Furthermore, addition of anti-μ antibodies to the cultures almost completely prevents colony formation by B cells from normal mice (Kincade and Ralph, 1976; Kincade *et al.*, 1979). While cloning of all $sIgD^+$ cells from marrow is prevented by addition of anti-δ to the cultures, less mature, sIg^- cells from that site are unaffected. This suggests that IgD is probably not acquired efficiently during culture. Maturation of newly formed B cells and their immediate precursors must therefore be minimal once they are dispersed in semisolid agar, and the method can be used to assess numbers of B-lineage cells that have reached this milestone in their development.

It has been suggested that some of the cells within B-cell colonies progress to an Ig-secreting stage (Claesson *et al.*, 1978; Poulson and Claesson, 1980). However, few attempts have been made to optimize and exploit this to study the diversity of antibody specificities. In one study it was found that cells taken from colonies could be specifically triggered by antigen to yield antibody-secreting cells (Pillai and Scott, 1981).

There have been relatively few reports involving the cloning of human B lymphocytes (Radnay *et al.*, 1979; Bobak and Whisler, 1980; Muraguchi *et al.*, 1980a; Kuritani *et al.*, 1980). A principal difficulty has been in obtaining T-independent mitogens that would efficiently stimulate human B cells in dispersed conditions without having first to activate them in liquid culture. However, it is already apparent that cloning procedures that have diagnostic utility will soon be developed (Muraguchi *et al.*, 1980b).

In summary, procedures now available permit the detection and characterization of relatively small numbers of functional B cells. By definition, cloning assays reveal the maximum diversity of lymphocyte populations, and the possibility that these resolve functionally specialized cell sets is considered in some detail below. There are obvious theoretical and practical advantages and disadvantages to each of the three systems described here. The relatively low cloning efficiency of the semisolid agar method and the absence of colony-forming cells in CBA/N mice suggest that particular categories of B cells may not be detected. This possible disadvantage is offset for many purposes by the fact that the incidence of functional B cells can be determined for many cell suspensions in a single experiment. The number of samples that can be processed by other cloning methods is considerably more limited.

V. Formation of B Cells in Cell-Transfer Assays

The simplest way to demonstrate the presence of B-cell precursors in cell suspensions is to inject them into irradiated or otherwise compromised recipient animals. This approach derived from the long-used practice of adoptively transferring immune responses (Cochrane and Dixon, 1962). It will be argued below that only those cell suspensions and tissues that contain committed B-lineage precursors have given rise to B cells in culture. In mammals, the only convincing demonstration that multipotential stem cells can experimentally follow this avenue of differentiation was the transfer of yolk sac cells to irradiated mice (Tyan and Herzenberg, 1968; Paige *et al.*, 1979). However, exceedingly complex events may take place between the time of cell injection and final readout of the experiment, and results thus obtained may be subject to multiple interpretations.

In the past, the transferred suspensions were usually very heterogeneous cell mixtures. Bone marrow grafts, for instance, may contain recirculating B and T cells, newly formed lymphocytes, Ig-synthesizing pre-B cells that are in the process of giving rise to B cells, committed B-cell precursors of different kinds, and multipotential stem cells that can potentially repopulate all blood cells. Most likely, these would differ in self-renewal and proliferative potential, and it is easy to imagine that the relative contribution of each to the B cells that emerge in the recipient would continuously change with time after transfer. Furthermore, if there are any niches or factors that commonly affect these cells and precursors of all the other hematopoietic lineages present in the inoculum, competition situations could arise. The com-

plexity of precursors of B cells and accessory cells that may influence their differentiation is considered in detail in the following sections along with available methods for resolving and physically separating them.

The recipient animals are a second major source of variability. To begin with, it is clear that a fraction of cells of the B lineage, helper T cells, and other cell types capable of influencing immune responses survive (hematopoietic) lethal irradiation exposures. Unless appropriate markers are used, and in certain circumstances, a majority of the regenerating B-cell activity can derive from host rather than transferred cells (Pilarsky and Cunningham, 1975). Donor and host can be discriminated by cytogenetic markers like the T6 chromosome (Micklem *et al.*, 1966), but this requires that the emerging cells of interest be in division. Alloantigenic disparities between recipient and injected cells can also be used (Mitchell and Miller, 1968), but one must determine that these differences do not impose an undesirable selective pressure on the transferred precursors. Immunoglobulin allotypes have also been used to advantage in studies involving mice, rats, and chickens (Tyan and Herzenberg, 1968; Hunt, 1980; Ivanyi and Makings, 1978), but the same reservations apply as for other genetic markers. Yet another solution is to take advantage of a partial immunodeficiency that preferentially affects B cells in a mutant strain of mice (Kincade *et al.*, 1978a; Paige *et al.*, 1979). CBA/N strain mice lack cells that can be detected by cloning in semisolid agar cultures, but these are formed from donor cells following irradiation and grafting with hematopoietic cells from normal CBA/H-T6T6 strain mice. It was determined that all the cells within the B-cell colonies bore the distinctive chromosome marker of the donor inoculum.

That surviving remnants of the host immune system can affect emerging B cells in irradiated recipients was apparent from studies of idiotype expression in the progeny of transferred hematopoietic cells (Kaplan *et al.*, 1978). The regenerating humoral immune system was reported to be influenced by injection of mitogens or thymocytes (Goidle *et al.*, 1976; Sherr *et al.*, 1978), whereas in other studies development of different parameters of B-cell diversity were not obviously affected by mitogens or T cells (Rosenberg and Cunningham, 1977; Van Muiswinkel *et al.*, 1975). In light of these findings, it would be unwise to assume that the kinetics of B-cell formation in this situation approximate those of normal developing or steady-state conditions.

Despite these complications, transfer models of some utility have been devised to enumerate and characterize B-cell precursors. Lafleur *et al.* (1972a) distinguished the immediate precursors of B cells from

typical B cells on the basis of time required after cell transfer before immunization with sheep red blood cells (SRBC). The assay was said to be quantitative for populations of marrow and spleen cells that were large (rapidly sedimenting) and that seemed to express surface Ig (Lafleur *et al.*, 1972b). In a different study, where the thymus cell-independent antigen DNP-polymerized flagellin was used, a delay between transfer and immunization improved the performance of bone marrow but not of spleen cells (Stocker *et al.*, 1974). However, differences in numbers of marrow cells injected were not proportionally reflected in the magnitude of the antibody responses obtained. It has been customary to plot log numbers of injected cells against the log of antibody titers, plaque-forming cell number, etc., in adoptive transfer assays (Celada, 1967; Stocker *et al.*, 1974; Janeway, 1975). Slopes of 1 are considered to represent the contribution of a single cell type that is limiting in the immune response, whereas larger slopes are indicative of some form of interaction between injected cells that is favored by high cell doses.

When bone marrow, or any tissue that contains sIg^+ cells, is used as a source of donor cells, it is possible that some of the B-cell activity measured in the recipients is due to the presistence and/or expansion of these B cells. However, studies of colony-forming B cells arising from transplants of normal CBA/H-T6T6 marrow to immunodeficient CBA/N mice suggest that this problem is insignificant with the cell doses that are usually employed (Paige *et al.*, 1979). Functional B cells emerged at the same rate regardless of whether the marrow cells derived from normal mice or anti-μ-suppressed mice that were deficient in B cells. Physical removal of B cells with anti-Ig-coated plastic dishes yielded the same result. It is important to note that with this system clonable B cells are formed with equivalent efficiency in irradiated normal and in irradiated CBA/N recipients. This suggests that all components of microenvironments necessary for B-cell differentiation are intact in the defective mice. The characteristics of cells that give rise to B cells under these conditions are discussed in detail in Section IX of this review. For now, it will suffice to say that a dose-response relationship exists between numbers of cells injected and numbers of clonable B cells that can be recovered from the recipient spleens 8–10 days later. At that time point, slopes just over one are obtained and a particular type of marker-bearing precursor is detected. When the assay is prolonged, it appears that other cell populations contribute to the emergence of functional cells.

Normally, chimerism is short-lived and inefficient when cells are injected into unirradiated recipients (Celada, 1966). This would be

consistent with the notion that sessile stem cell populations are not forced out of their niches by recirculating stem cells (Micklem *et al.*, 1975b). Mutant W/W^v mice have macrocytic anemia, are deficient in CFU-s, and can be efficiently grafted with hematopoietic stem cells without prior irradiation (Russell, 1979; Harrison and Astle, 1976). Thymocytes were virtually completely replaced by this transplantation, whereas there were indications that some host-type B cells persisted for months without replacement with donor cells. Similarly, immunodeficient CBA/N mice can be successfully grafted with donor cells without prior irradiation, and the resulting B-cell chimerism is long-lived (Scher *et al.*, 1975b; Volf *et al.*, 1978; Paige *et al.*, 1979). There was little if any penetration of the donor cells into CFU-s, CFU-c, or T-cell compartments consistent with the selective nature of the X^{id} mutation.

In summary, there is evidence (detailed below) that stem cells and committed B-cell precursors give rise to functional B cells on transfer to appropriate recipients. However, the rate of emergence of B cells may be influenced by numerous factors and meaningful exploitation of these models requires definition and/or fractionation of the transferred cells.

VI. Relationship between B-Lymphocyte Precursors and Other Hematopoietic Lineages

The existence of a common stem cell for all blood cells has been assumed for some time although formal proof of that statement has been gradually and relatively recently acquired. Experiments by Ford *et al.* (1966) and Micklem *et al.* (1966) used the T6 chromosome marker to demonstrate that most dividing lymphoid and hematopoietic cells of irradiated mice transplanted with bone marrow derived from donor cells. This marker is known to be present in all somatic cells, so it was not known whether individual lineages were restored by different, precommitted precursors or by a common stem cell pool. A proportion of cells in animals that survive sublethal irradiation carry distinctive abnormal karyotypes, and this has been utilized in a number of studies to assess relationships between stem cells and their descendants (Wu *et al.*, 1968; Nowell *et al.*, 1970; Abramson *et al.*, 1977).

Most analyses of stem cells in rodents in the last 20 years have also employed the *in vivo* spleen colony-forming unit (CFU-s) assay devised by Till and McCulloch (1961). Limiting numbers of hematopoietic cells are transferred to lethally irradiated animals, splenic foci develop to macroscopic size within 8–10 days. A linear relationship exists between numbers of injected cells and numbers of developing

spleen colonies, and this suggests that individual foci are clones. This was further supported by studies of colonies that arose in animals grafted with cytogenetically marked stem cells (Becker *et al.*, 1963; Barnes *et al.*, 1968). When unique metaphases were found in individual colonies, almost all of the dividing cells within the same focus were also marked. It should be emphasized that, while such results demonstrate the clonal nature of a majority of proliferating cells in spleen colonies, it cannot be assumed that all cells in proximity to these (including nondividing cells) originate from the same stem cell. In many studies the cellular composition was determined for splenic foci derived from various types of stem cells (Curry and Trentin, 1967; Metcalf and Moore, 1971). Individual colonies are predominantly erythroid, granulocyte-macrophage, megakaryocytic, or mixed in terms of morphologically distinguishable cell types. The existence of mixed colonies that are presumably of clonal origin argues for their derivation from multipotential stem cells. It is interesting to note that some stem cells may, after injection, give rise to more than one spleen colony (Barnes *et al.*, 1968).

Induced cytogenetic markers can be found in individual spleen colonies as well as lymphoid tissues of grafted animals, and Wu *et al.* (1968) took advantage of stem cell-deficient mutant mice to expand numbers of such marked stem cells. After a significant period of time, the penetration of these clones into the various lymphoid and hematopoietic pathways was assessed. In one such study, chimerism was found in T cells only, in myeloid cells only, or in B cells plus T cells and myeloid cells (Abramson *et al.*, 1977). This result suggested the existence of truly multipotential stem cells, stem cells capable of giving rise only to nonlymphoid cells, and stem cells restricted to T-lineage differentiation. The first two categories of cells seemed capable of spleen colony formation. Because a selection pressure was applied in the form of the stem cell-deficient mice, the proportion of stem-cell clones of these three types does not necessarily reflect their relative incidence in normal tissues. Another consideration is the possibility that some types of stem cells might be particularly radiosensitive and therefore lost during the induction of the chromosome markers. For these reasons the existence of stem cells with different capabilities, for example, lymphoid stem cells or B-restricted stem cells, cannot be ruled out.

CFU-s obtained from a wide variety of tissues and stages of development have been characterized with respect to physical properties, cycling characteristics, seeding efficiencies, and self-renewal potential (Haskill and Moore, 1970; Moore *et al.*, 1970; Metcalf and Moore,

1971; Micklem *et al.*, 1973). Heterogeneity is apparent in terms of all these parameters, and it should be cautioned that the potentiality of CFU-s from one tissue, such as the yolk sac, is not necessarily equivalent to that of CFU-s obtained from other organs, such as fetal liver, spleen, and marrow. In adult marrow, most CFU-s are normally in a noncycling or G_0 state, but their mitotic activity can be influenced by a variety of nonspecific and also by some apparently specific substances (Lajtha, 1979; Wright and Lord, 1979). Models have been proposed to explain the control over stem cell self-renewal as opposed to differentiation along one of several pathways (Curry and Trentin, 1967; Till *et al.*, 1964), and the principal dispute is over the degree to which localized stimuli or "hematopoietic inductive microenvironments" influence stem-cell behavior.

Committed progenitor cells of the granulocyte-macrophage pathway can be cloned in semisolid agar cultures where their proliferation and maturation is dependent on the continuous presence of colony-stimulating factors or activities (CSA) (Metcalf, 1977). Modifications to the basic technique also permit the propagation of clones of eosinophil, erythroid, and megakaryocytic cells. Physical and other properties of cells that form such colonies allow them to be distinguished from the *in vivo* CFU-s (reviewed in Metcalf and Moore, 1971). Johnson and Metcalf (1977) discovered that use of an appropriate spleen-conditioned medium permitted formation of colonies that may be more closely related to CFU-s. Individual foci could contain mixtures of erythroid, neutrophil, macrophage, eosinophil, and megakaryocytic cells. There was a degree of self-renewal of these colony-forming cells *in vitro*, and a small proportion of cells formed spleen colonies when taken from culture and injected into lethally irradiated animals (Metcalf *et al.*, 1979). Lymphocytes were not found within these colonies, and cells belonging to T or B lineages have thus far not been shown to originate from these stem cells (F. Melchers, personal communication). The cells responsible for these mixed-morphology *in vitro* colonies might be equivalent to the myeloid-restricted stem cells referred to above or, alternatively, suitable environments may not have been found for them to express their lymphoid capability.

Studies of this kind lead to the conclusion that all blood cells ultimately derive from a common stem cell. Some of these cells are capable of giving rise to spleen colonies and some of the cells within spleen colonies are capable of lymphoid differentiation. On the other hand, an unknown proportion of spleen colonies probably derive from cells with more limited options. This possibility is schematically depicted in Fig. 1.

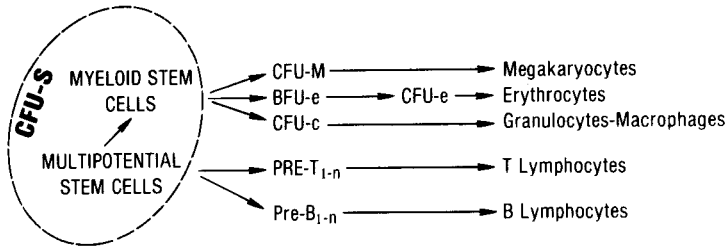

FIG. 1. A scheme depicting possible lineage relationships between lymphoid and other hematopoietic cells. Stem cells detected by the *in vivo* spleen colony-forming unit (CFU-s) assay are assumed to include restricted precursors of erythroid and myeloid cells and multipotential stem cells capable of giving rise to lymphocytes.

Cells dissected from spleen colonies have been used to restore humoral immunity to irradiated secondary recipients, and it was assumed that the time required was that due to the transition of stem cells (which self-renew to a degree within the splenic foci) to functional B cells (Trentin *et al.*, 1967; Yung *et al.*, 1973; Rozing *et al.*, 1977). Extensive examinations of spleen colonies has not revealed any that had predominantly lymphoid morphology (Curry and Trentin, 1967; Metcalf and Moore, 1971). However, the possibility of a minor entry of stem cells into B and T pathways would be difficult to rule out, particularly if the newly formed cells rapidly migrated elsewhere.

One study concluded that some B cells emerged within spleen colonies and that these derived from the same stem cell that made the clone (Lala and Johnson, 1978). This was based on the ability of small numbers of functional B cells to be detected by cloning in semisolid agar cultures and T6T6 chromosome typing of cells in *in vitro* B-cell colonies and the spleen colonies from which they were taken. However, another series of experiments done with similar methodology yielded quite different results (Paige *et al.*, 1979). The rationale of the latter studies was that CBA/N mice have no clonable B cells but provide suitable microenvironments in which normal precursors can mature to functional cells (Kincade, 1977; Kincade *et al.*, 1978b). The validity of the model was tested with chromosomally marked CBA/H-T6T6 hematopoietic cells injected alone or mixed with CBA/N cells into lethally irradiated CBA/N mice. In all cases all the clonable B cells that developed in the recipients derived from the transferred T6T6 marked cells. CBA/N mice have normal numbers of CFU-s, so, when equal numbers of T6T6 and CBA/N bone marrow cells were injected, half of the spleen colonies developed from CBA/N stem cells. It was reasoned that the CBA/N type spleen colonies could have clonable B cells within them only if there was contamination by normal

T6T6 stem cells or committed precursors that were present in the injected inoculum. Indeed, approximately half of the spleen colonies were of T6T6 origin, but a larger number contained functional B cells, and there was no relationship between the colony type and the presence of clonable B cells. Furthermore, numbers of functional B cells were higher in the spaces between colonies than within the splenic foci. Therefore, these results suggest that there is probably no spatial relationship in irradiated, grafted spleens between CFU-s that give rise to macroscopic foci and emerging B cells.

This conclusion would seem to accord with the results of Owen (1979), who was unable to find $c\mu^+$, sIg^- cells in foci or white pulp areas of spleens of mice recovering from irradiation and grafting with bone marrow at a time when these pre-B cells were easily detectable in regenerating marrow. It is known that the majority of B cells that arise in irradiated, marrow-transplanted mice come from sIg^- cells (Paige *et al.*, 1979). Therefore, if expression of the Ig synthetic characteristics of pre-B cells is essential for B-cell formation, it must be argued either that clonable B cells found within spleen colonies came from cells that first traversed the bone marrow or that they quickly lost their pre-B cell characteristics on lodging in the spleen. The latter possibility is consistent with findings that sIg^- cells can complete their maturation within splenic fragments isolated in culture (Teale *et al.*, 1979; Teale and Mandel, 1980).

The incidences of spleen colony-forming cells and B-cell precursors were compared in fetal liver at different stages of gestation and found to be quite different (Paige *et al.*, 1981a). Graded numbers of cells from 13- and 16-day fetal liver were transferred to irradiated mice, and total numbers of clonable B cells per spleen were determined 9 days later. With both types of graft there was a direct relationship between numbers of injected cells and numbers of resulting B cells, and the slopes of the curves on a log/log plot were just over 1. However, approximately 10 times as many 13-day cells had to be transferred to produce the number of B cells obtained with 16-day grafts. This indicates that, at this early time point after grafting, a relatively simple relationship exists between injected precursors and their functionally mature B-cell progeny and also that these precursors were considerably more numerous at the later gestational stage. In contrast, the incidence of CFU-s in 13- and 16-day liver was approximately the same, and a given dose of young fetal liver resulted in the same number of myeloid progenitor cells (CFU-c) in recipients as did older liver. The relationship of spleen colony-forming cells to the myeloid lineage is therefore much closer than it is to cells of the B-cell pathway. It is

noteworthy that at a slightly longer assay interval, 14 days, the B-cell repopulating potentials of 13- and 16-day fetal liver were indistinguishable. This point is considered in a later section in relation to possible differences in B-cell precursors.

An even more dramatic dissociation of CFU-s and B-cell precursors was obtained by using a cytotoxic monoclonal antibody. This rat anti-mouse brain antibody (19B5) lyses a substantial proportion of fetal liver or bone marrow cells in the presence of complement, but CFU-s are not obviously affected (Shinefeld *et al.*, 1980). The ability of fetal liver or bone marrow suspensions to generate B cells after transfer to irradiated CBA/N mice was suppressed by immune elimination with 19B5, and this was apparent even when the recipient animals were examined 6 weeks after grafting. Control experiments were done to show that accessory cells were not the target of the 19B5 antibody (Paige *et al.*, 1981a). Again these results suggest that stem cells do not quickly give rise to large numbers of B cells in irradiated recipients.

The yolk sac is an extraembryonic tissue in which Ig synthesis or other indications of B-lineage differentiation have generally not been detected (Owen *et al.*, 1975; Phillips and Melchers, 1976; Andrew and Owen, 1978; Melchers and Abramczuk, 1980). However, this is the earliest site where CFU-s and cells capable of mixed *in vitro* colony formation have been found, and, according to one hypothesis, all hematopoietic stem cells derive from cells that emerge there (Moore and Metcalf, 1970; Metcalf *et al.*, 1979; Moore and Owen, 1967; see discussion below). Paige *et al.* (1979) transferred suspensions of 10-day yolk sac and 12-day fetal liver from CBA/H-T6T6 mice containing equivalent numbers of CFU-s to irradiated CBA/N recipients. Clonable B cells promptly emerged in liver-grafted animals whereas 8 weeks were required for expansion of newly formed B cells in recipients of yolk sac. That the yolk sac contained pluripotential stem cells was evident from finding the T6T6 marker in CFU-s, CFU-c, T cells, and B cells of grafted mice. Therefore, if the CFU-s assay is any indication of numbers of multipotent stem cells, a considerable interval must be required for them to generate B cells and a majority of the B cells that develop soon after grafting must derive from some type of committed progenitor population rather than pluripotential stem cells.

B-lineage precursors and stem cells may also be partitioned by transfer of cells into CBA/N mice without prior irradiation (Paige *et al.*, 1980). Grafted fetal liver or marrow cells from normal donors find niches that are uniquely available in these mice as a result of the X^{id} mutation (Volf *et al.*, 1978). This results in long-term chimerism, and, particularly in the case of fetal liver grafts, there is a substantial pro-

duction and expansion of donor-type B cells. CBA/N mice are not deficient in numbers of CFU-s, and in the absence of irradiation there is no significant penetration of this compartment, CFU-c, or T cells by the injected cells. Bone marrow grafts also result in the production of normal B cells, but colonization of the CBA/N marrow and growth in numbers of functional B cells is less efficient than with fetal liver transplants (Paige *et al.*, 1980). It is possible that multipotent fetal liver stem cells are induced to enter the B lineage selectively in this situation. Alternatively, and perhaps more likely, the emerging B cells may derive from a class of B-cell precursors (which are deficient and/or defective in CBA/N mice) that has substantial proliferative/differentiative potential.

There is some evidence for the relationship of lymphoid and hematopoietic cells in humans (Fialkow, 1976). Allelic forms of the enzyme glucose-6-phosphate dehydrogenase (G6PD) are encoded on the X chromosome, and individual somatic cells of heterozygous women express only one of the alleles of the enzyme. Malignancies may result from the transformation of a pluripotential stem cell such that a majority of the cells belonging to several lineages are marked by the same enzyme allele. Chronic myelocytic leukemia (CML) is such a disease, and although T cells may rarely be replaced by cells related to the leukemic clone, there is good reason to believe that B cells in affected individuals derive from a cell that is common to erythroid, megakaryocytic, and granulocyte-macrophage lineages (Fialkow *et al.*, 1978; Martin *et al.*, 1980). Patients with polycythemia vera, on the other hand, have increased numbers of circulating platelets, erythrocytes, and granulocytes, which must originate from a common, abnormal stem cell, but lymphocytes are unaffected (Adamson *et al.*, 1976). Such observations can be used to infer the existence of stem cells that have certain and multiple differentiation options. As with the investigations of animals, however, the relative frequencies of various stem cell types in normal circumstances and the possibility of cells with different destinies are not elucidated.

These observations do not discount the notion that lymphoid and myeloid cells derive from a common stem cell. Rather, they emphasize that no close precursor–product relationship between such stem cells and cells of the B lineage has ever been experimentally demonstrated. Similar conclusions came from aging and competitive repopulation studies where the behavior of CFU-s did not correspond well with lymphoid repopulating potential of cell suspensions (Micklem *et al.*, 1975; Harrison, 1980). This means that we have little idea of the kinetics or magnitude of the contribution of stem cells to development

and maintenance of the humoral immune system. The difficulties involved in identifying putative inductive microenvironments that might be critical to this transition and in discerning when committed stem cells for T and B pathways diverge are obvious.

VII. Formation and Maturation of B Cells in Culture

Yoshida and Osmond (1971) enriched large transitional lymphocytes in suspensions of guinea pig bone marrow cells by discontinuous sucrose density gradient centrifugation and then observed the progression of these into small lymphocytes *in vitro*. In the following decade, many studies demonstrated that at least some of the processes involved in murine B-lymphocyte formation can occur in culture. There is no evidence that multipotential hematopoietic stem cells can be directed into the B lineage *in vitro*, but the possibility now exists for resolving important cellular interactions and stimuli required for differentiation of committed precursor cells. Culture techniques should also make it feasible to enumerate and manipulate relatively small numbers of these cells and assess the diversity of their antibody-forming potential.

In the studies cited in Table I these events appeared to occur spontaneously. In some cases, addition of substances like LPS to the cultures was found to have no influence on B-cell formation (Ryser and Vassalli, 1974; Melchers, 1977a; Pike and Nossal, 1979). In contrast, the investigations listed in Table II suggest that various inducing agents augmented or were essential for B-cell maturation events. There are some very obvious differences in the approaches that were employed. Most of the studies listed in Table I were done with only partially enriched B-cell precursors. An exception is the work of Pike and Nossal (1979), where the FACS was used to prepare small sIg^- bone marrow lymphocytes, most of which were just hours away from becoming functional B cells (Nossal *et al.*, 1977).

Komuro and Boyse (1973) discovered that T-lymphocyte marker expression could be induced by short-term culture of preselected cell populations with appropriate agents, and many comparable studies have been done with cells of the B lineage. Typically, a very small subpopulation of cells is isolated from spleen or bone marrow by density gradient centrifugation. All constituents of the separation and culture media would have been rigorously screened to have a low background stimulation of marker display. Native and synthetic substances raising cyclic AMP induce expression of a series of B-lymphocyte markers whereas, reciprocally, induction of the plasma cell antigen, PCl, requires cGMP elevation (Hämmerling and Chin, 1977; Scheid *et al.*,

TABLE I

SOME EXAMPLES OF "SPONTANEOUS" MURINE B-CELL FORMATION/MATURATION *in Vitro*[a]

Study	Tissues examined	Culture conditions	Change noted
Owen *et al.*, 1974	14–15-Day fetal liver	Explant cultures	sIg^+ cells
Gelfand *et al.*, 1974	Newborn spleen and liver	Dispersed cell culture	sIg^+ cells
Osmond and Nosal, 1974	Adult bone marrow	Marbrook cultures	sIg^+ cells[b]
Ryser and Vassalli, 1974	Small marrow lymphocytes	Dispersed cell culture	sIg^+ cells[b]
Nossal and Pike, 1975	Adult bone marrow	Marbrook cultures	Adoptive DNP resp.[b]
Raff *et al.*, 1975	14–15-Day fetal liver	Explant cultures	sIg^+ cells
Owen *et al.*, 1975	Fetal liver and spleen	Explant cultures	sIg^+, Fc^+, $MBLA^+$ cells
Johnson *et al.*, 1976	Fetal liver and spleen	Explant cultures	Clonable B cells
Owen *et al.*, 1976	14-Day fetal liver	Dispersed/reaggregated cells	sIg^+ cells
Owen *et al.*, 1977	Fetal marrow	Explant cultures	sIg^+ cells
Melchers, 1977	13-Day fetal liver	Dispersed cell cultures	LPS resp. B cells
Stocker, 1977	Adult marrow, newborn spleen	Dispersed cell cultures	NIP precursor frequency
Burrows *et al.*, 1978	B-cell-suppressed marrow	Dispersed cell cultures	cIg^+ and sIg^+ cells
Lau *et al.*, 1979	Size-separated adult marrow	Dispersed cell cultures	Late LPS resp.
Pike and Nossal, 1979	FACS-separated adult marrow	Dispersed + thymus fillers	sIg^+ and LPS resp.
Teale *et al.*, 1979	FACS-separated newborn spleen	Klinman splenic focus assay	DNP precursors
Teale and Mandel, 1980	14-Day fetal liver, 15-day spleen	Splenic focus and explant	sIg^+ and DNP resp.
Kincade *et al.*, 1980	B-cell-depleted adult marrow	Dispersed cell cultures	Clonable B cells

[a] FACS, fluorescence-activated cell sorter; LPS, lipopolysaccharide; sIg, surface immunoglobulin; cIg, cytoplasmic Ig; DNP, dinitrophenyl.

[b] Absolute numbers of B cells may not have increased in these studies.

TABLE II

SOME EXAMPLES OF ENHANCEMENT (INDUCTION) OF B-CELL FORMATION/MATURATION IN CULTURE BY MITOGENS AND OTHER FACTORS[a]

Study	Source of cells	Inducing agents used	Change noted
Scheid *et al.*, 1975	Separated spleen	LPS, cAMP, etc.	C3 receptors
Hämmerling *et al.*, 1975	Separated neonate marrow	LPS, ubiquitin, PGE_1, cAMP, etc.	Ia antigens
Hämmerling *et al.*, 1976	Separated spleen and marrow	LPS	sIg, Ia, C3R
Hämmerling and Chin, 1977	Separated spleen and lymph nodes	Carbachol, cGMP, PGF_2, thymopoietin	PC.1
Watson, 1977	Separated marrow	LPS, cAMP, etc.	Ia antigens
Scheid *et al.*, 1978	Separated spleen, Peyer's patch	Carbachol, cGMP, cAMP, LPS, poly(A : U), PGE_1	C3R, PC.1
Fairchild and Cohen, 1978	B-cell-depleted marrow	LPS, cAMP	sIg, LPS response
Sitia *et al.*, 1979	Separated spleen	LPS	sIgD
Melchers, 1979	Placenta	Dextran sulfate	LPS resp. B cells
Melchers and Abramczuk, 1980	Placenta, fetal blood, liver	Dextran sulfate	LPS resp. B cells
Coutinho *et al.*, 1980	Fetal liver, sIg-bone marrow	Anti-idiotype antibodies	LPS resp. B cells
Kincade *et al.*, 1981	B-cell depleted marrow, fetal liver	Accessory cells	Clonable B cells

[a] LPS, lipopolysaccharide; PGE, prostaglandin E; sIg, surface immunoglobulin.

