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Preface

The initial impetus to create a work combining aspects of cellular immunology with their clinical applications grew from the editors' discussions of the area's needs with many of the leaders in the field over a period of time. From the nucleus of ideas that emerged, we have here attempted to create a unified and integrated coverage of the rapidly growing field of cellular immunology research and to trace out—from what seems at times a genuine plethora of important new findings—the many and often important clinical implications.

Because of this approach, the chapters of *Clinical Cellular Immunology* attempt to be more than critical reviews of research and clinical data, going beyond analysis to synthesize working hypotheses about the functional meaning of cellular immunological phenomena and their likely clinical significance. To accomplish this undertaking, the text begins first with a consideration of the molecular aspects of antigen recognition (Luderer and Harvey) and of the ensuing regulatory program initiation (Fathman). Then, the functional subsets of lymphocytes as they interact to produce and control the developing immune response are explored in detail (Sigel et al.), followed by a unique analytical dissection of the action of immunosuppressive agents on the sundry inductive and regulatory immunologic pathways (Sigel et al.).

A majority of the data and conclusions drawn by the authors in the previous chapters arise from work on murine systems, although wherever appropriate, human data has been introduced. But Keller et al., in an interesting dissection of the immunobiology of human non-Hodgkin's lymphomas, add an important dimension to the previous chapters as they demonstrate how the non-Hodgkin's lymphomas appear to represent various functional or prefunctional lymphoid subsets that have been locked at a particular differentiative state by the neoplastic process. Keller et al. then

bridge the gap between mouse and humankind with a good synthesis of functional and molecular evidence showing the general validity of the murine T and B subsets as models for human immunology.

Thorough analyses of cell-mediated immunity in autoimmune disease (Burek, Rose, and Lillehoj) and of tumor immunity (Specter and Friedman) are then presented. These chapters emphasize the contribution of different lymphoid subsets to frank disease. This is especially appropriate since increasingly convincing evidence suggests that pathologic alterations in the T cell regulatory network, especially that of T suppressor cells, are at least partially responsible for the onset of disease.

The elegant and effective means of cell–cell communication that have evolved to modulate the immune response possess real clinical significance, both therapeutically and (possibly) diagnostically. Thus, Maziarz and Gottlieb chose to discuss the group of molecules collectively present in leukocyte diazylates that are operationally termed transfer factor. This is a timely analysis since the literature on the subject is large and both experimental and clinical investigations have proceeded with various transfer factor preparations. The difficulties experienced in drawing clinical conclusions for the establishment of a regular therapeutic role for transfer factor are also likely to be experienced with other immunomodulatory substances (e.g., interferon) as clinical trials are begun. Since many therapeutic regimens will attempt to address replacement of a particular function of a dysfunctional cell population, the proper assay of defective cell function(s) becomes a necessity. To this end, Fudenberg et al. describe a battery of immunologic tests for the diagnosis and monitoring of defects in immunodeficiency.

In the final chapter, Guarnatta and Parkhouse summarize the experimental approaches to the hybridization of lymphocytes. It is appropriate to end the text with the technical aspects of hybridoma production since this technology is currently redefining the cell surfaces of the lymphocyte subsets and both neoplastic and normal cells. In addition to the obvious diagnostic significance, therapeutic possibilities exist for specific cytotoxic drug targeting to tumor (primary and metastatic) or for removal of deleterious lymphoid subpopulations, to cite only a few examples. It is our hope that this work will provide a meaningful explanation of cellular immunology as its special relevance to disease processes and clinical medicine begins to emerge.

Acknowledgments

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Clinical Cellular Immunology

Chapter 1

The T Cell Antigen Receptor

Structural and Functional Considerations

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1. Overview

Initiation of the immune response following antigenic challenge begins with the specific interaction of epitopes on the antigen- and plasma membrane-associated receptors found on the cell surface of lymphocytes. Certainly the interaction of antigen with both regulatory and effector thymus-derived lymphocytes (T cells) is a critical event in this process. According to clonal selection theories (Burnett, 1969), antigen initially interacts with a relatively small number of T cells that exhibit antigen receptors of the appropriate specificity. The availability of these T cell specificities reflects events hypothesized to have occurred during early ontogeny, thus,

establishing a repertoire of nonself-reactive T cell sets. These sets are capable of expansion when antigenic signal is present.

Analysis of the cellular events occurring in in vitro tissue culture correlates of the immune response has revealed that cell activation is apparently not the sole result of a soluble antigen reacting with specific T cell receptors. The immune response reflects, at least in part, the filling of T cell receptors by antigen associated with an additional cell surface (Rosenthal et al., 1975; Benacerraf, 1978). T cells recognize antigen through antigen-specific receptors, but must also simultaneously recognize self components on the surface of antigen-presenting cells or antigen-bearing target cells. Cell interactions between antigen-presenting cells and antigen-specific T cells, as well as other regulatory interactions that may occur between different T cell subsets, function as critical modulators in the initiation and maintenance of the immune response (Cantor and Boyse, 1977; Woodland and Cantor, 1978).

Membrane perturbations caused by antigen–receptor binding lead to multiple rapid biochemical changes in the T cell, indicating activation. One effect of T cell activation is the de novo production of DNA, and a subsequent proliferation of the cell. Other indications of activation include the release of specific factors that may have an effect on other T cells or antibody-forming cells, and the differentiation of the activated cell into an effector cell similar to those capable of killing antigen-bearing target cells. In order to better understand the activation of T cells in carrying out these regulatory and effector functions, an understanding of the antigen recognition event is required. One approach to this problem has been to gain a better understanding of T cell specificity. This has been investigated by measuring functional T cell specificities, as well as by measuring direct antigen–T cell interactions. Recent work goes even further in providing biochemical information on the nature of the antigen-specific T cell receptor. Data regarding T cell specificity and the molecular nature of antigen-specific receptors provides the basis for proposing routes of cellular interaction in the immune response. It is apparent that key answers to meaningful therapeutic manipulation of the immune system in autoimmune and immunodeficiency diseases, as well as tumor immunity, rest in a better understanding of lymphoid cell interactions.

This chapter will deal primarily with information about antigen-specific T cell receptors and the interaction of these receptors with antigen. Studies designed to elucidate these T cell specificities provide the information necessary to understand the

routes of cellular interaction that lead to a positive or negative immune response.

2. Functional and Physical Analysis of the T Cell Antigen-Specific Receptors

T lymphocytes, which are both regulatory and effector cells in the immune system, must be able to respond to external stimuli (antigens). This response process is accomplished at least in part by antigen binding to membrane-associated antigen-specific receptors. The purpose of this section is to review what is known about the functional specificity and location of these receptors and something of their binding characteristics. Section 3 will provide additional information about the molecular nature of the receptors.

2.1. Functional Studies

A number of different experimental approaches have been used to demonstrate that T cells possess antigen-specific receptors that are associated with their plasma membranes. Many of these studies draw comparisons between T cell specificities and antibody specificities for the same antigen in order to gain some insight into the T cell receptor structure. A particularly useful functional approach has been the in vitro investigation of blastogenesis. In this technique, T cells are induced to undergo blastogenesis in the presence of antigen; the final result can be detected by the de novo synthesis of DNA. This antigen-induced proliferative response can be shown to be T cell-dependent (Corradin et al., 1977; Maizels et al., 1980; Schwartz et al., 1979), and in some cases under Ir gene control (Lonai and McDevitt, 1974; Schwartz and Paul, 1976). The in vitro proliferative assay allows the measurement of T cell functional specificity and presumably the receptor specificity of various subpopulations of responding T cells. For instance, using proliferative assays one can determine the specificity of T subpopulations responding to various crossreacting antigens or different determinants on the same antigen (Maizels et al., 1980; Corradin et al., 1977; Schwartz and Paul, 1979). This approach has provided information about the T cell receptor repertoire relative to the B cell or antibody repertoire. In some systems the range of specificities that T cells exhibit appears to be equivalent to that of antibody reactivity, while in other cases, T cell specificity appears to include reactivity with antigenic moieties not recognized by antibody. In certain systems, T cells have been shown to react equally well with determinants

present on denatured and native forms of the same antigen, while antibody reactivity appears conformationally dependent, reacting only with the native form of the antigen (Chesnut et al., 1980). The discrepancy between the ability of antibody and T cells to recognize both forms of the antigen suggests that T cell receptor molecules are at least somewhat different from the antigen-combining site of antibody molecules or that their specificity repertoire is somewhat different.

It has been difficult to precisely demonstrate the eventual function of T cell's responding to antigen in proliferative systems. Often the general assumption is made that proliferating T cells detected in blastogenesis assays are progenitors of helper T cells. If this is correct, it is apparent that this assay is only measuring a portion of the T cell receptor repertoire.

Several investigators in the early 1970s demonstrated that immune nonresponsiveness either in some Ir gene-controlled systems (Gershon et al., 1973; Kapp et al., 1974; Paul and Benacerraf, 1977) or in some tolerance systems (Baston et al., 1975; Benjamin, 1975) was attributable at least in part to the induction of suppressor T cells. It is generally assumed that proliferating systems do not measure suppressor T cells, yet these cells too are antigen-specific and therefore, in terms of measuring the T cell receptor repertoire, must be considered. Suppressor T cell specificities have been defined in several Ir gene-controlled systems (Benacerraf, 1978; Adorini et al., 1979a) and in several systems designed to investigate tolerance induction (Baston et al., 1975; Benjamin, 1975). Therefore, lack of a proliferative T lymphocyte response to a particular antigen is not necessarily reflective of the lack of a T cell receptor of that specificity.

Even different subregions (determinants) of the same antigen can elicit different T lymphocyte subtypes. Particular immunogenic subregions of an antigen can show preferential activation of T helpers or T suppressors in vivo. Turkin and Sercarz (1977) utilizing a cyanogen bromide cleavage peptide of β-galactosidase (β-Gal), termed CB2, as the immunogen induced a sustained wave of suppression of the response to hapten-coupled native β-Gal carrier. Since immunization with β-Gal generates sequential waves of help and suppression, the CB2 peptide apparently possesses determinants that can preferentially cause suppression of the β-Gal response. Similar data utilizing the myelin basic protein by Swanborg (1975), Sweirkosz and Swanborg (1975), and Hashim et al. (1976) have revealed two distinct regions of the molecule; one region of the antigen causes encephalitis upon immunization, another re-

gion elicits a suppressive response that prevents encephalitis upon further challenge with myelin basic protein. Muckerheide et al. (1977) have demonstrated that a particular region of bovine serum albumin predominantly induces suppressor T cells capable of suppressing any potential helper activity upon subsequent challenge with the intact antigen. In the Ir gene-controlled response to hen eggwhite lysozyme (HEL), Adorini and coworkers (1979b) were able to demonstrate that different epitopes on the same antigen elicit different functional T cells in different strains of mice. Nonresponder mice immunized against HEL generate suppressor T cells predominantly specific for a determinant or determinants located on a small disulfide-bonded peptide containing both the *N*-terminal and *C*-terminal portion of the antigen. Immunization of these same mice with a form of the antigen that no longer contains this suppressive peptide elicits helper T cells. Responder mice immunized with the intact antigen generate helper T cells specific for the nonsuppressive peptide portion of the molecule (Adorini et al., 1979b).

Thus, it is apparent that several problems exist in functionally defining T receptor specificity. T receptor specificity may or may not be precisely the same as antibody specificity. The same antigen in different strains of mice may elicit functionally different populations of T cells, presenting problems in measuring these cell types. Even within the same animal, different portions of the same antigen may elicit different functional cell types at different times after immunization. Considering the difficulties in determining T receptor specificity based upon measurement of T cell function, others have attempted to measure antigen binding to T cells directly in an effort to understand the specificity of binding more clearly.

2.2. Direct Physical Measurements

Direct physical measurements of the antigen (ligand)–T cell receptor reaction has proceeded slowly, owing in part to the difficulty in demonstrating direct interaction between ligand and the T cell receptors on the cell surface and also because of the difficulty in isolating receptor-like molecules from T cells. Nonetheless, specific T cell subsets do bind protein (Roelants et al., 1974; DeLuca et al., 1979), haptene conjugates (Goodman et al., 1978; Warr et al., 1979), and synthetic polypeptide antigens (Luderer et al., 1979). In this section, we will dwell principally upon two immunogenetic systems: *Ir-GAT* controlling the immune response to GAT^{10} (Martin et al., 1971; Merryman et al., 1975) and *Ir-(T,G)-A-L* controlling the response to (T,G)-A-L (McDevitt et al., 1969).

The synthetic polypeptide GAT, an abbreviation for L-glutamic acid^{60}, L-alanine^{30}, L-tyrosine^{10} (superscript refers to mole percent), is a globular-like peptide created by random polymerization of the α-*N*-carboxy anhydrides of the three respective amino acids. The molecule possesses discrete sequences of poly-L-glutamic acid; poly-L-glutamic acid, L-alanine; poly-L-glutamic acid, L-tyrosine; and poly-L-tyrosine. The initial two sequences are concentrated in the *N*-terminus whereas the last two sequences are associated with the carboxy terminus. The synthetic polypeptide (T,G)-A-L, an abbreviation for (L-tyrosine, L-glutamic acid)-poly D, L-alanine-poly-L-lysine is a branched polymer. Its structure consists of a poly-L-lysine backbone with short poly-D,L-alanine side arms ending with glutamyl and tyrosyl residues (McDevitt and Sela, 1965).

Autoradiographic studies with radio-iodinated GAT (GAT*) (Kennedy et al., 1976) and (T,G)-A-L [(T,G)-A-L*] (Hammerling and McDevitt, 1974) both demonstrated detectable binding of the radioligand with the T cell plasma membrane. The GAT binding data have been confirmed and expanded utilizing a quantitative radioreceptor assay (Luderer et al., 1979). The interaction between ligand and receptor displays immunochemical specificity and in general maximal ligand binding at physiological temperature (37°C). Figure 1 illustrates the competitive displacement of GAT* by unlabeled GAT or two immunochemically unrelated antigens, ovalbumin or lactate dehydrogenase, utilizing the radioreceptor assay (Luderer et al., 1979). Neither of the unrelated antigens were capable of displacing GAT* from the T cell receptor whereas unlabeled GAT displaced GAT* depending upon concentration. Similar specificity data have been generated by autoradiographic technique for the T cell binding of both GAT (Kennedy et al., 1976) and (T,G)-A-L (Hammerling and McDevitt, 1974) and enzymatically for β-Gal binding (Swain et al., 1976).

A number of experimental approaches have been used to demonstrate the plasma membrane-limited nature of the T cell receptor. Utilizing autoradiography, it was determined that the binding of GAT* to T cells is surface-associated and that a polar cap tends to form over one end of the T cell (Dunham et al., 1972). Utilizing the radioreceptor assay, Luderer et al. (1979) permitted GAT* to maximally react with thymic T cells; the cells were then pulsed with excess cold ligand and the displacement of cell-bound ligand determined. This experimental protocol allowed—although partial capping of the GAT receptor complexes may have occurred—a majority of the GAT* receptor complexes should be located at the

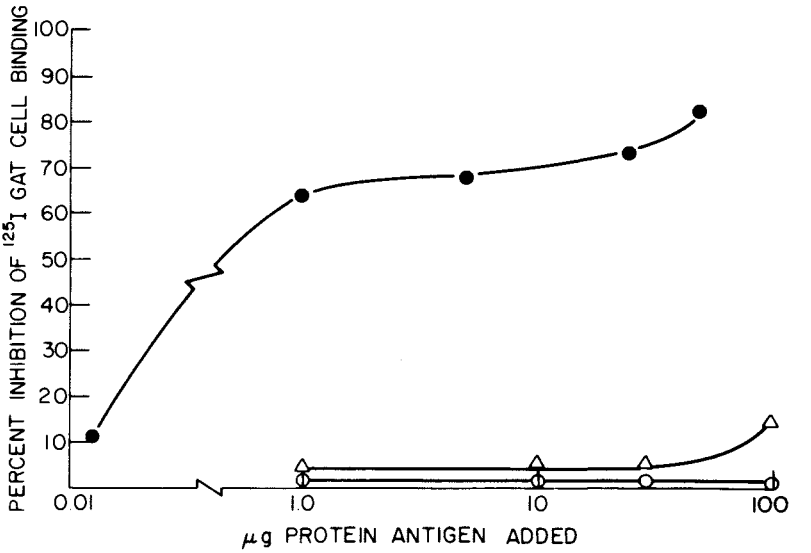

Fig. 1. Competitive inhibition of ^{125}I-GAT-thymocyte binding by unlabeled GAT, ovalbumin, or lactate dehydrogenase, utilizing the microradioreceptor assay. ●, GAT; Δ, ovalbumin; ○, lactate dehydrogenase. Adapted from Luderer et al. (1979).

cell's surface, and therefore capable of partial exchange with unlabeled GAT. If, on the contrary, a majority of the cell-associated counts were internalized, they would be nonexchangeable. As can be seen by the data presented in Fig. 2, more than 70% of the total cell-bound counts, after sham treatment with cell culture media, could be exchanged by excess unlabeled GAT. The amount of cell-bound GAT* displaced was dependent upon the concentration of unlabeled GAT added. The control consisting of unlabeled immunochemically noncrossreactive ovalbumin did not displace cell-bound GAT*. Additional supportive data for the plasma membrane-limited nature of the T cell receptor was presented by Swain et al., (1976) utilizing the natural antigen β-Gal. These authors demonstrated that almost all of the thymocyte receptors for β-Gal were trypsin-sensitive, indicating that the β-Gal receptors were accessible to trypsin attack.