1978). In some situations, substances like thymopoietin that induce T markers have an inhibitory effect on B-cell surface marker display. The most commonly used readout for such studies is the microcytotoxicity assay, and it could be argued that events unrelated to cell surface markers render cells more susceptible to lysis. However, absorption analysis suggested that an increment in antigen density occurred as a consequence of short-term induction (Hämmerling *et al.*, 1975). For T-lymphocyte antigens, it was determined that RNA and protein, but not DNA, synthesis were required for induction (Storrie *et al.*, 1976). By sequential separation of precursor populations, Hämmerling and colleagues (1976; Sitia *et al.*, 1979) were able to characterize the cells on which particular markers could be induced and propose a possible order for B-cell maturation events.

A protocol that has recently been used to assess the requirements for B-cell formation in culture is illustrated in Fig. 2. It is based on the observation that acquisition of sIg coincides with the ability of newly formed B cells to clone in semisolid agar whereas less mature cells give rise to colony-forming B cells after culture in liquid medium (Kincade *et al.*, 1980a). In the case of bone marrow precursors, the sIg^+

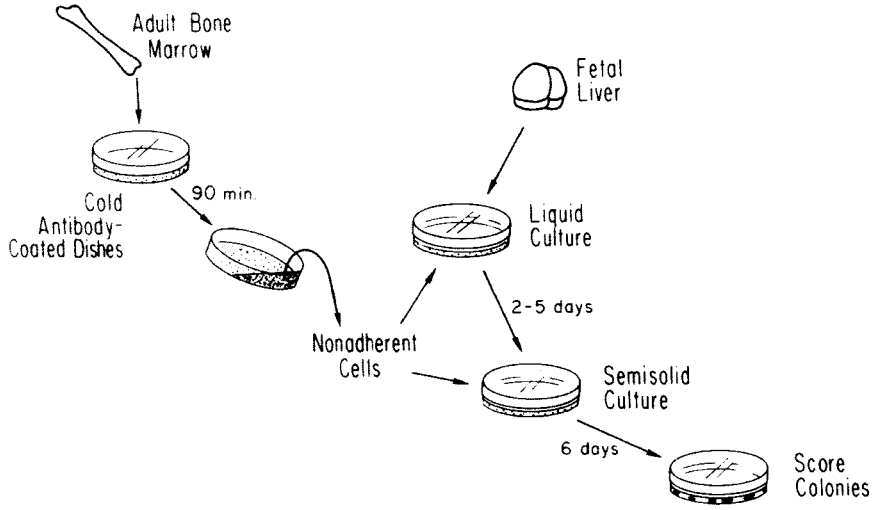

FIG. 2. An experimental protocol for demonstrating the maturation of B-cell precursors *in vitro*. Bone marrow suspensions are depleted of B cells by adherence in the cold to anti-Ig-coated plastic dishes. These contain very few functional cells that can respond directly to mitogens in semisolid agar cultures. Clonable B cells emerge, however, from such suspensions and from fetal liver cells when they are first held in liquid cultures (where cells settle to relatively high densities).

B cells were first removed on anti-μ antibody-coated plastic dishes before holding the remaining cells in liquid culture. After an appropriate interval (usually 48 hours), the cells were then harvested and plated in semisolid agar. Since there were always some remaining clonable B cells after the B-cell depletion, it was important to establish that these did not simply proliferate or mature during the liquid preculture period. That this was not the case was suggested by the fact that similarly B-cell-depleted lymph node cell suspensions did not generate functional B cells in culture (Kincade *et al.*, 1981a). Others have found that this tissue lacks cells with any characteristics of B-cell precursors (Raff *et al.*, 1976; Lafleur *et al.*, 1972a; Lau *et al.*, 1979). The relative size of precursors in marrow that could give rise to clonable B cells during liquid preculture was determined by separating the cell suspensions by velocity sedimentation at unit gravity. It was obvious that while the cloning efficiency of small, rapidly sedimenting cells was slightly increased by liquid culture, a majority of the functional cells that emerged as a result of this treatment arise from large precursors (Kincade *et al.*, 1981a).

There is considerable precedent for cells in culture being dependent on or influenced by adherent, macrophage-like cells. For example, it is known that adherent cells favor the maturation and proliferation in culture of erythroid, neutrophil-macrophage, and megakaryocytic precursors and thymocytes (Kurland *et al.*, 1980; Williams *et al.*, 1981; Beller and Unanue, 1978). Furthermore, adherent marrow cells elaborate a soluble factor that specifically inhibits division of hematopoietic stem cells (Wright *et al.*, 1980). The possible importance of adherent cells in the formation of B cells in culture was tested by first depleting marrow suspensions of B cells with antibody-coated dishes and then removing adherent cells by passage through Sephadex G-10 columns. Functional B cells did not emerge from cultures of these doubly depleted cells unless adherent cells from bone marrow or peritoneal exudates were added. Equivalent restoration was obtained when adherent cells were obtained from normal and immunodeficient CBA/N mice. The latter are a good practical source of accessory cells because they are incapable of contributing any clonable B cells. These experiments suggest that adherent accessory cells can dramatically augment the formation of B cells from sIg^- precursors (Kincade *et al.*, 1981a). Representative results are illustrated in Table III.

Among the known effects of adherent cells is their ability to facilitate the number and size of B-cell colonies, particularly when other potentiators are not added to the cultures (Kurland *et al.*, 1977). It therefore seemed possible to explain these results in terms of carry-over of mac-

TABLE III
INFLUENCE OF FILLER CELLS ON MATURATION OF FETAL AND ADULT PRE-B CELLS *in Vitro*

Cell suspension	Initial clonable B cells	Clonable B cells recovered after liquid culture	
		Cultured with	No.[a]
Unseparated bone marrow	8640	—	—
Bone marrow depleted of B cells	311	Medium alone	1467
		10^5 Adherent PEC[b]	1451
		10^6 CBA/N marrow	1744
Bone marrow depleted of B cells and adherent cells		Medium alone	300
		10^5 Adherent PEC	1160
		10^6 CBA/N marrow	788
16.5-Day fetal liver		Medium alone	1415
		10^5 Adherent PEC	1948
		10^6 CBA/N marrow	4061
16.5-Day fetal liver depleted of adherent cells		Medium alone	1190
		10^5 Adherent PEC	1295
		10^6 CBA/N marrow	6259

[a] Numbers of colonies per 10^6 initial cells. Adult marrow was held for 48 hours and fetal liver was held for 96 hours in liquid precultures.
[b] PEC, peritoneal exudate cells.

rophages or their products to the semisolid agar cultures. Control experiments were done in which adherent cells and B-cell precursors were held in separate liquid cultures and then added together just before plating. This established that the accessory cells needed to be present when new B cells were being formed.

A series of recent studies have established that some of the functions attributable to adherent macrophages result from the presence of small numbers of dendritic cells in the cultures (Steinman and Witmer, 1978). The contribution of dendritic cells to the experiments described above was not ruled out, but the adherent cells that were used were 99% phagocytic and nonspecific esterase positive. It is noteworthy that, in several limiting dilution-type liquid culture assays, thymocytes are used as filler cells (Melchers, 1977a; Pike and Nossal, 1979). The possible role of thymic macrophages (Beller and Unanue, 1980) in such assays has not been determined.

It is possible that the adherent cells (presumably macrophages) merely improved culture conditions in the above experiments by conditioning the medium. On the other hand, it would be worthwhile to look for more specific mediators of accessory cell function. Tumor ne-

crosis serum has been used to induce surface marker expression on immature B cells (Hoffmann *et al.*, 1977). More recently, M. Scheid (unpublished observations) discovered that the active moiety is most likely the macrophage product, interleukin 1 (Gery and Waksman, 1972). Nonspecific macrophage stimulation *in vivo* is thought to cause the sequential transition of newly formed small B lymphocytes into large blasts and then into typical small B cells with shortened response kinetics (Howard *et al.*, 1978; Shortman and Howard, 1979). It remains to be seen whether one or more macrophage-derived factors can influence several stages in B-lineage differentiation, beginning perhaps with sIg^- precursors.

Committed progenitor cells of the B lineage are present in fetal liver by 12 days of gestation, whereas substantial numbers of functional B cells do not emerge in that organ until at least day 17 of gestation (Paige *et al.*, 1979; Johnson *et al.*, 1976). If accessory cells are important for the final stages of precursor maturation, the availability of these could limit B-cell development during embryonic life. However, unlike adult bone marrow, depletion or addition of adherent cells had no influence on the emergence of functional B cells from liquid cultures of fetal liver (Table III). It is possible that in fetal liver sufficient immature precursors of adherent cells are present that can mature during the liquid preculture period and obscure their importance. It was also noted that where LPS either had no effect or slightly stimulated adult cultures it consistently inhibited cultures of fetal liver (Kincade *et al.*, 1981a). Both of these observations might be attributable to differences in the nonlymphoid cell composition of fetal and adult tissues. Another possibility, which will be considered later in the context of other observations, is that B-cell precursors in fetal tissues are intrinsically less adherent cell-dependent and more LPS sensitive than their adult counterparts.

In conducting these experiments, it was found that maturation of fetal pre-B cells *in vitro* could be enhanced substantially by addition of adult CBA/N bone marrow cells to the cultures. Marrow cells from these clonable B-cell deficient mice had no influence when added to cultures of normal adult marrow, and it did not matter if the CBA/N cells were depleted of adherent cells before addition to the fetal liver cultures. This suggests that fetal liver is deficient in some type of nonadherent accessory cell that is common in adult marrow. The development of this cell population during ontogeny could have a significant influence over the number of functional B cells produced. However, when culture conditions were optimized by use of adult CBA/N marrow cells, the age of the donor embryo was important both

in terms of the number of B cells formed and the time required for their emergence. This accords with findings of others that committed B-cell precursors appear in waves at various stages of embryogenesis in particular tissues (Melchers, 1977b; Melchers and Abramczuk, 1980).

Constituents of the culture medium could be an inadvertent source of inductive stimulation in experiments of this kind. For example, it is customary to add 2-mercaptoethanol to culture medium to promote lymphocyte viability, and it is known to act via its favorable effect on albumin (Claesson *et al.*, 1979). In this respect 2-ME is a nutrient, but it has also been said to be mitogenic for resting small lymphocytes (Goodman and Weigle, 1977), and it has been found to increase B-cell surface marker expression in short-term cultures (M. Scheid, unpublished observations). This was an essential component of liquid cultures in which clonable B cells were emerging only when adequate numbers of adherent cells were unavailable (Kincade *et al.*, 1981a), whereas clonal expansion of mitogen-activated B cells is absolutely dependent on this substance (Metcalf, 1976).

In some culture studies it was possible to observe B-cell formation and maturation in the absence of serum (Raff *et al.*, 1975). However, it has generally been found that inclusion of carefully selected batches of fetal calf serum (FCS) is essential to obtaining good B-cell recovery (Melchers, 1977a; Melchers and Abramczuk, 1980; Kincade *et al.*, 1981a). There is an interesting parallel in the behavior of the 70Z/3 pre-B lymphoma cell line, where background expression of sIg was highly dependent on batches of FCS (Paige *et al.*, 1978). This result could not be explained in terms of endotoxin contamination of certain sera and batches that were optimal for induction of the cell line were also superior in supporting B-cell formation in fetal liver organ cultures (C. Paige, unpublished observations). More recently, it was found that the cell line proliferates normally in a specially designed serum-free medium (Iscove and Melchers, 1978) but sIgM could not be induced with LPS unless 1% serum was added to the cultures (Paige *et al.*, 1981b). The serum requirement for differentiation of early erythroid lineage cells was largely replaceable by a 35,000 dalton glycoprotein (Iscove, 1978), and it is hoped that the distinguishing features of good and bad serum lots of B-lineage cell cultures can be similarly defined.

At the present time it is seldom possible to distinguish between factors and manipulations that make up for nutritional inadequacies of cell cultures and those that mimic important signals for regulating B-cell formation. Indeed, the degree to which observations derived

with tissue culture methods can be generalized to the situation *in vivo* must always be questioned. However, the methods now available permit at least the terminal events in B-cell emergence to occur under controlled circumstances, and this undoubtedly will lead us to a better understanding of the regulation of these processes. Furthermore, markers that have recently been defined with monoclonal antibodies now make it possible to isolate committed B-cell precursors in a high degree of purity. The culture techniques allow relatively small numbers of these to be manipulated and should ultimately provide information about the minimal signals necessary for their proliferation and/or differentiation.

VIII. *Microenvironments for B-Lymphocyte Formation*

A. In Birds

Development of the humoral immune system in birds is highly dependent on a single tissue, the bursa of Fabricius (Glick *et al.*, 1956). The concept of separate origins for cells that mediate humoral and cell-mediated immune responses first derived from studies of the roles of the bursa and thymus in chickens (Warner *et al.*, 1962; Cooper *et al.*, 1966). Much is known about the colonization of that organ by stem cells, the development of bursal lymphocytes, and the onset of Ig synthesis. Questions remain, however, about the orogin of these stem cells, their nature, and the degree to which B cells can emerge in the absence of presumably specialized microenvironments.

Basophilic cells in the circulation of chick embryos between day 7 and day 15 of incubation enter the bursa, invade the epithelium, and give rise to B lymphocytes (Moore and Owen, 1965, 1966; Metcalf and Moore, 1971; Le Douarin *et al.*, 1975; Houssaint *et al.*, 1976). The first IgM bearing cells appear in the bursa from about day 13 of incubation; unlike the case with mammals, this is not preceded by a prolonged period of cytoplasmic μ-chain synthesis (Thorbecke *et al.*, 1968; Kincade and Cooper, 1971; Lydyard *et al.*, 1976). In one study, chromosomally marked 13–14-day bursal cells were injected into sublethally irradiated embryos, and there was little tendency of these to give rise to T cells (Weber and Alexander, 1978). This might suggest that the stem cells that enter this tissue are already predestined to become B cells. On the other hand, in transfilter organ culture experiments it has been shown that populated embryonic bursas can serve as a source of cells that enter thymus rudiments and give rise to lymphocytes (Jotereau *et al.*, 1980). The evidence to date suggests that these

continue to differentiate along the T cell line (N. Le Douarin, personal communication).

Surgical bursectomy, performed at around day 17 of incubation, results in a profound immune deficiency. Such animals were found to be agammaglobulinemic, to lack plasma cells and germinal centers, and to fail to produce specific antibody (Van Alten *et al.*, 1968; Cooper *et al.*, 1969). Permanently agammaglobulinemic chickens can consistently be prepared by bursectomy and X-irradiation at hatching (Cooper *et al.*, 1966), embryonic injections of anti-μ antibodies followed by bursectomy at hatching (Kincade *et al.*, 1970), some treatment protocols employing testestosterone (Warner *et al.*, 1969), or bursectomy combined with cytotoxic drug treatment (Linna *et al.*, 1972; Toivanen *et al.*, 1972). A deficiency of sIg^+ B cells also results from embryonic bursectomy (Kincade *et al.*, 1971), and this is less severe the later the operation is performed (Kincade *et al.*, 1973). Animals surgically bursectomized in the late embryonic–newly hatched period are atypical in a number of respects. The full range of Ig isotypes may not be expressed, and individual animals may fall into a stable pattern of selective IgA deficiency or IgG and IgA deficiency (Kincade *et al.*, 1973; Kincade and Cooper, 1973). Antibody responses, primary responses in particular, are often defective (Jankovic and Isakovic, 1966; Rose and Orlans, 1968). Antibodies made by these animals may have limited diversity (Huang and Dreyer, 1978; Ivanyi, 1975), and recovery from tolerance is impaired (Ivanyi and Salerno, 1972). This might suggest that if very few B-cell clones leave the bursa before it is removed, there is little ability to add to or diversify them later.

All these observations lead to the conclusion that the bursa is obligate to the formation of B cells in birds. However, it has been reported that at least some vestige of the humoral immune system was present in chickens whose bursas were removed at an extremely early stage (Fitzsimmons *et al.*, 1973; Jankovic *et al.*, 1975). In one such study, tissues destined to become bursa were destroyed by cauterization at 65–70 hours of incubation (Ewert and Cooper, 1979). No sIg^+, Ia^+ cells were found in surviving animals within 2 weeks of hatching. A small number of B cells were detectable, however, when tests were performed at a later time (M. D. Cooper, personal communication).

Some bursectomy procedures result in the formation of active suppressor T-cell populations that are capable of preventing B-cell differentiation (Blaese *et al.*, 1974; Grebenau *et al.*, 1976; Kermani-Arab and Leslie, 1977). For example, spleen cells taken from bursectomized chickens suppressed development of the humoral immune system when transferred to young chickens with intact bursas. As a crucial test

of how well tissues other than the bursa can support the stem cell to B-lineage transition, the effect of bursectomy in athymic chickens should be investigated.

B. In Mammals

It has been obvious for some time that the formation of B cells is not restricted to a single tissue in mammals (Stutman, 1973; Nossal and Pike, 1973). Possible candidates for mammalian equivalents of the bursa of Fabricius can now be evaluated in terms of the earliest sites where Ig genes are expressed, the effect of tissue ablation, the distribution and frequency of precursors that can give rise to B cells, and localization of cells that express early B-lineage markers.

In vivo spleen colony-forming units (CFU-s) and granulocyte-macrophage progenitors (CFU-c) have first been detected within the yolk sac of murine embryos (Moore and Metcalf, 1970). There is also an appreciable incidence of cells which with appropriate conditioned medium form mixed morphology colonies composed of megakaryocytes, erythrocytes, eosinophils, granulocytes, and macrophages (Johnson and Metcalf, 1977; Metcalf *et al.*, 1979). Numbers of CFU-s and CFU-c reportedly increase during explant culture of isolated yolk sacs (Perah and Feldman, 1977; Moore and Metcalf, 1970), but neither Ig-synthesizing or LPS-responsive B-lineage cells appear (Owen *et al.*, 1975, 1976; Melchers and Abramczuk, 1980). Nor did yolk sac cells function in a transfer assay designed to detect the immediate precursors of B cells (Phillips and Melchers, 1976).

A number of observations suggest, however, that hematopoietic stem cells capable of lymphoid differentiation are also present within the blood islands of this extraembryonic tissue. Grafting yolk sac cells into 8–10-day embryos *in situ* resulted in animals that were chimeric for T cells and CFU-s (Weissman *et al.*, 1976). Irradiated recipients given yolk sac cells developed donor-type Ig, and the thymus and other lymphoid organs were repopulated by chromosomally marked cells of donor origin (Tyan and Herzenberg, 1968; Moore and Metcalf, 1970; Stutman, 1976; Paige *et al.*, 1979). In one study, repopulation of thymus grafts by yolk sac cells took longer than with fetal liver grafts and did not occur in ^{89}Sr-treated recipients (Stutman, 1976). This was interpreted to mean that transit through bone marrow was important to the thymus seeding capability of yolk sac stem cells. Clonable B cells developed from CBA/H-T6T6 yolk sac cells placed in irradiated CBA/N mice but did not consistently appear in appreciable numbers until 8 weeks after grafting (Paige *et al.*, 1979).

The incidences and time of appearance of CFU-s and CFU-c in murine yolk sac were consistent with the hypothesis that hematopoietic stem cells arise *de novo* only within extraembryonic tissues and subsequently migrate via the bloodstream to colonize various lymphoid and hematopoietic organs (Moore and Metcalf, 1970; Moore and Owen, 1967; Metcalf and Moore, 1971). Hematopoiesis has not been observed in organ cultures of isolated fetal liver when the tissue was obtained from embryos of less than 11 days of gestation (28 somites) (Moore and Metcalf, 1970; Johnson and Jones, 1973; Cudennec *et al.*, 1981). This did occur, however, when liver from slightly older embryos was cultured, when embryos were cultured along with intact yolk sacs, or when the liver rudiments were allowed to be colonized by yolk sac cells migrating across a membrane *in vitro*. Early liver rudiments also became hematopoietic when grafted into irradiated mice with an appropriate source of stem cells (Johnson and Moore, 1975). The dependence of intraembryonic B-lymphocyte development on yolk sac-derived cells has not been thoroughly investigated. Organ cultures of 11-day and older fetal liver fragments, but not 12-day yolk sac, developed $c\mu^+$ cells (Owen *et al.*, 1976).

At approximately the same stage of development (11 days of gestation), Andrew and Owen (1978) were able to detect some $c\mu^+$ cells in umbilical blood, and this leaves open the possibility that stem cells are not induced to follow B-lineage differentiation in the liver. Formal proof of that would require showing that prelymphoid hepatic rudiments will support differentiation of cells of this lineage without first being colonized by precommitted B-cell precursors that arise elsewhere. Without such a demonstration, it is not certain that the fetal liver contains bursal equivalent microenvironments.

In studies performed to date, cells expressing the 14.8 series of antigens were first detectable in 12-day fetal liver. At this stage no cells bearing this antigen were detected in either yolk sacs or embryonic blood (E. S. Medlock and P. W. Kincade, unpublished observations). As is demonstrated in the following section, cells capable of quickly giving rise to clonable B cells under liquid culture circumstances can be completely removed by monoclonal antibodies directed to this marker. The same was found to be true for cells that give rise to the B cells formed within 10 days, but not those arising later in irradiated recipient mice. In contrast, it has been reported that a wave of B-cell precursors appears within the ectoplacenta and circulating blood before their expansion in liver (Melchers and Abramczuk, 1980). Reconciliation of these findings will require determining whether precursors able to mature under the liquid culture conditions employed by Melchers are positive for this B-lineage antigen. It is noteworthy

that dextran sulfate was added to these cultures along with thymus filler cells. The rationale for this is that certain data suggest that immature cells of the B lineage are uniquely responsive to this mitogen (Gronowicz *et al.*, 1974).

The significance of the rare $c\mu^+$ cells in early embryonic blood (Andrew and Owen, 1978) is also not clear. All the cells with these pre-B characteristics in adult marrow express the 14.8 antigen, and their numbers greatly exceed Ig-synthesizing cells in early fetal liver (Landreth *et al.*, 1981b). The relationship suggested between these cells in Fig. 4 is seemingly in conflict with the observation of $c\mu^+$ but not 14.8 antigen$^+$ cells in the early embryonic blood. It is possible that a very early wave of B-cell differentiation occurs before this antigen system is activated. Otherwise, it would have to be concluded that a possibly important and precocious event in differentiation of the humoral immune system occurs first within the fetal liver.

Since Volpe *et al.* (1979) discerned that thymocytes of amphibians, like those of birds and mammals, derive from immigrant cells, the concept of extrinsic colonization of lymphoid rudiments by stem cells has not been seriously questioned. However, studies by Dieterlen-Lievre and her colleagues using chicken and quail embryos have raised the possibility that some site other than the yolk sac might provide stem cells relevant to intraembryonic lymphoid and hemopoietic differentiation in birds (Dieterlen-Lievre, 1975; Lassila *et al.*, 1978; Martin *et al.*, 1979; Beaupain *et al.*, 1979). Exchange grafts were made between very early blastoderms of quail and chick embryos such that the fate of cells in the yolk sac of one species could subsequently be determined in embryos of the other species that developed in association with it. The origin of cells could be discerned in this situation because of distinctly different nuclear morphologies in stained preparations. Essentially all the bursal cells that developed were of the same type as the embryo, although a small number of yolk sac cells were present for a time within the embryos. The same conclusion resulted from analysis of hemoglobin markers and when the yolk sacs and embryos differed only for sex and Ig allotype. These results have been used to argue that, while the yolk sac stem cells of birds probably have the same potential as those arising from some site within the embryo, the latter normally contribute most to the lifelong production of lymphoid and other blood cells. Collections of basophilic cells observed in the dorsal mesentery of quail embryos 4–5 days old are possible candidates for such stem cells (Dieterlen-Lievre, personal communication). Comparable studies have not been performed with mammals, and it should be noted that there are many physiologic differences between these species. For example, the liver

is not the major hematopoietic tissue in birds that it is in murine embryos (Kingsbury *et al.*, 1956; LeDouarin, 1975). Given the present uncertainty about the nature of stem cells and committed precursors that might be present in early embryonic blood, the question of where stem cells relevant to the humoral immune system originate should be regarded as open. As previously discussed, the yolk sac of mammals clearly contains cells able to give rise to B and T lymphocytes. It remains to be formally established that similar stem cells can or cannot arise in intraembryonic tissues.

By biosynthetic labeling, immunoglobulin synthesis has been detectable within 10–12-day embryos and in large cells in fetal liver from 13 days of gestation (Melchers *et al.*, 1975). The early appearance of cells with detectable cytoplasmic, but not surface, Ig in embryonic liver and then spleen and bone marrow has also been documented (Raff *et al.*, 1976; Owen *et al.*, 1974; Andrew and Owen, 1978). There is general agreement that cells with sIg detectable by immunofluorescence first emerge in fetal liver between 16 and 17 days of gestation (Nossal and Pike, 1973; Owen *et al.*, 1974; Bruyns *et al.*, 1976; Andrew and Owen, 1978; Teale and Mandel, 1980). However, studies employing surface labeling of cells with lactoperoxidase (Melchers *et al.*, 1976) or rosetting with anti-Ig coated RBC (Rosenberg and Parish, 1977) suggested that very small quantities of sIg might be displayed in an unstable fashion on the surface of immature B-lineage cells. Another report indicated that rosetting was more sensitive than autoradiography, and this permitted some large marrow lymphocytes to be detected with anti-Ig (Yang *et al.*, 1978). The curious tendency of lightly rosetting cells in fetal liver to be removed with carbonyl iron (Rosenberg and Parish, 1977) should perhaps be given further consideration. Studies of cell lines also suggested that some μ chains could be released from pre-B cells (Levitt and Cooper, 1980). It is a characteristic of B cells that have just acquired small quantities of sIg to be sensitive to exposure to anti-Ig antibodies (Kincade *et al.*, 1970; Lawton *et al.*, 1972; Raff *et al.*, 1975). However, while the emergence of small lymphocytes with B-cell markers may be prevented, numbers of sIg^- lymphocytes and cIg^+ cells are not affected (Kearney *et al.*, 1978; Burrows *et al.*, 1978; Osmond and Gordon, 1979). This would indicate that if Ig (μ chains or complete IgM molecules) is displayed in small amounts on B-cell precursors, it may have little functional significance.

Between 13 and 16 days of gestation, there is a dramatic increase in the incidence of committed B-cell precursors in fetal liver. This was apparent from titrations of cells in liquid cultures that could give rise to LPS-responsive B cells (Melchers, 1977b). Using adult thymus cells

as fillers, it was estimated that these expanded approximately 100-fold over this interval. The relative incidence of B-cell precursors capable of giving rise to clonable B cells within 9 days of transfer to irradiated recipients was also determined by titration (Paige *et al.*, 1981a). These increased approximately 10-fold over the 13–16-day interval. Of particular interest is the fact that the frequency of stem cells (CFU-s) and cells capable of giving rise to myeloid progenitor cells (CFU-c) did not change during this period of embryonic liver development (Metcalf and Moore, 1971; Paige *et al.*, 1981a). A perhaps more gradual increase occurred in numbers of cells that could respond in the Klinman splenic focus assay (Teale and Mandel, 1980). Cells displaying the 14.8 antigen also expand during this time (unpublished observations). The problem noted elsewhere in this review in standardizing gestational stages should be sufficient caution to prevent direct comparison of these various data. The results do show, however, that substantial proliferation occurs prior to the emergence of cells with easily detectable sIg.

It has been acknowledged for some time that the bone marrow is a major site of production of small lymphocytes in adult rodents (Osmond and Everett, 1964). Extensive studies employing autoradiography alone or in combination with cell surface marker analysis have contributed much of what we know about the final steps in B-cell formation in adult mammals (Osmond, 1975; Rosse, 1976). When a single injection of radiolabeled thymidine is given to an animal, all cells in DNA synthesis that are exposed to the isotope become labeled. Autoradiography and grain count analysis can then be used to follow the fate of these cells, since the amount of radioactivity is halved by each division and labeled cells are lost only through death or migration of the cells to other tissues. Typical small lymphocytes, which comprise approximately a quarter of marrow nucleated cells, are not immediately labeled, but increasing numbers of them become so with time after pulse exposure. Studies by Osmond and Nossal (1974a,b) first established a relationship between these newly formed, small lymphocytes and cells of the humoral immune system of mice. They found that sIg^+ cells in that site did not themselves divide but were instead derived from recently formed sIg^- small lymphocytes, which in turn were the progeny of cycling large cells. The data suggested that the sIg density gradually increased on these cells and that a random loss (through exit or death) of marrow B cells occurred during this maturational sequence.

Cells with detectable cytoplasmic, but not surface, μ chains in the marrow are both large and small (Cooper *et al.*, 1976). Owen *et al.*

(1977) learned that the large ones were among the cycling marrow lymphocyte population. More recent observations suggest that large, dividing Ig^- cells give rise to large $c\mu^+$ cells, which then may divide only once to yield two daughter cells destined to acquire surface Ig (Landreth *et al.*, 1981a). That the cells that precede Ig-synthesizing cells are cycling is also consistent with the finding that considerable time is required for recovery from hydroxyurea treatment (Rusthoven and Phillips, 1980). It is also noteworthy that $c\mu^+$ cells reappear before sIg^+ cells after cyclophosphamide treatment (Burrows *et al.*, 1978). In other studies, it seemed that receptors for Fc and complement as well as Ia antigens are all acquired soon after sIgM is displayed on small marrow lymphocytes (Yang *et al.*, 1978; Lala *et al.*, 1979).

It should be noted that while marrow is a major site of B-cell formation and includes precursor cells of various stages of maturity, a significant fraction (7–20%) of the lymphocytes in that tissue are long lived and the incidence of such cells tends to increase with age (Miller and Osmond, 1975). Some of these have been accounted for as being recirculating B and T cells. An additional population of recently formed null cells have not been further identified (O. W. Press *et al.*, 1977; Yang *et al.*, 1978). The production of small lymphocytes in murine bone marrow has been calculated to be approximately 10^8 cells per day (Osmond, 1975). This rate is maximal in young adults and subsequently declines with age (Miller and Osmond, 1975). It is not obviously influenced by suppressive doses of anti-μ antibodies (D. Osmond, personal communication) but is abnormally low in gnotobiotic animals (Osmond, 1979). These observations would suggest that numbers of sIg^+ lymphocytes do not feed back to regulate the rate of B-cell formation, whereas environmental antigenic stimulation can play a role. This may relate to other studies that indicate that nonspecific stimulation, perhaps acting via macrophages, causes the sequential transition of newly formed B cells to large cells and then to small typical B cells that have improved response capability in short-term assays (Howard *et al.*, 1978; Shortman and Howard, 1979). The apparent importance of macrophages in augmenting the formation of B cells in culture has been illustrated and discussed elsewhere in this review.