Physical binding data from a number of systems suggests that there are no obvious differences in the ligand binding properties of the total T cell populations in systems under direct Ir gene control. Thus equivalent numbers of naive antigen-binding T cell populations could be observed in both responder and nonresponder animals (Kennedy et al., 1976; Hammerling et al., 1973; Luderer et al.,

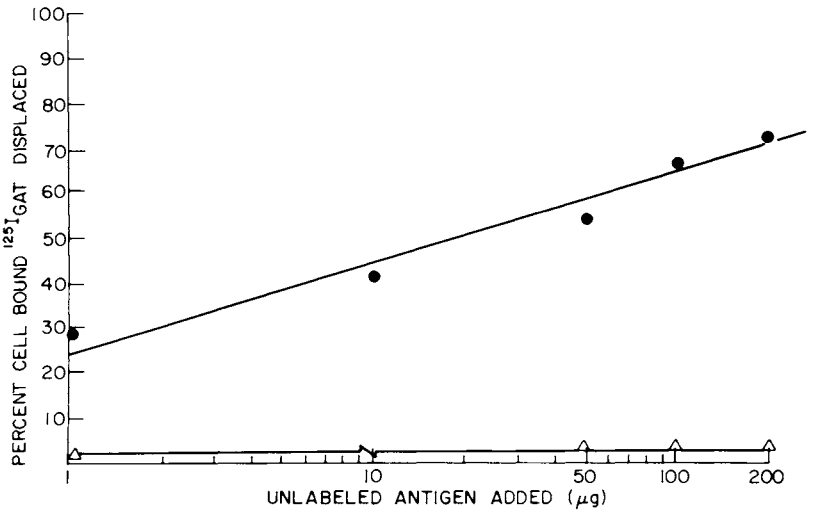

Fig. 2. Displacement of thymocyte-bound ^{125}I-GAT by unlabeled GAT or ovalbumin, utilizing the microradioreceptor assay. ●, GAT, Δ, ovalbumin. Adapted from Luderer et al. (1979).

1979). The demonstration of equivalent antigen-binding T cells in nonimmunized responder and nonresponder mice strongly argues that nonresponsive individuals possess a cell-recognition mechanism for the antigen against which it is nonresponsive.

Although responders and nonresponders possess T cells with antigen receptors that are reactive against the same antigen, the data presented thus far do not reveal how the ligand–T cell receptor influences—if it influences at all—the initiation of the regulatory program. If ligand binding is to influence or cause the initiation of the regulatory program, then it might be predicted that the receptors for a given antigen on a T cell from a nonresponder animal should be qualitatively different from the T cell receptor on responder T cells. Thus, recognition in the nonresponder should proceed through different antigenic determinants than those determinants interacting with responder T cell. This question of T cell receptor fine specificity was addressed in the GAT system, utilizing the microradioreceptor assay (Luderer et al., 1979). Copolymers of glutamic acid, alanine or glutamic acid, tyrosine which represent different antigenic determinants within the GAT terpolymer, were utilized to measure their ability to competitatively displace GAT* T cell binding on responder and nonresponder thymocytes. Figure 3 demonstrates the ability of copolymers poly-L-glutamic acid^{60}–L-alanine^{40} (GA) or poly-L-glutamic acid^{90}–L-tyrosine^{10} (GT) to

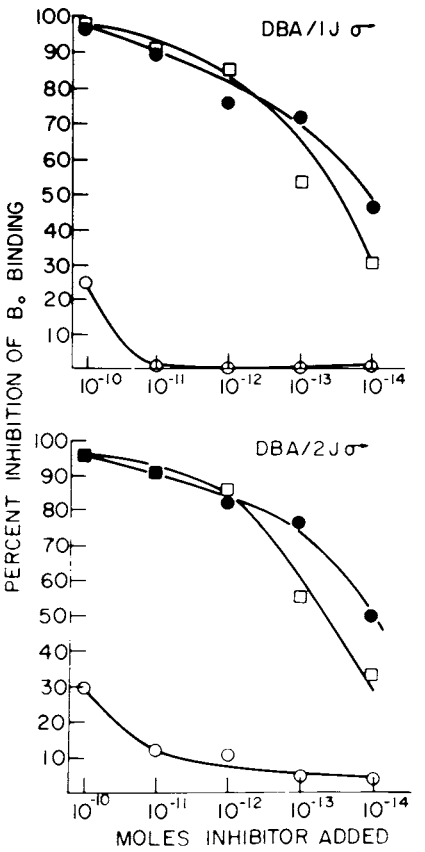

Fig. 3. Competitive displacement of ^{125}I-GAT from 1×10^6 thymocytes by GAT (○), GA (○), and GT (□) utilizing the microradioreceptor assay. Data are calculated as the percent inhibition of B_0 binding. (Top) DBA/1J nonresponders to GAT; (bottom) DBA/2J responders to GAT. Adapted from Luderer et al. (1979).

competitively displace GAT* from responder (DBA/2J) or nonresponder (DBA/1J) mice thymic T cells in the GAT* radioreceptor assay. Without regard to response type, the thymocyte GAT receptor exhibited preferential reactivity against GT. Thus the T cell GAT receptor in naive mice is preferentially reactive against the GT determinants of the GAT terpolymer *irrespective* of the genetically determined responder status. These results suggest that the basis of genetically controlled immune responsiveness or nonresponsiveness does not lie at the level of the T cell ligand receptor. Therefore once the T cell receptor has been filled, the salient feature of the nonresponder must lie somewhere distant from the receptor itself

or involve secondary reactions at the membrane-ligand junction that could not be measured utilizing the radioreceptor assay (Luderer et al., 1979).

The T cell receptor specificity data of Luderer et al. (1979) are substantially corroborated by the in vitro IgM plaque-forming response data of Howie et al. (1977) and Howie (1977). Their demonstration of apparently identical GAT-specific helper (Howie et al., 1977) or suppressor (Howie, 1977) T cells in both responder and nonresponder mice implies the absence of quantitative *or* qualitative differences in the T cell receptors of responders and nonresponders. Similar data presented by these authors for (T,G)–A–L indicate that the identity of T cell receptor specificity in responder and nonresponder animals is not limited to the GAT system.

2.3. Conclusions

Both functional and direct ligand–T cell binding studies demonstrate that T cells recognize antigen in a specific fashion. The specificity of this recognition may be similar to or different from the apparent predominant antibody specificity generated against the immunizing antigen although these findings appear dependent upon the antigen in question. The precise T cell antigen recognition specificity must account for the contribution of the various T cell subpopulations and genetic restrictions encoded in the host's genome. However, it has only recently become feasible to address these issues and their resolution is not immediately forthcoming. Ir genes affect the antigen driven functional expression of T cells in both in vitro functional assays and in vivo immune responses. However, the contribution of Ir genes to ligand binding on the T cell surfaces is not obvious in that no qualitative binding diferences or differences in receptor specificity appear to exist in naive whole T cell populations between genetically responsive and nonresponsive mice.

3. Serological and Biochemical Analysis of T Cell Antigen-Specific Receptors

3.1. Serological Approaches

Several lines of evidence suggest that B cells (progenitors to antibody-forming cells) recognize antigen via membrane-bound immunoglobulin (Ig) (Warner, 1974; Mitchinson, 1969; Bach, 1973).

This provides B cells with a specificity equivalent to their antibody product. Since T cells seem to possess an equally fine specificity in their recognition of antigen and because of theoretical considerations regarding the generation of large numbers of different possible specificities, many investigators made initial attempts to demonstrate that T cells also utilize antibody molecules as antigen specific receptors. Use of anti-Ig reagents to detect Ig on the surface of T cell populations met with questionable success for a number of years suggesting that typical Ig determinants were present at very low concentrations or not present at all on the surface of this cell type (Rabellino et al., 1971; Nossal et al., 1972; Raff et al., 1970). Most of the reagents used in these studies consisted of antibodies reactive with determinants on the constant region of Ig molecules.

Commencing with some work in the early 1950's and with increasing efforts throughout 1960–1980, it became apparent that antisera could be raised against isolated antibody populations that would recognize variable (V) region associated determinants (Slater et al. 1955; Kunkel et al., 1963; Oudin and Michel, 1963; Capra and Kehoe, 1975). More precisely, many of these antisera have been shown to recognize determinants intimately associated with the antigen combining site of the immunizing antibody population (Capra and Kehoe, 1975). Predominantly in the mouse, investigators have been able to show that these determinants (termed idiotypic determinants) provide an excellent marker for a particular V region; that portion of the antibody molecule responsible for binding antigen. Between mice of the same inbred strain, antibody molecules of the same specificity were demonstrated (in some cases) to share idiotypic determinants. In fact, anti-idiotypic reagents have been used to map the inheritance of V region genes (Pawlak et al., 1973; Eichmann, 1973). Because of the correlation between the antigen specificity of antibodies and the idiotypic determinants they carry, several investigators began to probe the effect anti-idiotypic reagents had on T cell populations. The rationale for these experiments was that perhaps T cells utilized the antigen-binding portion of antibody as part of their antigen-specific receptors.

3.2. Ability of Anti-Idiotypic Reagents to Induce Functional T Cell Populations

Early experiments (Nisonoff and Bangasser, 1975; Eichmann, 1974; Eichmann and Rajewsky, 1975; Binz and Wigzell, 1977), indicated that in vivo administration of anti-idiotypic reagents induced functional T cell populations. Nisonoff's laboratory has spent many

years examining the A/J mouse antibody response to the hapten azobenzene arsonate (ABA). They have found that some of the antibody produced by A/J mice to this hapten bear a particular set of idiotypic determinants that occur on a portion of the anti-ABA antibodies obtained from every A/J mouse (Owens et al., 1977). Normal A/J mice injected with anti-idiotypic antibody directed against this population and subsequently immunized with ABA coupled protein do not produce anti-ABA antibodies bearing the customary set of idiotypic determinants. Additional experiments indicated that the lack of idiotype-positive antibodies in these animals owed to the induction of a specific suppressor T cell population (Owen et al., 1977). One interpretation of these experiments was that anti-idiotype had interacted directly with the antigen specific receptors on that cell population and caused an expansion of that cell type. However, in vivo experiments can create some doubt about how direct interactions have occurred, if indeed they did occur. Subsequent experiments from several laboratories were able to show that direct treatment of isolated cell populations in vitro with anti-idiotypic antibodies could affect T cell function (Harvey et al., 1979; Eichmann et al., 1978). though such experiments strongly suggest that interaction of anti-idiotypic reagents with functional T cell populations is through direct binding to idiotypic determinants on the surface of these cells, it is not necessarily possible to state that these determinants are indeed part of the antigen-binding structure.

An interesting set of experiments that suggest that anti-idiotypic antibodies can stimulate T cells in the same fashion as antigen lends credence to the idea that idiotypic determinants are associated with the T cell antigen-specific receptor. The A/J mouse antibody response to group A streptococcal carbohydrate (A-CHO) is characterized by a predominant set of idiotypic determinants (Eichmann, 1973). Anti-idiotypic antibodies raised against a portion of this antibody population are capable of inducing both helper and suppressor T cells when administered to a naive A/J mouse (Eichmann and Rajewski, 1975). Perhaps of more interest are experiments in which anti-idiotypic reagents were shown to be able to substitute for antigen in culture and elicit an anti-A-CHO specific response (Eichmann et al., 1978). Anti-idiotypic antibodies added to cultures resulted in the production of anti-A-CHO antibodies. Though it is not entirely possible to say that all of the cells responding in culture were of the correct specificity, the fact that an antigen-specific response was obtained strongly suggests that a population of T cells of the correct specificity were stimulated by

the anti-idiotype. Because of the correlation between idiotypic stimulation and production of antibody reactive with A-CHO, the simplest interpretation of these experiments is that idiotypic determinants are part of A-CHO specific T cell receptors.

3.3. Additional Molecular Analysis of the T Cell Receptor Structure

The effects of anti-idiotypic antibodies on T cell populations strongly suggests that their antigen-specific receptors are composed in part of Ig V region structures. This observation suggests that T cell diversity is generated from the same set of genes responsible for B cell diversity. However, T receptors still do not appear to be composed of a typical Ig molecule though they do appear to contain a portion of an Ig molecule. Several investigators have performed experiments that provide some evidence about the identity of other portions of the molecule.

Predominantly two routes have been taken in the attempt to isolate material to allow further structural characteriztion. One involves analysis of immunologically active factors that are released by T cell populations (Mozes and Haimovich, 1979; Germain et al., 1979; Green et al., 1980). This approach has been used by several groups, with one of the most extensively studied systems being the mouse response to ABA. This system is attractive because of idiotypic markers described earlier. In addition, Green's laboratory has shown that ABA-specific suppressor T cells that can suppress an ABA-specific delayed-type hypersensitivity reaction (DTH) are inducible. Analysis of suppressor factors isolated from this T population has shown that T suppressor factors (which are thought to be released antigen receptor molecules) bear, in addition to idiotypic determinants, antigenic determinants coded for by genes in the major histocompatibility complex (H-2) of the mouse. In particular, I-J determinants have been found associated with suppressor factors. I-J antigens are coded for by genes in the *I* subregion of the H-2 complex and have been shown to be present on the surface of some T suppressor cell populations (Taniguchi et al., 1976). In Green's experiments, a suppressor factor preparation was obtained by physically disrupting an ABA-specific T suppressor cell population and then subjecting this lysate to subsequent adsorptions. Adsorbed or unadsorbed suppressor factor was then injected into A/J mice in order to measure the factors' ability to affect a subsequent DTH reaction to ABA. Unadsorbed factor could inhibit ABA-specific DTH approximately 90%. This ability to inhibit DTH was reduced to 15%

if the factor preparation was first adsorbed over a sepharose column to which anti-idiotype had been coupled. In addition, passing the factor preparation over a column to which anti-I-J antibodies had been coupled was equally effective at removing its ability to inhibit DTH. These experiments indicate that presumed suppressor T cell receptors are composed in part of V region gene products and in part by gene products of the major histocompatibility complex. In addition to the analysis of materials that demonstrate biological activities, such as suppressor factors, some attempts have been made to isolate receptor material from antigen-binding T cell populations to allow further biochemical analysis of receptor material. Goodman has reported isolation of antigen-binding material from a population of A/J mouse splenic T cells (Goodman et al., 1980). These authors found that immunization of A/J mice with ABA coupled to mouse Ig (a myeloma protein was used in this case) leads to the generation of a T suppressor population in the spleen of these mice. It was possible to enrich for this ABA-specific T population by passage of spleen cells over plastic petri dishes coated with ABA-derivativized protein. These cells were subsequently intrinsically labeled and the membranes solubilized. Fractionation of this material over antigen (ABA-bovine gamma globulin–sepharose) and polyacrylamide gel electrophoresis of adsorbed and eluted material has led to the isolation of a 92,000 dalton component. This material does not bear classical Ig determinants. Goodman et al. have been unsuccessful in demonstrating I-J or idiotypic determinants on this molecule. However, it should be pointed out that the ABA-specific T cells isolated from antigen-coated plates demonstrate specific suppressive activity, are stained with anti-idiotypic reagents and reaction with anti-idiotype decreases their ability to bind to the ABA-coated plates (Lewis and Goodman, 1978). These observations lead to the conclusion that this specific cell population expresses antigen-specific receptors whose ability to bind to antigen appears to be inhibited by anti-idiotypic reagents.