The bone-seeking isotope ^{89}Sr has been used experimentally to ablate bone marrow more or less selectively and test its importance in B-cell differentiation. It was found that such marrow-suppressed animals could support the generation of functional B cells after whole-body irradiation and reconstitution with hematopoietic cells (Phillips and Miller, 1974; Kincade *et al.*, 1975; Rozing *et al.*, 1975). It should be remembered, however, that the grafted cells were in each case

mixtures of undifferentiated stem cells and committed precursors of possibly several kinds. Thus, while it can be said that the marrow is not obligate to the final stages in formation and expansion of B cells, the necessity for stem cells to interact with that tissue has not been evaluated. Much the same problem applies to the designation of any tissue in mammals as the equivalent of the bursa of Fabricius.

There is some controversy as to the role of the spleen in B-cell formation and maturation. For example, $c\mu^+$, sg^- cells are not found in that tissue under normal circumstances or during regeneration from irradiation, although they are detectable in marrow (Burrows *et al.*, 1978; Owen *et al.*, 1979). In contrast, apparently sIg^- cells from spleen were able to give rise to antigen-responsive B-cell clones in the Klinman splenic focus assay (Teale *et al.*, 1979). Lafleur *et al.* (1972a,b) considered the spleen to be a source of large immature "pre-B" cells, but these were described as being sIg^+. In addition, there are small numbers of cells in adult spleen that can be induced to express B-cell markers during short-term culture with appropriate factors (Scheid *et al.*, 1975; Hämmerling *et al.*, 1976). There is reason to believe that many of the newly formed small lymphocytes that leave the marrow migrate to the spleen (Brahim and Osmond, 1970), and some of these might not be fully mature. Also, when immature cells from marrow were injected into the bloodstream, they completed the process of acquiring B-cell markers in the spleen (Ryser and Vassalli, 1974; Yang *et al.*, 1978). Therefore, to reconcile these observations it is only necessary to suppose that the intracellular pool of μ chains declines rapidly after cells leave marrow. An alternative possibility would be that, under certain circumstances, it is possible to form functional B cells without a prolonged period of $c\mu$ expression.

IX. Antigens Expressed on B-Lymphocyte Precursors

A. Antigens Defined by Conventional Antibodies

The field of immunogenetics has revealed a large number of serologically detectable, polymorphic structures on murine lymphocyte surfaces (McKenzie and Potter, 1979). Display of some of these seems to correspond to stages of differentiation, and these have been generally referred to as differentiation antigens (Boyse and Old, 1969). Some, but not necessarily all, may also correspond to particular cell lineages, but many others are shared by cell types that have no obvious common features. Elsewhere in this review, antigens are considered that coincide with B-lineage maturational stages or with the ability of B cells to

respond in various ways, or both. Conventional heteroantibodies have been occasionally used to study murine B- lymphocyte emergence, but standardization of such reagents is extremely difficult (Ryser and Vassalli, 1974; Owen *et al.*, 1975; Gorczynski, 1977). Only very recently have antigens been identified that are expressed on relatively undifferentiated lymphocyte precursors, and such determinants should be invaluable in identifying sites of origin of such early B lineage cells. In addition, these make it possible for the first time to enumerate and manipulate both B cells and their precursors.

Genes within the midportion of the murine major histocompatibility complex (MHC) encode Ia antigens that are expressed on most B cells, subpopulations of macrophages, and some T cells (Goding *et al.*, 1975; Hämmerling, 1976; Schwartz *et al.*, 1976). Cells with detectable cytoplasmic, but not surface, Ig lack detectable Ia; B cells bearing Ia are few in number and express a low density of antigens in fetal or newborn mice, and full expression occurs some weeks after birth (Kearney *et al.*, 1977; Mond *et al.*, 1980a). It has been reported that small numbers of Ia^- B cells are present in adult tissues and a series of induction experiments suggested that these might represent a normal intermediate stage in B-cell maturation (J. L. Press *et al.*, 1977; Hämmerling *et al.*, 1976). Other studies suggest that most B cells express at least small amounts of Ia and that these antigens are acquired in parallel with sIg by newly formed B cells in marrow (Mond *et al.*, 1980b; Lala *et al.*, 1979). Essentially all clonable B cells of adult mice are eliminated by anti-Ia + complement treatment (Kincade *et al.*, 1978b), whereas marrow precursors able to give rise to B cells during liquid culture are not affected (Kincade *et al.*, unpublished observations). These antigens are also not demonstrable on CFU-s or CFU-c of the mouse by cytotoxic elimination (Kincade *et al.*, 1978b; Basch *et al.*, 1977) whereas comparable antigens are present on the myeloid cells of humans (Winchester *et al.*, 1977).

Genes corresponding to several lymphocyte antigens are located in the *Tla* region, distal to the *H-2D* end of the MHC of the mouse (Flaherty, 1978; Klein *et al.*, 1978). In addition to TL antigens, which are normally only displayed on thymocytes, there is a series of antigens called Qa, which are expressed by peripheral lymphocytes (Stanton and Boyse, 1976; Flaherty *et al.*, 1978; Hämmerling *et al.*, 1979). Qa-2 is especially interesting because of its distribution and appearance during ontogeny. It is demonstrable on T cells and B cells belonging to several functionally distinct categories as well as CFU-s and CFU-c (Kincade *et al.*, 1980), but not erythrocytes, brain, kidney, or epidermal cells. The immediate precursors of B cells in adult marrow are also

Qa-2$^+$ (Kincade *et al.*, unpublished observations). However, this antigen is not demonstrable on any cells of fetal or newborn animals, and longer than 4 weeks may be required for full expression on all tissues. That is, both undifferentiated and fully mature cells express Qa in adults, but the time of appearance corresponds with the chronological age of the animal. This can be contrasted with the first appearance of H-2 antigens which have been consistently demonstrated by day 16 of gestation and have a wider cellular representation (Edidin, 1972). There is evidence that Qa-1 and Qa-4 antigens are also displayed on some B-cell populations, but developmental studies have not been done (Nell *et al.*, 1980; Kincade *et al.*, unpublished observations).

Lyb-2 is a well characterized B-lymphocyte alloantigen for which three alleles are known (Sato and Boyse, 1976; Shen *et al.*, 1977; Sato *et al.*, 1977; Taylor and Shen, 1977). The molecule is 40,000–45,000 daltons in size but can be distinguished from MHC-linked antigens by the fact that it is not associated with β-2-microglobulin (Tung *et al.*, 1977). All B cells but not antibody-secreting cells in adult mice display Lyb-2, and the number of positive cells in marrow exceeds the number of sIg$^+$ cells (Yakura *et al.*, 1980; Sato and Boyse, 1976). All clonable B cells in adult tissues express Lyb-2 as do the sIg$^-$ cells in marrow, which can quickly give rise to B cells (Kincade *et al.*, 1980a, unpublished observations). However, the same conditions of immune elimination did not affect the B-cell precursors in 16-day fetal liver. This marker would be the one of choice for studying B cells and their immediate precursors in adult murine tissues, but it would not be suitable for analysis of comparable events during embryogenesis.

B. New Monoclonal Antibodies

The 19B5 antibody described in another section of this review was extremely useful in resolving putative stem cells from the earliest members of the B lineage (Paige *et al.*, 1981a). However, a large proportion of cells in hematopoietic tissues are killed by this cytotoxic antibody (Shinefeld *et al.*, 1980), and it was therefore not suitable for localizing and enumerating relatively rare B-cell precursors. An ideal marker would have a more restricted cellular representation and yet be present from a very early stage of embryonic development.

Another approach to this problem was to prepare a panel of monoclonal antibodies directed to B-cell antigens (Kincade *et al.*, 1981c). Rats were immunized with murine B-lymphoma cell lines. The immune rat spleen cells were then fused with enzyme-deficient, nonsecreting, murine plasma cell tumors. Clones were selected whose antibody bound to the immunizing cell lines but not to erythrocytes,

thymocytes, or T lymphoma cells. Six of these antibodies bound to idiotypic or isotypic determinants of immunoglobulin (Kincade *et al.*, 1981b). A set of three clones were characterized that produced antibody to stem cells (CFU-s), certain myeloid cells, and peripheral T cells as well as B cells. Another set of five clones (typified by the prototype clone, 14.8) made antibodies that similarly bound to normal B cells and established B-lineage cell lines (Table IV). A small proportion of peripheral T cells were recognized by 14.8 antibody, but most nonlymphoid cell types were not. A notable exception is the myelomonocytic leukemia line, WEHI-3, which also expresses the T-cell marker Thy-1 (Ledbetter and Herzenberg, 1979).

Genetic polymorphism has not been apparent for the antigen detected by these antibodies, and it is interesting that two B cell lines did not display this marker whereas all the B cells in the strains of origin were positive. This suggests that some distinctions of normal cells are lost by tumor cells in culture and would correspond to the absence of HLA antigens on a human B-cell line (Singer and Williamson, 1979). Of special interest is the fact that pre-B cell lines were positive, and it is clear that this antigen is displayed on normal B-cell precursors as well (Kincade *et al.*, 1981c, see below).

The tentative conclusion that this group of monoclonal antibodies are directed to the same structure derives from the similarity of immunofluorescence staining patterns obtained by labeling 13 established cell lines and examining them with a FACS. Two antibodies

TABLE IV

DISTRIBUTION OF A B-LYMPHOCYTE ANTIGEN DETECTED BY A GROUP OF MONOCLONAL ANTIBODIES[a]

Positive cell types
Established cell lines: RAW 118 (lymphoma), 18.8 (pre-B lymphoma), 70Z/3 (pre-B lymphoma), W 279 (B lymphoma), 38C-13 (B lymphoma), WEHI-3 (Thy-1$^+$, myelomonocytic leukemia)
Normal tissues: Bone marrow (24%), spleen (49%), lymph nodes (49%), thymus (1%), sIg$^+$ cells (100%), T cells (25% of those in lymph nodes), B-cell precursors (see text)
Negative cell types
Established cell lines: EL-4 (T lymphoma), RAW 264 (macrophage tumor), P 815 (mastocytoma), GM 86 (erythroleukemia), 416B[b] (stem cell line), W 231 (B lymphoma), K56F (B lymphoma)
Normal tissues: Macrophages, erythrocytes, neutrophils, brain, stem cells (CFU-s), myeloid progenitor cells (CFU-c)

[a] Comparable results were obtained by immunofluorescence or absorption testing of antibodies from clones 14.8, 177.17, 385.11, 54.20, and 83.4.

[b] Under certain circumstances, a minority of these cells are antigen positive.

with very similar specificities to these were independently developed in another laboratory (Coffman and Weissman, 1981a,b). The most notable difference in those and the ones summarized in Table IV is that no reactivity for peripheral T cells was found. One of these clones was found to precipitate a 220,000 dalton glycoprotein, and it has been determined that several of the clones in the 14.8 group also recognize a molecule of this size (J. S. Tung and P. W. Kincade, unpublished observations). This is an important finding because it relates these B-lymphocyte antigens to a previously described family of high molecular weight antigens present on T and B cells, which have in turn been found to be identical to a polymorphic lymphocyte antigen system designated Ly-5.

Trowbridge and colleagues (1975) first noted that a major target of rabbit anti-mouse lymphocyte sera was a series of antigens of approximate 200,000 molecular weight. It seemed that B cells must express the larger (220,000 MW) form, and it was later found that all of these shared at least some antigenic determinants (Trowbridge and Mazauskas, 1976; Trowbridge, 1978; Tung *et al.*, 1981). The Ly-5 antigen system was originally thought to encode antigens that were restricted to T cells because of their greater susceptibility to alloantiserum plus complement-mediated lysis (Komuro *et al.*, 1975). Scheid and Triglia (1979) later used a rosetting procedure to demonstrate than an antigen or antigens determined by this genetic locus were displayed on myeloid cells, granulocytes, macrophages, natural killer cells, T cells, and B cells, but not on erythrocytes, teratocarcinoma, or neuroblastoma cells. Michaelson *et al.* (1979) learned that allele-specific anti-Ly-5 antisera precipitated several spleen cell antigens, including a 220,000 MW species. Biochemical, cross-precipitation, and peptide analyses then revealed that the so-called T-200 family of molecules described by Trowbridge actually bear Ly-5 antigen determinants (Omary *et al.*, 1980; Siadak and Nowinski, 1980; Tung *et al.*, 1981). Congenic mouse strains differing for Ly-5 alleles are now available, and there are a number of monoclonal rat anti-mouse antibodies that probably recognize this family of lymphocyte antigens (Trowbridge, 1978; Ledbetter and Herzenberg, 1979). At least four of the 14.8 series of monoclonal antibodies, one developed by Coffman and Weissman (1981b) and one directed at human cells (Dalchau and Fabre, 1981), recognize the member of this family that is preferentially expressed on B cells.

Aside from the cellular representation of the 14.8 series of antigens shown in Table IV, initial studies have been made of the tissue distribution of antigen-bearing cells (Medlock and Kincade, 1981). Antigen-positive cells were not detected in fetal yolk sac,

placenta, or circulating blood whereas they were easily found in the liver at 12 days of gestation. The incidence of these cells rose from approximately 3% to 16% between day 16 of gestation and birth. Examinations of cryostat sections of newborn spleen revealed collections of antigen-bearing cells, 3–4 cell layers thick, distributed around the central arteries of developing periarteriolar lymphoid sheaths (PALS). These cells predominated in PALS of adult spleen and in cortical primary follicles and germinal centers of lymph nodes. Antibody-coated plastic dishes have been utilized to deplete and/or enrich selectively for cells bearing these antigens (Landreth *et al.*, 1981b). Essentially all the B-cell precursors from fetal liver or adult marrow that are capable of giving rise to clonable B cells in the system depicted in Fig. 2 are removed by this adherence procedure, whereas numbers of CFU-s and CFU-c in the suspensions are not diminished. The ability of such suspensions to regenerate functional B cells on transfer to irradiated or immunodeficient mice is dramatically reduced by removal of antigen-bearing cells, but only if the assays are performed within 10 days of grafting. At longer intervals after grafting, the contribution of these particular cells to the rapid expansion in B-cell numbers is not obvious (Table V). This would indicate that 14.8 antigen-positive precursors of B cells may have limited proliferative potential and that some type of less mature precursors account for most of the B cells that emerge from 2 weeks of grafting onward. As noted elsewhere in this review, such cells still have little obvious relationship with CFU-s (Paige *et al.*, 1981a). It should be cautioned, however, that the true proliferative potential of 14.8 antigen-positive cells could have been underestimated if they were incompletely removed by the antibody-coated dishes, and similar studies have to be performed with positively selected cells.

TABLE V
MATURATION OF B-CELL PRECURSORS *in Vitro* AND *in Vivo*

16-Day fetal liver suspension	Clonable B cells recovered after residence in: Liquid culture, 4 days	Unirradiated CBA/N spleen, 10 days	Irradiated CBA/N spleen, 10 days	Irradiated CBA/N spleen, 19 days
Undepleted	2031[a]	349	5202	626,000
Depleted of 14.8^+ cells	127	62	1565	687,000

[a] All data are expressed as numbers of clonable B cells per 10^6 initial cells.

Remarkably enriched lymphocyte populations can be obtained by recovery of cells from 14.8 antibody-coated dishes. One such preparation is shown in Fig. 3. This was made by first depleting sIg^+ B cells from adult marrow with monoclonal anti-μ, allowing the remaining cells to incubate in 14.8 antibody-coated dishes, and finally mechanically removing the specifically adherent cells. This suspension was composed of greater than 97% lymphocytes and included small leptochromatic lymphocytes and large, pale "transitional" cells. Approximately half of these cells contained μ chains. Fetal liver cells from 16-day embryos where only 3% of the cells are 14.8 antigen$^+$ have been similarly enriched so that 97% of the cells were lymphocytes. Some morphological differences have been noted in the fetal and adult cells, and a much lower percentage of the fetal lymphocytes at this

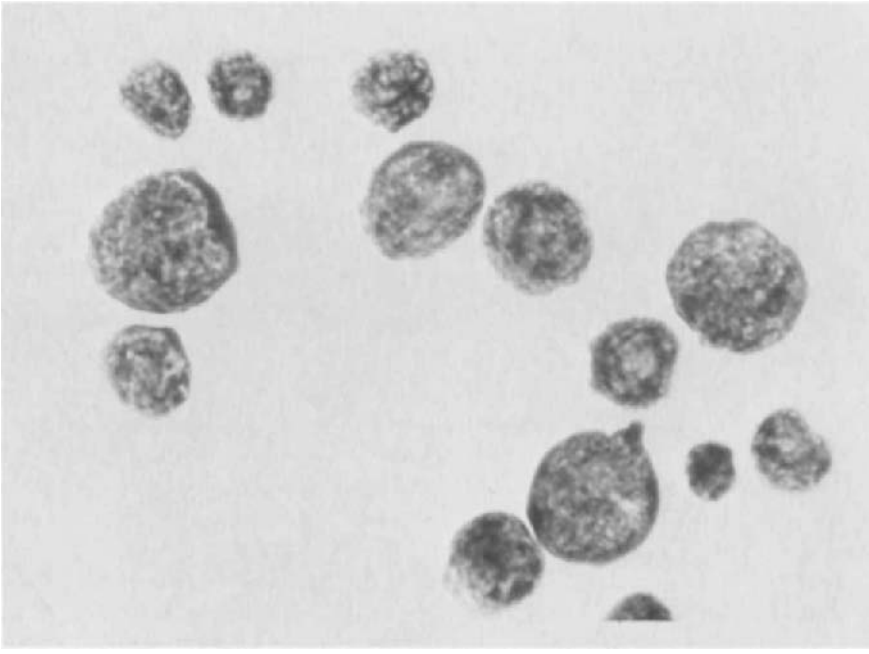

FIG. 3. A positively selected preparation of bone marrow B-cell precursors. B-cell-depleted bone marrow suspensions were allowed to adhere to plastic dishes coated with the 14.8 monoclonal antibody. Specifically adhering cells were mechanically removed, cytocentrifuged, and stained with Jenner–Giemsa stain. All the cells that can give rise to B cells in culture or quickly after transfer to irradiated mice are included in such selected populations. (Preparation made and photographed by K. Landreth and G. Lee.)

stage synthesize detectable μ chains (Landreth *et al.*, 1981b, and unpublished observations). Numbers of these cells considerably exceed numbers of $c\mu^+$ cells in fetal liver and precede the appearance of sIg^+ cells in that site by at least 4 days.

C. A Possible Sequence of Marker Acquisition

The above observations suggest that appropriate markers will now permit the resolution of various B-cell precursors and allow them to be experimentally manipulated. A possible series of maturational stages that might occur between undifferentiated stem cells and the earliest functional B cells is depicted in Fig. 4. The various cell types are distinguished both by their apparent display of surface antigens described in this section as well as by the apparent time required for their transition to B cells under experimental circumstances. The very long time required for substantial B-cell recovery from irradiation in yolk sac-grafted animals and the striking differences in the incidence and locations of CFU-s and B-lineage precursors form the basis for the distinction of the first two cell types. The differential effects of removal of 19B5 antigen$^+$ cells (Paige *et al.*, 1981a) and depletion of 14.8$^+$ cells (Kincade *et al.*, 1981e) on precursors that give rise to B cells in irradiated mice suggest a possible subdivision of these early B-cell precursors. Both or neither of these cell types could be irrevocably committed to differentiate along the humoral immune line. The permanent and selective chimerism achieved with unirradiated CBA/N mice (Paige *et al.*, 1980) argues for the existence of a committed B stem cell,

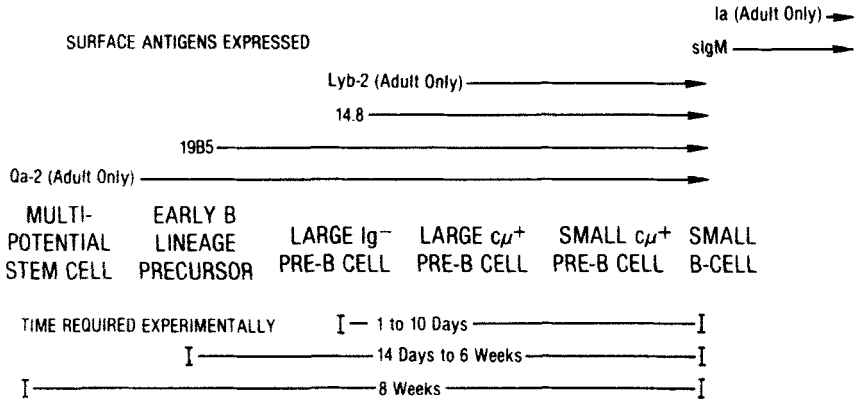

FIG. 4. Some possible categories of B-cell precursors. Cells are resolved by their apparent expression of surface antigens, the time required for formation of functional B cells in experimental circumstances, and postulated changes in size and cytoplasmic μ synthesis (adult marrow only).

but the characteristics of these have not been determined. Cells displaying 14.8 antigen considerably exceed numbers of Ig-synthesizing cells in fetal liver, and in bone marrow approximately half of the sIg^-, 14.8^+ lymphocytes are $c\mu^+$. The remaining cells in the series are so placed because of the presumed events that take place in adult bone marrow and the results of culture and transplantation experiments discussed in other sections.

X. Functional Heterogeneity of B Lymphocytes—Specialized Subpopulations

A. Apparent Cell Sets

The emergence of B cells with antibody specificities for an extremely large array of antigens was documented in an earlier volume of this series (Sigal and Klinman, 1978). B cells are also extraordinarily diverse in terms of physical properties, display of cell surface markers, and responses in various assays. This heterogeneity may be attributable in part to cells differing with respect to maturity, antigen experience, or recent stimulation by specific and nonspecific factors. However, in recent years much has been said about differential responsiveness that might reflect functional specialization of B-cell sets. Any consideration of B-cell development should at least acknowledge this possible dimension of B-cell heterogeneity.

Playfair and Purves (1971) and Gershon (1974) introduced the terms B1 and B2 to denote cells that could be activated to antibody secretion without T-cell help or only in the presence of T-cell help. Many others have examined responses of B cells to the same hapten presented on T-independent carriers, e.g., Ficoll, and on T-dependent carriers, e.g., ovalbumin. Gorczynski and Feldman (1975) observed an apparent difference in the size of cells responding to haptens presented on different carriers. This was true for marrow and spleen but not lymph node cells and might be explained in terms of differential recruitment of immature (Lafleur *et al.*, 1972a) or nonspecifically activated (Howard *et al.*, 1979) cell sets. Jennings and Rittenberg (1976) demonstrated that the magnitude of immune responses to TNP-T4 (a T-cell-independent antigen) and TNP-KLH (a T-cell-dependent antigen) in culture were additive when both antigens were used together and responses to the individual antigens could be selectively inhibited by high doses. In other studies using limiting dilution analysis in liquid cultures, it was shown that simultaneous immunization with two such different antigens stimulated very nearly the sum of the number of B cells that were activated when the antigens were used separately

(Quintans and Cosenza, 1976; Lewis and Goodman, 1977; Tittle and Rittenberg, 1978). Memory B1 and B2 cells are also differentially affected by allogeneic T-cell help (Golding and Rittenberg, 1980). More recently it was found that hapten-specific B-cell sets could also be independently stimulated with presentation on different carriers in the Klinman splenic focus assay (Fung and Kohler, 1980).

Mosier and colleagues (1977, 1979) studied the appearance of B cells in ontogeny that responded to different T-independent (TI) antigens. On the basis of these findings and other distinctions, they proposed subdivision of these antigens into two types. Type 1 includes haptenated *Brucella* or LPS, and cells responding to them emerge by the time of birth and are present in partially immunodeficient CBA/N mice. Type 2 includes haptens conjugated to Ficoll, dextran, and pneumococcal vaccine, and responsiveness to them develops some days later. Among other differences, antigens in the first category are taken to be good mitogens. TI-1 antigens apparently can elicit antibody from the same subsets as T-dependent (TD) antigens, but in a T-cell independent fashion. Galanaud *et al.* (1975, 1976) found that responses to nonmitogenic antigens or haptenated SRBC were sensitive to the drug azothioprine, whereas responses to mitogenic carriers like LPS were resistant. Similarly, Kunkl and Klaus (1980) found that the drug cyclosporin A can be used to inhibit selectively antibody production to DNP–Ficoll without impairing responses to DNP–LPS or DNP–*Brucella*.

Independence of B-cell sets in formation of antibody to hapten presented in various ways has been demonstrated with primary (IgM) and memory (IgG) responses (Quintans and Cosenza, 1976; Tittle and Rittenberg, 1978). Thus, there may be at least four populations of B cells (B1 virgin, B2 virgin, B1 memory, and B2 memory) with similar antibody specificities but distinct triggering requirements. However, if there is selectivity at the level of antibody responses, the same does not hold for proliferative responses. There are circumstances in which antigens of one type cause expansion (priming for memory responses) in cells that would not make antibody to them (Tittle and Rittenberg, 1980; Mosier, 1978; Scott *et al.*, 1979). The complexity of this problem is evident by the fact that it has been necessary to evoke even more populations of B cells to explain certain findings (Fung and Kohler, 1980; Tittle and Rittenberg, 1980).

B. Implications of the CBA/N (X^{id}) Mutation

Many observations and speculations on functional B-cell diversity center on studies of partially immunodeficient CBA/N strain mice.

These were first discovered and described on the basis of their X chromosome-linked inability to form antibody to pneumococcal S III antigen and low serum IgM levels (Amsbaugh *et al.*, 1972, 1974). The mutation was later mapped and termed X^{id} (Berning *et al.*, 1980). It was soon learned that CBA/N mice were unresponsive to certain other TI antigens whereas at least some antibody was made on immunization with TD antigens (Scher *et al.*, 1973, 1975b; Janeway and Barthold, 1975; Cohen *et al.*, 1976). However, responses were measured to the TI-1 type of immunogens discussed above (Mosier *et al.*, 1976; Mond *et al.*, 1978).

Newborn CBA/N mice have approximately normal numbers of splenic sIg^+ cells, but these fail to develop to normal adult values (Kincade *et al.*, 1978a; Scher *et al.*, 1975a). Furthermore, the B cells of adult X^{id} mice lack many of the characteristics associated with normal mature populations. They develop a persistent population of sIg^+ cells that have large amounts of μ relative to δ and that would be typical of spleen cells of young normal mice (Finkelman *et al.*, 1975; Scher *et al.*, 1976, 1980). Ia antigens are expressed on these cells, and on average the density is higher than normal (Scher *et al.*, 1975a; Kincade *et al.*, 1978a; Mond *et al.*, 1980a). Spleen cells from adult defective animals neither provoke proliferative responses across minor histocompatibility differences nor respond to stimulation by intact anti-Ig antibodies, whereas normal mature B cells do (Ahmed and Scher, 1976; Sieckman *et al.*, 1978).

Because CBA/N mice fail to develop many of the B-cell populations and functional characteristics of normal adult mice, it seemed for a time that the mutation might represent a maturational block in a single lineage of B-cell differentiation. According to this interpretation, these mice would possess all B-cell functions that are proximal to this point and lack all those that emerge later in the sequence. Further, their B cells might be representative of intermediate stages in differentiation in adults and typical of B cells present at any one time in immature animals. The finding that B cells capable of colony formation in semisolid agar were never detectable in any tissue of fetal or adult CBA/N mice was not easily reconciled with this view (Kincade, 1977). As detailed elsewhere in this review, the clonable B cells that emerge in the liver of normal embryos at 17 days of gestation are "immature" in the sense that Ia, Qa-2, and IgD are not expressed, and the cells are hypersensitive to capping of their surface Ig. Maturation of B-lineage cells within semisolid cultures seems to be minimal, and B-cell function was most efficiently restored in unirradiated CBA/N mice by grafting with fetal liver cells (Kincade *et al.*, 1978a; Kincade and Paige,

1979). These observations suggested that the mutation of these mice must affect an early stage of B-cell development.

The disease results from an X-linked mutation, and, according to the Lyon hypothesis (1974), one of a pair of X chromosomes in heterozygous female mice should be randomly inactivated in somatic cells during embryonic life. Such animals should therefore be mosaics of normal and defective B cells which in turn arose from equal numbers of normal and defective stem cells. The incidences and total numbers of clonable B cells in young female offspring of the cross CBA/N × CBA/H were almost exactly half that of their male littermates (Kincade *et al.*, 1978a). In more mature mice, numbers of these cells tended to increase, presumably because of the influence of environmental antigen stimulation. A similar gene dosage effect was noted in other studies employing heterozygous carrier female animals (Amsbaugh *et al.*, 1972; Scher *et al.*, 1975a).

The deficiency in clonable B cells did not correspond well with numbers of sIg^+ cells in the F_1 mice, and there was reason to suspect that many of the residual B cells of CBA/N mice are intrinsically defective (Kincade *et al.*, 1978a). B cells of these mice could be activated by several B-cell mitogens, but only when the cells were held at high density in liquid cultures (Kincade, 1977). When activated blasts were dispersed in semisolid agar cultures, they died within a few days without further proliferation. Artificial mixtures were prepared between CBA/N and normal CBA/H-T6T6 spleen cells and then cultured at different cell densities with LPS. The proportion of responding normal cells, as evidenced by T6T6 marked metaphases, steadily increased as the cultures became more dilute. From the percentage of cells that proliferated under the most optimal conditions, it appeared that CBA/N mice had one-third to one-fifth as many responsive B cells as the normal mice (Kincade and Paige, 1979). Others reported an extraordinarily low incidence of LPS responsive cells using limiting dilution liquid culture conditions (Huber and Melchers, 1979).

There have been other suggestions that the T-cell-dependent antibody responses of these mice were not completely intact. Scher and colleagues (1979), for example, found that, while primary IgM responses to SRBC antigen were subnormal, the primary IgG titers to this antigen were practically nil. This was substantially increased by priming, but others had noted that a particular, presumably high-affinity, component of HRBC cross-reactive antibody was missing from SRBC responses of these mice (Gershon and Kondo, 1976). The avidity question was addressed in another series of experiments where hapten T-dependent carrier conjugates were employed, and it was considered to be normal (Stein *et al.*, 1980).

The tolerance susceptibility of CBA/N B cells has also been examined. When B cells from adult animals were exposed to TNP–hapten presented on LPS before challenge in liquid cultures with the same antigen, CBA/N cells were more tolerance sensitive than those of normal mice (McKearn and Quintans, 1980). However, normal tolerance thresholds were noted when TNP–*Brucella* was employed as tolerogen and immunogen. Metcalf *et al.* (1980b) used similar haptens presented on mouse immunoglobulin or ovalbumin to tolerize before testing residual responses in the Klinman system with the T-dependent antigen, hapten–KLH. Conditions were used such that virtually all the hapten-specific B cells from newborn mouse spleen and less than 10% of adult B cells would be tolerized. Adult CBA/N cells were intermediate in sensitivity, and their resistant clones comprised approximately a third of the response.