3.4. T Cell Hybridomas as an Approach to Studying the T Cell Receptor Structure

The second route being taken to investigate receptor structure takes advantage of recent advances in somatic fusion techniques. As well as techniques that allow fusion of B cells with myeloma cells, procedures for fusing T cell populations with a T lymphoma now exist (Goldsby et al., 1977). Several groups have reported the construction of T cell lines that retain the ability to bind antigen

and carry out some T cell functions (Goldsby et al., 1977; Taniguchi et al., 1979; Kontiainens et al., 1978). This approach is particularly attractive because it circumvents all the problems of dealing with small numbers of specific T cells in heterogeneous cell populations. Theoretically, extremely lage numbers of cells of a specific T cell clone could be obtained, thus allowing isolation of receptor material and characterization of that material by traditional biochemical methods. Again in the ABA system, Ruddle has reported the construction of T cell hybrids capable of forming rosettes with ABA-coupled red blood cells (Ruddle et al., 1980). The rosetting could be inhibited by soluble antigen, indicating that the phenomena was specific. Further work on these lines indicates that they produce an antigen-specific factor that inhibits a primary in vitro antibody response to ABA (Goodman et al., 1980). It would appear that this approach should soon yield useful information as to the nature of the T cell receptor.

3.5. Conclusions

Based on an ever accumulating body of data from a number of different systems, several characteristics of T cell antigen-binding molecules can be suggested. Consistently, T cells fail to demonstrate antigen-binding molecules that bear classical (i.e., C region) Ig determinants. They do, however, appear to bear molecules that have determinants associated with the Ig antigen-binding site (idiotypic determinants). In general the idiotypic determinants expressed on T cells are those associated with heavy-chain V regions. In addition, increasing evidence supports the notion that antigen receptors are composed in part of molecules encoded by the major histocompatibility complex. Conflicting evidence still exists about the chain structure and exact size of these antigen-binding molecules. Forthcoming evidence, particularly from work on T cell hybrids, should shed light on these problems.

4. Physiologic Constraints in the Recognition Function of the T Cell Antigen Receptor

4.1. General Antigen Processing Cell (APC) Requirements

The filling of the T cell receptor by soluble ligand, although responsible for the antigenic specificity of the activated T cell, is not totally adequate for T cell triggering. This is readily apparent in in vitro proliferative experiments where removal of APCs greatly dimin-

ishes or prevents antigen-specific educated T cell proliferation (Cline and Sweet, 1968; Waldron et al., 1973; Rosenstreich and Rosenthal, 1973; Seeger and Oppenheim, 1970). The role of the APCs extends beyond a dependence for support of proliferation and involves an obligatory APC uptake of antigen followed by close physical interaction of the APCs containing the processed immunogenic moiety for presentation to the T lymphocyte (Cline and Sweet, 1968).

4.2. APC Antigen Processing

APCs ingest soluble proteins by pinocytosis. In the case of human serum albumin and keyhole limpet hemocyanin, approximately 20% of the initial protein phagocytosized by the APC is resistant to catabolism, with approximately 33% of the uncatabolized antigen present as a stable immunogenic moiety on the presenting cell membrane (Unanue et al., 1969; Schmidtke and Unanue, 1971). [Also see: Ellner and Rosenthal (1975), Ellner et al. (1977), and Rosenthal et al. (1975).] This stable surface antigen is capable of inducing educated T cells to respond to the specific presenting antigen (Unanue and Cerotini, 1970; Scmidtke and Unanue, 1971, Unanue and Askonas, 1968). Other data have indicated that the APC-associated antigen may be unnecessary for the induction of T lymphocyte proliferation or lymphokine production (Ellner and Rosenthal, 1975; Ellner et al., 1977). For example, guinea pig APC obtained from peritoneal exudate cell preparations were pulsed with 2,4-dinitrophenyl guinea pig albumin, washed, and cultured 24 h at 37°C. Residual surface-bound material (Ellner and Rosenthal, 1975) was removed by trypsin and the treated APCs were then mixed with purified immune T cells obtained from (syngeneic) peritoneal-exudate cells. Trypsinization failed to alter lymphokine production (measured by migration inhibition factor) (Ellner et al., 1977), T cell immune clusters (an early step in antigen-dependent T cell interaction) (Ellner et al., 1977), or T cell in vitro proliferation (Ellner and Rosenthal, 1975). These data support the concept that APC-surface-bound, trypsin-sensitive antigen is not the sole source of immunogenically active material, and suggests the possible directed return of APC-processed antigen to the T cell surface after cell–cell interaction by a process similar to exocytosis (Rosenthal et al., 1975).

4.3. APC–T Cell Interaction Structures

Rosenthal et al., (1975) cites two types of APC–T binding reactions: an antigen-dependent reaction and an antigen-independent reaction (Table 1). It is of particular significance that activation of T cell

Table 1
Characteristics of Antigen-Dependent and Antigen-Independent APC–Lymphocyte Binding

Antigen-independent phase (1st phase)	Antigen-dependent phase (2nd phase)
Will bind T or B lymphocyte	Will not occur if antigen-independent phase is blocked by cytochalasin-B
Reversible	Irreversible
Allogeneic and syngeneic lymphocytes will bind to APC	Only syngeneic lymphogytes will bind (with exception of MLR)
Not blocked by alloantisera	Blocked by alloantisera
Blocked by cytochalasin-B	Not reversed by cytochalasin-B or mitogycin
Ca^{2+} required	Carrier specific
APC only must be metabolically active	Both APC and lymphocytes must be metabolically active

[a]Adapted from Rosenthal et al. (1975).

DNA synthesis or migration-inhibition-factor production by APC-containing antigen requires the cooperation of APC and T cells that are syngeneic (Shevach, 1976; Rosenthal and Shevach, 1973; Ben-Sasson et al., 1974). Thus, while antigen-independent binding of lymphocytes occurs approximately equally in both allogeneic and syngeneic APC–T cell combinations, antigen-dependent binding is observed only in syngeneic combinations (Lipsky and Rosenthal, 1975). Rosenthal et al. (1975) regard the first phase of APC–T cell binding as antigen-independent, followed by the antigen-dependent period yielding activation. The antigen-dependent reaction occurs only if the APC and lymphocyte share identity of membrane-linked histocompatibility-associated determinants and the APC has antigen for which the lymphocyte possesses immunospecific receptors. The interaction structure that controls effective APC–T cell interaction appears to be the *I*-region-coded Ia antigens (Rosenthal and Shevach, 1973; Erb and Feldman, 1975; Shevach, 1976). Since the *I* region contains genes that control immune responses, the interaction of APC and T cell mediated by *I* region products suggests that Ir genes may exert their regulatory role through modulation of cell–cell interactions. Recent data from Baxevanis et al. (1980) demonstrating *I* subregion specific inhibition of the proliferative immune response to poly (Glu^{40}, Ala^{60}) and poly (Glu^{51}, Lys^{34}, Tyr^{15}) are consistent with this hypothesis. [Also see Schwartz et al. (1979), and Longo and Schwartz (1980).]

4.4. Conclusions

APC are required to generate T cell proliferative immune responses to soluble ligands. The interaction that occurs between cooperating APC and T cells during antigen presentation is critically dependent upon *I*-region-coded Ia antigens. These cell surface molecules appear to function as interaction structures and suggest that Ir genes may exert their regulatory function through modulation of cell–cell interactions.

5. Cytotoxic T Cell Ligand-Binding Sites Are Critically Affected by Recognition Structures on Other Cells

5.1. Introduction

Antigen recognition at the level of the cytotoxic T cell reveals unique molecular restraints imposed upon the ligand-bearing target. These restraints are similar to the cooperative restraints placed between the educated T cell and the antigen-presenting cell described previously. The experimental systems utilized to dissect effector T recognition explore the cytolytic reaction of cytotoxic T lymphocytes (CTLs) against solid-phase ligand-bearing targets consisting of virally infected cells, neoplastic cells, chemically modified cells, or cells differing at minor histocompatibility loci. This observed killing effect of CTL on their relevant targets in vitro is thought to correlate with in vivo responses to viral and tumor antigens and has direct bearing on theories of immune surveillance. Cytotoxic T lymphocytes have been classically described as the subset of T cells (Zinkernagel and Doherty, 1977) chiefly responsible for the allograft response (Klein, 1975).

5.2. Ligand–Histocompatibility-Antigen Display

When immune murine CTLs are reacted with target cells identical to the immunizing type, cytotoxic activity against the target is efficient only when CTLs and target *share* either *K* or *D* regions of H-2 (Zinkernagel and Doherty, 1974; Zinkernagel and Doherty, 1975; Blanden et al., 1975; Koszinowski and Ertle, 1975). The demonstration of histocompatibility restrictions between killer and target have been rather extensively confirmed (reviewed by Zinkernagel and Doherty, 1977) and are found to influence the reaction between CTLs and cellular targets that are neoplastic (Blank et al., 1976;

Schrader et al., 1975), chemically modified (Shearer et al., 1977), or histoincompatible at minor H loci (Bevan, 1975; Gordon et al., 1975). Thus, the interaction of immunizing cell and virgin T cells yields an educated CTL population (both active and memory) whose recognition of the target cell is restricted to identical (or cross-reactive) ligand on the cell's surface (Zweerink et al., 1977) and cellular histocompatibility antigens K or D. It is of interest that *I* region compatibility necessary for T helper function (Katz and Benacerraf, 1975; Kindred and Shreffler, 1972), antibacterial protection (Zinkernagel et al., 1977), delayed type hypersensitivity (Miller et al., 1975), and in vivo T cell proliferation (Rosenthal and Shevach, 1973) is not required for efficient CTL killing of viral and haptenated targets (Zinkernagel and Doherty, 1977). These findings suggest that H-2 subregion identity required for a particular T cell function, probably relates to the different types of signals transmitted by T cells to target cells via the distinctly different self-markers employed (Zinkernagel et al., 1977).

Recently the histocompatibility requirements between target and CTL have been extended to man by the initial observations of Tursz et al. (1977). These authors utilized the Daudi cell line, which originated from a Burkitts' lymphoma (B cell), but does not express HLA antigens. The Epstein–Barr (EB) viral genome-coded antigenic determinants (as in other EB-virus-infected cells) are expressed at the Daudi cell surface. Cytotoxic lymphocytes from patients with infectious mononucleosis could specifically kill HLA-positive, EB-viral genome-carrying cell lines (Svedmyr and Jondal, 1975) but were unable to kill HLA-positive EB-viral genome-negative or HLA-negative EB-viral genome-positive (Daudi) targets. Definitive data for HLA restriction of human CTL-mediated killing were provided, utilizing female CTL killing of Y-chromosome-bearing targets (Goulmy et al. 1977), CTL killing of viral targets (McMichael et al., 1977), and chemically modified target CTL killing (Dickmeiss et al., 1977). Figure 4 illustrates the HLA-restricted CTL killing of influenza-virus-infected human cells (after McMichael et al., 1977). It can be seen that only virally infected targets that are *HLA-B* identical to the CTLs are efficiently lysed.

5.3. Molecular Relationships and Models of Interaction Structures

The relationship of the cellular ligand and histocompatibility "display" recognized by CTLs has been successfully probed by the utilization of antisera directed against the relevant ligand or histo-

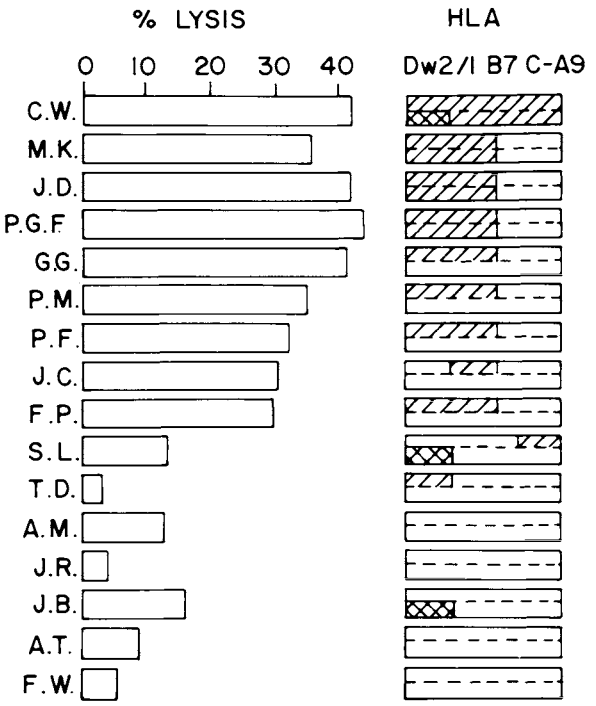

Fig. 4. HLA restriction of cell-mediated lysis of influenza type A/X31-infected cells. Individual C.Ws peripheral blood lymphocytes were sensitized to autologous lymphocytes infected with influenza type A/X31, and tested for cytotoxicity against the target cells shown. Results are expressed as the percent specific target-cell lysis measured as ^{51}Cr release (left side of figure). The degree of HLA compatibility is illustrated on the right side of the figure. Sharing of HLA DW2, or B7, C-A9 is represented by diagonal hatching either as one haplotype (top half only) or two haplotype compatibility (both halves). Sharing of HLA DW1 is shown by crosshatching. The HLA A B D type of target cells were: C. W. (Donor) 9 (W24), 7, W2/9(W24), 7, W1; M.K.: 3, 7, W2/3, 7, W2; J.D.: 3, 7, W2/2, 7W2; P.G.F.: 3, 7, W2/3, 7 W2; G.G.: 2, 3, 7, W14, –, W2; F.P.: 3, 7, W2/1, 8, –; S.L.:9 (W24), W30, 13, W15, W1 –; T.D.:2, 2, 12, W40, 2, –; A.M.:2, 21, – /W32, W40, –; J.R.:2, 2, 12, 27, –, –;J.B.:W30, W32, W15, –, W3, W1; A.T.:2, 11, W40, W22, –, –, F.W.: 1, 8, W3/1, 8, –. After McMichael et al. (1977).

compatibility antigens. Utilizing well-defined anti-H-2 antiserum, the specific lysis of virally infected target cells (Koszinowski and Ertl, 1975) or neoplastic target cells (Schrader et al., 1975) by reactive CTLs could be inhibited by relevant anti-H-2 sera. This inhibition

was absent when normal serum or irrelevant anti-H-2 serum* was employed to block cytotoxicity. The blocking of CTL activity by relevant anti-H-2 sera is apparently not a result of inhibition of the antigen-specific receptor(s) on the CTLs (Schlessinger and Chaouat, 1975; Nabholz et al., 1974). Steric hindrance by the histocompatibility antigen–antibody complexes could be a cause of the observed target-killing inhibitions. However, this event seems somewhat unlikely in view of the findings that cells expressing viral antigens, but lacking H-2 antigenic determinants, cannot be lysed by CTLs reactive against cells virtually identical to the ineffective targets, with the sole exception of possession of histocompatibility antigens (Zinkernagel and Oldstone, 1976; Doherty et al., 1977). Corollary experiments utilizing specific antiviral sera to block effector CTLs lysis of virally infected target cells has been demonstrated with poxvirus Koszinowski and Thomssen, 1975; Senik and Neauport-Sautes, 1979), vesicular stomatitis (Hale et al., 1978), and influenza (Effros et al., 1979). These data strongly suggest that at least some of the CTLs are recognizing viral antigens.

The requirement for at least partial identity of histocompatibility type between CTLs and their target suggested two mutually exclusive models to explain the *K*- and *D*-region restriction of virus-specific CTL and CTL-target reactions in general (Zinkernagel and Doherty, 1977).

5.3.1. Intimacy or Dual Recognition Interaction Model Cytotoxic T cells express virus-specific receptors for viral antigens on the target cell surface. For lysis, a second signal coded by *H-2K* or *H-2D* must be recognized (i.e., self to like-self) (Fig. 5).

5.3.2. Altered Self Hypothesis Virus-specific cytotoxic T cells posseses receptors reactive to a virus-*modified* cell-surface structure coded by *H-2K* or *H-2D* (Fig. 5).