CBA/N mice do not make IgM antibodies to the hapten phosphorylcholine (PC) on primary immunization, even when it is presented on TI-1 type carriers, to which they are generally good responders (Mond *et al.*, 1977). However, these mice do mount substantial titers of IgE anti-PC antibodies during primary immune responses and can make normal amounts of IgG or IgA antibody on repeated challenge (Kishimoto *et al.*, 1979; Clough *et al.*, 1981).

It was recently found by one group that responsiveness to a particular moiety of endotoxin, the mitogenicity of Fc fragments of human immunoglobulin, and the antigen TNP–Ficoll were all substantially recovered in aged CBA/N mice, whereas recognition of the mitogen 2-ME was never acquired (Fidler *et al.*, 1980). It was suggested that development of certain B-cell functions might be severely delayed rather than aborted entirely. A slight retardation in the development of B cells responding to TI-1 type antigens was also noted in neonate defective mice (Mosier *et al.*, 1977). In contrast, clonable B cells were never detectable in spleen, lymph nodes, or bone marrow of X^{id} mice, even when the animals were over 2 years old (Kincade, unpublished observations).

Ia antigens and Qa-2 are generally not confined to a particular functionally restricted subpopulation of B cells although cytotoxic conditions can be found where some types of responses are more sensitive than others (Frelinger *et al.*, 1978; Kincade *et al.*, 1980b; Mond *et al.*, 1979; Bick and Shreffler, 1979). The availability of CBA/N mice, however, stimulated the development of a number of antibodies that discriminate B-cell sets. Huber *et al.* (1977) immunized CBA/N mice with normal cells to produce a noncytotoxic antiserum that reacted with approximately half the normal adult B cells. This antiserum (anti-Lyb-3) also had an adjuvant effect on suboptimal dose immuniza-

tions with SRBC and seemed to obviate the requirement for T-cell help. Ahmed *et al.* (1977) absorbed an alloantiserum with X^{id} cells until a similar pattern of reactivity was obtained and this serum (anti-Lyb-5) was cytotoxic. Among the specificities in the anti-Lyb-5 serum was one that blocked *in vitro* responses to haptenated Ficoll, and polymorphisms of this determinant segregated along with genes for Ig heavy-chain allotypes (Subbaro *et al.*, 1979). This antigen was designated Lyb-7. All of these determinants have in common their absence from CBA/N B cells, late appearance in ontogeny, and expression on only a proportion of B cells in adult mice. It has generally been true that antigen-positive cells can mediate immune responses of which CBA/N mice are incapable. An antigen with similar distribution was discovered to be a private specificity encoded by *I* region genes (Huber, 1979). Very recently a monoclonal antibody was prepared that binds to most CBA/N B cells and B cells in neonates but labels only some of the B cells (which are Lyb-5$^-$ in normal adult spleen (J. Kung, personal communication). This marker might therefore correspond to those (B1) populations that are not detected by the Lyb-3, Lyb-5, Lyb-7, and IaW39 antisera.

The T cells of immune-deficient CBA/N mice are reportedly normal in mitogen responses, in cytotoxic activity, and in helping T-dependent humoral responses (Scher *et al.*, 1975b, 1979; Janeway and Barthold, 1975). T cells from these mice did not, however, provide nonspecific augmentation to a polyclonal LPS response (Goodman and Weigle, 1979b). CBA/N macrophages are normally sensitive to endotoxin, release normal amounts of LAF, prostaglandin, and factors that enhance proliferation of B-cell clones, and they are equivalent to other mice in acting as accessory cells for pre-B cell maturation (Kincade, 1977; Rosenstreich *et al.*, 1978; Kincade *et al.*, 1981a). Similarly, numbers of CFU-s, CFU-c, and CFU-m (megakaryocyte) are normal, and irradiated X^{id} mice support the formation of B cells from immature precursors as well as CBA/H mice (Kincade, 1977; Paige and Kincade, unpublished observations).

The lesion of CBA/N mice may affect important interactions between particular B-cell sets and accessory cells. It was recently found that a proportion of normal B cells bind to adherent macrophages in culture and that X^{id} mice lack such B cells (O'Toole and Wortis, 1980). It was reported that macrophage-presented antigen selectively activates Lyb-5$^+$ B cells and that CBA/N mice are unresponsive to antigen delivered in this way, even when antigens are chosen to which they are responsive in soluble form (Boswell *et al.*, 1980a,b). It should be noted that in another study the most efficient cells in presenting TNP–Ficoll were B cells rather than macrophages (Kirkland *et al.*, 1980).

Some similarities have been noted in the human Wiscott–Aldrich syndrome and the CBA/N lesion (Cooper, 1976). In both immune deficiencies, responses to complex polysaccharide antigens are poor. Levels of IgE are elevated in patients with this disease, and X^{id} mice can mount impressive primary IgE antibody responses (Waldmann *et al.*, 1972; Kishimoto *et al.*, 1979). The human disease is also characterized by thrombocytopenia. The defective mice were recently found to have a slightly reduced platelet number, and these showed diminished ADP and thrombin-induced aggregation in comparison to controls. Electron microscopic examination of platelets from these animals did not, however, reveal any ultrastructural abnormalities (M. Long and P. Kincade, unpublished observations).

All of these observations point to the possible existence of functionally restricted B-cell subpopulations. It remains to be seen whether these are stable cell sets comparable to T cells specialized for help or suppression (Cantor and Boyse, 1975). If they are, we need to address the question of their developmental interrelationships. It has been proposed that such commitment might occur early in development to yield separate lineages of differentiating B cells (Kincade, 1977; Kincade *et al.*, 1978a). One analysis of irradiated mice grafted with limiting numbers of neonatal spleen cells would be consistent with the existence of separable categories of B cells at this early stage (Quintans *et al.*, 1979). It is also not clear whether the antibody specificity repertoires of different B-cell populations would be equivalent. In the case of B1 and B2 responses to the hapten PC, the same idiotype was produced (Quintans and Cosenza, 1976; Fung and Kohler, 1980). However, it is now known that idiotypes are not necessarily clonal markers (Reth *et al.*, 1979; Estess *et al.*, 1980), and either fine specificity analysis of antibodies elicited from different B-cell sets or new monoclonal antibodies directed to "idiotopes" should be employed to study this question.

The apparent B-cell heterogeneity discussed in this section might be, at least in part, attributable to relatively trivial differences in maturity, cycle status, or recent encounters with specific and nonspecific stimuli (Shortman and Howard, 1979; Melchers *et al.*, 1980). To this end, the physical characteristics and response kinetics of cells that are detected in various assays should be rigorously studied. Solution of this problem also requires a better understanding of the functional relationships between categories of B cells and helper cells. Recent observations indicate that T-cell dependency is a matter of degree and some T-cell-derived factors influence even those responses that were formerly thought to be completely T-cell independent (Goodman and Weigle, 1979a; Mond *et al.*, 1980b).

The CBA/N model has been of unquestioned utility in dissecting B-cell diversity, and observations made with it should continue to fuel speculation in this area. However, it seems unlikely that the mutation of these mice neatly partitions stages of maturity such that all of their B cells are typical of subpopulations found in young normal mice. On the contrary, the mutant gene product may be expressed widely in the humoral immune system and yet differentially affect various responses.

XI. Important Uncertainties and Promising Sources of New Information

A major objective of this review is to call attention to commonly held assumptions that are inadequately supported by experimental observations. It is therefore fitting to conclude with a restatement of these and other important questions in the context of new approaches that seem capable of elucidating them.

At the top of such a list would be the issue of the nature and number of stem cells. The use of irradiation-induced chromosome markers has provided evidence for stem cells common to both lymphoid and hematopoietic cells, T-lineage restricted cells, and myeloid-committed stem cells. In addition, the grafting experiments described with CBA/N mice could be used to argue for the existence of committed stem cells of the B lineage that have extensive proliferative/differentiative potential. However, these studies provide no evidence for the relative frequency of stem cells of the various kinds and major questions remain about the efficiency with which they would be detected in various assays. The analysis of marker distribution in clonal human malignancies can also suggest different types of stem cells but provides no insight into their incidence and normal behavior. No doubt our repertoire of markers will continue to increase, and these will permit substantial enrichment of particular categories of cells. For example, cells bearing one antigen but lacking several others can be brought to relative homogeneity by sorting procedures and then placed in several *in vivo* and *in vitro* situations. The obvious caveat is that the act of labeling, selecting, and transferring these cells may influence their behavior. However, we should soon know the minimum capabilities of many cells with given cell surface marker phenotypes.

If our knowledge is incomplete about the types of stem cells that might exist, we have considerably less idea as to their life histories and the progression of their progeny through various stages of differentiation. It appears that some of the B-cell differentiation events must

occur very early in embryonic life. However, it is not clear whether committed precursor cells arising at that time persist or are replaced by similar cells arising at later stages of development. Documenting the time required for a given cell type to give rise to B cells on transfer to irradiated recipients tells us nothing about the kinetics of progression through various compartments under homeostatic circumstances. It has been possible in the case of anemic W mice and B-cell-deficient CBA/N mice to achieve chimerism without the use of irradiation. Appropriate exploitation of these models could provide insight into the homing and fate of selected cell populations under more normal conditions than those pertaining in whole body-irradiated animals.

It is now clear that a number of the processes involved in B-cell formation can take place in cell culture. Since all the tissues and cell suspensions that have been used to initiate such cultures already contained some cells with B-lineage characteristics (e.g., synthesis of Ig), we cannot be certain whether stem cells were committed to this pathway *in vitro*. Appropriate application of new monoclonal antibodies that recognize B-lineage antigens should make it possible to resolve this point and then move on to the more interesting question of what signals are necessary to achieve this important differentiation step.

The cell culture approach also should make it possible to define the nature and minimal number of factors and interactions necessary for progression of cells through various differentiation stages. However, it is now obvious that this requires both a better understanding of the constituents of culture medium (particularly the fetal calf serum) and a high degree of enrichment of the cells of interest. For example, positively selected cells that lack sIg but bear B-lineage markers should be placed in association with physically separated accessory cells of different types (nonadherent bone marrow, peritoneal exudate macrophages, thymocytes, etc.) to determine which allow the maximum expansion and/or differentiation of these precursors in culture. It would simplify interpretation of the results of such experiments if serum-free medium and purified factors (lymphokines and monokines) could be successfully employed.

The problems in identifying sites in mammals that are functionally equivalent to the avian bursa of Fabricius have been discussed in some detail. More important than implicating a particular tissue, however, is describing the microanatomy associated with the emergence of early B-lineage cells. The precedent of studies describing close associations of stromal elements with differentiating erythroid, myeloid, and thymus cells (Bessis and Breton-Gorius, 1962; Curry and Trentin, 1967; Trentin, 1971; Westen and Bainton, 1979; Wekerle and Ketelsen,

1980) suggest that the location of B-lineage cells within the architecture of hematopoietic tissues is a timely question. The structure and development of bone marrow have been thoroughly described, and it is of some interest that gradients of stem cells and proliferating cells may exist from the endosteal surface to the center of the bone shaft (Weiss, 1976; Chen and Weiss, 1975; Lord *et al.*, 1975; Shackney *et al.*, 1975). Preliminary immunofluorescence observations suggest that Ig-synthesizing cells are scattered in sections of embryonic liver (E. S. Medlock and P. W. Kincade, unpublished observations). However, these may have migrated from their point of origin, and the structure of cells proximal to them needs to be determined. Furthermore, the availability of new monoclonal antibodies directed to perhaps earlier appearing and certainly more frequently expressed markers might be uniquely advantageous to such analyses. It would seem probable that either through chance or deliberate design, some of the new monoclonal antibodies that are being produced by many laboratories will be specific for determinants on consitituents of the putative microenvironments in which B cells and their precursors are formed. Once the basic microanatomy of cellular interactions is described, we should be in a position to ask questions about the nature of cellular recognition processes that govern the migration of stem cells and their progeny (Butcher *et al.*, 1979).

The advent of cell fusion technology (Kohler and Milstein, 1976) provided a seemingly easy solution to resolution of complex cellular populations, and one could only encourage the preparation of additional monoclonal antibodies to additional cell surface antigens. It should be noted that such antigens are not always distributed in a predictable fashion (Springer, 1980). For instance, the cellular representation of Thy-1 antigen is quite different in rats and mice, and in both species it is present on cells with few obvious common features (Crawford and Goldschneider, 1980). Despite this, antibodies to this antigen have been used to highly enrich stem cells of the rat (Goldschneider *et al.*, 1980), and even antibodies directed to broadly distributed markers can be used to advantage (Paige *et al.*, 1981). Many of the more useful antibodies now available for distinguishing functionally restricted B-cell sets were either made in immunodeficient animals or rendered specific through extensive cross-absorption protocols. Almost by definition, these may not be uniform and of sufficient avidity for some purposes. It is hoped that the conventional reagents will soon be replaced by monoclonal antibodies of similar as well as additional reactivities. Many of the highly specific functions of precursors and mature B cells must be mediated by cell-surface recep-

tors for factors and aside from the utility of the new monoclonal reagents, these may be useful in identifying some of these structures.

A provocative recent example of antibodies directed to functional receptors comes from the work of Coutinho and colleagues (1978, 1980; and Forni *et al.*, 1979). It was found that anti-idiotypic antibodies (including some monoclonal antibodies) cross-reacted with some non-Ig cell surface determinant and that such reagents could promote the maturation, proliferation, and terminal differentiation of cells of the B lineage. This finding has potential implications for the influence of maternal and adult idiotypic networks on B-cell development. Among the unexpected observations was the fact that $c\mu^+$, sIg^- "pre-B" cells were unlabeled by the anti-idiotypic antibodies whereas functional B-cell precursors in fetal liver and sIg^- marrow populations were. Until these observations are reconciled, it remains possible that cells synthesizing only μ chains are not representative of an obligate stage of B-cell differentiation.

The new observations on molecular aspects of Ig gene rearrangement and expression need to be correlated with normal changes in cell surface phenotypes and stages of differentiation. The possibility of error in gene splicing has been suggested, and it remains to be determined how many cells undergo abortive rearrangement of both chromosomes and what their fate would be. Some insight into these questions should come from isolation of cells bearing B-lineage antigens at various stages of development and examining their Ig gene configurations.

The CBA/N model is likely to provide interesting observations for some time to come, and there are other single-gene mutations (including Sl, W, Me, Lpr) that have not been adequately studied from the point of view of early B-lineage abnormalities. Development of autoimmune disease in the New Zealand mouse strains is certainly attributable to more than one gene, but the model could provide insight into important immunoregulatory phenomena (Kincade, 1981b). For instance, preliminary observations suggest that functional B cells are not formed normally in marrow cultures under circumstances that seem optimal for other mouse strains (H. Jyonouchi and P. W. Kincade, unpublished observations). Analyses of naturally occurring human immunodeficiency and autoimmune diseases should also continue to contribute to our understanding of fundamental problems (Good and Finstad, 1980).

Established cell lines and tumors have been given less attention in this review than might be justified (Warner *et al.*, 1979; Ralph, 1979). Particularly interesting as experimental models are those that seem not

to be completely frozen in differentiation, but rather are responsive to certain inductive stimuli (Fu *et al.*, 1978; Paige *et al.*, 1978; Boss *et al.*, 1979; Knapp *et al.*, 1979; Siden *et al.*, 1979; Burchiel and Warner, 1980; Gronowicz *et al.*, 1980; Isakson *et al.*, 1980). It was very recently learned that an established, nonmalignant cell line that can form CFU-s and enter certain hematopoietic lineages (Dexter *et al.*, 1979) is able to give rise to cells expressing lymphocyte alloantigens within irradiated recipients (M. P. Scheid and P. W. Kincade, unpublished observations).

Fetal tissues provide a convenient source of B-cell precursors that are not contaminated with mature B cells, and it is important to study the very earliest B-lineage differentiation events. However, it is not necessarily true that fetal precursor cell characteristics and mechanisms through which B cells are formed in embryos are identical to those of adult bone marrow. It was noted, for example, that fetal liver precursors colonized the marrow of adult CBA/N mice better than normal marrow cells and tended to be more rapidly sedimenting (Paige *et al.*, 1979, 1981a). It was demonstrated here that adult type pre-B cells were absolutely dependent on adherent accessory cells for maturation *in vitro* whereas this dependence was not obvious for fetal liver cells. At least two antigens (Lyb-2 and Qa-2) that were found on adult marrow precursors were not observed on comparable embryonic cells and newly formed B cells in marrow were Ia^+, whereas fetal B cells were not. Enriched cells from fetal liver that bear B-lineage antigens differ morphologically from the majority of cells similarly isolated from adult marrow (K. Landreth, unpublished observations). Some of these differences may relate to differences in the total cellular composition of fetal and adult hematopoietic tissues rather than to intrinsic differences in the precursor cells. It may also be that cells that predominate in embryos represent relatively rare intermediate stages of adult B-cell differentiation. However, the possibility must be seriously considered that the early waves of B-cell formation that occur in fetal tissues may take place through processes somewhat different from and perhaps simpler than those that occur in adult life.

Finally, models have been proposed for a likely sequence of size changes and marker acquisition by differentiating B cells (Strober, 1975; Nossal *et al.*, 1977; Hämmerling *et al.*, 1976; Scher *et al.*, 1977). The principal limitations of these is that they do not detail embryonic events, explain the apparent functional heterogeneity of all B-cell sets, or identify those changes and cellular interactions that are obligate to the emergence of B cells. Data derived from all the sources cited above should soon provide compelling reasons for updating those hypotheses.

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Structural Aspects and Heterogeneity of Immunoglobulin Fc Receptors[1]

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I. Introduction

Receptors for immunoglobulin (Ig) on cells of the immune system provide an important link between the humoral and cellular aspects of immunity by enabling effector cells, such as macrophages and polymorphonuclear leukocytes, to identify microorganisms and other antigens recognized as "foreign." In addition, it seems likely that Ig receptors serve other, more complex functions in the immune response since the receptors are also found on the surfaces of a variety of immunocompetent cells such as T and B lymphocytes. The involvement of Fc receptors in such phenomena as idiotype suppression can be inferred from the necessity of an intact Fc domain on the anti-idiotypic antibody for suppression and generation of suppressor T cells. Thus, the study of Fc receptors and the cellular interactions they can trigger is of clear importance to an understanding of the regulation of the

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immune response. At the level of the individual cell, binding of Ig to its receptor can modulate cell behavior dramatically. Cross-linking of the IgE Fc receptor, for example, causes explosive degranulation of mast cells and basophils, with important physiological consequences. Of no less interest are the macrophage functions triggered by interaction of IgG Fc receptors with immune complexes or opsonized particles. Secondary events to these receptor–ligand interactions include phagocytosis or internalization of the bound ligand (Silverstein *et al.*, 1977), antibody-mediated cytolysis of target cells (Cerrottini and Brunner, 1974), and the release of soluble mediators of inflammation including prostaglandin (Humes *et al.*, 1980), leukotriene (Rouzer *et al.*, 1980), and hydrolytic enzymes (Gordon *et al.*, 1974; Werb and Gordon, 1975).

The mechanisms by which immune complexes mediate these activities through interaction with Fc receptors are not known. Clearly, detailed information on the structure of the receptor molecule(s) is necessary to understand these processes in detail. Accordingly, in the last few years an increasing number of investigators have studied the properties of Fc receptors (FcR) from a number of cell types. In this review, we will attempt to evaluate and clarify work on FcR structure as well as to examine the recent data that suggest the existence of FcR heterogeneity. We will limit our consideration largely to results published since the comprehensive review by Dickler (1976).

II. Specificity and Heterogeneity of Fc Receptor Activity

A. Mouse Fc Receptors for IgG_1, IgG_{2b}, and IgG_{2a}

1. Subclass Specificity and Trypsin Sensitivity

The first observation that mouse macrophages have more than one class of FcR for IgG was by Kossard and Nelson (1968), later confirmed by Askenase and Hayden (1974). In these studies, the formation of rosettes with antibody-coated erythrocytes was examined using both normal and trypsin-treated macrophages. Erythrocytes sensitized with "early" antisera failed to bind to trypsinized macrophages although they formed rosettes with untreated cells. On the other hand, erythrocytes sensitized with hyperimmune mouse serum formed rosettes regardless of trypsin treatment of the macrophages. Askenase and Hayden (1974) found further that rosette formation with "early" antisera was inhibited by an IgG_{2a}-specific antiserum, suggesting that the

mouse IgG subclass that bound to the trypsin-sensitive site was IgG_{2a}. More recently, binding studies of monomeric ^{125}I-labeled mouse myeloma proteins to mouse macrophages and macrophage-like cell lines confirmed the presence on macrophages of a trypsin-sensitive binding site specific for IgG_{2a} (Unkeless and Eisen, 1975; Heusser *et al.*, 1977). These studies were extended by Diamond *et al.* (1978), who employed different subclasses of monoclonal anti-erythrocyte antibodies to show that IgG_{2a}-coated red cells did not bind to trypsinized macrophages, in contrast to IgG_{2b}-coated cells. Thus, the specificity of IgG_{2a} for a trypsin-sensitive receptor was not affected by the formation of immune aggregates. Similar findings were also obtained using IgG_{2a} covalently attached to CNBr-activated Sephadex beads (Unkeless, 1977).

2. *Competition Experiments*

Another set of observations providing evidence for the existence of multiple FcR's on mouse macrophages is the demonstration of lack of inhibition of binding of one IgG subclass by different subclasses of IgG. Walker (1976) found that binding of labeled monomeric mouse IgG_{2a} to macrophages or macrophage cell lines was not inhibited by aggregated IgG_{2b} or IgG_1, nor was binding of labeled aggregated IgG_{2b} inhibited by aggregated IgG_1 or monomeric IgG_{2a}. The lack of competition by IgG_{2a} and aggregated IgG_{2b} was interpreted as evidence for the existence of separate sites. Similar results were reported by Heusser *et al.* (1977), who reached much the same conclusion—that monomeric IgG_{2a} bound to one site and aggregated IgG of all subclasses, including IgG_{2a}, bound to a distinct, trypsin-resistant site. Apart from the question of whether IgG_{2a} aggregates bind to a trypsin-resistant site, these results are in general agreement with conclusions drawn by Diamond *et al.* (1978) and Diamond and Scharff (1980), who found that binding to macrophages of IgG_1 and IgG_{2b}-coated erythrocytes could not be inhibited by aggregated IgG_{2a}, nor IgG_{2a}-coated erythrocytes by aggregated IgG_1 or IgG_{2b}. Based on these competition experiments, it appears that there are at least two receptors, one specific for IgG_{2b}/IgG_1 aggregates, and another, trypsin-sensitive receptor that binds mouse IgG_{2a}. Matters may be yet more complex, however. Mouse IgG_3, which does not bind as a monomer (Unkeless and Eisen, 1975), binds when presented as an immune complex. Diamond and Yelton (1981) reported that binding of erythrocytes coated with an anti-erythrocyte IgG_3 to macrophages could not be inhibited by aggregated IgG of any mouse subclass except IgG_3, suggesting the existence of a separate (trypsin-resistant) IgG_3 receptor on mouse macrophages.

Other evidence, however, indicates that the specificity of these Fc receptors is not absolute, especially when monomers of myeloma proteins are used to compete against each other. Segal and Titus (1978) reported that monomeric mouse IgG_{2a}, IgG_{2b}, and IgG_1 compete equally for binding to receptors on the $P388D_1$ macrophage cell line. A similar study was published by Haeffner-Cavaillon *et al.* (1979), who found preferential association of IgG_{2a} with one receptor and IgG_{2b} with the other, albeit with marked cross-specificity. Interestingly, they found that canine IgG discriminated between the IgG_{2b}/IgG_1 and IgG_{2a} Fc receptors, since it would inhibit only mouse IgG_{2a} binding. Unlike Segal and Titus (1978), they found no competition by mouse IgG_1. The reason for the discrepancies between these results and the findings discussed above, which suggest the existence of two functionally distinct receptor sites, is not clear. However, a possible explanation may relate to the use of monomeric vs. aggregated IgG in these binding and competition experiments. The high degree of cross-reactivity of monomeric IgG_{2a} and IgG_{2b} binding (Segal and Titus, 1978; Haeffner-Cavaillon *et al.*, 1979) was not observed when immune complexes or aggregates were employed (Diamond *et al.*, 1978; Walker, 1976; Heusser *et al.*, 1977). Thus, it is possible that complex formation results in alterations in affinity or in the actual conformation of the Fc domain of IgG such that subclass specificity in binding to cellular FcR is restored.

3. *Fc Receptor-Negative Mutants and Variants*

Although the binding and competitive inhibition studies are not unequivocal, the clear consensus is that mouse macrophages have at least two, and perhaps three, distinct FcR activities—one that is trypsin-resistant and appears to bind IgG_{2b} and IgG_1 aggregates preferentially, a second that is sensitive to trypsinization and specific for IgG_{2a}, and a third that binds IgG_3 aggregates. If there are indeed distinct FcR proteins, it should be possible to differentiate among them genetically by deriving cell lines in culture that lack one or the other receptor activities. To this end, Unkeless *et al.* (1979) have selected for mutants that bound few IgG-coated erythrocytes in populations of mutagenized J774 macrophages. A series of clones were isolated that were largely deficient in the ability to bind IgG_{2b}-coated erythrocytes but exhibited a normal capacity to bind monomeric IgG_{2a}. The defect was, however, pleiotropic. Many of these clones also had a partial C3b receptor defect and were characterized by abnormal surface morphology and reduced adhesiveness.

Another intesesting set of variants was isolated by Diamond *et al.* (1979), who used a variation of the clever "Trojan horse" selection

scheme originally devised by Muschel *et al.* (1977). In order to isolate variants incapable of ingesting IgG_{2a}-coated erythrocytes, the erythrocytes were loaded with the toxic adenosine analog tubercidin, and fed to J774 cells. Some of the surviving clones obtained in this manner were able to ingest mouse IgG_{2b}- and IgG_1-coated erythrocytes normally, but although IgG_{2a}-coated cells bound, they were not ingested (Diamond and Scharff, 1980). These results demonstrate that IgG_{2b}/IgG_1 and IgG_{2a} receptors are independently coupled to the signal-transducing mechanism or contractile apparatus of the macrophage, which facilitates the phagocytosis of FcR-bound opsonized particles. Ordinarily, all mouse IgG subclasses are known to mediate the ingestion and lysis of erythrocytes (Ralph *et al.*, 1980; Diamond and Yelton, 1981). Apparently no cell lines deficient in IgG_{2a} binding per se were isolated using the tubercidin selection system. However, one cell line variant of J774 has been isolated that is selectively deficient in the ability to bind IgG_3-coated erythrocytes, which provides further evidence for the existence of an IgG_3-specific receptor (Diamond and Yelton, 1981).

4. Monoclonal Anti-Fc Receptor Antibody

An independent approach to the question on FcR specificity and heterogeneity was afforded by the isolation of a monoclonal rat anti-mouse FcR antibody (Unkeless, 1979). This antibody, designated 2.4G2 IgG, was derived from the fusion of the $Hprt^-$ mouse myeloma P3U1 with the spleen cells of a rat immunized with the mouse macrophage cell lines J774 and $P388D_1$. The resulting hybrids were selected in HAT medium and the clone producing 2.4G2 IgG was identified by screening culture supernatants for the ability to inhibit rosette formation by erythrocytes coated with monoclonal anti-erythrocyte IgG_{2b}. Several lines of evidence strongly suggested that 2.4G2 IgG was directed against the trypsin-resistant receptor for IgG_{2b} and IgG_1 aggregates. First, the monovalent 2.4G2 Fab fragment (prepared by papain digestion of 2.4G2 IgG purified from ascites) was shown to inhibit rosette formation by erythrocytes coated with either monoclonal anti-erythrocyte IgG_1 or IgG_{2b} but not IgG_{2a}. Second, the binding of ^{125}I-labeled 2.4G2 Fab to macrophages was not affected by prior trypsin treatment of the cells. Third, 2.4G2 Fab did not interfere ($<10\%$) with the binding of $[^{125}I]IgG_{2a}$ monomer under conditions were 2.4G2 binding sites were completely saturated (Mellman and Unkeless, 1980). In addition, the IgG_1/IgG_{2b} FcR-negative J774 mutants described above (Unkeless *et al.*, 1979) were almost totally deficient in the ability to bind 2.4G2 Fab.

Since by definition, the monoclonal 2.4G2 IgG recognized only one

antigenic site. ^{125}I-labeled antibody was used to determine the distribution of the antigen on different cell types. In addition to cells of macrophage origin, 2.4G2 IgG was found to bind only to lymphoid cells and cell lines that rosetted with IgG-sensitized erythrocytes, which suggests that macrophage and lymphocyte FcRs are structurally if not genetically related. 2.4G2 Fab did not bind to FcR negative cells or to macrophages of species other than the mouse, including rat, rabbit, and guinea pig (Unkeless, 1979). It is noteworthy that the 2.4G2 antigen was expressed on the S49.1 T cell line, which does not bind IgG_{2a} (Heusser *et al.*, 1977; Anderson and Grey, 1978; Mellman and Unkeless, unpublished results). Taken together, these data demonstrate that 2.4G2 IgG recognizes an FcR for IgG_1/IgG_{2b}, and moreover, that this receptor is functionally distinct from the trypsin-sensitive IgG_{2a} receptor.

5. *Other Evidence for Fc Receptor Heterogeneity*

The receptors for IgG_{2a} and IgG_{2b}/IgG_1 on mouse macrophages can also be differentiated by temperature sensitivity to rosette formation at 4°C, where the IgG_{2a} receptor is largely inactive, and by the greater sensitivity of the IgG_{2b}/IgG_1 FcR to inhibition by cytochalasin B (Diamond *et al.*, 1978). The receptors can also be capped differentially by plating macrophages on rabbit IgG immune complex-coated cover slips (Michl *et al.*, 1979). Such macrophages have IgG_{2b}/IgG_1 receptors effectively cleared from their apical surfaces, but still bind IgG_{2a}-coated erythrocytes. Moreover, this residual binding is trypsin sensitive.

Diamond *et al.* (1978) found that the inhibition of binding of rabbit IgG-coated erythrocytes to mouse macrophages required the addition of *both* aggregated IgG_{2a} and IgG_{2b}. Thus, they concluded that rabbit immune complexes were bound by both the trypsin-resistant IgG_{2b}/IgG_1 FcR and trypsin-sensitive IgG_{2a} FcR. Similar conclusions were deduced from the study of binding of rabbit immune complexes to IgG_{2b}/IgG_1 FcR negative J774 variants (Unkeless *et al.*, 1979). Relevant to this conclusion are the recent observations of Sulica *et al.* (1979), who studied the effect of *Staphylococcus aureus* protein A on the binding of rabbit IgG to macrophages. While protein A inhibited the the binding of rabbit IgG immune complexes, the binding of monomeric IgG was actually enhanced. In light of the demonstration that rabbit IgG monomer binds to the IgG_{2a}-specific receptor (Unkeless, 1977; Unkeless *et al.*, 1979), these results imply that the IgG_{2a} receptor interacts with different domains of the rabbit IgG Fc region than does the IgG_{2b}/IgG_1 receptor, which binds primarily rabbit IgG

immune complexes. It should be pointed out, however, that protein A inhibits the binding of both IgG_{2b}- and IgG_{2a}-coated erythrocytes to J774 cells (Diamond and Yelton, 1981).

Further evidence that the IgG_{2a} and IgG_{2b}/IgG_1 mouse Fc receptors may interact with different domains of IgG comes from the work of Diamond *et al.* (1979), who studied the ability of aggregated mutant myeloma proteins to inhibit the binding to macrophages of IgG_{2a} or IgG_{2b}-coated erythrocytes. A mutant IgG_{2b} myeloma protein with a deleted C_H3 domain still inhibited IgG_{2b}-coated erythrocyte rosettes, whereas another variant protein, with a crossover such that the C_H3 domain contained IgG_{2a} sequence and the C_H2 domain contained largely IgG_{2b} sequence, inhibited partially both IgG_{2a} and IgG_{2b}-mediated rosetting. This data, as well as that of Sulica *et al.* (1979) with protein A, suggested that different receptors may bind different domains of the Fc piece, and that the same antibody may interact with different receptors through different contact regions. This may account for the discrepant results in studies of binding of IgG fragments to Fc receptors, which have implied that either the C_H2 domain (Alexander *et al.*, 1976; Ovary *et al.*, 1976; Tsay and Schlamowitz, 1978; Johanson *et al.*, 1981), or the C_H3 domain (Yasmeen *et al.*, 1976; Okafor *et al.*, 1974; Ramasamy *et al.*, 1975; McNabb *et al.*, 1976) was involved.

B. Guinea Pig Fc Receptors for IgG

The guinea pig has two IgG subclasses, IgG_1 and IgG_2. Although IgG_2 was initially described as the only cytophilic subclass (Berken and Benacerraf, 1966), the subsequent quantitative binding studies of Leslie and Cohen (1974, 1977) revealed that both IgG_1 and IgG_2 bound to guinea pig macrophages in a specific fashion. Moreover, IgG_1 did not compete for the binding of IgG_2 and although IgG_2 inhibited IgG_1 binding, its K_i was lower than the K_a of either subclass. Similar results were obtained using inhibition of rosette formation: IgG_1 could not block the binding to guinea pig macrophages of IgG_2-coated erythrocytes, and both subclasses inhibited the rosetting of IgG_1-coated erythrocytes (Alexander *et al.*, 1978). These results suggest that guinea pig macrophages, like the mouse macrophages, have multiple Fc receptors. Apparently, both the IgG_1 and IgG_2 FcR's are expressed on the plasma membranes of guinea pig macrophages, since all cells can be shown to bind erythrocytes sensitized with either IgG_1 or IgG_2. On the other hand, FcR-positive guinea pig T lymphocytes can be subdivided into two populations that bear receptors for guinea pig IgG_1 (47%) or IgG_2 (66%) based on the inhibition by aggregated IgG_1 or IgG_2 of rosette formation using rabbit IgG-coated ox erythrocytes

(Ricardo, 1980). The simultaneous addition of aggregated IgG of both subclasses inhibited rosetting by >90%. No evidence was obtained for the expression of both the IgG_1 and IgG_2 FcR's on individual T cells. The existence of T cells bearing FcR's exclusively for one or the other IgG subclass may have important implications with regard to the regulation of the immune response, since FcR-positive T cells are classically thought of as being suppressor T cells. It is of obvious interest to determine whether analogous mouse T cell subpopulations exist that express FcR's specific for different mouse IgG subclasses.

C. Fc Receptors for IgG on Human Cells

The binding of human IgG to human peripheral blood monocytes was first demonstrated by Huber and Fudenberg (1968). They showed that monocytes were capable of binding erythrocytes coated with either anti-Rh_0 IgG or with nonspecific IgG bound to the erythrocytes by chromic chloride or diazotized benzidine. Erythrocytes coated with Fab fragments of IgG were not bound by the monocytes. Inhibition experiments using human myeloma IgG's of different subclasses demonstrated that IgG_1 and IgG_3 inhibited rosette formation more effectively than IgG_2 and IgG_4. Similarly, Fc fragments derived from human IgG_1 and IgG_3 myeloma proteins inhibited rosette formation between human monocytes and anti-Rh_0(D)-coated human erythrocytes (Okafor *et al.*, 1974). Confirming these rosette inhibition studies by direct binding assays of radiolabeled IgG, several groups have shown that human IgG_1 and IgG_3 bind with higher avidity to mononuclear cells than do IgG_4 and IgG_2 (Alexander *et al.*, 1978; Hay *et al.*, 1972; and Huber *et al.*, 1971).

In addition to monocytes, receptors for IgG have been demonstrated on peripheral blood neutrophils (Messner and Jelinek, 1970). As with monocytes, inhibition of rosette formation was demonstrated using myelomas of IgG_1 and IgG_3 subclasses. These investigators showed that IgG's against bacterial antigens or anti-Rh_0 Ripley bound to both neutrophils and monocytes whereas conventional anti-Rh_0 IgG bound only to monocytes, similar to the findings of Huber *et al.* (1969). This finding suggested the possibility of differences among receptors on neutrophils and monocytes.

A human leukemic cell line, U937, derived from a patient with histiocytic lymphoma, has been used to study the binding of human IgG. High affinity for IgG_1 ($K_a = 2.9 \times 10^8$ liters/mole) and IgG_3 was demonstrated in this cell line as well as lower affinity for IgG_4 (Anderson and Abraham, 1980). IgG_1 and IgG_3 myelomas were equivalent in their ability to compete for the IgG_1 binding site. Double reciprocal

inhibition experiments that used all four human IgG subclasses indicated that U937 has a single Fc binding site. In addition, preparations of human placental membranes have been shown to bind IgG with subclass specificity similar to that shown by others with monocytes, i.e., human IgG_1 and IgG_3 bound better than IgG_2 or IgG_4 (McNabb *et al.*, 1976). Only the Fc portion bound to the placental membrane preparations. Using a purified fraction of placental plasma membrane vesicles, these investigators more recently demonstrated that IgG_1 and IgG_3 bound to the placental FcR more avidly than did IgG_2 and IgG_4 (Van der Meulen *et al.*, 1980).

The binding of human IgG to the human promyelocytic leukemia cell line HL-60 has been studied (Crabtree, 1980). HL-60 cells were shown to possess approximately 20,000 Fc receptors per cell and to bind human IgG_1 and IgG_3. Interestingly, one-half as much IgG_1 complexed with protein A bound to HL-60 cells compared to uncomplexed human IgG_1. Since the binding of human IgG_1 to the $P388D_1$ mouse macrophage line was totally inhibited by protein A, the authors suggest the possibility that there are two types of Fc receptors on HL-60 cells.

D. Fc RECEPTORS FOR IgE, IgA, AND IgM

The most intensively and thoroughly studied immunoglobulin receptor is the IgE-specific FcR found on mast cells and basophilic granulocytes (for review, see Metzger, 1978). IgE binds to this receptor with high avidity ($K_a \geq 10^{10} M^{-1}$) and dissociates extremely slowly (Kulczycki and Metzger, 1974). The high association constant has facilitated the isolation of the IgE FcR by affinity chromatography from both rat basophilic leukemia cells and rat peritoneal mast cells in several laboratories (Kanellopoulos *et al.*, 1979; Kulczycki and Parker, 1979; Kulczycki *et al.*, 1979; Froese, 1980). The basophil IgE receptor has been purified to homogeneity in milligram quantities (Kanellopoulos *et al.*, 1979). The receptor appears to be similar on both cell types and is generally agreed to be a glycosylated integral membrane protein consisting of one or possibly two polypeptides with molecular weights of 45,000–55,000. In addition, through the use of bifunctional cross-linking reagents, the IgE FcR has been found to be closely associated with another smaller (~30,000) molecular weight peptide (Holowka *et al.*, 1980). It is interesting to note that the macrophage/lymphocyte FcR for IgG seems to be structurally similar to the IgE receptor (see below).

Considerable attention has been devoted to understanding the role of the IgE receptor in triggering the release of mast cell or basophil

granule content. While incompletely understood, it is evident that degranulation is potentiated by receptor aggregation in the plane of the membrane as a consequence of extracellular "bridging" by a multivalent ligand. Metzger and co-workers have used both anti-receptor antibody and chemically cross-linked IgE complexes to show that the aggregation of at least two receptors is necessary to elicit a response (Segal *et al.*, 1977; Isersky *et al.*, 1978). Since a more detailed consideration of IgE receptor function is beyond the scope of the present paper, the reader is referred to the following reviews: Metzger, 1978; Ishizaka and Ishizaka, 1978; Sobotka *et al.*, 1978.

In addition to the high-affinity basophil/mast cell IgE Fc receptor, IgE Fc receptors have been described on lymphocytes (Gonzalez-Molina and Spiegelberg, 1978; Yodoi and Ishizaka, 1979) and on macrophages (Dessaint *et al.*, 1979; Melewicz and Spiegelberg, 1980). The number of lymphocytes bearing IgE FcR increases dramatically in rats infected with *Nippostrongylus brasiliensis*, which induces high levels of IgE. Yodoi *et al.* (1979) subsequently observed that IgE would induce IgE receptors on lymphocytes *in vitro* and that this induction was dependent on protein and RNA, but not DNA, synthesis. A similar phenomenon was observed for lymphoid cells that bear IgA Fc receptors, which are present in large numbers (up to one-third of peripheral θ-bearing lymphocytes) in mice that bear IgA-secreting plasmacytomas (Gebel *et al.*, 1979; Hoover and Lynch, 1980). IgA Fc receptors have also been reported on subpopulations of monocytes and granulocytes through use of rosetting techniques, and there was no inhibition of IgA rosettes by IgG, or vice versa (Fanger *et al.*, 1980). Receptors for IgM on lymphocytes have been well described, particularly for human cells [see Dickler (1976) for review], and are thought to define a subset of T helper cells (Moretta *et al.*, 1977). Of particular interest is the observation that T lymphocytes can modulate from expression of IgG receptors to IgM Fc receptors upon exposure to IgG immune complexes (Pichler *et al.*, 1978; Moretta *et al.*, 1978). There is also a report on the presence of IgM receptors on macrophages from cultured bone marrow cells and splenic macrophages (Roubin and Zolla-Pazner, 1979).

Interestingly, several nonlymphoid cell types have been found to express FcR for IgG. Best known are receptors that mediate the transfer of maternal Ig to progeny. Such diverse tissues as neonatal rat intestinal epithelial cells (Rodewald, 1976, 1980; Guyer *et al.*, 1976), the placental syncytial trophoblast (McNabb *et al.*, 1976), fetal rabbit yolk sac membrane (Cobb *et al.*, 1980; Schlamowitz, 1976), and fetal chick yolk sac (Linden and Roth, 1978) express FcR. A preliminary

report has appeared which suggests that retinal pigmented epithelial cells also bear FcR for IgG (Elner, 1981). In addition, it is apparent that fibroblasts infected with herpes virus express FcR activity (Baucke and Spear, 1979). It is interesting to speculate whether these diverse receptors are related and have common structural determinants.

III. Isolation and Characterization of IgG Fc Receptors

A detailed knowledge of the structure of the Fc receptor(s) for IgG would be of great value in understanding the mechanism whereby the binding of immune complexes signals the release of mediators of inflammation, phagocytosis, and other cellular phenomena. Accordingly, there have been numerous reports on the isolation and characterization of FcR's. Since there has been considerable disagreement among these published reports, it may be worthwhile to discuss criteria by which these results should be evaluated. Ideally, the protein isolated should be purified to homogeneity and should have FcR activity and specificity consistent with the activity on the cell surface. Similarly, the distribution of the molecule should reflect the distribution of the receptor on different cell types. The yield and recovery of the protein should be specified, and should be at least reasonably consistent with the number of binding sites detected on the cell surface, assuming that the receptor is primarily a cell-surface molecule. If a sufficient quantity of pure protein could be prepared, the production of sera that both immunoprecipitate specifically the immunogen and block receptor activity would constitute conclusive evidence for isolation of the desired molecule.

The principal method used to isolate Fc receptors has been affinity chromatography of a nonionic detergent lysate over IgG or IgG aggregates coupled to a solid support. These studies have been modeled after the successful isolation of the IgE Fc receptor (see above). However, the use of affinity chromatography to purify the Fc receptor for IgG has resulted in conflicting reports of molecular weight (M_r). Part of this confusion may relate to the probable presence of multiple FcR's or FcR activities on individual cell types. In addition, however, the avidity of the IgG FcR'(s) for its ligand(s) is probably not as high as the mast cell-basophil IgE-specific FcR for IgE. IgG's of different subclasses have been estimated to bind with K_a's of $10^5\ M^{-1}$ to $10^8\ M^{-1}$ whereas IgE binds with a K_a in excess of $10^{10}\ M^{-1}$ (Kulczycki and Metzger, 1974). Thus, efforts to purify the IgG FcR have been hindered both by the low affinity of the FcR molecule for IgG and by the

necessity—in order to preserve receptor activity—of using mild detergents that may not remove nonspecifically adsorbed proteins from the affinity matrix.

A. Fc Receptor Purification Using the Anti-Receptor Monoclonal Antibody, 2.4G2

In contrast to the use of immobilized IgG as an affinity adsorbent, the availability of the anti-FcR monoclonal antibody 2.4G2 (see above) offered several distinct advantages and one major disadvantage as an immunoadsorbent for FcR isolation from cell lysates. The disadvantage was that, by definition, 2.4G2 recognized *one* antigenic site; thus, other receptors or receptor-associated molecules might be discarded during the purification. On the other hand, the major advantages were that the 2.4G2 Fab fragment had a high K_a, $\sim 1 \times 10^9 M^{-1}$, and that the antigen–antibody complex was not disrupted by sodium dodocyl sulfate–Nonidet P-40 (SDS-NP40) or deoxycholate-containing buffers, thus allowing stringent washing procedures. Accordingly, using 2.4G2 Fab coupled to Sepharose 4B, a one-step >5000-fold purification of FcR to apparent homogeneity was effected from NP40 detergent lysates of J774 macrophages (Mellman and Unkeless, 1980). Typically, antigenic activity was recovered with an efficiency of >50%. The degree of purification attained was in good agreement with the theoretical yield based on the saturation binding value of 2.4G2 Fab to the J774 cell surface (about 600,000 sites per cell, which corresponds to ~0.01% of total cell protein). The antigen was displaced from the immunoadsorbent using a pH 11.5 0.5% deoxycholate buffer described by McMaster and Williams (1979), and after neutralization, the eluate could be analyzed immediately for antigenic activity. The purification procedure was carried out in the presence of the protease inhibitors phenylmethyl sulfonyl fluoride and trasylol and took only 1–2 hours, thus minimizing opportunity for proteolytic degradation.

1. Structure of the Purified Receptor

The presumptive FcR isolated from J774 cells exhibited two diffuse polypeptides of 60,000 and 47,000 M_r following SDS–polyacrylamide gel electrophoresis. The presence of oligosaccharide on the receptor was demonstrated by the following observations: the antigen bound to concanavalin A; the p*I* of the purified protein was broad (pH 4.6–5.8) and was shifted to more alkaline values by neuraminidase treatment; and the antigen was labeled by the galactose oxidase/NaB[^{3}H]$_4$ procedure. Trace amounts of the ^{125}I-labeled antigen were also isolated from other FcR-bearing cells of B cell, T cell, null lymphocyte, and mac-

rophage lineage for purposes of comparison. The ^{125}I-labeled glycoprotein displayed significant M_r heterogeneity, ranging from 45,000 M_r (thioglycolate-elicited mouse peritoneal macrophages) to >70,000 M_r (B-cell line WEHI 231).

The basis for the multiple polypeptides from J774, and the divergent M_r for antigens from different Fc-receptor bearing cells is not clear, but may reflect proteolysis and/or altered glycosylation of the same gene product. We favor the interpretation that the two polypeptides are related forms of the same receptor because (*a*) both peptides bear the 2.4G2 antigenic determinant; (*b*) trypsinization of macrophages does not alter the number of 2.4G2 binding sites; (*c*) trypsinization of ^{125}I-labeled macrophages decreases the amount of the 60,000 and increases the amount of the 47,000 M_r peptide; (*d*) and preliminary two-dimensional analysis of both tryptic and chymotryptic peptides suggests an overall similarity between the two bands (I. S. Mellman and J. C. Unkeless, unpublished results).

Since detergent is required to solubilize the 2.4G2 antigen from J774 membranes, it is apparent that the receptor is an integral protein of the plasma membrane (Steck, 1974). Moreover, in detergent-free solution, the FcR forms large aggregates similar to the "protein micelle" structure commonly observed for membrane proteins in aqueous solution (Simons *et al.*, 1978). Such behavior is consistent with the FcR being an amphipathic molecule, with a hydrophilic ectodomain and a hydrophobic domain normally buried in the membrane. In the presence of NP40, however, the FcR aggregates appear to be disrupted as indicated by their sedimentation velocity in sucrose density gradients (I. S. Mellman and J. C. Unkeless, unpublished results).

2. *Fc Receptor Activity of the Purified 2.4G2 Antigen*

The isolated receptor from J774 cells, after dialysis to remove deoxycholate, was iodinated *in vitro* using chloramine-T. The purified, labeled protein bound with high avidity to IgG; approximately 50% bound to rabbit anti-DNP immune complex-coated Sephadex beads whereas <0.5% bound to beads bearing the corresponding $F(ab')_2$ complexes (Mellman and Unkeless, 1980). The purified receptor would also hyperagglutinate IgG-coated erythrocytes, which demonstrates the multivalent nature of the aggregates. This effect was particularly dramatic when monoclonal anti-erythrocyte antibodies, which do not hemagglutinate, were used. However, the subclass specificity of the purified receptor was different from that expected based on the inhibition by 2.4G2 Fab fragment of IgG_{2b} and

IgG_1 immune complexes (see above). As determined both by binding of ^{125}I-labeled FcR to IgG-coated particles and by hyperagglutination of IgG-coated erythrocytes, the purified receptor bound to IgG_{2a} in addition to IgG_{2b} and IgG_1. IgG_3 was not recognized. The binding of IgG_{2a} by the purified J774 receptor may, however, be an *in vitro* artifact. Preliminary results with Fc receptor isolated by 2.4G2 Fab affinity chromatography from the S49.1 T cell line, which does not bind IgG_{2a} (I. S. Mellman and J. C. Unkeless, unpublished results; Anderson and Grey, 1978), show that the purified T-cell FcR, in the absence of detergent, binds to IgG_{2a}. Similarly, in the presence of 0.5% NP40 (which disrupts the FcR aggregates) ^{125}I-FcR from J774 cells binds only IgG_{2b}- and IgG_1-coated Sephadex beads (I. S. Mellman and J. C. Unkeless, unpublished results). Thus, it seems quite likely that the 2.4G2 antigen is in fact the IgG_1/IgG_{2b} FcR.

The purified FcR has been used as an immunogen in rabbits. Immunoprecipitation studies using either ^{125}I-surface labeled or [^{35}S]methionine-labeled macrophages and the lymphoid Fc receptor-bearing S49.1 T cell line showed the antisera were specific for the same peptides precipitated by 2.4G2 IgG. Furthermore, the rabbit antiserum totally inhibited IgG-mediated rosette formation on J774 cells at a dilution of 1 : 1000 (I. S. Mellman and J. C. Unkeless, unpublished results). Thus, it has been possible to purify the antigen using a monoclonal antibody from a fusion in which the rat spleen cells were immunized with viable macrophages, and subsequently produce specific antisera to the purified protein.

B. Fc Receptor Purification Using IgG Affinity Adsorbents

1. Mouse Macrophages

Several groups have purified FcR's from macrophages by affinity chromatography over IgG affinity columns and obtained results largely in accord with the properties of the protein isolated using the monoclonal anti-FcR antibody as immunoadsorbent. Loube *et al.* (1978) isolated a 57,000 M_r protein by chromatography of NP40 lysates of [^{125}I]lactoperoxidase labeled $P388D_1$ cells over human IgG_1 or mouse IgG_{2a} Sepharose. No such peptide was isolated after chromatography over IgM and Fab-coupled columns. Since a general protein label was not used, the degree of purification could not be assessed, nor was there any demonstration of FcR activity of the isolated proteins. A similar approach was employed subsequently to isolate FcR that had been shed into the medium by $P388D_1$ cells (Loube and Dorrington, 1980). This material, labeled with [^{35}S]methionine, displayed two well

separated peptides of 62,000 and 58,000 M_r, as well as a variable amount of a 12,000 M_r peptide. One-dimensional peptide maps of these components, though of low resolving power, suggested that they were related.

Substantially similar results to those reported by Dorrington's group have been reported by Kulczycki *et al.* (1980), who isolated ^{125}I-labeled or [^{14}C]glucosamine-labeled FcR by chromatography of NP40 lysates of labeled rabbit alveolar macrophages over rabbit IgG coupled to Sepharose. The bound protein, after washing with 1% NP40 in a pH 8 borate saline buffer, was eluted with 0.5 *M* acetic acid/1% NP40 and immediately neutralized. The labeled receptor had a broad electrophoretic mobility, centered at $M_r \sim 50{,}000$. About 50–70% of the material thus isolated could then rebind to IgG-Sepharose compared to a background binding to BSA, Fab, or $F(ab')_2$ Sepharose of about 5–15%. Again, since a general protein label was not used, the degree of purification obtained is not known. The demonstration of rebinding to IgG-Sepharose, although circular in reasoning, is important and is in agreement with the results of Mellman and Unkeless (1980), who observed that the isolated, detergent-free J774 Fc receptor was inactive at pH 4.3, but regained full activity when reneutralized to pH >5.

Lane *et al.* (1980) have isolated ^{125}I-labeled Fc receptor from thioglycolate-elicited peritoneal macrophages by chromatography of NP40 lysates over Sepharose coupled with various IgG preparations or non-IgG controls. Elution of the bound protein was accomplished either with acetic acid or SDS. Use of aggregated human IgG or rabbit IgG complexes as the immunoadsorbent resulted in the isolation of two closely spaced proteins of 67,000 and 52,000 M_r. However, only the 52,000 M_r peptide was isolated from an aggregated mouse IgG_1 column, and the 67,000 M_r peptide was the predominant peptide isolated from an aggregated IgG_{2a} immunoadsorbent. Trypsinization removed the 67,000 M_r peptide preferentially. Thus, the authors concluded that there are two structurally different proteins, one specific for IgG_{2b}/IgG_1 and the other specific for IgG_{2a}. However, rigorous proof of the distinctiveness of these two proteins must reside in tryptic mapping or sequence studies. It should be noted that the two peptides isolated by Lane *et al.* (1980) look very similar to those isolated by Loube and Dorrington (1980), who used a similar isolation protocol employing human IgG_1, supposedly the human analog of mouse IgG_{2a}. In addition, they found similarity in the peptide maps of their 62,000 and 58,000 M_r proteins.

Schneider *et al.* (1981) reported a partial purification of IgG-FcR

from NP40 lysates of P388D$_1$ cells labeled with ^{125}I by lactoperoxidase-catalyzed iodination. They coupled myeloma proteins of IgG_1, IgG_{2a}, and IgG_{2b} subclasses, pooled mouse Ig and rabbit IgG, to Sepharose for use as affinity adsorbents, and used the same acetic acid elution followed by rapid neutralization described previously (Kulczycki *et al.*, 1980) to obtain Fc binding protein which could be used for rebinding studies. Unlike the results described by Lane *et al.* (1980), they found that substantially the same peptides were isolated from all IgG immunoadsorbents. Furthermore, material isolated by chromatography IgG_{2a} or IgG_{2b} Sepharose would rebind equally well to either subclass, and rebinding was inhibited equally well by either subclass. The reasons for the discrepancies between Schneider *et al.* (1981) and Lane *et al.* (1980) are not obvious, since the methods used by both groups were very similar.

Moreover, the conclusion of Lane *et al.* (1980) that the 60,000 M_r protein is the IgG_{2a}-specific FcR and the 52,000 M_r protein is the IgG_1/IgG_{2b} FcR is difficult to reconcile with the results obtained for FcR's purified using 2.4G2 (Mellman and Unkeless, 1980; see above). First, identification of the larger of the two J774 FcR peptides, which is the major species, as the IgG_{2a} FcR is inconsistent with the small number of IgG_{2a} binding sites ($\sim 1 \times 10^5$ per cell) relative to the number of binding sites for immune complexes and 2.4G2 Fab (4 to 6 $\times$ 10^5 per cell). Second, trypsinization of J774 cells did not decrease the number of ^{125}I-2.4G2 Fab binding sites although the 60,000 M_r protein was apparently completely converted to the 47,000 M_r peptide. This result strongly suggests that the 2.4G2 antigenic site was preserved in the lower molecular weight proteolytic degradation product. Finally, although the one- and two-dimensional gels presented by Lane *et al.* (1980) and by Mellman and Unkeless (1980) are similar, it is significant that the monoclonal anti-FcR antibody 2.4G2 immunoprecipitated only a single, broad 45,000 M_r glycoprotein from thioglycolate-elicited macrophages—the cell type used by Lane *et al.* for their FcR isolation by ligand affinity chromatography. Further genetic and biochemical studies will be needed to resolve these conflicts.

2. *Fc Receptors from Human Mononuclear Cells*

Results comparable to those discussed for mouse macrophage and lymphocyte FcR have been reported for human FcR's isolated from mononuclear cells (Cunningham-Rundles *et al.*, 1978, 1980). In the first publication (Cunningham-Rundles *et al.*, 1978) NP40 lysates of surface iodinated pooled mononuclear cells (80–90% lymphocytes and 10–20% monocytes) were incubated either with IgG, $F(ab')_2$, or uncon-

jugated Sepharose, and washed in PBS. Two peaks of radioactivity, 60,000 and 120,000 M_r, which were present only after incubation with IgG Sepharose, were observed in nonreducing gels. Reduction of the sample resulted in only a 60,000 M_r peptide which was not isolated from an FcR-negative cell line Molt 4. In the later paper, after nitrogen bomb cavitation, protein solubilized by an unspecified method was passed over a column of heat-aggregated IgG and eluted with pH 2.3 glycine. As before, a peptide of 60,000 M_r was isolated and, in non-reduced gels, a dimer of 120,000 M_r. However, there were no controls using either Fab or $F(ab')_2$ immunoadsorbents or non-receptor-bearing cells, nor was any activity of the purified protein described, nor were any quantitative purification data included. The NH_3 terminus was identified as glycine, but, as glycine was used to elute the material from the column, the result is suspect without any quantitative data or controls. An antiserum elicited using the purified protein as immunogen inhibited T-cell rosettes 50% at a 1 : 30 dilution, but the antiserum was not used for immunoprecipitation.

3. *Other Cell Types*

Although there seems to be reasonable consensus among several laboratories that the purified and active FcR is a glycoprotein of 50,000–70,000 M_r that is not altered by reduction, this is by no means universally accepted. Rather than affinity chromatography, Bourgois *et al.* (1977) have used a coprecipitation system of immune complexes formed overnight in the presence of labeled NP40 lysates of various cell types. To prevent proteolysis, phenylmethyl sulfonyl fluoride, 100 m*M* iodacetamide, and trasylol were added. In this and subsequent reports (Sire *et al.*, 1980; Kahn-Perles *et al.*, 1980) the FcR was described as a single-chain 110,000–120,000 M_r peptide that was extremely sensitive to proteolysis. After proteolytic cleavage the peptides remained associated by disulfide bonds. After reduction peptides of 90,000, 75,000, 46,000, and 23,000 were observed. The 120,000 M_r protein was observed from lysates of mouse macrophages, thymus cells, B cells, and fibroblasts (Bourgois *et al.*, 1977), rabbit lymphoid cells (Sire *et al.*, 1980), and the P815 mastocytoma cell line (Kahn-Perles *et al.*, 1980), but not on the L5178Y T lymphoma line. The relation of the 120,000 M_r peptide to the 50,000–70,000 M_r FcR protein discussed above is unclear. No activity has been demonstrated for the 120,000 M_r protein. The presence of this peptide on mouse fibroblasts which do not bear FcR's or react with the monoclonal mouse anti-FcR antibody 2.4G2, is also a source of concern.

A comparable coprecipitation method was used by Neauport-Sautes

and Fridman (1977) and by Joskowitz *et al.* (1978) to isolate a molecule termed immunoglobulin binding factor (IBF) secreted by allogeneic activated T cells and by the L5178Y lymphoma. The L5178Y lymphoma line was characterized by Bourgois *et al.* (1977) and by Unkeless (1979) as being FcR negative, but this apparently was not the case for the line used by Neauport-Sautes and Fridman (1977). Supernatants of [^{3}H]fucose-labeled L5178Y cells bound to IgG-coated and, to a lesser extent, to IgM-coated erythrocytes. The crude fucose-labeled supernatant medium upon chromatography on Sephadex G-200 separated into two peaks. Approximately 50% of the total radioactivity in both the void volume peak and the peak with a M_r of 140,000 bound to IgG-coated erythrocytes. This value is surprisingly high for what should be a minor cell surface component. In acrylamide gels, peptides of 40,000 M_r and 20,000 M_r were observed after reduction; without reduction an 80,000 M_r protein was observed. No quantitation of the amount of protein secreted and/or purified was given, but the Sephadex G-200 pools containing IgG binding activity suppressed an *in vitro* anti-sheep red blood cell (SRBC) response. In a subsequent paper, Joskowitz *et al.* (1978) described the purification of IBF by affinity chromatography from unlabeled activated T cells. This material was then chromatographed on an anti-Ia affinity column, eluted, and iodinated. When anti-Ia of the appropriate haplotype was employed, peptides of 40,000 and 17,000 M_r were detected. They thus concluded that the FcR (or immunoglobulin binding factor) bears determinants for both IgG and Ia. However, since the starting material was not pure (and in fact no criteria for purity were ever presented) subsequent isolation of Ia specificities is not proof of association.

Frade and Kourilsky (1977) also analyzed FcR from membranes of L5178Y cells. Total membranes labeled with ^{14}C-labeled amino acids were dissolved in deoxycholate and after dialysis, were fractionated on Sephadex G-200. As described by Neauport-Sautes and Fridman (1977), a high percentage of material eluting in the first two peaks bound to IgG-coupled Sepharose; after chromatography of either of the pooled peaks on IgG-Sepharose followed by IgM-Sepharose, a homogeneous glycoprotein of 110,000 M_r was found that gave rise, on reduction, to subunits of 56,000, 36,000, 25,000, 18,000, and 15,000 M_r. The amount of material so isolated is difficult to reconcile with the extremely small number of receptors on lymphoid cells. For example, the S49.1 mouse T cell line, a relatively rich source of FcR, binds only 7 ng of 2.4G2 Fab/10^6 cells (Unkeless, 1979), which is ~0.01% of total cell protein. The association of membrane proteins with IgG-Sepharose in the absence of detergent might represent "nonspecific"

interactions of hydrophobic domains of membrane proteins with the hydrophobic domains of the Fc piece of IgG.