These two models focus directly on one central experimental point: Does the T cell recognize its target via one or two receptors? Schrader et al. (1975) approached the receptor question by following the membrane-surface redistribution (patching) and consequent polar capping of H-2 antigens on both P388 and EL4 cells. Both cell lines bear Rauscher-leukemia-virus (RVL) viral-surface antigens. The surface redistributions of membrane or membrane-

*That is, anti-H-2 serum directed against an alternate allelic gene product of the *K* or *D* region.

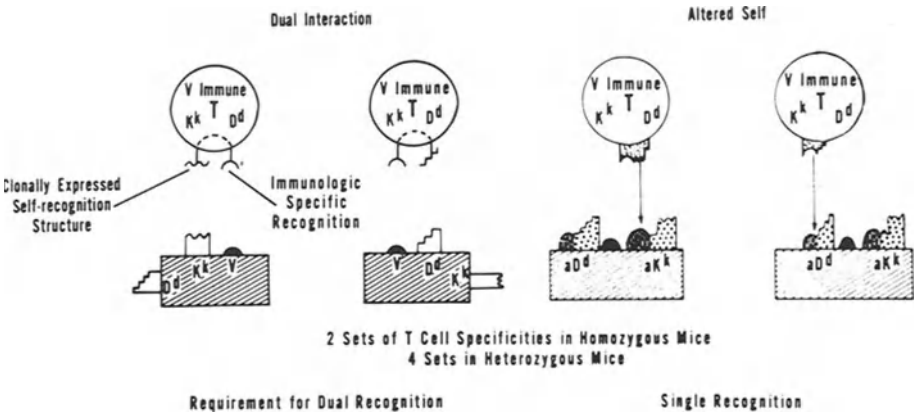

Fig. 5. The dual interaction and altered-self hypothesis developed to explain the apparent *H-2K* and *D* restriction of virus-specific T cell cytotoxicity. See text for detailed description. (V) Viral antigen on cell surface; (K^k D^d) histocompatibility antigens coded by the *K* or *D* region of H-2; (aK^k, aD^d) altered *K* or *D* histocompatibility antigens. After Zinkernagel and Doherty (1977).

associated protein is induced by the cross-linking of surface components by antisera directed against those components (Taylor et al., 1971; Raff and DePetris, 1973). Patching occurs following the addition of bivalent antibody and at 37°C is rapidly followed by capping. Co-capping of H-2 antigens and RLV antigens in EL-4 or P388 cells was repeatedly observed by Schrader et al. (1975). Since trapping effects could occur as a result of the cooperative nature of capping (Edelman et al., 1973), the capping inhibitor NaN_3 (Taylor et al., 1971; Edidin and Weiss, 1972) was utilized to limit the surface reaction to patch formation. Patch formation is a better indication of the physical association of cell-surface molecules (Edelman et al., 1973). The finding that H-2 and RVL antigenic determinants copatch strongly suggests close physical association between these two moieties (Schrader et al., 1975). Another attempt to unravel the physical relationship of histocompatibility antigens and viral determinants utilizes a technique known as lysostripping (Bernoco et al., 1973; Hauptfeld et al., 1975). This technique utilizes patch formation and subsequent endocytosis to clear cells of a particular membrane protein. Pretreatment of EL-4 cells, with rabbit anti-RLV or a purified goat anti-gp70 of murine leukemia virus reduced subsequent complement-mediated lysis of the cell by anti-$H\text{-}2^b$ antiserum. Cells not displaying RLV antigens were unaffected by anti-RLV pretreatment (Henning et al., 1976). These data

again suggest a close physical association between histocompatibility and viral antigens and are additionally supported by the observation of: (1) selective incorporation of H-2 antigenic determinant into Friend virus (FV) particles (Bubbers and Lilly, 1977) and (2) the association of FV and H-2 antigenic determinants on the target cell surface (Blank and Lilly, 1977). Similar data on the associations of vaccinia viral antigens and H-2 antigens on the cell surface were reported by Koszinowski and Ertle (1975). The majority of cell-surface data are suggestive of close physical association or interaction of viral and H-2 determinants possibly on the same molecule. It is of interest that target-cell coating with nonreplicating virus can render the cell susceptible to lysis by CTLs specific for the virus. Thus Schrader and Edelman (1977), utilizing ultraviolet-irradiated Sendai virus (SV), suggested that T cells recognize an antigenic determinant formed by the physical association of H-2 antigens and preformed antigens of the noninfectious SV. The CTL killing these authors observed was subject to H-2 restriction.

The HLA restriction data in humans for viral-infected human targets (McMichael et al., 1977) or Y-antigen human targets (Goulmy et al., 1977) localizing homology requirements to *HLA-B* and *HLA-A*, respectively, indicates that in man, as in mouse, the serologically defined histocompatibility regions (*H-2K*, *H-2D*, and their human counterparts *HLA-B*, *HLA-A*) (Hood, 1976) define similar CTL restrictions. However, it must be recognized that the unequivocal identification of the target–antigen complex recognized by CTLs requires the direct evidence of binding of a purified substance to CTLs or the demonstration that the purified substance is capable of *blocking* CTL killing of target cells. Although there is little doubt concerning the close physical association of viral and H-2 antigens at a target–cell surface, the precise recognition determinants reactive with the CTLs receptor(s) remain unknown. Because of this dilemma, it is impossible to decide whether CTLs view targets via one or two receptors.

5.4. Conclusions

Recognition and lysis of virally infected cells, neoplastic cells, chemically modified cells, or cells differing at minor histocompatibility loci are critically dependent upon the sharing of *K* or *D* region coded (or their human equivalent in man) histocompatibility antigens *and* the expression of relevant "foreign" antigen(s) (e.g., viral, tumor-associated) on the target cell surface. Effective killing can only be observed in cases where CTL and target *share K* or *D*, but

not *I* region-coded, antigens. Although either appropriate anti-H-2K, H-2D, and antiviral reagents can effectively block CTL expression, it is not clear whether CTLs view their targets as altered histocompatibility antigen-bearing cells or whether histocompatibility antigens and foreign determinants are discreet surface moieties not located on the same molecule or macromolecular complex. Further understanding of the mechanisms of CTL recognition should greatly advance our understanding of the host's immune response to virus and neoplasia, thus providing a more rational basis for the clinical manipulation of the host both prophylactically and therapeutically.

6. Summary

An understanding of the T cell recognition structure has been gained via several different experimental approaches. Some of these approaches have addressed the question of which forms of an antigen are capable of activating T cells, whereas other approaches have examined direct T cell–antigen interaction, and still others have demonstrated that, at least in some cases, recognition of antigen by T cells requires simultaneous recognition of other self-antigens.

Attempts to measure functional T cell repertoires has illustrated that one method of measuring T cell function is not sufficient to enumerate the entire T cell repertoire. It is also apparent that different functional classes of T cells can exhibit different specificity repertoires and that different types of T cells (i.e., suppressor T cells vs helper T cells) can recognize different forms of the same antigen. Additionally, helper and cytotoxic T cells require not only antigen recognition for activiation but also recognition of relevant histocompatibility antigenic determinants on the surface of an antigen presenting cell. No such observation has been made for suppressor T cell antigen recognition, although one might predict a form of histocompatibility restriction in view of the mapping of T suppressor activity to the *I-J* subregion of H-2. It is therefore plausible to suggest that different molecular configurations of the T cell receptor may exist on these three different cells, thus serving to restrict immune responsiveness in a logical and controlled fashion to cells of only the relevant surface phenotype.

Serological and biochemical analysis of T cell receptors indicates that, at least in the case of suppressor T cells, Ig constant region determinants are not found, but idiotypic determinants do

compose a portion of the antigen receptor. Idiotypic determinants predominantly associated with the V_H region of Ig appear to be present on the receptor and interaction of anti-idiotypic antibodies with these cells can lead to activation. Additional studies indicate that again in the case of receptor materials isolated from suppressor T cells, antigens of the MHC (I-J determinants) contribute to a portion of the receptor. Further biochemical analysis of receptor material is not complete though molecular weights as high as 92,000 have been reported for presumed receptor materials.

It is clear that major steps in the understanding of the T cell antigen receptor have been taken. The recent advances in the cloning of antigen specific T cells (Watson, 1979; Serendi et al., 1981), the production of T cell hybridomas, and the possibilities arising from application of recombinant DNA technology to elucidate fundamental information on the T cell receptor(s) derived from a pure antigen reactive T cell clone may revolutionize our understanding of immune recognition and regulation.

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Chapter 2

Regulation of the Immune Response

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1. Introduction

When I sat down to write this chapter, I was reminded of the story of how three blind men attempted to describe an elephant (Fig. 1). All of their descriptions were accurate yet no one, hearing these descriptions, would conjure up the vision of an elephant. So it is with a book such as this. Each chapter provides accurate descriptions of a portion of the whole yet it may not leave any of us with an overall view of the "principles of clinical cellular immunology." Thus, several "basic" chapters, including this one, have been written in an attempt to provide the reader with an understanding of the basic immunological phenomena that have led to the advances illustrated in the more clinically oriented chapters and to the concepts of clinical cellular immunology to which this book is addressed.

There are many advantages to studying immunological phenomena that relate directly to man. Unfortunately, the field of

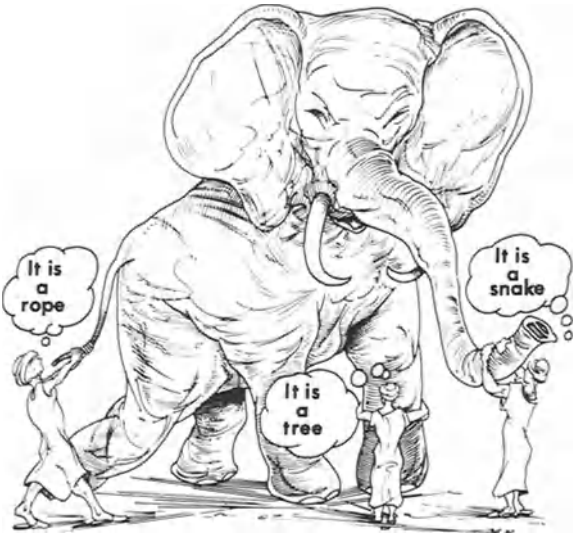

Fig. 1.

immunogenetics does not lend itself to studying outbred populations such as man nor species that have evolved a reproductive system that yields a limited number of progeny following mating. We are therefore left with the unenviable task of attempting to find immunologically relevant genetic-control mechanisms in experimental animals and trying insofar as is possible to relate these to similar phenomena that appear to occur in man. I will therefore attempt to define genetic control of immune responsiveness as it has been demonstrated in experimental animals and correlate these findings with phenomena that have been demonstrated in experimental situations in man. Despite the fact that the bulk of this chapter will be dedicated to descriptions of immune response genes linked to the major histocompatibility complex of several species, such immune response genes have not yet been clearly identified in man. Many of the phenomena that have been associated with immune response genes in experimental animals have, however, also been demonstrated in man and I will address these points at the end of the chapter.

The first clearly defined immune response gene was the *PLL* gene described by Benacerraf and co-workers in the early 1960s (Kantor et al., 1963; reviewed by McDevitt and Benacerraf, 1969). This gene, which controls the immune response to poly-L-lysine (PLL) in guinea pigs, is inherited as an autosomal dominant trait.

The *Ir-1* gene described by McDevitt and Sela in the mid-1960s describes an analogous immune response gene in mice that determines responsiveness to the synthetic polypeptide, (T,G)–A—L (McDevitt and Sela, 1965). In addition to demonstrating autosomal dominant inheritance of the *Ir-1* gene (or cluster of genes), McDevitt and his co-workers were able to demonstrate linkage of the *Ir-1* gene to the mouse major histocompatibility complex (MHC) (McDevitt and Tyan, 1968). In the ensuing 15 years, a variety of antigens have been used to demonstrate MHC-linked immune responsiveness in several species of experimental animals. In general, the antigens used to define immune response (Ir) genes can be divided into three categories: (1) synthetic polypeptides with limited structural heterogeneity, (2) weakly immunogenic alloantigens, and (3) more complex multideterminant antigens, which, when administered in limiting doses, seem to allow only the most immunogenic determinants to be recognized.

Although the demonstration of genetic control of immune responsiveness has been admirably served by these three classes of antigens, it has always been implied that the pathways by which immune responses are mediated toward complex natural antigens not under apparent Ir gene control do not differ fundamentally from those described for the antigens listed above for which Ir gene control can be demonstrated. Sela (1969) hypothesized that there is strain-dependent regulation of responsiveness to natural antigenic determinants within a protein. Some earlier observations tended to support this premise, in particular, findings by Arquilla and Finn (1963, 1965), who demonstrated that the specificity of antibodies produced in different animals in response to the same antigen appeared to be under genetic control. In these experiments, they demonstrated that antibodies raised in strain-2 guinea pigs seemed to bind to separate portions of the insulin molecule than did those antibodies raised in strain-13 guinea pigs. This suggested to them that strain-2 guinea pigs produced antibodies to portions of the insulin molecule to which strain-13 animals were unable to respond.

Similar results have been obtained in mice, using the branched-chain synthetic polypeptide, (PHE,G)–pro-L (McDevitt and Benacerraf, 1969). In these experiments, two different strains of mice, both of whom responded well to (PHE,G–pro-L, produced antibodies with different specificities. One of the strains, DBA/1, responded mainly to the (PHE,G) portion of the immunogenic macromolecule whereas antibodies in an equally high responder, strain SJL, seemed to be mainly directed toward the pro-L antigenic

portion. Recent studies on a variety of complex naturally occurring antigens have demonstrated the truth of Sela's hypothesis. There does appear to be genetic control of responsiveness to antigenic determinants (epitopes) on complex proteins.

In this chapter, which is principally about genetic control of immune responsiveness, first I will outline the original experiments that defined the major-histocompatibility-complex-linked genetic control of immune responsiveness. Second, I will discuss genetic control of immune responsiveness to more complex naturally occurring proteins. Third, I will attempt to outline the complex genetic interactions that seemingly regulate immune response to these naturally occurring proteins.

Never has the adage "Chance favors the prepared mind" been more apt than in relation to the discoveries that led to the description of the genetic control of MHC-linked immune responsiveness. The ability of Benacerraf and his co-workers to make use of the observation that there seemed to be a possible genetic restriction on the ability of guinea pigs to respond to random copolymers of L-amino acids, and the realization by McDevitt and Humphrey (McDevitt and Sela, 1965) that their inability to raise anti-(T,G)–A—L antibodies in a particular strain of rabbits might be because these rabbits were genetically nonresponders, led to what has been a major breakthrough in immunology. We are indebted to both these groups for their findings, which have led to so much fruitful investigation into such a complex phenomenon as the genetic control of the immune response.

2. Ir Genes in Guinea Pigs

2.1. The PLL Gene

Original studies by Maurer and his associates demonstrated that random copolymers of two or three L-amino acids were capable of inducing antibody formation in guinea pigs and rabbits; however, all animals of a given species did not seem to recognize these copolymers as antigens (Pinchuck and Maurer, 1968). Taking advantage of these observations, Benacerraf and his associates (reviewed in Benacerraf and Katz, 1975) studied the responsiveness of outbred Hartley-strain guinea pigs to immunization with poly-L-lysine that had been derivatized with DNP. They demonstrated that only 40% of immunized animals developed delayed-type hypersensitivity and antibody responses to this antigen, DNP–PLL (Kantor et al., 1963). They then carried out breeding studies in these

outbred guinea pigs to characterize the genetic transmission of the capacity of these animals to respond to PLL. The results of their original studies, which indicated that the trait was transmitted genetically as a unigenic mendelian dominant trait, have become the hallmark of such MHC-linked Ir genes. Additionally, they studied the responsiveness in inbred strain-13 and strain-2 guinea pigs and found a striking phenomenon. Strain-13 guinea pigs were uniquely unable to respond to DNP–PLL whereas 100% of strain-2 guinea pigs (and the F_1 derived between strain-2 and strain-13 guinea pigs) could respond to DNP–PLL both by delayed-type hypersensitivity and antibody response. The ability of guinea pigs to recognize poly-L-lysine and hapten conjugates of *PLL* as antigens is under the control of a single autosomal dominant gene; which is now called the *PLL* gene. The *PLL* gene controls responsiveness not only to poly-L-lysine but to poly-L-arginine, a copolymer of L-glutamic acid and L-lysine GL, protamine, and hapten conjugates of these polypeptides (Benacerraf and Katz, 1975). Responsiveness conferred by the *PLL* gene is characterized by both cellular immunity and the synthesis of significant levels of specific antibodies. Animals that lack the *PLL* gene neither develop delayed-type hypersensitivity nor produce significant levels of antibodies.