Takacs (1980) has reported isolation of FcR from human lymphoblastoid cell lines, which represented 4–5% of total cytoplasmic membrane protein. NP40-solubilized membrane proteins were chromatographed on a column containing immune complexes or aggregated IgG, and bound material eluted with pH 2.5 glycine-HCl (which removes a 46,000 M_r peptide), and then SDS, which eluted more of the 46,000 M_r peptide and some contaminating protein. The material was a glycoprotein, distinguishable from actin by isoelectric focusing, and could agglutinate erythrocytes coated with subhemagglutinating amounts of IgG. Antisera directed against the protein were reported to inhibit IgG-mediated rosette formation, but Fab or $F(ab')_2$ fragments were not tested for ability to inhibit FcR. Furthermore, binding of the anti-FcR antibody did not correlate well with binding of immune complexes by lymphoblastoid cell lines (Elliott and Takacs, 1979). For example, although the putative receptor was found to be a major constituent (>4% of total cell protein) of WT-18 cells, only 5% of these cells were found to bind IgG immune complexes whereas 98% bound the $F(ab')_2$ fragment of the anti-receptor antibody. Thus the function in the cell of this protein has not been unequivocally demonstrated.

Thoenes and Stein (1979) reported the isolation of a low M_r FcR from B type chronic lymphocytic leukemia (CLL) cells through the use of a somewhat different method of solubilizing membrane proteins. Since meaningful results could not be obtained using NP40 lysates, membranes from surface-iodinated CLL cells were prepared from cells disrupted by nitrogen cavitation and then incubated in 80 m*M* EDTA, 50 m*M* 2-mercaptoethanol, and 20 m*M* Tris-HCl at pH 7.3. Solubilized proteins were then subjected to coprecipitation using human IgG or $F(ab')_2$ aggregates followed by sheep anti-human antiserum. A 28,000 M_r polypeptide was identified that was precipitated only by intact IgG. A similar protein could not be isolated from membrane extracts of T-type CLL cells. This peptide exhibited a single peak after isoelectric focusing (p*I* 5.5) and was reported to have an aminoterminal glycine. However, no quantitative data were presented. There was no estimation of the amount of material isolated, and all the experiments presented were based on ^{125}I-labeled material, which makes evaluation of the purity of the peptide difficult. Preparation of an antiserum was reported that inhibited rosette formation 60–80% at 1 : 30 dilution and stained FcR-bearing leukemic cells but not T-type leukemias, peripheral T cells, or erythrocytes. No attempt was made to immunoprecipitate receptor using this antiserum.

Suzuki *et al.* (1980a,b) have also used B-type CLL cells as a source of FcR, but were able to employ NP40 or Triton X-100 to solubilize intrinsic membrane proteins. Lysates of iodinated cells were first passed over protein A-Sepharose (to remove IgG) and then chromatographed on heat-aggregated IgG-Sepharose. After washing, the bound protein was eluted with 6 *M* urea. A single, nonglycosylated protein (M_r 30,000 and p*I* 6.5) was isolated which was shown, after dialysis, to be capable of rebinding to IgG. A similar polypeptide was not found in lysates of the FcR-negative cell line, Molt 4. From 10^{12} CLL cells, they obtained 60–70 mg of the purified protein. The putative FcR did not bind to $F(ab')_2$ or Fab fragments and did appear to inhibit the formation of rosettes by 40–60% at concentrations of 20–40 μg of receptor per milliliter. Comparable inhibition was reported for $F(ab')_2$ fragments purified from an antiserum raised against the isolated protein.

The remarkable finding in the second paper (Suzuki *et al.*, 1980b) is that the same protein (identified by tryptic peptide maps and amino acid analysis) was eluted from a phosphorylcholine-Sepharose column. Furthermore, the IgG and phosphorylcholine binding protein exhibited phospholipase A_2 activity, releasing fatty acid from the 2 position of phosphotidylcholine in a Ca^{2+}-dependent reaction with a pH optimum of 9.5. The activity of the phospholipase was stimulated five- to sixfold by roughly stoichiometric amounts of Fc fragment. The protein isolated by Suzuki *et al.* (1980a,b) may well be the same as that characterized by Thoenes and Stern (1979) and is clearly differentiated from the FcR's isolated from mouse macrophages by Mellman and Unkeless (1980), Loube and Dorrington (1980), Kulczycki *et al.* (1981), and Lane *et al.* (1980) by lower M_r, absence of carbohydrate, and sharp isoelectric point. The relation of this protein to other human FcR's on mononuclear cells that have been characterized must await further clarification.

4. Herpes Virus-Induced Fc Receptor

The Fc receptor induced by infections of fibroblasts by herpes simplex virus has been purified by Baucke and Spear (1979) by passage of NP40 lysates of [^{35}S]methionine-labeled cells over columns of immobilized immune complexes, followed by elution with 2–3 *M* KSCN. They obtained two glycoproteins, of M_r 80,000 and 65,000, which were not isolated from uninfected cells, or $F(ab')_2$ control columns; the same proteins were isolated from two different species, HEp-2 cells (a human carcinoma line) and BHK-21 cells (hamster). Pulse-chase analysis revealed a precursor–product relationship between a protein slightly lower in M_r than 65,000 and the 80,000 M_r

peptides. The relative ease with which Baucke and Spear (1978) identified the peptides in [^{35}S]methionine-labeled cells may be due, in part, to the inhibition of most host protein synthesis by the viral infection. The 80,000 M_r protein was found as well on the herpes simplex virion, where it is a minor constituent relative to major viral glycoproteins (Para *et al.*, 1980). The virions bound immune complexes and, interestingly, upon infection of HEp-2 cells at high multiplicities of infection in the presence of cycloheximide, the HEp-2 cells gained the ability to bind peroxidase immune complexes. The role this FcR plays in the viral life cycle is not clear, but it has been proposed to protect infected cells from immune cytolysis (Costa and Rabson, 1975; Adler *et al.*, 1978). It would be of interest to determine whether there are structural homologies between the herpes-induced FcR and macrophage receptor.

IV. Conclusion

The extensive literature of the identification, isolation, and characterization of FcR's provides a clear demonstration of the difficulties in studying cell-surface receptors. In particular, it has shown that defining specific receptors simply on the basis of their ligand-binding activities may lead to some confusion, depending on the nature of the ligand chosen for study. Similarly, the subsequent use of these ligands as affinity reagents for the purification of solubilized FcR can pose problems. Clearly proving that polypeptides isolated in this way are, in fact, receptors is also difficult, since simply demonstrating the ability of the putative FcR to rebind IgG is somewhat circular in reasoning. Thus, it becomes extremely important to show that the purity, yield, specificity, cellular distribution, and immunogenicity of the isolated material is consistent with its being an FcR. To date, the only isolation attempt to have met all these criteria is that which was effected using the anti-IgG/IgG$_{2b}$ FcR monoclonal antibody 2.4G2. In addition, since this FcR was isolated on the basis of its antigenicity, the subsequent demonstration of its avidity for IgG Fc regions represents a totally independent property of the molecule. Several reports agree fairly well with the data obtained using 2.4G2. Thus, it is probably fair to conclude that at least one macrophage FcR is a glycoprotein, consisting of one or two polypeptides of M_r 45,000–60,000, and that the same antigen, at least, in the mouse, is formed on lymphoid cells as well.

It also seems clear that the many years of experience in FcR isolation hold important lessons for those involved more recently in isolating other cell surface receptors. Typically, ligand-mediated affinity

chromatography has been employed and, more often than not, few or no other criteria are applied to substantiate the identification of the isolated polypeptides. There are some notable exceptions to this situation including the isolation of receptors for acetylcholine (Lindstrom *et al.*, 1978), asialoglycoproteins (Kawasaki and Ashwell, 1976; Pricer and Ashwell, 1976), and transcobalamin II (Seligman and Allen, 1978). In these cases, not only has a functional receptor protein been purified to homogeneity, but antisera have been raised against the isolated proteins which were shown to inhibit receptor function. Thus, since this approach seems to be generally applicable, similar data should be gathered for other putative receptor preparations before accepting their identification as receptors.

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The Autologous Mixed-Lymphocyte Reaction

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I. Introduction

This review considers the reaction between T lymphocytes and autologous cells. Although emphasis is placed on studies carried out with human lymphocytes, investigations in experimental animals that corroborate or extend findings obtained in man will be discussed. A considerable body of evidence suggests that T lymphocytes recognize determinants on autologous cells specified by genes of the major histocompatibility complex (MHC). Whenever possible, the autologous

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mixed-lymphocyte reaction (MLR) will be related to the recognition of self MHC determinants that occurs in the normal immune response.

During the past 10 years, there has been a growing recognition that immune reactions to foreign antigens depend upon presentation of foreign determinants in association with autologous MHC determinants. Lymphocyte receptors for autologous MHC determinants, which play an essential role in the immune response to foreign determinants, are clonally distributed (Janeway *et al.*, 1976). The term MHC restriction is used to describe the requirement for histocompatibility between interacting lymphocyte populations, between lymphocytes and monocytes as well as the consequences of the associative recognition of foreign antigens with autologous histocompatibility determinants (Zinkernagel and Doherty, 1979). Thus, lymphocyte receptors for self MHC determinants are important in both the afferent and efferent limbs of the immune response.

Although immune recognition of autologous determinants has been rapidly incorporated into contemporary immunological thinking, it is important to recognize that this concept represents a radical departure from orthodox immunological theory that had been accepted for nearly a century. At the end of the last century, Ehrlich and Morgenroth (1900) were studying the antibody response of goats immunized with allogeneic erythrocytes. These investigators observed that antibodies induced by allogeneic erythrocytes reacted with the immunizing erythrocytes and other allogeneic erythrocytes, but never with autologous erythrocytes. These investigators were the first to contrast the ease of inducing immunity to foreign antigens with the difficulty in inducing immunity to autologous antigens. Based on these results, Ehrlich proposed the doctrine of "horror autotoxicus." According to this concept, the normal immune system, despite its capacity to respond to a vast repertoire of foreign antigens, cannot react to autologous antigens. Furthermore, Ehrlich predicted that immune reactions to autologous antigens, if such occurred, would lead to disease.

Later on in this century, the study of tissue and organ transplantation, governed by cell-mediated immunity, supported the concept that the immune response was activated by foreign, but not by autologous, cells and tissues. Furthermore, the *in vitro* correlate to transplantation immunity, the allogeneic MLR appeared to demonstrate reactivity to allogeneic but not autologous lymphocytes. Thus, the discrimination between self and nonself appeared to be an empirical fact of cell-mediated and humoral immunity and a fundamental tenet of immunological theory. The discovery that immunological tolerance could be induced by administering foreign antigen to fetal or neonatal

animals suggested a mechanism to explain the failure of the immune system to react to autologous antigens. Self-tolerance, established during the ontogeny of the immune system, might preclude immune reactions to autologous antigens.

In 1962, Burnet extended his clonal selection theory of immune reactivity to explain the lack of immunological reaction to autologous antigens. He suggested that self-tolerance resulted from clonal deletion of lymphocytes with receptors for autologous antigens during early development. According to his thesis, autoantibodies resulted from the appearance of "forbidden clones," which arose by somatic mutation.

Despite the heuristic value of clonal deletion of autoreactive lymphocytes as an explanation for self-tolerance, evidence obtained in the last 10 years has challenged this concept. Two lines of evidence weigh heavily against clonal deletion: one, based on the identification of normal lymphocytes that bind autologous antigens or can be activated to express autoreactivity; the other, derived from MHC-restricted interactions among autologous cells in the immune response.

Bankhurst and his colleagues (1973) identified in the blood of normal individuals B lymphocytes that bind human thyroglobulin. Thyroglobulin-binding lymphocytes have also been found in lymphocyte preparations from normal mice (Clagett and Weigle, 1974). Normal human lymphocytes also include cells that bind mammalian DNA (Bankhurst and Williams, 1975). The frequency of thyroglobulin- or DNA-binding lymphocytes was comparable to the frequency of lymphocytes that bind conventional foreign antigens. Finally, a subpopulation of T lymphocytes has been found in both experimental animals and man that bind autologous erythrocytes (Charreire and Bach, 1975; Baxley *et al.*, 1973).

In addition to the demonstration of lymphocytes that bind autologous antigens, the existence of autoreactive lymphocytes has been demonstrated by the appearance of autoantibodies or autoaggressive lymphocytes following polyclonal activation of lymphocytes. Thus, incubation of lymphocytes from normal mice with lipopolysaccharide *in vitro* stimulates the appearance of autoerythrocyte plaque-forming cells (Hammarstrom *et al.*, 1976) and by the secretion of anti-poly(A) antibodies (Fischbach *et al.*, 1978). Incubation of normal murine T lymphocytes with allogeneic effect factor has been reported to activate cytotoxic T lymphocytes with specificity for autologous cells (Altman and Katz, 1980). Polyclonal activation of B cells *in vivo* also leads to the expression of autoreactive lymphocytes. Thus, patients with chronic bacterial (Williams and Kunkel, 1962) or parasitic (Greenwood,

1974) disease frequently have autoantibodies in their serum. Similarly, mice given the polyclonal activator, lipopolysaccharide, develop anti-DNA antibodies (Izui *et al.*, 1977) and antibodies that are cytotoxic to autologous spleen cells (Primi *et al.*, 1977). Finally, autoaggressive lymphocytes have been generated in culture after incubation with rat brain (Orgad and Cohen, 1974) or testicular tissue (Wekerle and Begemann, 1976). Thus, considerable evidence has been collected that normal lymphocytes not only bind autologous antigens, but also can be activated to express autoreactivity.

The other line of evidence suggesting immune recognition of autologous cells comes from studies of cell cooperation and MHC-restricted immune responses. Cell cooperation between T and B lymphocytes, discovered more than 15 years ago, was initially thought to reflect antigen-linking of cooperating cells: B lymphocytes recognizing haptenic determinants and T lymphocytes recognizing carrier determinants (Mitchison, 1970). Subsequently, genetic restriction required for cell cooperation between T and B lymphocytes suggested direct interaction between the cooperating cells. The first clue that recognition of MHC determinants was involved in cell cooperation came from experiments that attempted to restore immune competence to nude mice. Kindred and Shreffler (1972) found that a normal anti-sheep red blood cell (SRBC) response could be restored in nude mice by administering thymocytes from normal donors only if the donors shared the same H-2 determinants as the nude mice. Katz and his associates employed congenic mice to map the MHC determinants required for cell cooperation. These studies revealed that cell cooperation required B and T lymphocytes to share the same *I* region of the MHC (Katz and Armerding, 1976). Shevach (1976) showed that the normal interaction of T cells with antigen-pulsed macrophages also required that T cells and macrophages have the same *I*-region genes. The induction of helper T lymphocytes *in vitro* (Erb and Feldmann, 1975) and the transfer of delayed-type hypersensitivity *in vivo* (Miller *et al.*, 1976) also required *I*-region identity of T cells and macrophages. As interactions of T lymphocytes with allogeneic cells did not induce suppressor activity (Kindred, 1975), it appears that the failure of cell cooperation results from a failure of T cells to interact positively with allogeneic determinants on non-T lymphocytes.

Recognition of MHC determinants has also been shown to be a requirement for activity of effector T lymphocytes. Cytotoxic T lymphocytes express MHC restriction, reflecting the capacity of T lymphocytes to recognize autologous MHC determinants. The determinants recognized by cytotoxic T lymphocytes are coded for by the *H-2D*

or *H-2K* region of the murine MHC as expressed by the thymus gland (Zinkernagel *et al.*, 1978). The studies of cell cooperation and MHC-restricted cytotoxicity support the concept that T-cell recognition of autologous MHC determinants is essential for the interaction of T lymphocytes, B lymphocytes, and macrophages in the immune response.

In addition to cell recognition of autologous MHC determinants, regulatory products of T and B lymphocytes react with determinants on autologous cells. Thus, factors produced by suppressor T lymphocytes act only on cells that share *I*-region determinants with the suppressor T lymphocytes (Tada *et al.*, 1976). Auto-anti-idiotypic antibody recognizes surface determinants on autologous B lymphocytes. Auto-anti-idiotypic antibody has been shown to "down regulate" antibody production by autologous B lymphocytes (Goidl *et al.*, 1979). Displacement of cell-bound auto-anti-idiotypic antibody by hapten reactivates antibody secretion.

In summary, immunological thinking has changed dramatically in the 1970s with regard to the nature of self-tolerance. Today, clonal deletion of autoreactive lymphocytes is not believed to be the only mechanism of self-tolerance. Weigle and his associates (1975) have argued convincingly that T-cell tolerance to autologous antigens and the activity of suppressor T cells are important mechanisms that contribute to self-tolerance. While autoaggressive immune reactions may be pathological and be normally suppressed, the existence of lymphocytes that recognize autologous determinants seems clear. Finally, a series of important studies over the 1970s suggest that recognition of autologous MHC and idiotypic determinants plays a critical role in the regulation of the normal response. How the adaptive features of autorecognition are expressed while the maladaptive consequences of autoreactivity are repressed is not entirely clear. What is clear, is that the immune response is driven as much by recognition of self as of nonself determinants.

II. The Autologous MLR in Man

A. Lymphocyte Proliferation in Culture

The capacity of human blood lymphocytes to proliferate in culture was first reported by Nowell (1960), who recognized that the plant lectin phytohemagglutinin (PHA) stimulated lymphocyte transformation and mitosis. Subsequently, Bain and her associates (1963) and Hirschhorn and his associates (1963) reported that lymphoblasts ap-

peared in cultures containing lymphocytes from two unrelated individuals. This reaction termed the allogeneic MLR was rapidly recognized to be an *in vitro* correlate of the allograft reaction. The rules of transplantation immunology established by tissue grafting were observed in the allogeneic MLR. Thus, the degree of mixed lymphocyte reactivity reflected the histocompatibility of the lymphocyte donors, and no allogeneic MLR was detected in cultures containing lymphocytes from identical twins. Genetic analysis revealed that the HLA-D locus that codes for the determinants that stimulate the allogeneic MLR was distinct but closely linked to the HLA-A, -B, and -C loci that code for the histocompatibility determinants detected by serological means. Studies that identified the responding and stimulating cells in the allogeneic MLR showed that non-T lymphocytes and monocytes stimulated allogeneic T-lymphocyte proliferation. This conclusion was supported by the finding that the gene products of the HLA-D locus were expressed on non-T lymphocytes and monocytes, but were absent or weakly expressed on resting T cells (Mann and Sharrow, 1980). Further study of the allogeneic MLR revealed that the proliferative response was followed by the generation of cytotoxic T lymphocytes that express specificity for the allogeneic gene products of the HLA-A, -B, or -C loci. In addition, the allogeneic MLR was found to possess two cardinal attributes of an immune response: memory and specificity (Fradelizi and Dausset, 1975). An unexpected observation made in the course of the study of the allogeneic MLR was the extraordinary frequency of allospecific T lymphocytes (10^{-2}) in contrast to the frequency of antigen-specific B lymphocytes (10^{-5} to 10^{-6}). These characteristics and other considerations concerning the allogeneic MLR have been thoroughly reviewed by Dupont *et al.* (1976).

B. Lymphocyte Proliferation Induced by Autologous Neoplastic Cells

The early studies of human lymphocyte proliferation induced by autologous cells involved cocultures of lymphocytes with autologous neoplastic cells. This approach was chosen because transplantation techniques used in animals to identify tumor-associated antigens could not be employed in man. Human lymphocytes were cultured with a variety of neoplastic cells including Burkitt's lymphoma, renal and breast carcinoma, seminoma, and osteogeneic and liposarcoma (Vanky and Stjernsward, 1971). Fridman and Kourilsky (1969) studied the proliferative response of lymphocytes, taken from 10 patients with acute leukemia during remission, in culture with cryopreserved autologous leukemic blast cells. The neoplastic cells were obtained be-

fore treatment and stored in liquid nitrogen. Leukemic cells from 6 patients with acute lymphoblastic leukemia and from 1 patient with acute myeloblastic leukemia stimulated tritiated thymidine ([^{3}H]Tdr) incorporation by autologous lymphocytes. The authors, noting the similarity of the reaction to the allogeneic MLR, suggested that their observations might be due to antigenic differences between leukemic and normal cells.

Another strategy to identify tumor-specific antigens involved the coculture of neoplastic cells with normal lymphocytes from an MHC-identical sibling. M. L. Bach *et al.* (1969) showed that the leukemic cells from 2 out of 3 patients with acute leukemia stimulated [^{3}H]Tdr incorporation by lymphocytes from MHC-identical siblings. Cells from the leukemic patients, obtained during remission, did not stimulate lymphocytes from the normal siblings. The authors believed that their results suggested that either leukemic cells possessed leukemia-associated antigens or that blast cells expressed differentiation antigens not found on normal cells.

C. Lymphocyte Proliferation Induced by Lymphoid Cell Lines

Continuous cultures of lymphoblastoid cells derived from normal subjects or patients with a variety of diseases share certain morphological and biochemical characteristics with neoplastic cells. Most of these cell lines express characteristics of B-lymphocyte lineage and were referred to as B-lymphoblastoid cell lines (B-LCL). Several groups studied the capacity of B-LCL to stimulate lymphocyte proliferation. B-LCL were found to stimulate autologous lymphocytes as vigorously as they stimulated allogeneic lymphocytes (Green and Sell, 1970; Weksler and Birnbaum, 1972). This observation was compatible with the concept that B-LCL expressed a differentiation antigen not found on normal B lymphocytes. There was little evidence that new HLA determinants were expressed by B-LCL, that fetal calf serum used to cultivate the B-LCL or factors released from B-LCL explained their capacity to stimulate autologous lymphocytes. As B-LCL could be established by infection of lymphocytes with Epstein–Barr virus (EBV) and most B-LCL contained the EBV genome (Klein *et al.*, 1968), the possibility that EBV antigens caused B-LCL stimulation of autologous lymphocytes was carefully considered. It was shown that lymphocytes from donors not sensitized to EBV reacted as strongly to B-LCL as did lymphocytes from EBV immune donors (Weksler, 1976). Furthermore, lymphocyte proliferation stimulated by B-LCL was not inhibited by antiserum to EB viral capsid or membrane antigen. Fi-

nally, B-LCL that did not possess the EBV genome stimulated autologous lymphocyte proliferation. The capacity of differentiation antigens on proliferating cells to stimulate autologous lymphocyte proliferation was suggested by the finding that human lymphoblasts induced by mitogen stimulated autologous lymphocyte proliferation (Weksler, 1973; Bluming *et al.*, 1975). The lymphoblast-associated antigens might be cell cycle-specific determinants that were expressed during restricted periods of the cell cycle of normal cells (Fox *et al.*, 1971).

Cells of different lineage do not express the same differentiation antigen. Lymphoid cells of the B lineage express determinants that stimulate allogeneic and autologous lymphocyte proliferation whereas lymphoid cells of the T lineage do not stimulate proliferation of either allogeneic or autologous lymphocytes (see Table I). LCL derived from T-lymphoid cells failed to stimulate lymphocyte proliferation, in striking contrast to the capacity of B-LCL to stimulate lymphocyte proliferation (Royston *et al.*, 1974; Weksler, 1976). Thus, T lymphocytes or T-LCL did not stimulate allogeneic lymphocyte proliferation although non-T lymphocytes or B-LCL from the same donor did. Subsequently, it was found that Ia-like antigens appear to be the major stimulating determinant for lymphocyte proliferation. The fact that these antigens

TABLE I
LINEAGE OF HUMAN LYMPHOID CELLS THAT STIMULATE T-LYMPHOCYTE PROLIFERATION

Stimulating cells	Responding cells	Proliferation	References
Allogeneic or autologous B-LCL[a]	Peripheral blood lymphocytes (PBL)	++++	Hardy *et al.*, 1970; Weksler and Birnbaum, 1972
Mitogen-activated autologous PBL	Peripheral blood lymphocytes	++	Weksler, 1973; Bluming *et al.*, 1975
Allogeneic T-LCL	Peripheral blood lymphocytes	−	Royston *et al.*, 1974; Weksler, 1976
Allogeneic non-T lymphocytes	T Lymphocytes	++++	Kuntz *et al.*, 1976; Ilfeld *et al.*, 1977
Allogeneic T lymphocytes	T Lymphocytes	−	Kuntz *et al.*, 1976
Autologous non-T lymphocytes	T Lymphocytes	++	Opelz *et al.*, 1975; Kuntz *et al.*, 1976
Autologous T lymphocytes	T Lymphocytes	−	Opelz *et al.*, 1975; Kuntz *et al.*, 1976

[a] LCL, lymphoblastoid cell line.

are well expressed on B-lymphoid cells but poorly expressed on T-lymphoid cells confirms the importance of cell lineage in the expression of determinants capable of stimulating lymphocyte proliferation.

D. Lymphocyte Proliferation Induced by Autologous Blood Cells

Background thymidine incorporation occurred in autologous cultures that served as controls for the allogeneic MLR. The first clue that this phenomenon represented an interaction between autologous lymphocyte subpopulations came from studies that investigated [^{3}H]Tdr incorporated by control cultures containing cells from only one individual. Raising the number of mitomycin C-treated cells incubated with a constant number of autologous cells increased [^{3}H]Tdr incorporation (Etheredge *et al.*, 1973). Subsequently, Opelz *et al.* (1975), showed that [^{3}H]Tdr incorporated by unfractioned lymphocytes in culture could be dramatically altered by changing the ratio of T and non-T cells. When the number of non-T cells added to a constant number of autologous T cells was decreased, [^{3}H]Tdr incorporation fell. In contrast, [^{3}H]Tdr incorporation rose when the number of non-T cells added to a constant number of T cells was raised. This observation and studies that employed different lymphocyte subpopulations as responding and stimulating cells suggested that non-T cells stimulated [^{3}H]Tdr incorporated by autologous T cells. T cells and non-T cells that are the responding and stimulating populations in the allogeneic MLR play a similar role in autologous cultures. For this reason, the reaction between T lymphocytes and autologous non-T lymphocytes was termed the autologous MLR. Autologous non-T cells stimulated approximately one-third as much [^{3}H]Tdr incorporation as did allogeneic non-T cells in cultures (Kuntz *et al.*, 1976).

Although gamma irradiation can denature proteins, irradiation damage did not explain the capacity of non-T cells to stimulate autologous T-cell proliferation. Mitomycin C-treated (Opelz *et al.*, 1975), as well as untreated (Kuntz *et al.*, 1976; Katz and Fauci, 1979), non-T cells also stimulated the proliferation of autologous T cells. Techniques used to isolate T cells did not activate them (Opelz *et al.*, 1975; Kuntz *et al.*, 1976). Thus, the interaction of T cells with sheep erythrocytes was not required for T cells to respond to autologous non-T cells. T cells obtained by negative selection (depletion of non-T cells) also were stimulated to proliferate by autologous non-T cells. In addition, unfractionated lymphocytes were stimulated by autologous non-T cells. Another potential artifact considered was the role of allogeneic serum components routinely used to support the autologous MLR. In fact,

when the autologous MLR was performed in autologous serum, a greater proliferative response was observed than that observed in allogeneic serum (Kuntz *et al.*, 1976; Ilfeld *et al.*, 1977).

E. Cells That Participate in the Autologous MLR

1. The Responding Cell

Elegant studies by Lohrman and Whang-Peng (1974) using chromosomally marked T cells showed that T lymphocytes are the proliferating lymphocyte subpopulation in the allogeneic MLR. Similarly, the early studies of the autologous MLR revealed that T lymphocytes are the proliferating cells in this reaction (Opelz *et al.*, 1975; Kuntz *et al.*, 1976).

The techniques used to prepare T cells have identified characteristics of the T cells that proliferate in the autologous MLR (Table II). Autoreactive T lymphocytes are medium-size cells. Fractionation of T cells on Percoll or bovine serum albumin (BSA) density gradients revealed that low-density T cells were most responsive to autologous non-T cells and to concanavalin A (Con A), while both low and high density fraction responded to alloantigen and PHA (Stobo and Loehnen, 1978; Smith and Knowlton, 1981). T lymphocytes that rosette immediately with sheep red blood cells (SRBC) are referred to as "early" rosette-forming cells whereas the remaining T cells will form "late" rosettes if incubated for 1 hour with SRBC. "Early" rosette-forming cells comprise 20–30% of the peripheral blood lymphocytes and were shown to be enriched for autologous responsive T cells as compared with the "late" rosette-forming T cells (Yu, 1978). Therefore, autoreactive T lymphocytes appear to have a high affinity for SRBC.

TABLE II
CHARACTERISTICS OF AUTOREACTIVE T LYMPHOCYTES

Characteristics	References
1. Are low density	Stobo and Loehnen, 1978; Smith and Knowlton, 1981
2. Form early rosettes with sheep red blood cells	Yu, 1978
3. Express receptor for autologous erythrocytes	Palacios *et al.*, 1980; Tomonari *et al.*, 1980; Fournier and Charriere, 1981
4. React with OKT_4 monoclonal antibody	Kozak *et al.*, 1981
5. Respond to concanavalin A	Fournier and Charriere, 1978; Sakane and Green, 1979; Smith and Knowlton, 1981

A subpopulation of human T lymphocytes bind autologous erythrocytes (Baxley *et al.*, 1973). These autologous rosette-forming T cells (Tar) were shown by several groups to contain the autoreactive T-cell population (Palacios *et al.*, 1980; Tomonari *et al.*, 1980; Fournier and Charriere, 1981). Thus, Tar cell preparations were more responsive and Tar-depleted preparations were less responsive than the original T-cell preparation to autologous non-T lymphocytes. Although T lymphocytes depleted of Tar cells did not respond well in the autologous MLR, Palacios and his co-workers (1980) found these cells to be more responsive in the allogeneic MLR than were the original unfractionated T cells. Thus, T cells that form rosettes with autologous erythrocytes are enriched with respect to autoreactive T cells and depleted with respect to alloreactive T cells. Other techniques can be used to distinguish autoreactive T cells from alloreactive T cells. Auto- and allo-activated T cells can be fractionated after a primary MLR using Percoll gradient density centrifugation. The OKT series of monoclonal antibodies have been used to characterize auto- and alloreactive T cells. The OKT4 monoclonal antibody is believed to define a subset of T cells endowed with helper function and the capacity to induce suppressor function, and OKT5 and OKT8 antibodies define T-cell subsets with suppressor-cytotoxic effector function (Reinherz *et al.*, 1979). Ninety percent of autoactivated T cells prepared in this manner reacted with the monoclonal antibody OKT4, but only 10% reacted with OKT5 or OKT8 (Kozak *et al.*, 1981). In contrast, 60% of normal T cells are stained by OKT4 and 20–30% with OKT5 or OKT8. Alloreactive T cells and fractions depleted of autoreactive cells had a normal T-cell profile. Thus, autoactivated T lymphocytes appear to fall in the helper-inducer class of T lymphocytes. This conclusion correlates with the phenotype of the murine autoreactive T cells. Pasternak *et al.* (1980) and Altman and Katz (1980) reported that treatment of murine T cells with anti-Lyt 1 antibody and complement eliminates the capacity of T-cell preparations to respond to autologous non-T cells. The Lyt 1 T cell is a helper-inducer cell.

Autoreactive and alloreactive T cells also differ in population size. Limiting dilution analyses has revealed that the number of autoreactive T cells in the blood is significantly less than the number of alloreactive T cells (Kozak *et al.*, 1981). Thus, the frequency of autologous responding units was 3×10^{-4}, and the frequency of allogeneic responding units was 4×10^{-3}.

The relationship between autoreactive T cells and other subsets of T cells has been determined by eliminating an activated subset by the technique of Zoschke and Bach (1971), which makes use of

bromodeoxyuridine (Budr) and light to delete a proliferating population. Elimination of Con A-responsive T cells by this method eliminated the autoreactive T cell population (Smith and Knowlton, 1981). This indicates that the autoreactive T-cell population lies within the Con A-responsive T-cell population. Sakane and Green (1979) using this technique have shown that eliminating autoreactive T cells from a T-cell population eliminated those T cells activated by Con A to become suppressor cells. Other results that suggest a close relationship between autoreactive T cells and Con A-responsive T cells are the findings that Con A expands the autorosetting population of T cells in culture (Fournier and Charreire, 1978) and that all the Con A-induced suppressor cells are found within the autorosetting population (Sakane *et al.*, 1980). A diagram illustrating the relationship between autoreactive, alloreactive, Con A-responsive, autorosetting lymphocyte, and suppressor cells induced by Con A is shown in Fig. 1.

Recent evidence suggests a heterogeneity of autoreactive T lymphocytes. Hausman and her associates (1980) have reported two distinct T-cell populations responding in the autologous MLR. One T-cell population reacts with autologous macrophages and participates in the proliferative response to conventional antigens. This subset can be identified with a monoclonal antibody, T-29. This autoreactive T-cell population comprises about 10% of all T lymphocytes. The other autoreactive T cell population lacks the determinant identified by the T-29 antibody and is stimulated by autologous B lymphocytes.

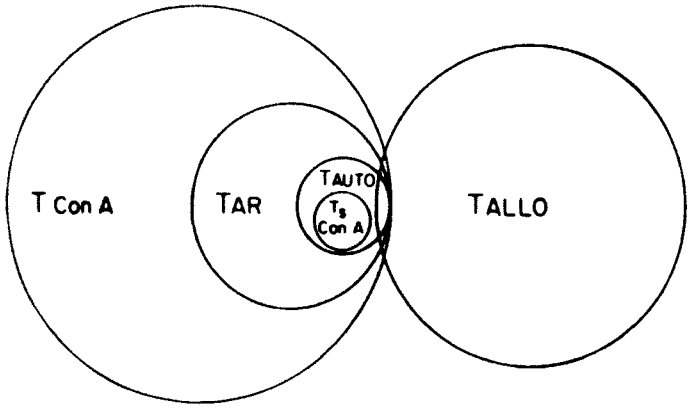

FIG. 1. Relationship between T cells that are activated by Con A ($T_{Con\ A}$), by allogeneic cells (T_{Allo}), by autologous cells (T_{Auto}), T cells that form rosettes with autologous erythrocytes (T_{ar}), and T cells that develop suppressor activity in the presence of Con A ($T_{s\ Con\ A}$). The area of the circles is not intended to reflect the size of the T cell subpopulations.

2. *Stimulating Cell*

The cells that stimulate the autologous MLR have been the focus of a great deal of attention and of controversy. The earliest studies showed that the stimulating cells were present in the non-T-cell preparation (Opelz *et al.*, 1975; Kuntz *et al.*, 1976). As non-T lymphocytes are a heterogeneous population of cells including B lymphocytes, null (L, K) lymphocytes, and monocytes, it was not clear whether all or only certain cell types were capable of stimulating autologous T lymphocytes. It was originally shown that removal of phagocytic or adherent cells increased the stimulating capacity of non-T-cell preparations. B and null lymphocytes were separated based on the capacity of B lymphocytes to form rosettes with mouse erythrocytes and the capacity of null lymphocytes to form rosettes with antibody-sensitized human erythrocytes (Ripley rosettes). Thus, preparations enriched for B or null lymphocytes could be obtained by positive or negative selection. Null cells obtained by either technique stimulated autologous T cells better than did B lymphocytes (Kuntz *et al.*, 1976).

A cell preparation that stimulates autologous T cells more strongly than the original non-T-cell population must be enriched for the most stimulating cells. However, preparations that stimulate less strongly than the original non-T-cell preparation may contain cells that are intrinsically less stimulatory or contain a smaller percentage of the most stimulatory cells. Another fact that contributes to the contradictory conclusions drawn from studies of the cells that stimulate autologous T cell proliferation comes from the method of purification. Thus, differences were obtained when a stimulatory cell preparation was obtained by positive or negative selection. When a population contains three or more cell types, positive selection is the only method for identifying the most stimulatory cell. When a preparation obtained by negative selection is more stimulatory than the original population, one can only conclude that the cells depleted were not the most stimulatory cells. We have summarized the studies that have investigated the type of cell in the non-T-population that stimulates the autologous MLR (Table III). As one can see from Table III, cells isolated by positive selection usually stimulated poorly. Only macrophages prepared by positive selection were more stimulatory than the original preparation (Beale *et al.*, 1980). On the other hand, preparations depleted of null cells (Sakane *et al.*, 1978b) or of B cells (Gottlieb *et al.*, 1979; Kuntz *et al.*, 1976) were more stimulatory than the unfractionated non-T-cell preparation. These results, although not establishing which cell type in the null or B depleted preparation is most stimulatory, are consistent

TABLE III
LINEAGE OF STIMULATORY CELLS IN THE AUTOLOGOUS MIXED LYMPHOCYTE REACTION[a]

Null lymphocytes		B lymphocytes		Macrophages		
Enriched	Depleted	Enriched	Depleted	Enriched	Depleted	References
++	−	+	+++	ND	+++	Kuntz *et al.*, 1976
ND	ND	−	++	ND	ND	Takasugi *et al.*, 1977
ND	ND	ND	ND	−	+++	Smith, 1978
−	+++	ND	ND	+	ND	Sakane *et al.*, 1978b
ND	ND	−	ND	ND	ND	Semenzato *et al.*, 1979
ND	ND	+	+++	−	ND	Gottlieb *et al.*, 1979
+	ND	−	ND	+++	+	Beale *et al.*, 1980

[a] Table III compares the results of several investigators who have identified the cell that stimulates autologous T lymphocytes. The stimulation by unfractionated non-T cells is taken as ++. A preparation that is more stimulatory is identified by +++, and a preparation that is less stimulatory is identified by +. Little or no stimulation is identified by −. It should be noted that if preparations depleted of a subpopulation increased its stimulatory capacity, it was concluded that the depleted cell was not the principal stimulatory cell.

with the conclusion that macrophages are the most stimulatory cells. However, in three studies (Smith, 1978; Sakane *et al.*, 1978b; Gottlieb *et al.*, 1979) macrophages obtained by positive selection were not more stimulatory than the original non-T-population. It should be noted that the stimulatory capacity of macrophages is critically dependent upon their number. Above or below an optimal concentration of macrophages their capacity to stimulate declines (Beale *et al.*, 1980). Addition of macrophages to non-T cells has frequently been reported to inhibit the autologous MLR (Fernandez and MacSween, 1980a). The reason for discrepancy between positively and negatively selected cell preparations is not obvious. It is possible that perturbation of the cell surface that occurs as a consequence of positive selection may alter the stimulatory capacity of the cells. It is difficult to determine whether the stimulatory capacity of preparations that were equal or less than the original non-T-cell population reflects contamination with the most stimulatory cells or the intrinsic but weaker capacity of null cells (Kuntz *et al.*, 1976; Beale *et al.*, 1980) or of B cells (Gottlieb *et al.*, 1979).

Stobo and his associates have identified a subpopulation of macrophages that stimulate autologous T cells. This subpopulation of mac-

rophages expresses a 120,000 MW cell-surface determinant that has been identified by a monoclonal antibody called Mac-120 (Raff *et al.*, 1980). Approximately 40% of peripheral blood macrophages react with Mac-120. The macrophage is the only cell in the non-T-cell preparation that expresses this determinant. Macrophages that carry the Mac-120 determinant stimulate the T lymphocyte identified by the T-29 monoclonal antibody (Hausman *et al.*, 1980). Thus, one component of the autologous MLR reflects the proliferation of T-29-positive T cells stimulated by Mac-120-positive macrophages.

The autologous MLR is far more sensitive to corticosteroids than is the allogeneic MLR. Thus, administration of hydrocortisone prior to obtaining blood for *in vitro* study or addition of hydrocortisone to mixed lymphocyte culture preferentially inhibits the autologous MLR as compared to the allogeneic MLR reaction (Ilfeld *et al.*, 1977; Katz and Fauci, 1979; Hahn *et al.*, 1980). Yu and his associates (1978) have found that preincubation of T cells had little or no effect, whereas preincubation of the non-T-cell preparations with methylprednisolone inhibited its capacity to stimulate autologous T cells. Studies by MacDermott and Stacey (1981) suggest that macrophages within the non-T-cell preparation are affected by corticosteroids.

F. Antigens That Stimulate the Proliferation of Autologous T Cells

Gene products of the HLA-D locus of the MHC or the closely related D-related (Dr) antigens are thought to be the determinants that stimulate the allogeneic and autologous MLR. These antigens have a restricted tissue distribution being expressed primarily on hematopoietic cells. HLA-D determinants are identified by their capacity to stimulate lymphocyte proliferation, and the closely related Dr antigens are identified by serological techniques. Dr antigens have been identified on B cells, macrophages (Wernet, 1976), null cells (Niaudet *et al.*, 1979), a subpopulation of T cells (Greaves *et al.*, 1979), and activated T cells (Ko *et al.*, 1979; Greaves *et al.*, 1979). Using more sensitive techniques, small amounts of Dr antigens have been detected on most blood T cells (Mann and Sharrow, 1980). Evidence that Dr antigens stimulate the autologous MLR comes from the inhibition of the autologous MLR by specific anti-Dr antiserum. Gottlieb and her co-workers (1979) were able to inhibit the autologous MLR using a Fab fragment of a rabbit anti-human Dr antigen antiserum. Huber and co-workers (1981) demonstrated that the continuous presence of heterologous anti-Dr antibodies, as well as the pretreatment of stimulator cells with specific antibody and complement, would inhibit

proliferation of the autologous MLR. The finding that specific Fab antiserum blocks autologous non-T cell-induced proliferation lends support to the stimulatory capabilities of Dr antigens. Bergholz and his associates (1977) correlated the degree of inhibition of the autologous MLR by specific anti-HLA-D antiserum with the expression of anti-Dr antigens on non-T cells. The autologous MLR was inhibited by 79% if the cells were homozygous for the Dr specificity of the antiserum and was inhibited 58% if the cells were heterozygous for the Dr specificity of the antiserum. Finally, the appearance of Dr antigens on activated T cells and the simultaneous acquisition of the capacity to stimulate autologous T cells suggest that Dr determinants stimulate the autologous MLR (Indiveri *et al.*, 1980). This study demonstrated that incubation with PHA led to the appearance of Dr antigens on T cells that can stimulate autologous T-lymphocyte proliferation. This reaction was shown to possess memory and specificity. The stimulation of autologous T cells could not be attributed to residual PHA and was partially blocked by monoclonal anti-Dr antibody.

Although considerable evidence suggests that Dr determinants are critical for stimulation of the autologous MLR, Tomonari and his associates (1980) investigated the shared antigens on allogeneic lymphocytes required to produce a secondary response after a primary autologous MLR. Self recognition was not found to be restricted to one specific locus, but complete HLA identity was necessary for autologous MLR primed lymphocytes to respond to allogeneic cells as self in a secondary response. In Japan certain haplotypes are very common, and the authors believed that the determinants that stimulate the autologous MLR are closely linked to the Dr locus, and use of donors that share complete HLA identity with the donors of the primary culture increases the possibility that this determinant will also be shared.

The HLA-D locus is probably analogous to the murine *I* locus. It was therefore of interest to consider whether reactivity in the autologous MLR could be controlled by immune response genes. One study tried to correlate the level of the autologous MLR with Dr phenotype (Dock and Davey, 1980). No relationship between high, medium, and low responders in the autologous MLR and Dr phenotype was found. More extensive studies and examination of quantitative and qualitative differences in Dr expression, autologous MLR activity, and immune response awaits investigation.

G. The Autologous MLR as an Immune Phenomenon

Lymphocyte proliferation can result from immune or nonimmune mechanisms. The allogeneic MLR exhibits characteristics of an im-

mune response, memory and specificity. The autologous MLR also has these classical attributes of an immune response (Weksler and Kozak, 1977). As has been noted in the allogeneic MLR (Fradelizi and Dausset, 1975; Sheehy *et al.*, 1975), a secondary response in the autologous MLR is characterized by an altered rate of response. In the primary autologous MLR [^{3}H]Tdr incorporation peaked on day 6 or 7, whereas in secondary cultures maximum [^{3}H]Tdr incorporation occurred on day 3. The accelerated response following rechallenge with autologous cells suggested that "memory" had developed in the autoreactive T-cell population. Specificity was demonstrated in the autologous MLR by comparing the magnitude of response in a secondary culture stimulated by cells from the original donor or by cells from an unrelated donor. T cells activated in the autologous MLR incorporated more [^{3}H]Tdr when rechallenged with autologous non-T cells than when challenged with allogeneic non-T cells, despite the fact that allogeneic non-T cells stimulate more [^{3}H]Tdr incorporation in primary cultures than do autologous non-T lymphocytes. The generation of memory and specificity during the autologous MLR was confirmed by Sakane and Green (1979) and Zier *et al.* (1979). In addition, Sakane and Green (1979) confirmed the specificity by eliminating either auto- or allo-reactive T cells, using Budr and light according to the method of Zoschke and Bach (1971). Eliminating autoreactive T cells by this method did not impair the allogeneic reactivity of the residual T-cell population. Conversely, the autologous MLR was well preserved after elimination of alloreactive T cells. However, cross-reactivity between autoreactive and alloreactive T cells was suggested by the fact that removal of allogeneic T cells did diminish the autologous MLR by approximately 30% (Sakane and Green, 1979) and by the fact that alloactivated T cells respond with secondary kinetics to autologous non-T cells (Weksler and Kozak, 1977). Further evidence for this distinct specificity of auto- and alloreactive T cells was obtained by separating auto- and alloreactive T cells by Percoll density centrifugation (Kozak *et al.*, 1981). This technique permits the auto- and alloreactive T cells to be isolated after mixed lymphocyte culture. Thus, after an autologous MLR, all the autoreactive T cells were recovered in the low density fraction whereas less than 5% of autoreactive cells were recovered in the high density fraction that contained 97% of alloreactive T cells. In general, cross-reactivity was seen more clearly when the specificity of the secondary response was studied following an allogeneic MLR.

Clearly the only way of determining whether an individual cell expresses cross-reactivity or whether the observed cross-reactivity results

from the use of a heterogeneous population where the possibility of recruitment exists, is to clone auto- and allo-reactive T cells. If autoactivated clones were restimulated by allogeneic and autologous cells, the cross-reactivity would exist at the level of the individual T cell.

H. Effector Activity Generated during the Autologous MLR

1. Effector-Cell Function Generated during the Autologous MLR

The proliferative response observed in the autologous MLR may represent the afferent limb of an immunological response. The efferent limb of this immune response was investigated by studying suppressor, helper, and cytotoxic activity of cells generated during this reaction. Suppressor T lymphocytes activated during an autologous MLR have been shown to suppress both T and B lymphocyte reactions.

T lymphocytes activated during the 6 days of an autologous MLR were shown by Smith and Knowlton (1979) to suppress the proliferation of T cells and the generation of cytotoxic T lymphocytes (CTL) in an allogeneic MLR. Significant suppression was seen when autoactivated T cells represented as little as 10% of the responding T-cell population in an allogeneic MLR. No suppression was detected if autoactivated T cells were treated with mitomycin C. Autoactivated T cells did not suppress the allogeneic MLR when added 24 hours before the end of the culture period. Whether autoactivated T cells can directly influence the activity of CTL or whether the effect on CTL is secondary to the inhibition of T-cell proliferation is not certain.

B-cell function is also suppressed by T cells activated in an autologous MLR. Thus, addition of autoactivated T cells to peripheral blood lymphocytes (PBL) suppressed their plaque-forming cell (PFC) response to formalinized staphylococci by 50–100% (Innes *et al.*, 1979). Suppressor activity was inhibited by irradiating the autoactivated cells. Autoactivated T cells showed no MHC restriction, as they suppress the PFC response of PBL from the same or unrelated donors. Neither supernatant from an autologous MLR nor T lymphocytes incubated in the absence of non-T cells inhibited the PFC response.

The relationship between autoactivated T cells and suppressor cells was strengthened by the findings of Sakane and Green (1979). These investigators reported that elimination of autoreactive T cells by the addition of Budr to an autologous MLR followed by exposure to light prevented the induction of suppressor cells by Con A. This result indicates that the Con A-induced suppressor cells lie within the population of autoreactive T cells (Fig. 1). Evidence has suggested that Con

A-induced suppressor cells were developed only in the presence of the cell population required for an autologous MLR. Thus, Con A does not induce significant suppressor activity in the absence of an autologous MLR (Innes *et al.*, 1979). Recent studies by Smith and Knowlton (1981) have shown that elimination of Con A-responsive T lymphocytes by Budr and light abrogated the autologous MLR, although the reaction to allogeneic cells and PHA was retained. Furthermore, when autoreactive T lymphocytes were eliminated by Budr and light there was no effect on the response of T lymphocytes to allogeneic cells, PHA, or Con A. In fact, the response to Con A was increased by eliminating the autoactivated T cells. This, perhaps, was a consequence of eliminating Con A-induced suppressor cells contained within the autoreactive T cell population that normally limits the proliferative response in culture. Thus, it appears that suppressor cells generated in an autologous MLR and Con A-induced suppressor cells are drawn from the same subset of T lymphocytes (Fig. 1).

Not all T cells activated in the autologous MLR become suppressor cells. Helper activity has also been generated during the autologous MLR. Hausman and Stobo (1979) have found that autoactivated T cells, isolated by BSA density centrifugation, possessed helper activity manifested by their capacity to augment pokeweed mitogen (PWM)-induced immunoglobulin production by autologous B cells. Little helper activity was found if autoreactive T cells were eliminated from an autologous MLR by Budr and light. High-density T cells not activated in the autologous MLR did not provide helper activity.

The generation of CTL in autologous MLR has been studied by several investigators. Vande Stouwe *et al.* (1977) did not detect any CTL after an autologous MLR when autologous or allogeneic PBL or LCL were used as targets. T cells incubated with autologous B-LCL did not acquire any specific cytotoxic activity. However, these cells did show nonspecific cytotoxicity for a number of B-LCL. Although the autologous MLR did not induce specific CTL, this reaction did provide the proliferative stimulus required for the generation of specific CTL. Thus, the addition of heat-treated allogeneic PBL (which themselves did not induce proliferation or generate CTL cells) to an autologous MLR resulted in the appearance of specific CTL. The CTL generated were specific for the allogeneic cells added to the autologous MLR. No cytotoxic reactivity was generated for autologous cells. Subsequent studies showed that irradiated, allogeneic T cells that fail to induce CTL also stimulated the generation of specific CTL when added to an autologous MLR (Weksler *et al.*, 1980). These experiments demonstrate that the autologous MLR can provide the proliferative stimulus

required to generate CTL in the presence of cells that express determinants that can stimulate CTL differentiation but lack the determinants necessary to induce the proliferation of helper T cells required for the differentiation of precursors to CTL. This strategy could be employed to produce specific CTL for leukemic and other neoplastic cells by adding tumor cells to an autologous MLR. Specific CTL obtained in this manner could be grown up with T-cell growth factor to obtain tumor-specific CTL in numbers that would offer therapeutic potential.

Although the autologous MLR does not generate specific cytotoxicity for autologous cells, Miller and Kaplan (1978) suggested that autologous human B lymphoblasts induced by lipopolysaccharides were lysed by T cells activated in the autologous MLR. Autologous PBL or autologous non-T cells were not lysed. In contrast, Tomonari (1980) demonstrated that nonspecific cytotoxic cells were generated during an autologous MLR which lysed a wide variety of target cells. These cells killed autologous B-cell lines, autologous and mitogen-induced lymphoblasts, and nonlymphoid cell lines (e.g., squamous cell carcinoma). Normal autologous and allogeneic non-T cells were less susceptible to cytotoxic attack. We have reconciled some of these differences with respect to the generation of nonspecific cytotoxic cells in the autologous MLR. We found that the source of serum used to support the autologous MLR is critical. Pooled allogeneic serum did not support the development of nonspecific cytotoxic cells during the autologous MLR whereas autologous serum did. The mechanism by which autologous serum facilitates the generation of nonspecific cytotoxic activity is not clear.

2. *Factors Produced by the Autologous MLR*

Immunoregulatory factors are produced during the autologous MLR. Chiorazzi *et al.* (1979) have demonstrated that supernatant medium from the autologous MLR contain helper factors for immunoglobulin production by autologous PBL or tonsillar lymphocytes. Yu *et al.* (1980) showed that supernatant medium from autologous MLR contained helper factors not only for the development of antibody-producing cells, but also for the differentiation of cytotoxic cells. Supernatant medium, obtained 48 hours after the initiation of an autologous MLR, facilitated the proliferation and differentiation of CTL in cultures containing irradiated allogeneic T cells or TNP-modified autologous cells (Weksler *et al.*, 1980). In the absence of supernatant neither cell proliferation nor differentiation of CTL occurred. In the absence of allogeneic T or TNP-modified autologous cells, the super-

natant did not stimulate cell proliferation or differentiation of CTL. Helper activity in the supernatant medium was not removed by passage over insolubilized anti-Ia or anti-Ig antibodies (Yu *et al.*, 1980). These factors are active with allogeneic as well as autologous T cells and thus appear to express no histocompatibility restriction. Finally, neither lymphocyte-inhibiting factor (Arvilommi and Rasanen, 1980) nor interferon (Manger *et al.*, 1980) has been detected during the autologous MLR.

III. Alterations in the Autologous MLR during Aging and in Human Disease

When the autologous MLR was described by Kuntz *et al.* (1976), a potential role for this reaction in the regulation of the immune response was suggested. Evidence for an immunoregulatory role of this reaction includes the generation, during the autologous MLR, of helper activity (Hausman and Stobo, 1979), of suppressor activity (Sakane and Greene, 1979; Innes *et al.*, 1979; Smith and Knowlton, 1979), and of cytotoxic activity (Miller and Kaplan, 1978; Tomonari, 1980) as well as the generation of soluble factors with immunoregulatory function (Yu *et al.*, 1980; Weksler *et al.*, 1980; Lattime *et al.*, 1981). The impaired autologous MLR observed in patients with diseases associated with abnormal immunological regulation lends additional support for an immunoregulatory role of the autologous MLR. Immune disturbances have been documented in systemic lupus erythematosus, infectious mononucleosis, multiple sclerosis, aging, Sjögren's syndrome, chronic lymphocytic leukemia, and Hodgkin's disease, and in each of these states the autologous MLR is altered (Table IV).

A. Systemic Lupus Erythematosus

Several studies have reported an impairment in the autologous MLR of patients with systemic lupus erythematosus (SLE); SLE is a multisystem disease in which a variety of autoantibodies are found. It has been suggested that the presence of autoantibodies in patients with SLE is due to impaired regulation of B cells by regulatory T cells (Steinberg and Klassen, 1977). Specifically, a deficiency of suppressor T cells in patients with SLE has been reported (Sakane *et al.*, 1978a). The autologous MLR in cultures from 15 patients with inactive SLE was only one-half that observed in cultures from 38 normal subjects (Sakane *et al.*, 1978b). No autologous MLR could be demonstrated in 18 patients with active SLE. These SLE patients were not receiving immunosuppressive drugs or more than 10 mg of prednisone per day. Despite the marked impairment of the autologous MLR in these pa-

TABLE IV
AUTOLOGOUS MIXED LYMPHOCYTE REACTIVITY DURING AGING AND HUMAN DISEASE

Group studied	Response[a] (% of control)	Cellular basis of alteration	Reference
Systemic lupus erythematosus (active)	10	T and non-T	Sakane *et al.*, 1979
Systemic lupus erythematosus (inactive)	17	Non-T	Kuntz *et al.*, 1979
Systemic lupus erythematosus (inactive)	52	T	Sakane *et al.*, 1978
Infectious mononucleosis	15	Non-T	Moody *et al.*, 1978
Multiple sclerosis	600	Non-T	Birnbaum and Kotolinek, 1981
Aged (♀ over 50 years)	500	ND[b]	Fournier and Charreire, 1981
Aged (over 65 years)	33	ND	Fernandez and MacSween, 1980
Sjögren's syndrome	36	ND	Miyasuka *et al.*, 1980
Chronic lymphocytic leukemia	1	ND	Smith *et al.*, 1977
Chronic lymphocytic leukemia	23	ND	Kuntz, 1977
Hodgkin's disease	18	T	Engleman *et al.*, 1980

[a] In order to compare the results obtained by different investigators, the autologous MLR in experimental subjects has been expressed as a percentage of the autologous MLR in healthy individuals reported in the same study. Healthy individuals between 20 and 40 years of age usually served as controls.

[b] ND, not determined.

tients, the allogeneic MLR response was normal. When the T-lymphocyte preparations were fractionated into T-gamma and T-gamma-depleted preparations, the T-gamma preparations from patients with SLE were impaired in their response to both autologous and allogeneic non-T lymphocytes. In contrast, lymphocyte preparations depleted of T-gamma cells responded normally to allogeneic lymphocytes, but not to autologous lymphocytes. Lymphocytes from 6 patients with active SLE did not generate an autologous MLR or normal suppressor activity after incubation with Con A. Sakane and his associates (1978b) suggested that the defect in T-gamma-mediated autologous reactivity might form the basis for the failure to generate Con A-induced suppressor cells, calling attention to the finding of Moretta and his colleagues (1977) that T-gamma cells mediate suppression. Kuntz *et al.* (1979) also reported that lymphocytes from patients with SLE were impaired in their response to autologous non-T cells but had a normal response to allogeneic non-T cells. In this study the autolo-

gous MLR in 7 patients with SLE was only 17% of that observed in healthy age-matched controls. Although T cells from patients with SLE respond normally to allogeneic cells, non-T cells from these patients did not stimulate allogeneic T cells normally. The cellular basis of the impaired autologous MLR was studied in 3 patients and their HLA-identical healthy siblings. T lymphocytes from these patients and their siblings responded normally to non-T cells from the healthy siblings. In contrast, non-T cells from the patients did not stimulate T cells from patients or their healthy siblings normally. Thus, non-T cells from the patients with SLE stimulated only 25% as much [^{3}H]Tdr incorporation by T cells as did non-T cells from the healthy sibling.

Two patients with SLE and their identical twins were studied by Sakane *et al.* (1979). Both T-gamma-enriched and T-gamma-depleted preparations from the active SLE patient did not respond to the non-T cells from her healthy sibling. In addition, non-T cells from the patient failed to stimulate T cells from the healthy sibling. In contrast, the patient with inactive SLE demonstrated a defect in the T-gamma response only. T-gamma depleted cells from inactive SLE responded normally and non-T cells stimulated normally in the autologous MLR. Thus, in the patient with active SLE defects in both the responding and stimulating populations of the autologous MLR were suggested.

B. Infectious Mononucleosis

Infectious mononucleosis (IM) is a viral disease associated with a transient production of autoantibodies (Charlesworth *et al.*, 1978; Thomas and Phillips; 1973) and a depression of cell-mediated immunity (Mangi *et al.*, 1974). In nine patients with acute IM the autologous MLR was found to be markedly depressed (Moody *et al.*, 1979). As was observed in SLE, T cells from patients with IM reacted normally to allogeneic cells despite an impaired autologous MLR. The autologous MLR returned to normal during convalescence. The cellular basis of the impaired autologous MLR in patients with IM was studied by mixing cryopreserved T or non-T lymphocytes obtained at the time of acute illness with T or non-T cells obtained from the same subject during convalescence. Non-T cells obtained during acute disease did not stimulate the proliferation of T cells obtained during acute disease or during convalescence. In contrast, T cells obtained during acute disease responded normally to non-T cells obtained during convalescence. Thus, the cellular basis of the impaired autologous MLR in IM was due to an impaired capacity of non-T cells to stimulate T-cell proliferation. The failure of non-T cells from patients with acute IM to stimulate autologous T cells was not due to suppressor activity in the

non-T cells. Mixing experiments showed that non-T cells obtained during acute disease did not suppress the capacity of convalescent non-T cells to stimulate T-cell proliferation.

C. Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease associated with autoantibodies to nerve cells. Both viral and autoimmune phenomena have been reported to play a role in MS (Norrby, 1978; Sheremata *et al.*, 1974). Birnbaum and Kotolinek (1981) have studied the autologous MLR in patients with MS. They observed a striking increase in the autologous MLR of patients with active MS when compared to age- and sex-matched controls or patients with other neurological diseases. The elevated autologous MLR during acute MS returned to normal levels following therapy with adrenocorticotropic hormone. The cellular basis of the altered autologous MLR in MS was determined by studying non-T cells obtained from the same patient before and after therapy. Non-T cells obtained before therapy induced a greater than normal proliferative response in T cells obtained before or after therapy. In contrast, non-T cells obtained after therapy induced a significantly lower proliferative response in T cells obtained before or after therapy. It should be noted that steroids are known to suppress the autologous MLR by depressing the capacity of non-T cells to stimulate autologous T cell proliferation (Yu *et al.*, 1978). These studies did not directly compare the response of T cells from patients with active MS to the response of T cells from healthy HLA-identical siblings. Thus, it remains possible that the responding T-cell population may also contribute to the increased autologous MLR. The recent report of Reinherz and co-workers (1980) suggests that the ratio of OKT4+ to OKT5+ T cells is markedly increased in patients with MS. As we have preliminary evidence that autologous reactive T cells appear to be included in the OKT4+ T-cell population (Kozak *et al.*, 1981), it is possible that the relative enrichment of the T-cell population with the OKT4+ subpopulation may contribute to the increased autologous MLR in these patients.