In contrast to responsiveness controlled by the *PLL* gene that is carried by strain-2 but not strain-13 guinea pigs, responsiveness to a random copolymer of L-glutamic acid and L-tyrosine (GT) (Bluestein et al., 1971) and to limiting doses of hapten-derivatized guinea pig albumin (Green et al., 1972) is under the control of *Ir* genes present in strain-13 but absent in strain-2 animals. All F_1 animals between strain-2 and strain-13 respond to each of these antigens, again illustrating the dominant character of these responses (Table 1).

2.2. PLL Gene Control of T Cell Responses

Although guinea pigs lacking the *PLL* gene are incapable of mounting an immune response to DNP–PLL, they can, nevertheless, recognize this antigenic conjugate as a hapten when it is coupled to a highly immunogenic carrier molecule (McDevitt and Benacerraf, 1969). Results of antigen-induced in vitro T cell proliferative assays first carried out by Green et al. (1966) demonstrated that the DNP–PLL conjugate did in fact serve as a hapten in nonresponder guinea pigs. Immunization of nonresponder animals with complexes of DNP–PLL complexed with foreign albumin induced antibody synthesis against DNP–PLL but could not be shown to elicit

Table 1*

INHERITANCE OF SPECIFIC *Ir* GENES AND OF THE MAJOR HISTOCOMPATIBILITY LOCUS OF STRAIN 2 AND STRAIN 13 GUINEA PIGS BY $(2 \times 13)F_1$ AND BACKCROSS ANIMALS

	Strain			$(2 \times 13)F_1 \times 13$		$(2 \times 13)F_1 \times 2$	
Antigens[a]	2	13	$(2 \times 13)F_1$	50%[b]	50%	50%	50%
DNP-PLL	+[c]	−[c]	+	+	−		
GL	+	−	+	+	−		
GA	+	−	+	+	−		
GT	−	+	+			+	−
BSA 0.1 μg	+	−	+	+	−		
HSA 1 μg	+	−	+				
DNP-BSA 1 μg	+	−	+	+	−		
DNP-GPA 1 μg	−	+	+			+	−
Major H locus							
Strain 2	+		+	+	−		
Strain 13		+	+			+	−

[a] DNP, dinitrophenyl; PLL, poly-L-lysine; GL, GA, GT, copolymers of glutamic acid and, respectively, L-lysine, L-alanine, and L-tyrosine; BSA, bovine serum albumin; HSA, human serum albumin; GPA, guinea pig albumin.

[b] Column identifies the same group of backcross animals.

[c] Plus indicates responsiveness and presence of major histocompatibility specificities: minus indicates nonresponsiveness and absence of major histocompatibility specificities of the inbred strains.

*Reproduced by permission from B. Benacerraf (1975) *Adv. Canc. Res.* 21, 121.

delayed-type hypersensitivity or to sensitize T lymphocytes to blast transformation in vitro in response to appropriate antigenic challenge. The findings on in vitro T cell proliferation to antigens controlled by the *PLL* gene and the immunogenicity in nonresponder guinea pigs of PLL coupled to an immunogenic carrier demonstrated two important concepts of the genetic control of immune responses: (1) The Ir gene control might be exerted at the level of the T lymphocyte, and (2) genetically nonresponder animals had antigen-specific B cells and could produce antibodies specific for the DNP–PLL conjugate.

2.3. The Role of MHC Products in the PLL Response

The final point I wish to make regarding the *PLL* gene is that alloantisera primarily directed against histocompatibility antigens on the cell surface can markedly diminish the blast transformation induced in responder lymph-node cells to antigenic challenge in vitro (Shevach et al., 1972). The most striking finding in these stud-

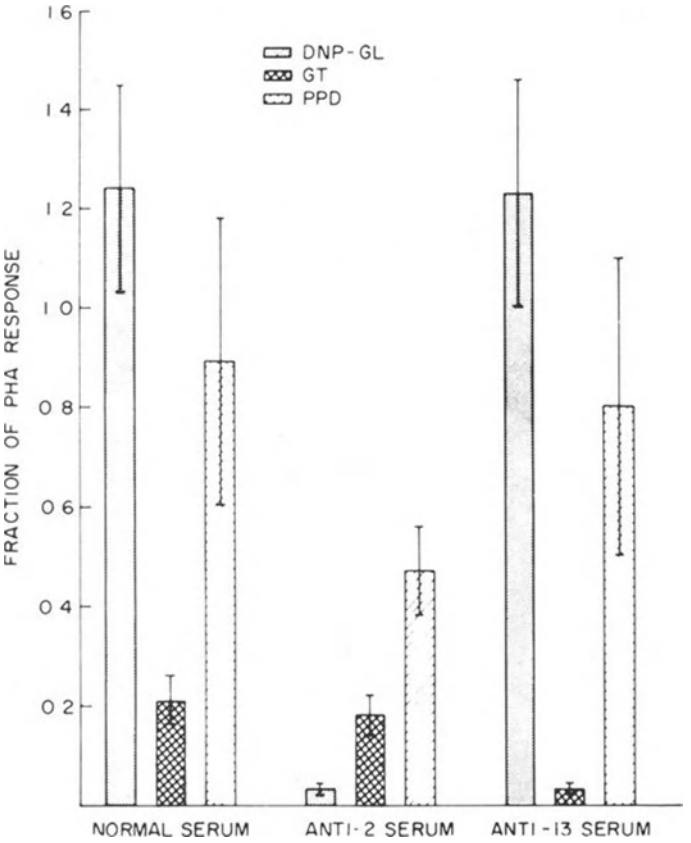

Fig. 2. The response measured as fraction of PHA response of strain 2×13 F_1 guinea pig peritoneal extracted T cells in response to stimulation with antigen either in the presence of normal serum anti-strain 2 serum or anti-strain 13 serum to 3 separate antigens, one of which, DNP-GL is under Ir gene control linked to the MHC of strain 2 whereas another, GT, is under Ir gene control linked to the MHC determinants of strain 13. Reproduced by permission from Shevach, E. (1972), *J. Exptl. Med.* **136**, 1207.

ies was that the alloantisera inhibited only the response that was linked to the histocompatibility antigens against which the serum was directed; that is, that anti-2 serum would inhibit the response of 2×13 F_1 guinea pigs to DNP–GL but not to GT, whereas antisera directed toward the alloantigens on strain 13 would inhibit the GT response of cells from these F_1 guinea pigs but would not affect their response to DNP–GL (Fig. 2). The conclusion reached by these investigators was that immune response genes produce a cell-

surface-antigen-associated product and that this product plays a role in the mechanism of antigen recognition by T lymphocytes. This hypothesis has not yet been disproven.

3. The *Ir-1* Gene

3.1. Introduction

Shortly after the original description of the *PLL* gene, McDevitt and Sela (1965) demonstrated the existence of an autosomal dominant immune response gene in mice, the *Ir-1* gene, which controlled the ability of certain strains of mice to respond to the branched-chain synthetic polypeptide, (T,G)–A—L. Their original observations are presented in Fig. 3.

3.2. (T,G)–A—L and Ir Gene Control

When strain-CBA and -C57 mice are immunized with (T,G)–A—L, a long, branched, multichain synthetic polypeptide composed of a backbone of poly-L-lysine with side chains of poly-D,L-alanine to which are randomly attached L-tyrosine and L-glutamic acid (Fig. 4), C57 mice mount a good immune response whereas CBA mice respond poorly as judged by antibody production. Crosses between these strains, and subsequent backcrosses, showed that the responsiveness was under the control of an autosomal dominant inherited gene or gene cluster (McDevitt and Tyan, 1968).

If CBA and C57 mice are immunized with (H,G)–A—L, a synthetic polypeptide in which histidine replaces tyrosine, the oppo-

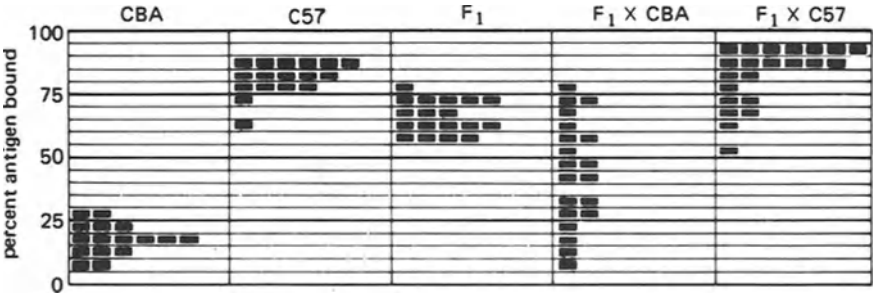

Fig. 3. The response following immunization with (T,G)-A-L of strain CBA, strain C57, the F_1 hybrid between these two pair and backcross animals measured as percent antigen bound using a radioimmunoassay. Reproduced by permission from McDevitt, H. O. (1965), *J. Exptl. Med.* 122, 517.

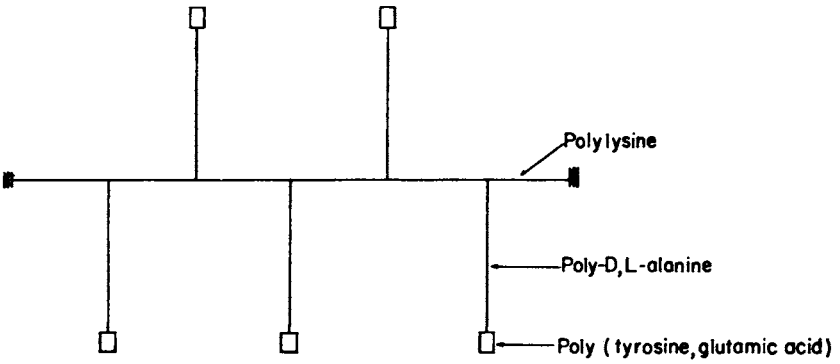

Fig. 4. A graphic representation of the synthetic polymer (T,G)-A-L. Reproduced by permission from McDevitt, H. O. (1965), *J. Exptl. Med.* **122**, 517. © 1965 by Rockefeller University Press. All rights reserved.

site results are obtained (McDevitt and Sela, 1967). Now CBAs become high responders whereas C57s do not mount an adequate response. In addition to demonstrating the unigenic control of responsiveness to these two polypeptides, these studies demonstrated that the product of this immune-response gene possessed reasonably highly developed discriminatory powers since (T,G)–A—L and (H,G)–A—L have very similar structures and antibodies raised against either of these polypeptides cross-react quite extensively with the other (McDevitt and Benacerraf, 1969).

3.3. Ir-1 *Control of T Cell Responses*

Subsequent investigations demonstrated that genetic control of the ability of mice to respond to these synthetic polypeptides resided in immunocompetent lymphocytes. The transfer of spleen cells from a high responder into a thymectomized irradiated high responder by low-responder F_1 mouse [which is capable of responding to (T,G)–A—L] and into an irradiated thymectomized low responder transferred the ability to make both a primary and secondary immune response to (T,G)–A—L. More importantly, at this same time, studies with congeneic strains of mice and linkage studies in segregating backcross populations showed that the ability to respond to (T,G)–A—L was linked to the H-2 locus (Table 2) (McDevitt and Tyan, 1968). This was in marked contrast to the hypotheses that preceded these experiments, which suggested that the effect of Ir gene control would be at the level of the expression of antibodies raised in response to the antigen. It was expected that this genetic control might reside in close linkage association

Table 2*

Failure to inhibit MLC with anti-Ia antisera directed against the nonstimulating Ia antigens of an F_1 stimulator cell[a]

Responder	Stimulator	2% Normal F_1 Serum	2% F_1 Anti-B10 Serum
		cpm ± S.E.M.	
B10.A	$B10.A_x$	370 ± 40	650 ± 10
B10.A	$B10.D2_x$	35,900 ± 1,400	12,900 ± 500
B10.A	$(B10.D2 \times B10.A)F_{1_x}$	16,200 ± 3,600	8,000 ± 1,400[b]
B10.D2	$B10.D2_x$	180 ± 30	240 ± 80
B10.D2	$B10.A_x$	5,600 ± 1,200	7,300 ± 900
B10.D2	$(B10.D2 \times B10.A)F_{1_x}$	7,100 ± 500	6,500 ± 300

[a] 4×10^5 B10.A or B10.D2 responder spleen cells were cultured with 4×10^5 irradiated B10.A, B10.D2, or $(B10.D2 \times B10.A)F_1$ stimulator spleen cells for 5 days in EHAA medium containing either 2% normal $(B10.A \times A)F_1$ serum or 2% $(B10.A \times A)F_1$ anti-B10 antiserum, pool no. 4. Stimulation was assessed by measuring the incorporation of a pulse of tritiated-thymidine, expressed here as the mean cpm ± S.E.M. for triplicate determinations.

[b] MLC significantly suppressed ($p < 0.05$) by F_1 anti-B10 serum compared to normal F_1 serum.

with the *Ig-1* or immunoglobulin gene locus. The finding of linkage of genetic control of immune responsiveness to the mouse major histocompatibility complex was quite unexpected and has revolutionized the field of immunogenetics.

3.4. Mechanisms of Ir-1 Gene Control

The next series of studies on the *Ir-1* gene attempted to elucidate the mechanism that was controlled by the *Ir-1* gene. A rather striking finding was made by Grumet (1972), who observed that there seemed to be a selective defect in immunologic IgG memory in nonresponder mice (Fig. 5). By immunizing strain-C3H.SW($H\text{-}2^b$) high-responder mice and strain-C3H($K\text{-}2^k$) low-responder mice with (T,G)–A—L in aqueous solution and studying the kinetics of antibody formation, it became apparent that both strains responded equally well during the first week after immunization and developed brisk primary responses consisting of IgM antibody. Following secondary challenge with aqueous (T,G)–A—L, however, only high-responder $H\text{-}2^b$ mice showed immunological memory producing high titers of IgG anti-(T,G)–A—L antibody. The low-responder $H\text{-}2^k$ mice continued to make a persistent low level of IgM anti-(T,G)–A—L antibody, but did not develop any demonstrable

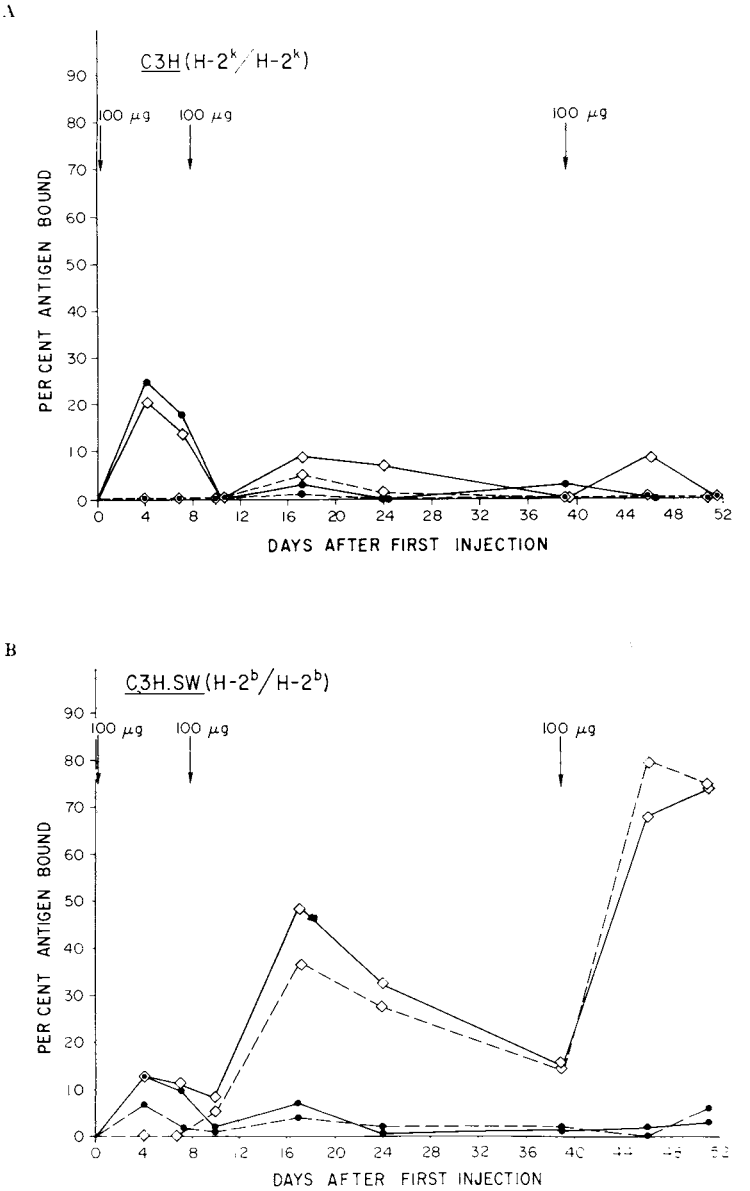

Fig. 5. The primary, secondary, and tertiary response of strain C3H, Panel A, and the congenic C3H.SW, Panel B, following immunization on three separate occasions with 100 μg of aqueous (T,G)-A-L. Reproduced by permission from McDevitt, H. O. (1973), *Hosp. Pract.* **8**, 61. Graph by A. Miller. © 1973 by H. P. Publishing Co., Inc.

IgG antibody to (T,G)–A—L. This finding suggested that a possible mechanism of the *Ir-1* gene was the control of the switch from IgM to IgG antibody production.

Recent studies by Singer et al. (1977) have raised apparently conflicting data that suggest that the in vitro primary IgM anti-hapten response to DNP conjugates of (T,G)–A—L is T-dependent and under autosomal dominant H-2-linked Ir gene control. Furthermore, their mapping studies suggest that the localization of this Ir gene control is identical to that of the *Ir-1* locus. These results are in conflict with Grumet's findings that the primary IgM response was T-independent and apparently not genetically controlled. One possibility for these seemingly contrary results is that different batches of the random copolymer (T,G)–A—L may vary in their relative dominance of two included amino acid sequences, one of which has been shown to be T-dependent and the other T-independent, both of which occur in the random copolymer (Schwarz et al., 1976).

As with the *PLL* gene, by immunizing with (T,G)–A—L complexed to a carrier protein such as methylated bovine serum albumin (M-BSA), nonresponder mice produce the same number of anti-(T,G)–A—L-antibody-producing cells as do responder mice immunized either with (T,G)–A—L or M-BSA (T,G)–A—L (McDevitt, 1968). Again, this demonstrated that nonresponder mice have the capability of producing anti-(T,G)–A—L antibody molecules and suggested that the defect in nonresponders might be faulty or incomplete recognition of the antigen at the level of the T cell. These findings coupled with the data obtained in the guinea pig led McDevitt and his colleagues to postulate that the Ir gene effect was exerted at the level of thymus-derived T cells rather than at the level of antibody-producing B cells. If this postulate were true, thymectomy should convert responder mice into nonresponder mice without affecting the response patterns of nonresponders.

3.5. Characteristics of the Ir-1 Gene

Studies by Mitchell et al. (1972), shown in Fig. 6, demonstrate that thymectomy, but not sham thymectomy, totally abolishes the secondary (T,G)–A—L response of strain-C3H.SW($H\text{-}2^b$) high-responder mice without demonstrably affecting their primary response, but does not affect the response of the low-responder strain-C3H ($H\text{-}2^k$) mouse. Additionally, adult thymectomy blocked the normal high response to methylated bovine serum albumin (T,G)–A—L, which was seen in sham thymectomized or normal low-responder

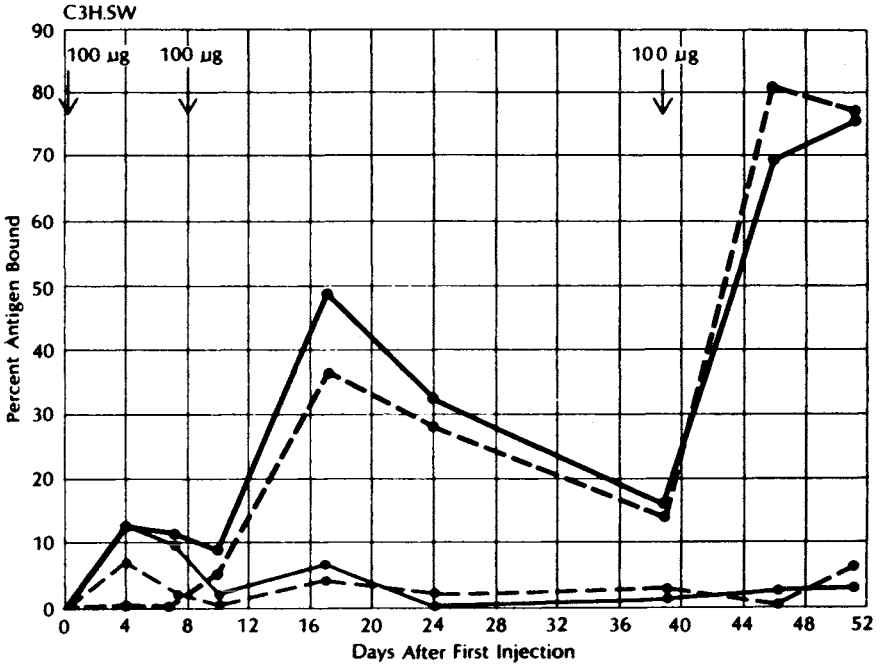

Fig. 6. The response of thymectomized and sham thymectomized animals of strain C3H.SW to 3 serial stimulations with aqueous (T,G)-A-L. Reproduced by permission from McDevitt, H. O. (1973), *Hosp. Pract.* 8, 61. Graph by A. Miller. © 1973 by H. P. Publishing Co., Inc.

mice. These data were consistent with the theory that the *Ir-1* gene effect was mediated by thymus-derived T cells. Thus these original experiments on both the *PLL* and *Ir-1* gene set the following criteria for immune response genes: (1) that they were autosomal dominant genes, (2) that their effect was mediated by immunocompetent cells, (3) that the effect of these genes seemed to be mediated by T cells, and (4) that cell-surface antigens controlled by MHC genes closely linked to (or identical to) Ir genes were involved in antigen recognition.

Studies in the past 10 years have demonstrated the validity of these original observations but have also demonstrated the almost unbelievable complexity of the system that is governed by these immune response genes.

In the next section, I would like to make use of observations on the genetic control of immune responsiveness to naturally occurring protein antigens. I think these antigens allow us to examine the complexity of genetic control of immune responsiveness yet

provide a unification of the concepts and experiments, that have been performed using synthetic polypeptides.

4. Genetic Control of Immune Responsiveness to Staphylococcal Nuclease

4.1. Introduction

Perhaps the most extensively studied system of major-histocompati bility-complex-linked genetic control of immune responsiveness to a naturally occurring protein has been the H-2-linked control of immune response to staphylococcal nuclease. Despite the fact that staphylococcal nuclease is a structurally complex naturally occurring protein antigen, a pattern of Ir gene control of humoral response to nuclease has been demonstrated (Lozner et al., 1974). In vitro T cell-proliferative response to nuclease has been studied, and a similar pattern of immune-response gene control has been observed (Sachs et al., 1978a,b). The availability of well-characterized polypetide fragments from nuclease has allowed a rather extensive and careful dissection of responsiveness to this protein antigen.

4.2. Staphylococcal Nuclease Molecule

Staphylococcal nuclease is an extracellular enzyme of *Staphylococcus aureus*. Anfinsen and his colleagues (1971) have determined not only the amino acid sequence but the three-dimensional structure of nuclease. An artist's interpretation of this structure is presented in Fig. 7., and several points of chemical and/or proteolytic cleavage are illustrated. Thus it is possible to examine Ir gene control of the response to fragments of the nuclease molecule, which consist of sections of serially arrayed amino acids (although in solution these fragments seem to exist in an equilibrium between a variety of three-dimensional configurations) (Sachs et al., 1972). Antinuclease inhibition of enzymatic activity is easily measured in a spectrophotometric assay that has been well described (Fathman and Sachs, 1976).

4.3. Ir-Nase

The initial studies on the immune response to staphylococcal nuclease examined primary responses to immunization of nuclease in complete Freund's adjuvant. Marked differences in re-

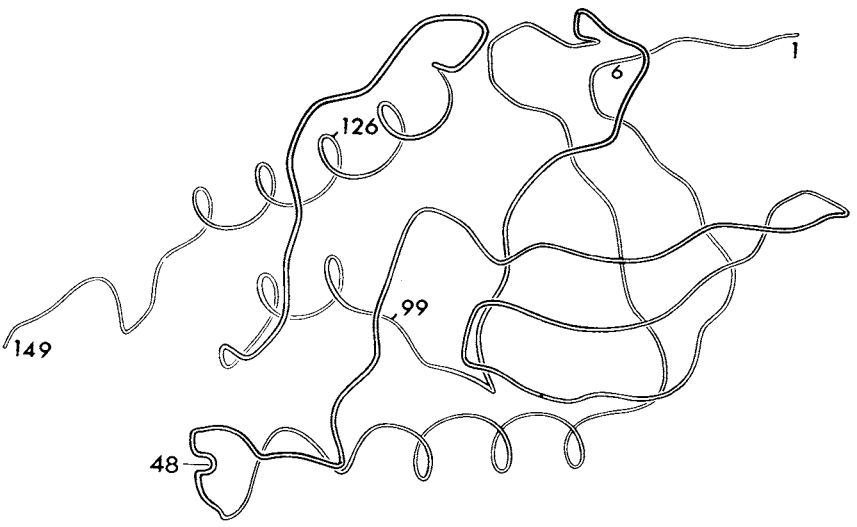

Fig. 7. An artist's representation of the three-dimensional structure of staphylococcal nuclease. The approximate positions of the residues at which specific modes of cleavage are available are indicated. Residues are numbered from the amino-terminal to the carboxy-terminal end of the molecule. Fragments are referred to by the numbers of the amino acids at each end (e.g., 1–126). Reproduced by permission of Sachs, D. H. (1976), *C. S. H. Symp. Quant. Biol.* 41, 295. © 1976 by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

sponsiveness were noted, and linkage studies demonstrated that there were H-2-linked immune-response genes governing the ability of certain strains of mice to mount an effective response to a primary immunization with nuclease (Lozner et al., 1974). Mapping studies for the responsiveness to nuclease are shown in Fig. 8. It can be seen that high responsiveness, which is characteristic of the $H\text{-}2^b$ haplotype strain, resides within a small region of the major histocompatibility complex, the *I-B* subregion. This demonstration of Ir gene-controlled responsiveness of antibody to a complex naturally occurring protein was somewhat unexpected. Subsequent studies have demonstrated that although the initial antibody response is under H-2-linked Ir gene control, hyperimmune response, as detected by studying antibodies raised following multiple immunizations, is controlled by non-H-2-linked genes. Thus it became possible for the first time to attempt to determine the genetic control of responsiveness to natural antigenic determinants (epitopes) within a protein. Sachs and his colleagues (1978a) selected certain fragments from the nuclease molecule and immu-

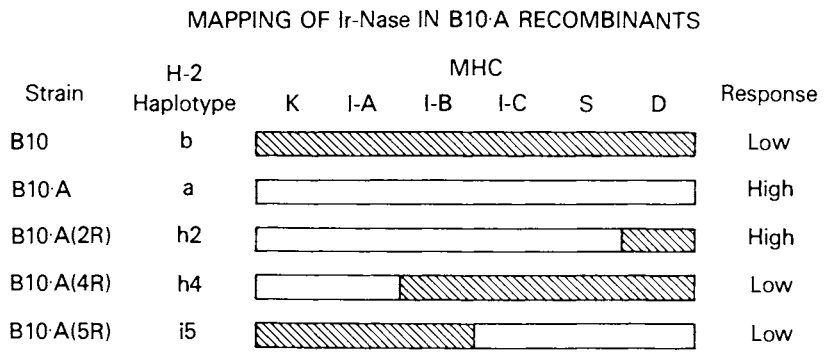

Fig. 8. A graphic representation of the immune response to immunization with staphylococcal nuclease in five different congenic and recombinant congenic mice. Haplotype B is represented by a slashed bar, haplotype A by an open bar. Response of each animal as indicated to the right under "response." Reproduced by permission of Sachs, D. H. (1976), *C. S. H. Symp. Quant. Biol.* **41**, 295. © 1976 by Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

nized a variety of strains of mice, which included both high responders and low responders to the intact molecule. One of the fragments selected, fragment 99 to 149, elicited responses that were characteristic of that to the intact nuclease molecule in that the high responders responded quite well whereas the low responders were unable to respond to this particular fragment of the intact molecule.

In striking contrast, responsiveness to a second fragment, fragment 1 to 126, demonstrated equal ability to respond in both a low-responder strain (B10) and in a high responder (strain-A/J mice).

Other differences and relative magnitudes of response were seen using other fragments of the nuclease molecule in the strains selected for study. The observation that fragment 99 to 149 elicited responses similar to that for the intact nuclease molecule suggested the hypothesis that this determinant carried the antigenic determinant, the response against which was under H-2-linked Ir gene control. Additionally, these studies suggested that the ability of the low-responder mice to respond to hyperimmunization with nuclease was probably resulting from the ability of these mice to recognize and respond to other immunogenic fragments on the native molecule. Thus it was concluded that H-2-linked Ir genes controlled the relative proportions of antibodies that were specific for different determinants on the same antigen molecule, a possibility that was, in part, raised by Arquilla and Finn (1963) several years before.

4.4. T Cell Responses to Nuclease Controlled by Ir-Nase

Striking similarities were found in the genetic control of antibody response and the antigen-induced T lymphocyte-proliferative response to nuclease (Schwartz et al., 1978a). The demonstration of this similarity in Ir gene-controlled T lymphocyte-proliferative response and antibody production depended upon the concentration of antigen used in the immunization protocol. Using higher doses of immunogen, however, led to results that were inconsistent with those seen at the lower-dose immunization schedule. The experimental findings observed following the higher-dose immunization suggested that the genetic control was more complex than the single Ir gene in the *I B* subregion, which had been postulated previously (Sachs et al., 1978a,b).

Again, the ability to make use of the fragments of the native molecule as stimulating antigens in the T lymphocyte-proliferative assay allowed confirmation of this hypothesis. The data obtained from studying the proliferative response to the fragments of the nuclease molecule suggested that although responsiveness appeared to be controlled almost exclusively by genes mapping within the Ir region, there appeared to be multiple genes within the Ir region that controlled T cell-proliferative responses to nuclease. Thus it was apparent confirmation of Sela's original hypothesis (1969) that there were multiple antigenic determinants residing internally within native proteins and that immune responsiveness to the entire protein involved component reactions to a variety of different determinants.

4.5. Anti-Idiotype Antibodies and Ir-Nase

One other finding deserves comment in the studies on staphylococcal nuclease. As had been previously demonstrated with the responsiveness controlled by the *Ir-1* gene, the specificity of the presumed receptors on T cells seems to be dissimilar from those on antibody molecules that have specificity for the intact molecule. Antinuclease antibody binding was highly confirmationally specific, whereas comparative studies of the immune response elicited in T cell-proliferative responses (comparing response to the nuclease fragment 99 to 149 with that to intact nuclease) would suggest that the T cell receptor is not confirmationally specific. Additionally, it was possible, using anti-idiotypic reagents raised against antibodies to nuclease, to study genetic control of immune response at the level of the antibody-combining site (Fathman and

Table 3*
Relationship of H-2 Haplotype to Expression of A/J Idiotype

Strain	*H-2* haplotype	*Ig-1* allotype	Inhibition of Inactivation	Significance of inhibition ($P < 0.05$)
			%	
A/J	a	Ig-1^a	66.9 ± 4.5	+
B10.A	a	Ig-1^b	5.1 ± 7.1	−
B10	b	Ig-1^b	5.4 ± 9.1	−
A.BY	b	Ig-1^a	42.0 ± 4.6	+

Pooled sera from immune animals were tested for the presence of A/J idiotype by comparing inactivating activity in the presence and in the absence of anti-idiotypic antibody.