D. Aging

Normal human aging is associated with a spectrum of immune abnormalities including impaired cell-mediated immunity, autoantibody formation, and an increased incidence of benign serum monoclonal immunoglobulins (Makinodan and Kay, 1980). Fournier and Charreire (1981) have studied the autologous MLR in subjects of varying ages. The autologous MLR in females over 50 years of age was increased

fivefold when compared to females below the age of 50. No increase in the autologous MLR was seen in male subjects over the age of 50. These authors had previously reported that the percentage of T cells that form rosettes with autologous erythrocytes (Tar) increases with age in females but not in males (Fournier and Charreire, 1977). As the autoreactive T cells are found in the Tar population (Palacios *et al.*, 1980), it was suggested that the increase in the number of Tar cells in women with age might explain their increased autologous MLR. In contrast, Fernandez and McSween (1980b) have reported a depressed autologous MLR in aged subjects. They found that the autologous MLR in 10 healthy subjects over 65 was only one-third that seen in young healthy controls. We have studied the autologous MLR in more than 50 healthy subjects over the age of 65 and found it to be depressed by approximately 50% when compared with healthy control subjects between the ages of 20 to 40 years (Moody *et al.*, 1981). The autologous MLR was depressed in both male and female elderly subjects in our study. The explanation for the different results obtained by Fournier and Charreire, and by Fernandez and McSween has not been established, but it should be noted that Fournier and Charreire used PBL as the responding population while Fernandez and MacSween (and we) used T cells as the responding population.

E. Sjögren's Syndrome

Sjögren's syndrome (SS) is a autoimmune disease characterized by lymphocyte infiltration of the lacrimal or salivary glands. This disease is associated with a variety of autoimmune phenomena. Antinuclear antibodies, rheumatoid factor, and polyclonal hypergammaglobulinemia are frequently observed, and in addition cell-mediated immunity is frequently impaired (Alspaugh *et al.*, 1976). Miyasaka *et al.* (1980) have studied the autologous MLR in patients with SS. In 25 patients with SS, the autologous MLR was depressed by approximately 64% as compared to the autologous MLR in 18 normal subjects. There was no correlation between the decreased autologous MLR and age, serum immunoglobulin levels, antilymphocyte antibody, or PHA response. There was a significant correlation between the decreased autologous MLR and the decreased response to Con A. These authors believed this finding to indicate a possible T-cell defect in the autologous MLR. However, the authors also pointed out that a non-T cell defect may also be present since B cells and macrophages from SS patients had a reduced capacity to stimulate allogeneic T cells. Thus, no definite conclusion can be made concerning the cellular basis of the impaired autologous MLR in patients with SS.

F. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a disease characterized by a monoclonal proliferation of immunoglobulin-bearing B cells in the peripheral blood and bone marrow (Salsano *et al.*, 1974; Fu *et al.*, 1974). Peripheral blood lymphocytes from patients with CLL respond poorly to Con A, PWM, and PHA (Smith *et al.*, 1972; Ruhl *et al.*, 1975) and to allogeneic lymphocytes (Kasakura, 1975; Han *et al.*, 1977). Kuntz (1977) studied the autologous MLR from patients and normal subjects in cultures with human AB serum. The [^{3}H]Tdr incorporated by patients with CLL in the autologous MLR was only 23% of that incorporated by lymphocytes from normal subjects. Similar findings were reported by Smith *et al.* (1977). These investigators established the autologous MLR using T-cell and non-T cell preparations in culture medium containing autologous sera. No autologous MLR was detected in cultures from 7 patients with CLL. In contrast, the autologous MLR was observed in each of the normal subjects. The lack of an autologous MLR in cultures from patients with CLL did not result from factors present in their serum. Serum from patients with CLL when added to cultures from normal subjects did not depress the autologous MLR and the autologous MLR was not restored in cultures from CLL patients if normal serum was used instead of autologous serum. The authors speculated that the lack of an autologous MLR in CLL patients could be a result of the monoclonal nature of the B cells or a defect in helper or suppressor T cells. Subsequently, Smith and Knowlton (1980) suggested that the fundamental defect is in the responding T-cell population based on the poor response of T cells from CLL patients to Con A and the presence of the autoreactive T cells within the population of T cells that respond to Con A. The authors further suggest that the normal ability of non-T cells from patients with CLL to stimulate in an allogeneic MLR was consistent with a T-cell defect. It is important to note that the non-T-cell population that stimulates autologous or allogeneic T cells may be different. Furthermore, Halper *et al.* (1980) have reported that B lymphocytes from patients with CLL were impaired in their capacity to stimulate allogeneic T cells.

G. Hodgkin's Disease

Immunological activity in patients with Hodgkin's Disease (HD) has been extensively studied. Although antibody responses may be normal in these patients (Brown *et al.*, 1967), T-cell-mediated immune reactions, such as delayed hypersensitivity (Aisenberg, 1962) and al-

lograft rejection (Miller *et al.*, 1969) as well as *in vitro* T lymphocyte responses (Brown *et al.*, 1967; Bjorkolm *et al.*, 1976), are depressed. Engleman *et al.* (1980) investigated the autologous MLR in 64 patients with HD. A marked depression in the autologous MLR was observed in newly diagnosed patients as well as in patients who had been in remission for as long as 15 years. The defect observed was not related to altered kinetics of the response, to impaired lymphocyte viability in culture, or to an effect of plasma from patients with HD. The cellular basis of the defect was investigated in two patients and their healthy MHC-identical siblings. T cells from the healthy siblings responded normally to the non-T cells of the patients, whereas T cells from the 2 patients were depressed in their response of non-T cells from their healthy siblings as well as their own non-T cells. Thus, an impaired response of T cells from patients with HD to autologous non-T cells appears to explain the defective autologous MLR in this disease.

It is clear that in several pathological states in which immune function is altered the autologous MLR is also altered. However, the cellular basis of the defect in the autologous MLR differs. In HD the defect is in the responding T-cell population. In other diseases, e.g., IM, and MS, a defect in the stimulating non-T cell population is found. In SLE, defects in both the T-cell and non-T cell preparations have been reported. Since the autologous MLR can generate immunoregulatory activity *in vitro*, it is tempting to speculate that the abnormalities in the autologous MLR may underlie the impaired immune responses in patients with several diseases.

IV. Autologous and Syngeneic Reactivity in Experimental Animals

A. The Isogeneic Lymphocyte Interaction in Mice

Although the autologous MLR has received considerable attention since 1975, a similar lymphoid cell interaction had been recognized by 1970 in experimental animals. Howe *et al.* (1970a) were the first to report a reaction between neonatal T cells and syngeneic spleen cells from adult mice. They termed this reaction the isogeneic lymphocyte interaction (ILI). We shall use this term to describe the reaction of thymocytes to lymphoid cells from syngeneic donors of a different age. In this reaction adult spleen cells stimulated the proliferation of syngeneic neonatal thymus cells. Fetal thymus cells expressed a low ILI. The maximum ILI was observed with thymus cells from animals 2–3 days old and spleen cells from syngeneic 12-week-old mice (Howe, 1973). After 1 week of age thymocytes rapidly lost their capac-

ity to respond to adult spleen cells. Neonatal thymocytes failed to respond to syngeneic cells from sources other than the spleen. Furthermore, spleen cells from neonatally thymectomized mice stimulated only 25% as much [^{3}H]Tdr incorporation by neonatal thymocytes as did normal spleen cells (Howe *et al.*, 1970b). They suggested that the ILI reflected the recognition of specific splenic antigens by neonatal thymocytes and that the loss of the ILI with increasing age reflects the acquisition of immunological tolerance.

In 1972, von Boehmer and Byrd suggested that the ILI reflects a normal recognition of antigens on spleen cells by the neonatal thymus, not a reaction that occurs before the acquisition of self-tolerance early in development. In fact, the autostimulatory antigens on spleen cells do not appear until mice are 3–8 weeks old (Ponzio *et al.*, 1975). In contrast, the antigens that stimulate allogeneic reactivity are present on neonatal spleen cells (von Boehmer and Byrd, 1972).

B. The Syngeneic MLR in Mice

Von Boehmer and Byrd (1972) also observed that adult spleen cells could stimulate the proliferation of adult thymocytes. This reaction between syngeneic lymphoid cell from animals of the same age was termed the syngeneic MLR (SMLR). This reaction appears to be comparable to the autologous MLR studied in outbred populations. Von Boehmer and Adams (1973) characterized the lymphocyte subpopulation that stimulates the SMLR. The stimulating cell was present in lymph node, spleen, peripheral blood, and peritoneal exudate cells from 10-week-old mice. The stimulatory cell was not an erythroid cell and did not require the presence of macrophages or granulocytes. Additional studies suggested that the stimulatory cells were mature B cells. Thymus, bone marrow, or peripheral T cells from adult mice as well as spleen cells from neonatal mice failed to stimulate syngeneic thymus cells (von Boehmer *et al.*, 1972; von Boehmer, 1974).

1. The Responding Cell

Ponzio *et al.* (1975) found autoreactive cells in the peripheral lymphoid organs of adult mice. Their studies demonstrated that lymph node cells from adult mice contain T cells that proliferate when cultured with splenic non-T cells from autologous or syngeneic animals. Responding T cells were first detected in lymph nodes from 8-week-old animals, whereas stimulatory cells were first detected in spleens from 3-week-old animals.

Smith and Pasternak (1978) have reported that spleens of adult mice also contain a population of nylon-wool-nonadherent T cells that re-

spond to syngeneic non-T cells. The responding T cells express the Lyt 1^+, 2, 3^- phenotype (Pasternak *et al.*, 1980). These cells were subsequently found in lymph nodes and thoracic duct cells (Pasternak *et al.*, 1980). Lattime *et al.* (1980) and Glimcher *et al.* (1980) confirmed the fact that the responding cell in the SMLR carries the Lyt 1^+, 2, 3^- phenotype.

2. *The Stimulating Cell*

Studies that compared the response of adult lymph node T cells (SMLR) and neonatal thymocytes (ILI) to syngeneic non-T cells suggested that the antigens that stimulate the ILI differ from those that stimulate the SMLR (Ponzio *et al.*, 1975). Bone marrow cells stimulate neither the ILI nor SMLR. Cells in the spleen or lymph nodes of mice more than 3 weeks of age stimulate syngeneic neonatal T cells, but not lymph node T cells. The antigen on these cells was termed murine differentiation antigen-1 (MDA-1). In contrast, spleen cells from mice more than 8 weeks of age stimulate both neonatal T cells and adult lymph node T cells. The antigen on these cells was termed MDA-2. The spleen cells that express these antigens appeared to be B cells, as treatment with anti-immunoglobulin sera and complement eliminated the ability of spleen cells to stimulate lymph node T cells or neonatal thymocytes. Thus, these studies support the conclusions of von Boehmer (1974; von Boehmer and Byrd, 1972) that the cells that stimulate the SMLR were included in the B-cell population. Finke *et al.* (1976) confirmed the earlier observations and showed by depletion with antisera specific for the various classes of Ig and complement that only B cells that expressed surface IgG_1 on the surface stimulated autologous and syngeneic reactivity. B cells that expressed other classes of immunoglobulin did not.

Recent reports have demonstrated that Ia-bearing cells are potent stimulators in the SMLR. Bocchieri and Smith (1980) and Ponzio (1980) have reported that specific anti-Ia antiserum blocked the SMLR. Preincubating non-T cells with anti-Ia antiserum also inhibited their capacity to stimulate syngeneic T cells. Both B cells and macrophages express the Ia determinant. The macrophage or cells of this lineage have been suggested to be the major stimulus of the SMLR. Nussenzweig and Steinman (1980) have reported that dendritic cells that lack markers carried by T cells, B cells, or macrophages but express Ia antigens are the most potent stimulators of syngeneic T-cell proliferation. Their studies suggest that the stimulatory capacity of dendritic cells cannot be attributed to exogenous antigens acquired *in vivo*, or to heterologous serum components *in vitro*. Other studies

have also suggested that the major stimulatory cell in the SMLR is an Ia-bearing macrophage-like cell (Lattime *et al.*, 1980).

Physiological concentrations of hydrocortisone (7.5×10^{-8} *M*) suppress the murine SMLR (Ting and Ranney, 1980). This concentration suppresses the SMLR by more than 90% without affecting the response to Con A or allogeneic cells. Higher concentrations of hydrocortisone suppressed all three responses. In contrast to studies in man (Yu *et al.*, 1978), pretreatment of either the stimulator or responder cells did not effect the SMLR. Hydrocortisone appeared to act by inhibiting an early event in the SMLR, as its effect is manifest only during the first 30 hours of a 96-hour culture.

C. The Syngeneic MLR in Other Species

In addition to the human and the mouse, the autologous or SMLR has been studied in several other species. Investigation of the SMLR in the guinea pig has provided considerable insight into the genetic restriction between stimulating and responding cells (Yamashita and Shevach, 1980). The SMLR was studied in strains 2, 13, and the $(2 \times 13)F_1$ guinea pigs. Strain 2 and strain 13 animals differ only in the *I* region of the MHC. Lymph node cells from either strain were stimulated to incorporate [^{3}H]Tdr by syngeneic peritoneal exudate cells (PEC). The SMLR in these animals showed memory and specificity. Thus, the peak of the proliferative response of T cells occurred 8 days after challenge in primary culture, but 4 days after rechallenge. Specificity was demonstrated by challenging T cells primed with syngeneic PEC with strain 2 or strain 13 PEC. A secondary response was observed only when primed T cells were rechallenged with PEC from syngeneic animals. These authors also observed that the receptors for self MHC determinants were clonally distributed. Thus, two distinct populations of autoreactive T cells exist in $(2 \times 13)F_1$ animals. Lymph node cells from F_1 animals, stimulated with PEC from F_1 animals, show a secondary response when challenged with cells from either parent. However, T cells from F_1 animals primed with PEC from one parental strain did not show a secondary response when challenged with PEC from the other parental strain. The genetic restriction observed in the SMLR is the same as that observed between T cells and antigen pulsed macrophages (Thomas and Shevach, 1977; Paul *et al.*, 1977).

Cells that stimulate the SMLR in the guinea pig are found not only in PEC, but also in adherent spleen cell preparations. Unfractionated spleen or lymph node cells as well as T cells stimulated autologous T cells poorly. The role of Ia antigens on the stimulatory cells was inves-

tigated by measuring the effect of treating the stimulatory cells with anti-Ia antiserum and complement. Although only 20–25% of the PEC population were lysed by anti-Ia antibody, the remaining cells did not stimulate syngeneic T cells. Moreover, when $(2 \times 13)F_1$ T cells were primed with PEC from strain 2 animals and rechallenged with strain 2 PEC, the secondary response was blocked by strain 2-specific anti-Ia antiserum, but not strain 13-specific anti-Ia antiserum. Treatment of the responding cells with anti-Ia antiserum had no effect on the guinea pig SMLR.

Milthorp and Richter (1979) have reported that the lymphocytes from New Zealand white rabbits express an autologous MLR. Peripheral blood leukocytes or spleen cells from rabbits were stimulated to incorporate [^{3}H]Tdr in culture with autologous mitomycin C-treated lymphocytes from the appendix, Peyer's patches, sacculus rotundus, and to a lesser, but still significant, extent from the spleen and the lymph nodes.

D. Effector Functions Generated in the Sygeneic MLR

Less is known about the generation of effector function in the SMLR than in the human autologous MLR. We have found no studies that comment on the generation of helper or suppressor activity in the SMLR. Unpublished studies from our laboratory have demonstrated that T cells activated during the SMLR suppress the proliferative response of syngeneic spleen cells to Con A or to allogeneic spleen cells (Gutowski and Weksler, unpublished observation).

No cytotoxic activity was generated during an SMLR toward syngeneic or allogeneic mitogen-induced lymphoblasts (Smith and Pasternak, 1978) or toward syngeneic and allogeneic transformed cells (Ponzio, 1980). However, as has been shown in the human autologous MLR (Vande Stouwe *et al.*, 1977), the SMLR can provide the proliferative stimulus required to generate CTL in culture with foreign cells that themselves cannot stimulate a proliferative response or the generation of cytotoxic cells. Thus, the addition of heat-treated allogeneic cells to an SMLR resulted in the generation of allo-specific CTL (Ponzio *et al.*, 1979). Purified T cells alone or T cells with either syngeneic stimulators or heat-treated allogeneic stimulators failed to generate CTL. *In vitro* generation of CTL requires cooperation between two subpopulations of responder T cells; Lyt 1^+ helper cells which respond to Ia determinants and Lyt 2, 3^+ cytotoxic cells that recognize foreign H-2K and D gene products (Cantor and Boyse, 1975). If Lyt 1^+ helper cells are not stimulated, CTL do not differentiate.

Although a soluble helper factor has not been demonstrated in the

SMLR as it has in the human autologous MLR (Yu *et al.*, 1980; Weksler *et al.*, 1980), the production of another T-cell product, T-cell growth factor (TCGF) has been reported in the SMLR (Lattime *et al.*, 1981). The proliferation of nylon-wool-nonadherent murine T cells induced by irradiated syngeneic nylon-adherent spleen cells and the production of TCGF is dependent on the expression of Ia antigens on the stimulatory cells. Thus, the addition of anti-Ia antisera inhibited both proliferation and the production of TCGF.

E. Alterations in the Syngeneic MLR with Age and Disease

Autologous or syngeneic reactivity has been demonstrated in a number of mouse strains including CBA/J (Howe *et al.*, 1970), BALB/c (Smith and Pasternak, 1978), C57B1/6 and DBA/2 (Nussenzweig and Steinman, 1980), and AKR, C3H, and C58 (Smith and Pasternak, 1978). However, the SMLR has been shown to be impaired in strains of mice with autoimmune disturbances.

The SMLR has been studied in the NZB mice, a strain with a variety of abnormalities that resemble the human disease SLE (Talal and Steinberg, 1974). Like the patients with SLE, NZB mice do not generate a normal SMLR. The SMLR was present but diminished in 4-week-old NZB mice but was absent in 23-week-old NZB mice (Smith and Pasternak, 1978). Splenic T cells from 4-week-old mice respond to syngeneic non-T cells from 23-week-old or 4-week-old donors. This suggested that the defect in the SMLR in NZB mice lay in an impaired response of T cells to syngeneic non-T cells. Subsequent studies by Glimcher *et al.* (1980) not only confirmed these findings, but also demonstrated a temporal relationship between the decline of the SMLR and the onset of autoimmune disease in other autoimmune strains. Thus, by 12 weeks of age, the MRL/lpr strain of mice manifests autoantibodies and substantial lymphoproliferation and fails to manifest an SMLR. Likewise, by 12 weeks of age, the (NZB × NZW)F_1 mouse manifests antinuclear antibodies as well as alterations in suppressor cell function and fails to show an SMLR. Reciprocal mixing experiments between young and adult responder or stimulator cells showed that the defect in the NZB, (NZB × NZW)F_1 and MRL/lpr strains resided in the responding T cell. No evidence for excessive suppressor activity mediating the impaired SMLR in autoimmune mice could be found.

Other studies have extended these findings to other autoimmune prone strains (Hom and Talal, 1981). An impaired SMLR was observed in the (NZB × NZW)F_1 MRL/lpr, and BXSB strains. A decrease in the SMLR was observed in these autoimmune susceptible strains before

the appearance of clinical symptoms. In (NZB × NZW)F_1 mice the SMLR is lower in females at 2 months of age than in normal C57B1/6 mice. A very weak SMLR was observed in 2-month-old BXSB mice and MRL/1pr mice. At 7 months of age the SMLR declines to almost undetectable levels. In the (NZB × NZW)F_1 mouse, the decreased SMLR is more pronounced in the females, whereas in the BXSB strain the SMLR is lower in the males. The degree of impairment in the SMLR thus parallels the development of autoimmune phenomena, as the (NZB × NZW)F_1 females, and BXSB males show an early onset of both impaired SMLR and autoimmune disease. In contrast, male and female C57B1/6 mice show no difference in the SMLR response. As in previous studies the impaired SMLR in autoimmune strains of mice was shown to be due to a failure of T cells to respond to syngeneic non-T cells.

The SMLR has been investigated in recombinant inbred lines derived from F_1 matings of mice with normal and impaired SMLR reactivity (Bocchieri *et al.*, 1981). These studies examined the SMLR in NZB ($H\text{-}2^d$) mice, in C58 mice ($H\text{-}2^k$), and in NZB × C58 recombinant inbred lines. Approximately one-half of the recombinant lines demonstrated an impaired SMLR and one-half demonstrated levels of reactivity comparable to the normal C58 parental strain. The level of SMLR was not correlated with H-2 phenotype or allotype of the immunoglobulin heavy or kappa light chain. Non-T cells from impaired $H\text{-}2^k$ recombinant inbred lines were capable of stimulating a SMLR when cultured with C58 T cells. Furthermore, SMLR reactive $H\text{-}2^d$ recombinant strains could respond to NZB non-T cells even though NZB T-cells did not respond to NZB non-T cells. NZB T-cells did not respond to any of the $H\text{-}2^d$ recombinant inbred lines. Thus, the defective SMLR in NZB mice and in recombinant inbred lines appears to be due to defects in the responding cell population. These authors also represent preliminary data that the impaired SMLR is not correlated with the presence of Coombs-positive hemolytic anemia or the production of anti-thymocyte autoantibody. On the one hand, autoantibodies were found in mice with a normal SMLR. On the other hand, mice with an impaired SMLR did not always have detectable autoantibodies. These important studies suggest that the genes that regulate the production of autoantibodies can be distinguished from genes that regulate the reactivity of T cells in the SMLR.

The SMLR has also been studied in the immunodeficient CBA/N mouse strain. CBA/N mice have an X-linked recessive trait that results in the failure of male and homozygous female animals to respond to a class of thymic independent antigens (Huber *et al.*, 1977). Dustoor *et*

al. (1981) have found that neonatal thymocytes from these mice do not respond to adult syngeneic spleen cells in the ILI. In contrast, T cells from lymph nodes of adult animals respond normally to autologous or syngeneic spleen cells. This defect was shown to reside in the stimulating cell population, since both male and female F_1 neonatal thymus cells respond to CBA/J cells. Therefore, CBA/N mice appear to lack MDA-1 but possess MDA-2 on the stimulating cell. The relationship of MDA-1 and MDA-2 to other known B cell surface markers has not been elucidated.

Finally, an interesting interaction between spleen cells from animals of different ages has been reported by Callard *et al.* (1979). The response of spleen cells from young (3–4 months) and from old (24–30 months) mice to syngeneic spleen cells from animals of the same or different ages were studied. All the animals were of the same sex. More [^{3}H]Tdr incorporation occurred when cultures contained spleen cells from animals of different ages. Serological evidence also suggested the presence of an antigen on spleen cells from old mice recognized by lymphocytes from young mice. Repeated injection of spleen and lymph node cells from old donors into young syngeneic animals lead to the appearance of an antibody in young recipients that reacted with more lymphocytes in old animals (7%) than in young mice (2%). These results suggested that the antigeneic repertoire on lymphocytes from young and old mice is different and that such differences can be recognized immunologically. These data are similar to the difference in idiotype repertoire expressed by lymphocytes from the mice of different ages (Goidl *et al.*, 1980). Anti-idiotypic antibody can also recognize differences between surface determinants on lymphocytes from old and young mice.

F. Other Autologous Interactions

Although the interaction of T lymphocytes with syngeneic hematopoietic cells has been most extensively studied, T cells have also been shown to recognize other autologous or syngeneic cells. It has been reported that epidermal cells from guinea pigs can be recognized by autologous PBL (Beucher and Saurat, 1979). PBL from guinea pigs were stimulated to proliferate in culture by mitomycin C-treated autologous epidermal cells.

Studies have also demonstrated that normal rat T cells can recognize and specifically react against other autologous tissues. CTL develop when rate spleen cells were cultured with syngeneic thymus reticulum cells (Cohen and Wekerle, 1972). These cells were specific for

the thymic epithelial targets. Similarly, CTL developed in cultures of T cells with syngeneic testicular preparations (Wekerle and Begemann, 1976). Heterologous serum factors do not appear to play a role in determining the specificity of the responding cells (Wekerle, 1977). The immunological nature of the reaction between lymphocytes and testicular cells was suggested by the demonstration of immunological memory in this system (Wekerle, 1978). Finally, incubation of lymphocytes with testicular cells primes T cells for an SMLR with hematopoietic cells (Wekerle, 1978). It was suggested that Sertoli cells, which appear to be of macrophage lineage, present in the testicular cell preparation may stimulate this reaction (Wekerle, 1978).

The reaction of lymphocytes with autologous cells has also been described *in vivo*. Thus, lymphocytes injected into the foot pads of mice induced enlargement of the regional lymph nodes. This reaction has been termed the syngeneic graft-versus-host reaction (SGvH). Carnaud *et al.* (1977) showed that spleen cells from adult thymectomized mice caused an SGvH reaction. Thus, enlargement of the regional lymph nodes followed transfer of spleen cells to syngeneic recipients. The SGvH reaction was greater if spleen cells were obtained from cyclophosphamide-treated donors (L'age-Stehr and Diamantstein, 1978). Gozes *et al.* (1978) found somewhat different results in studies of the SGvH. These investigators found that the SGvH reaction was induced only by spleen cells from old mice. Spleen cells from 104-week-old C57Bl/6 mice caused significant enlargement of the regional lymph nodes in young or old syngeneic recipients. In contrast, injection of spleen cells from young mice did not induce a SGvH in either young or old recipients. The capacity of spleen cells from old animals to produce a local SGvH reaction was eliminated by treating old spleen cells with anti-theta antiserum and complement.

V. Concluding Remarks

Studies of the autologous MLR in humans and comparable reactions in experimental animals offer overwhelming evidence that T lymphocytes are stimulated by autologous non-T cells to proliferate, to generate helper and suppressor activity, and, under appropriate conditions, to develop cytotoxic reactivity. Furthermore, in several disease states associated with immunological abnormalities, the autologous MLR is abnormal. These results suggested the importance of this reaction in

the regulation of the immune response. While the *in vitro* phenomena were not in doubt, the biological significance of the autologous MLR has been questioned. It had been suggested that the autologous MLR might result from perturbations of the responding or stimulating cells during cell fractionation or exposure to foreign sera in culture. These possibilities have been carefully evaluated and not found to contribute to the generation of the autologous MLR. Thus, this reaction occurs in unfractionated lymphocyte preparations, takes place when both stimulating and responding cells are prepared by negative selection, and occurs in the presence of autologous serum. There is no evidence that the autologous MLR is a laboratory artifact. In fact, the autologous MLR appears to be a good model for the cooperative interaction between cells involved in the immune response.

The early workers suggested that thymocytes that recognized syngeneic spleen cells had escaped self-tolerance, a view that reflected the dominance of the clonal deletion theory of self-tolerance in the early 1970s. At present, the existence of lymphocytes with receptors for autologous determinants not only is not questioned, but lymphocytes that recognize autologous MHC determinants are known to play a crucial role in the regulation of the immune response. The genetic restriction required for cell cooperation among lymphocyte subpopulations and mononuclear cells involves the recognition of MHC gene products. In man these determinants, specified by the HLA-D locus also stimulate the autologous MLR. Thus, the autologous MLR is stimulated by the same determinants that are involved in cell cooperation.

The generation of helper and suppressor activity during the autologous MLR suggested a role for this reaction in immune regulation. It is likely that helper and suppressor cells are drawn from distinct subpopulations of autoreactive T lymphocytes. It is possible that both helper and suppressor cells are activated by the same determinants in the autologous MLR. Alternatively, different subpopulations of autoreactive T lymphocytes that respond to monocytes or to non-T lymphocytes (Hausman *et al.*, 1980) may give rise to effector cells with different functions. Thus, it is possible that T lymphocytes activated by monocytes become helper cells, whereas T lymphocytes activated by non-T lymphocytes might become suppressor cells. Although little is known about the determinants that stimulate the differentiation of helper or suppressor cells, the possibility that Ia and idiotypic determinants stimulate helper and suppressor cells, respectively, should be considered.

The large proportion of T lymphocytes that recognize allogeneic cells (Bach *et al.*, 1969) has been difficult to explain. Hypotheses attempting to account for this observation (Jerne, 1971; Williamson, 1980) suggest that germ line genes specify receptors for all allogeneic MHC determinants. However, Bodmer (1972) has questioned such hypotheses. Individuals are not likely to be exposed to allogeneic determinants. Therefore, no evolutionary pressures necessary to maintain a repertoire of receptors for allogeneic MHC determinants are likely to exist. It appears more reasonable to suggest that T lymphocytes that express alloreactivity are generated following sensitization to chemical ligands, viral antigens, or developmental antigens presented in association with autologous MHC determinants. Cross-reactivity between altered self and allogeneic MHC determinants could explain alloreactivity. Such cross-reactivity has been demonstrated. Thus, cytotoxic T lymphocytes generated in response to Sendai virus lyse allogeneic cells as well as virus-modified syngeneic cells (Finberg *et al.*, 1978). Conversely, cytotoxic T lymphocytes generated in the allogeneic MLR lyse hapten-modified syngeneic cells (Lemonnier *et al.*, 1977). Finally, Sredni and Schwartz (1980) have reported that a T-cell clone from mice immunized with DNP-ovalbumin-modified syngeneic cells is stimulated to proliferate not only by DNP-ovalbumin-modified syngeneic cells but also by allogeneic cells. Thus, it appears that cross-reactivity between altered-self and allogeneic cells is expressed at the level of the individual T lymphocyte. If alloreactivity follows sensitization to antigen-modified autologous MHC determinants, one would predict that animals raised in an environment with little or no antigen exposure would not develop alloreactive T lymphocytes normally.

This review has summarized present knowledge of the autologous MLR. These studies strongly imply a biological role of the autologous MLR in the immune response. Today, the most pressing need is to establish the biological significance of this reaction *in vivo*. Will techniques that specifically alter the autologous MLR influence immune competence *in vivo*? Two possible approaches to this question should be considered in experimental animals. The first is to reconstitute the impaired autoreactive T-lymphocyte population in autoimmune-prone strains of mice. It would be expected that techniques that reconstitute this population would influence immune function, disease and lifespan. The second approach is to deplete normal mice of autoreactive T lymphocytes. If the autologous MLR plays an important role in the immune response *in vivo*, depletion of autoreactive T lymphocytes

would be expected to lead to impaired immune competence. We look to these experiments to establish the biological significance of the autologous MLR.

Note: Recently, an impaired autologous mixed-lymphocyte reaction was reported in patients with primary biliary cirrhosis [James, S. P., Elson, C. O., Waggoner, J. G., Jones, E. A., and Strober, W. (1980). *J. Clin. Invest.* **66,** 1305)].

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