*Reproduced by permission of Fathman, C. G. (1977), *J. Exptl. Med.* 145, 569. © 1977 by Rockefeller University Press. All rights reserved.

Sachs, 1976; Fathman et al., 1977). These studies suggested that the genes determining idiotypes of antibodies to nuclease are independent of H-2-linked Ir genes and, in fact, reside in close linkage with the *Ig-1* heavy-chain locus marker (Table 3). Recent studies on the localization of the *Ig-1* heavy-chain locus to chromosome 12 in the mouse would further tend to disprove simple associations between Ir genes (on chromosome 17) and immunoglobulin variable regions (Hengartner et al., 1978).

4.6. At Least Three Genes Control Immune Response to Nuclease

The immune response to staphylococcal nuclease is controlled by at least two separate sets of genes: (1) H-2-linked Ir genes that control the ability of the animal to generate a primary antibody response to nuclease and (2) genes linked to the *Ig-1* heavy-chain allotype locus that control the expression of idiotypes of antibodies raised in response to immunization with nuclease.

A third level of genetic control has been demonstrated following immunization with nuclease (Fig. 9). These studies (Pisetsky et al., 1978) demonstrated that the genes that controlled the overall magnitude of antibody response to nuclease were not linked to H-2 nor were they linked to the *Ig-1* heavy-chain locus. As can be seen in Fig. 9, strain-A.By ($H\text{-}2^b$) mice were incapable of mounting an effective primary response to immunization with nuclease yet by the time of the fifth serial immunization they were making as high titer antibodies to nuclease as were strain-A/J ($H\text{-}2^a$) high-responder mice. Furthermore, strain-BIO.A ($H\text{-}2^a$) high-responder mice, on

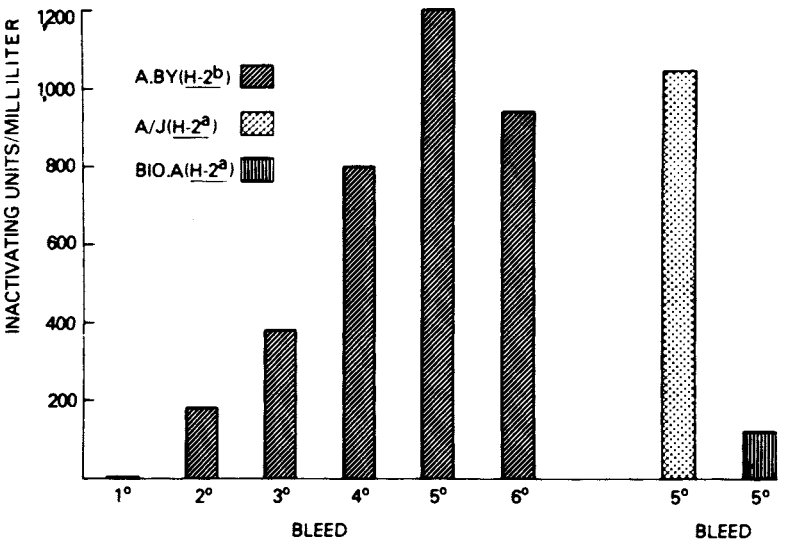

Fig. 9. The response to serial immunizations with staphylococcal nuclease of three separate strains of mice. Strain A.BY is a low responder following the primary immunization. Strain A/J and B10.A are both high responders following primary immunization. Reproduced by permission from Sachs, D. H. (1978), *J. Exptl. Med.* 147, 396. © by Rockefellerr University Press. All rights reserved.

another genetic background, were making quantitatively much less antibody at the fifth immunization than were either strain-A/J or strain-A.BY mice. It is thus apparent that the study of complex naturally occurring proteins such as staphylococcal nuclease allows a much more elegant dissection of the mechanisms of genetic control of immune responsiveness. This genetic control has been demonstrated to be exerted in at least three levels in response to immunization with staphylococcal nuclease: (a) H-2-linked Ir genes, (b) *Ig-1*-linked genes that determine the idiotype of antibodies raised in response to nuclease, and (c) a gene or genes that regulate the overall magnitude of antibody response that segregate independently from the H-2 and the Ig-1 locus.

5. Ia Antigens

5.1. Introduction

Before discussing the relationships that exist between cells that seem to be governed by immune response genes, I would like to de-

scribe the initial observations that led to the demonstration of immune-response-region-associated or Ia antigens. Following the demonstration of (1) the existence of MHC-linked immune-response genes and (2) the capability of antisera directed toward the MHC of guinea pigs to block T-dependent proliferative responses to antigens, the response against which was under Ir gene control, it seemed feasible to various groups of investigators that it might be possible to use immunocompetent lymphocytes as a source of antigen to raise antibodies against Ir gene products.

5.2. Anti-Ia Antisera and Ir-Gene-Controlled Responses

Reciprocal immunization of strains of mice who differed at the Ir region led to the production of antisera that permitted the detection of a group of polymorphic cell-surface structures that have been designated immune-region-associated or Ia antigens. Since the original description of these antigens in the early 1970s a variety of excellent reviews have been written that document historically as well as functionally the current data on Ia antigens [see *Transplantation Reviews* **30** (1976)]. Studies by Schwartz and his colleagues (1976) demonstrated that the in vitro antigen-induced proliferative responses of murine T lymphocytes can be inhibited by such antisera raised against products of the *I*-region loci (i.e., anti-Ia sera). When the response being studied was under the control of specific immune response genes, the anti-Ia inhibition showed haplotype specificity in a manner similar to that shown previously in guinea pigs, as described earlier in this chapter.

Mouse studies allowed further elucidation of genetic control of immune response. These antisera-mediated blocking studies demonstrated that in certain instances (in which an Ir gene could be mapped to a particular subregion of *I*) anti-Ia antisera specific for products of that subregion would inhibit activation. These results suggested to Schwartz and his colleagues (1976) that, as in the guinea pig, there was an intimate relationship between Ia antigens and molecules that mediate immune-response gene function. Studies originally performed by Hämmerling (1975) demonstrated that not only did immunocompetent lymphocytes express these Ia antigens, but a variety of other murine tissues, including macrophages, expressed Ia antigens. Although conflicting results were obtained initially, it has subsequently been determined that certain T lymphocytes also express Ia antigens (Fathman et al., 1975); the effect of these *I*-region gene products on cellular interaction and

cooperation is perhaps the foremost area of research in the field of genetics of immune responsiveness.

The major question that is yet unanswered is whether Ia antigens mediate Ir gene effects. It is generally agreed that the complexity of Ia antigens, coupled with the complexity of immune response region phenomena, make it unlikely that any simple view of the relationship between Ir genes and Ia antigens is correct.

6. Complementing Ir Genes

6.1. GL-Phenyl Ir Genes (α and β)

Another complication was introduced into the picture of genetic control of immune responsiveness following the observations in several species and in several laboratories that in certain antigen-responsive systems, under Ir gene control, F-1 hybrids between two low-responder parental strains may give responses higher than either parental strain alone (reviewed by Dorf, 1978). This apparent complementation was demonstrated to be linked to the H-2 complex (Stimpfling and Durham, 1973). Perhaps the most definitive experiment demonstrating the requirement for two H-2-complex immune-response genes to complement one another, in allowing responsiveness to an antigen, were the studies on the terpolymer GL-phenyl. Initially, the responsiveness to GL-phenyl had been demonstrated to be linked to the mouse H-2 complex, and preliminary mapping studies indicated that the *I-B* region might control responsiveness to GL-phenyl (Dorf et al., 1974). It soon became apparent that there were certain anomalies: First, certain recombinant mice carrying presumed low-responder alleles in the *I-B* subregion could respond to GL-phenyl, and F-1 hybrids derived from selected nonresponder strains also could respond to GL-phenyl (Dorf et al., 1975). Mapping studies performed on this Ir gene complementation have described two separate genes, alpha and beta. Each of these two complementing Ir GL-phenyl genes appears to be dominant, yet both must be present for responsiveness. Thus phenotypic nonresponders can lack both responder alleles of alpha and beta, or possess one or the other, and yet be incapable of mounting an immune response to GL-phenyl. Perhaps an even more striking finding was the demonstration that the dominant alpha and beta genes can function in both cis (on the same chromosome) or trans (on opposing chromosomes) position.

Recent studies by Schwartz and his colleagues (1976) have presented an elegant confirmation of the complementation re-

quired to obtain GL-phenyl response between the alpha and the beta genes, and have demonstrated that the T lymphocyte-proliferative immune response to GL-phenyl is under the control of two Ir genes as well. Schwartz et al. were able to map one gene, termed *Ir-GL-phenyl-alpha*, to the *I-C* or *I-E* subregion of the mouse H-2 complex and the other gene, termed *Ir–GL-phenyl-beta*, to the *I-A* subregion of the mouse H-2 complex.

6.2. Anti-Ia Antisera and GL-Phenyl α and β Genes

Finally, Schwartz et al. (1978b) were able to show that antibodies directed toward Ia antigens coded for by genes in either of the *Ia* subregions containing alpha or beta genes were capable of inhibiting the proliferative response of immune lymphocytes to in vitro challenge with GL-phenyl. This added further complexity to the genetic control of immune responsiveness and suggested that a complementing function mediated by two Ir gene products can be blocked by anti-Ia antisera directed against cell-surface molecules controlled by genes in either subregion. This type of Ir gene complementation has now been observed in many laboratories, and further complexity has been introduced with the observation that certain complementarities between responder alleles at two distinct loci appear to be restricted, in that all dominant beta genes do not necessarily complement all alpha genes. This phenomenon has been termed "coupled complementation" and suggests that all responder Ir genes for a given antigen in different haplotypes are not identical, and furthermore, that the mechanism of interaction between Ir gene products may be dissimilar, in certain instances resulting in considerable heterogeneity in complementing Ir gene funtions. It has become apparent following studies such as those cited above, that several immune-response gene functions can reside within the MHC, and it is becoming increasingly apparent that Ir gene function is expressed in more than one cell type.

6.3. Cells Involved in Ir Gene-Controlled Response

6.3.1. Introduction

The original investigations carried out by Benacerraf and McDevitt and their coworkers seemed to demonstrate that the immune-response gene effect they were describing resided exclusively in T lymphocytes. In fact, in a collaborative effort, these two investigators suggested that the product of the *I* gene locus might be the antigen-specific T cell receptor (Benacerraf and McDevitt, 1972). The arguments in favor of Ir gene

function residing in T cells were overwhelming, and the few anomalous results that initially trickled out suggested that immune-response region defects could occur in B lymphocytes and perhaps even in a cell as seemingly innocuous as the macrophage, were brushed aside. Indeed, the initial observations suggested that H-2-linked Ir gene responses were only observed following immunization with antigens that were T-dependent, were ablated by thymectomy, and were expressed as both cellular immunity and antibody production. Furthermore, it had been demonstrated for both the *PLL* and *Ir-1* gene that there were B cells in non responder animals capable of making antibodies to Poly-L-lysine as well as to (T,G)–A—L, respectively. Thus, expression of Ir genes in T lymphocytes (and only T lymphocytes) seemed likely.

6.3.2. Macrophage Ir Genes More recent studies have confirmed the earlier observations that immune-response-region gene defects can also result from faulty antigen presentation by macrophages and from the incapacity of B cells to respond to regulatory activity of T cells. The original observations of Rosenthal and Shevack (1973), that the *I*-region products on responder guinea pig macrophages are essential for antigen presentation in a T lymphocyte-proliferative response to antigen, have been confirmed. Recent studies by Schwartz and his colleagues (1978c) have demonstrated that in a murine T lymphocyte-proliferative assay, adequate antigen presentation of GL-phenyl by macrophages requires expression on the macrophage of both the alpha and beta *GL-phenyl* genes. Additionally, an elegant series of experiments by Singer and his colleagues (1978) have recently demonstrated that Ir genes in macrophages regulate the primary immune response to TNP–(T,G)–A—L and TNP–(H,G)–A—L. In these experiments, they depleted (B10 × B10.A) F-1 spleen cells of adherent cells. Immune responsiveness could be restored by the addition of spleen adherent cells from this F-1. Additionally, this system was designed so that the F-1 was composed of reciprocally responsive nonresponsive parents to different antigens [TNP–(T,G)–A—L and TNP–(H,G)–A—L]. Although spleen adherent cells from F-1 mice reconstituted the responses to both antigens, spleen adherent cells from each of the parental strains reconstituted responsiveness only to the antigen for which they were genetically responders. However, spleen cells from either parental strains were capable of reconstituting non-Ir-gene-controlled responses to a third antigen. Finally, the use of spleen adherent cells from recombinant strains allowed mapping of the genes controlling this antigen presentation

function of macrophages to the *K* or *I-A* regions of the responder H-2 complex. These findings were similar to the studies of Paul and his colleagues (1977), who demonstrated that immunization of 2 × 13 F-1 guinea pigs with ovalbumin resulted in the appearance of distinct clones of responding lymphocytes, each of which was capable of response to antigen presented on only one of the parental strain macrophages, where all clones of lymphocytes could respond to antigen presented on F-1 macrophages.

6.3.3. B Cell Ir Genes The first study that seemed to demonstrate that low-responder gene regulation resided in B cells was a study by Shearer et al. (1971), using limiting dilution assays to analyze the cellular control of immune response. Subsequently, studies by Munro and Taussig (1975) suggested that the immune-response gene defect could reside in B or T cells and was dependent on the production of both a T cell factor and a B cell-acceptor site of this T cell factor. Both acceptor and factor seemingly were under control of H-2-linked Ir genes. The studies of Munro and Taussig, together with the elegant studies by Berzofsky et al. (1977) (who demonstrated that Ir gene function affected the specificity of antibodies produced against native protein molecules), suggested that the ability of helper T cells to regulate or select B cell clones for antibody production may be a function of Ir genes.

7. Immunosuppressor Genes

7.1. *GAT* Is *Genes*

Experiments by various investigators over the past five years have illustrated another level of genetic control of immune responsiveness: specific *I* region gene(Is)-controlled suppression. The first demonstration of an H-2-linked Is gene was that of Kapp and her colleagues (1974), who made use of the response of inbred strains of mice to a random terpolymer, L-glutamic acid–L-alanine–L-tyrosine (GAT). H-2-linked genetic control of immune responsivenes to this antigen had been previously demonstrated. Like antigens controlled by the *PLL* and *Ir-1* gene, nonresponder mice to GAT were able to respond to GAT complexed to an immunogenic carrier, methylated bovine serum albumin (M-BSA). In their original study, which demonstrated a specific immune suppressor phenomenon, these investigators found that GAT failed to elicit a GAT-specific response in nonresponder mice, and if the nonresponder mice were previ-

ously immunized with GAT, they subsequently failed to respond to an immunogenic dose of GAT bound to M-BSA. These "nonresponder mice" in fact responded by raising GAT-specific T suppressor cells. They showed that B cells from such a nonresponder mouse who had been rendered unresponsive by in vivo GAT treatment could respond in vitro to GAT–M-BSA if exogenous carrier-primed T cells were added to the cultures.

These findings raised several questions concerning the nature of the immune response defect in nonresponder mice. Specifically, the universality of the occurrence of specific immune suppressor T cells raised by immunization of genetically nonresponder mice was questioned. Experiments by Debré and his colleagues (1976), using the copolymer L-glutamic-acid–L-tyrosine (GT), have demonstrated that this phenomenon of specific generation of immune suppressor T cells can be seen with other antigens and in other strains but is by no means universal. The important findings of studies on specific immunosuppression are that the control of this immune suppression is also H-2-linked and, in fact, immune suppressor or Is genes reside within the same subregions of the H-2 complex of the mouse as do the Ir genes. Not only are the Is genes antigen-specific, but immunosuppressive antigen-specific factors have been identified as the product of T lymphocytes from certain nonresponder mice primed with the specifc immunogen to which they are nonresponsive. Initial studies of these T-cell-derived suppressor factors have demonstrated that they possess *I*-region determinants and that they have antigen specificity. Several but not all of these antigen-specific immunoregulatory molecules have been demonstrated to bear determinants that are genetically controlled by a new subregion of *Ia*, the *I-J* subregion. Thus it appears that antigen-specific helper T cells are controlled by genes within the *I* region of the mouse H-2 complex, and the development of specific suppressor T cells is also controlled by genes residing in this region. It is not clear what, if any, relationship exists between these two different classes of immunoregulatory gene products.

7.2. *Complementing* Is *Genes*

Recent data have shown that the inability of nonresponder strains to develop helper T cells in these systems is not absolute. If the nonresponder animals to GAT, or to GT, are immunized with macrophages pulsed in vitro (or following in vivo cyclophosphamide or anti-IJ alloantiserum administration), these genetically defective animals are able to produce helper T cell responses. Thus it ap-

pears that in these "nonresponder systems," immunization with aqueous or "free antigen" seems to predominantly stimulate suppressor T cells and few, if any, helper T cells. Additionally, as with the alpha and beta complementation for response seen in the GL-phenyl system, GT-specific suppression is controlled by complementing *I*-region Is genes. These complementing genes can function in cis or in trans position. Again, the phenomenon of "coupled complementation" is seen in certain instances for GT suppression.

8. Genetic Control of Cellular Immune Responses

The *I* region of the mouse H-2 complex controls not only the immune response to soluble antigens but also, in perhaps a similar manner, controls recognition of cell-bound antigens: alloantigens, virally coded cell-surface antigens, and chemically modified self-antigens.

8.1. Mixed Lymphocyte Reactions

I would like to briefly outline experimental data in two of these systems that will provide only a general idea of the complex events under control of the *I* region of the H-2 complex. The first of these is the mixed lymphocyte reaction (MLR). When lymphocytes of two different strains of mice are grown together in culture, these lymphocytes undergo blastic transformation. By inactivating one of the pool of lymphocytes either by X-irradiation or mitomycin C, it is possible to assess the antigenic stimulatory determinants on the surface of the inactivated or stimulator cell that lead to blast transformation in the untreated responder population. Early studies in the mouse linked this lymphocyte activation response to the H-2 complex. Inherent in these observations is the fact that not only are the antigenic differences on the stimulator cell controlled by H-2 gene products, but recognition of these products must be controlled by similarly localized genes.

Recent studies have demonstrated that the strongest MLR-stimulating determinant in the mouse are products of the *I* region of the H-2 complex. It has been suggested that the strong MLR elicited by *I*-region incompatibility is a result of recognition of Ia-antigen differences. Genetic correlations between Ia antigens and MLR incompatibility have remained complete even when detailed studies on intra-*I*-region recombinants and the strain distribution of Ia antigens specificities are taken into account.

Table 4*

Response of Congenic Strains of Mice to Synthetic Polypeptide Antigens

Strain*	*H-2* type	Response	
		(T, G)-A--L	(H, G)-A--L‡
		%	%
A/J	a	10 (6–15)	66 (29–81)
A.BY	b	78 (62–87)	—
C57	b	69 (51–77)	0
C57Bl/10	b	32 (7–62)	—
B10.Br	k	7 (2–14)	28 (7–55)
C3H	k	10 (0–31)	44 (17–78)
C3H.SW	b	79 (52–91)	0

* Five males and five females of each strain.

‡ Responses to (H, G)-A--L were titered with (T, G)-A--L 509-^{125}I, by virtue of the extensive cross-reactions of these antigens, and therefore give lower per cent antigen bound values than with the homologous antigen (2).

The most direct evidence linking Ia antigens and MLR-stimulating determinants are the studies that have demonstrated blocking of stimulatory capabilities with the use of anti-Ia antisera. One of these studies makes use of an anti-Ia antiserum (αI-A.8) directed against a cross-reactive Ia determinant that is shared between two different strains of mice, B10 and B10.D2 (Schwartz et. al., 1976). When the antiserum is directed at Ia antigns of the stimulator cell, profound suppression of stimulation is observed (Table 4). Additionally, this antiserum blocks stimulating determinants on an F-1 cell only in the appropriate allogeneic combination. Thus when the antiserum is directed at the nonstimulatory parent [B10.D2 responders vs (B10.D2 × B10.A) F-1 stimulators], there is no apparent inhibition of stimulatory capability. However, when the same antiserum is directed toward the same (B10.D2 × B10.A) F-1 stimulator population and the responder strain is B10.A, inhibition is observed. These findings indicate that the antiserum inhibits stimulation by reacting with the Ia antigens of the stimulator cell, but when interacting with the responding cell does not inhibit responsiveness. The Ia antigens on F-1 cells seem to be separable on the cell surface so that antisera directed against Ia antigens of one haplotype do not interfere with the stimulatory capabilities of Ia antigens of the other haplotype.

As with the complementing Ir and Is genes seen in certain combinations in the mouse, it has been possible to demonstrate unique MLR determinants on certain F-1 hybrids that do not seem to be present on either parent and are controlled by genes of at least two interacting loci within the mouse H-2 complex (Fathman et al., 1977 Fathman et al., 1978). Again, these interacting genes seem capable of complementing in cis or in trans position to create this "hybrid" MLR determinant. The molecular configuration of the F-1 hybrid MLR determinant recognized in such situations has not been determined, but it seems to be composed of Ia antigens and under H-2-linked restriction of recognition (Fathman and Infante, 1979).

8.2. Cytotoxic T Cell Responses

Immune response genes have been demonstrated in the proliferative responses of mixed lymphocyte reactions and immune response genes seem to control cytotoxic T lymphocytes that are generated during such mixed lymphocyte reactions. In addition to the responses against alloantigens, detected in such cultures, being under Ir gene control, responses elicited by cells whose membranes have been altered either by chemical modification or viral infection to provide an effective stimulus to unmodified syngeneic lymphocytes (which leads to the generation of cytotoxic T cells) seem to be controlled by H-2-linked Ir genes.

The initial studies by Zinkernagel and Doherty (1974) on virally infected cells, as well as the studies by Shearer (1974) on chemically modified cells, led to the observations that the target of these cytotoxic T cells must be similarly modified and share in common with the responding cell a cell-surface antigen coded for by the major histocompatibility complex. Subsequently, these H-2-restricted responses have been seen for a variety of antigens, including minor histocompatibility alloantigens. Several lines of investigation have demonstrated that the altered target antigen alone is not sufficient for generation of cytotoxic responses to these modified syngeneic cells but that there also exists a requirement for a histocompatibility-linked Ir gene on the responder cell that controls this responsiveness (von Boehmer et al., 1978).

9. Human Immune-Response Genes

At the present time there is only very limited evidence for HLA-linked control of immune responses (Buckley et al., 1973; Pellegrino

et al., 1972; Levine et al., 1972; Greenberg et al., 1975; Spencer et al., 1976.) The most promising of these initial observations was that by Levine et al., who showed that the immune response to ragweed allergen E seemed to be closely associated with certain HLA haplotypes. These findings have subsequently been expanded and the original associations are not as good now as they were with the initial small series. Several attempts at purposeful immunization of volunteer subjects resulted in no clear association of HLA and immune responsiveness to the synthetic polyamino acids that had been used so effectively in experimental animals. (Scher et al., 1975; McMichael and McDevitt, 1977b). Although it has not yet been possible to directly demonstrate HLA-linked immune-response genes in humans, it has been possible to examine some of the phenomena that have been associated with H-2-linked immune-response genes in mice as they occur in experimental protocols using human cells. A recent meeting was held on genetic control of the human immune response sponsored by the Kroc Foundation and reported in a supplement of the Journal of Experimental Medicine, August, 1980. At this meeting there were several indications that there existed human immune response genes. One such indication was the association of antibodies to native DNA with HLA DRw3 (Griffing et al., 1980). The investigators examined the incidence of B lymphocyte alloantigens in patients who had elevated antibody titers to native DNA. There was a statistically significant ($P < 0.0001$) association between HLA DRw3 and the presence of antibodies to native DNA.It has been possible to demonstrate HLA correlation with lack of responsiveness following tetanus toxoid immunization (Sasazuke et al., 1978), and in vitro immune responsiveness to vaccinia virus (deVries, 1977). Both of these investigations demonstrated that there was haplotype association between the quantitative ability to respond to these two immunizing agents as demonstrated by in vitro secondary T cell-proliferative responses. Sasazuki et al., (1980) studied the genetic control of immune response in man by looking at T cell-proliferative responses to streptococcal cell wall antigens. Family analysis revealed that low response to streptococcal cell wall antigen seemed to be controlled by a single dominant gene that was closely linked to HLA. They suggested that this might be the first description of an HLA-linked immune suppressor gene. Thus, as has been recently demonstrated, there do exist good data supporting the presence of human immune response and immune suppressor genes.

9.1. Antigen-Specific Helper Factors in Humans

As described elsewhere in this book cooperation between thymus-derived T lymphocytes and non-thymus-derived B lymphocytes has been shown to be necessary for efficient antibody response to protein antigens. Several of these studies have suggested that supernatants of antigen-stimulated T cells can replace T cells for in vitro antibody responses mediated by B cells. There are two classes of such T cell-helper factors described in mice. One of these nonspecifically activates B cells (analogous to a polyclonal B cell-activator) and a second, antigen-specific factor has demonstrative antigen specificity and can induce B cells to produce antibodies directed only toward the antigenic determinants on the antigen used to elicit the T cell helper factor. Studies in murine systems have demonstrated that such antigen-specific factors can be absorbed on immunoabsorbent columns containing the antigen used to raise the factor, and can also be absorbed on immunoabsorbent columns containing antisera to Ia determinants expressed on the cell that produced the factor. Additionally, in mice, such antigen-specific factors (ASF) have been generated for antigens, the response against which is mediated by H-2-linked Ir genes.

Recent studies by Mudawwar et al. (1978) have very elegantly demonstrated that such antigen-specific factors exist in man and, furthermore, that these factors, in addition to having antigen specificity, can be removed by immunoabsorbent columns containing antisera to the DRw (Ia analogs) antigens of the cells donating the ASF. These studies were performed using purified human T cells that were immunized in vivo to tetanus toxoid. Following in vitro stimulation of these purified T cells, a helper factor was derived that induced proliferation and IgG synthesis of specific antitetanus toxoid antibodies from autologous B cell cultures. This antigen-specific factor could be absorbed on immunoabsorbent columns containing antisera to the DRw antigens of the donor of the cells used as a source of the antigen-specific factor. As in the experimental studies on mice, already outlined, passage of such antigen-specific factors over immunoabsorbent columns containing rabbit antisera reactive against heavy-chain determinants of human IgG or IgM did not result in loss of the antigen-specific factor activity. Finally, in studies very similar to those performed on mice, passage of this antigen-specific factor over immunoabsorbent columns containing anti-idiotypic antisera with reactivity toward the combining site of antitetanus toxoid antibodies resulted in an almost complete loss of antigen-specific factor activity, thus demon-

strating that such ASF have idiotypes (?V_H) in common with antibodies raised against tetanus toxoid.

9.2. Cytotoxic T Cell Responses in Humans

As mentioned briefly in Section 8.2, cytotoxic T lymphocytes generated against certain cell-surface antigens, whether they be virus encoded or chemically modified self-determinants or minor histocompatibility antigens, result in cytotoxicity that has certain histocompatibility restrictions imposed upon the priming and target cell. Very simply, this MHC restriction involves a requirement that the target cell must express the specific non-H-2 antigen; i.e., minor histocompatible antigen, viral component or chemically modified determinant, and, in addition, the same *H-2D* or *K*-region antigens as were present on the cells that initiated the immune response. A very elegant series of studies by Gordon et al. (1975) and von Boehmer et al. (1977) demonstrated (1) that cytotoxic T cells can be generated that specifically lyse syngeneic target cells carrying the minor histocompatibility antigen H-Y (male antigen), and (2) that this lysis is restricted by the MHC as outlined above; that is, cytotoxic T cells from $H\text{-}2^b$ female mice suitably primed to the Y antigen of $H\text{-}2^b$ males will only lyse cells from male mice carrying an $H\text{-}2D^b$ region. A very similar situation has been demonstrated in humans, where it has been shown that Y-antigen killing by T cells of a woman, who had previously rejected a bone marrow transplant from her brother, was restricted to the *HLA-A2* haplotype and the Y antigen in a manner very analogous to that described above for $H\text{-}2^b$ mice (Goulmy et al. 1977). In a similar manner, McMichael et al. (1977a) demonstrated that there was HLA restriction of cell-mediated lysis of influenza-virus-infected human cells. Finally studies by Shaw and Shearer (1978) demonstrated that human cytotoxic responses, observed in vitro, to trinitrophenylated autologous cells were in part restricted by HLA-A and/or HLA-B locus antigens. Thus, it has been possible in all three experimental systems demonstrated in mice to show analogous results in humans; that is, there is HLA restriction of cytotoxic T effector lymphocytes directed against virally infected, chemically modified, or minor transplantation antigens present on the target cell, and this restriction is identical to that seen in mice; that is, the cytotoxic T effector cell must "see" not only the antigen in question but also the identical MHC determinat on the target cell that it "saw" on the cell used to initiate the immune response.

10. An Hypothesis to Explain the Interrelationship Among Ir Genes, Ia Antigens, and MLR-Stimulating Determinants

As mentioned earlier in this chapter Ia antigens were initially discovered following purposeful immunization between Ir gene disparate strains of mice in an attempt to raise antisera reactive with products of Ir gene loci. Despite numerous experiments attempting to identify immune response gene products during the past decade, it has not yet been possible to attribute Ir phenomena to the serologically demonstrable Ia antigens. There have been many experiments performed that demonstrate close correlations between immune response phenomena and Ia antigens. Studies done recently in our laboratory utilizing the mutant B6.C-H-2^{bm12} (bm12) mouse have suggested that Ia antigens and Ir gene phenomena, as well as MLR-stimulating determinants encoded within the *I-A* subregion are different manifestations associated with the same product (Fathman et al., 1981). The bm12 mouse is a mouse that has a mutation in the *I-*A^b subregion. It is a gain and loss type of mutation in that there is reciprocal rejection of skin graft between bm12 and the parent C57BL/6 strain from which the mutant was derived. Additionally, the mutant seems to respond normally to certain antigens, the response against which is under Ir gene control including (T,G)–A—L and bovine type-2 collagen. It is, however, incapable of responding to other antigens including the loop of the A chain of beef insulin and cannot generate cytotoxic T effector cells to the H-2 restricted target antigen H-Y (Allan Rosenthal and Ian McKenzie, personal communication). Not only are there Ir gene defects, but the Ia antigens expressed on the mutant mouse are dissimilar from those on the parent C57Bl/6 cells. Recent studies done by McKean at the Mayo Clinic have suggested that the mutation in bm12 results in the production of an abnormal *I-*A_β polypeptide chain. This abnormality has been shown by tryptic peptide analysis and resides within the *I-*A^b beta chain (A^b_β). The studies carried out in our laboratory showed that the mutation that resulted in loss of certain serologically detectable *I-*A^b products on spleen cells of bm12 could be partially circumvented by trans-complementation by producing F_1 hybrids between the bm12 mutant and other strains of mice exhibiting normal *I-A* subregion gene products. These studies made use of the concept alluded to earlier that there exist hybrid *I-A* molecules on the surface of *H-*$2^a \times$ *H-*2^b

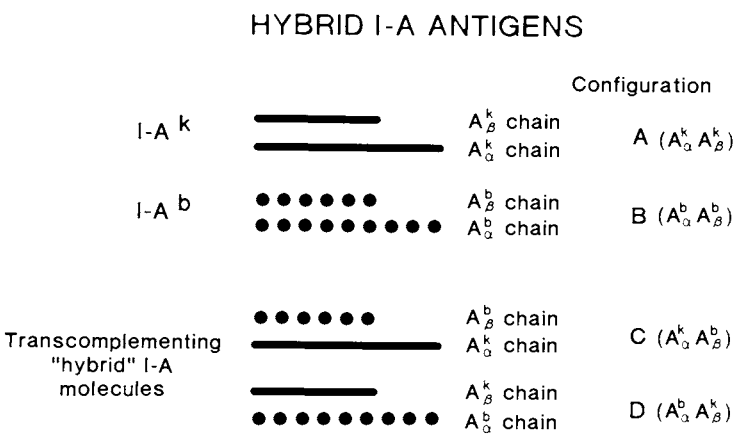

Fig. 10. An artist's representation of *I-A* products from ($H\text{-}2^a \times H\text{-}2^b$) F_1 mice. Reprinted by permission of *Immunological Reviews*.

heterozygous cells. Such hybrid *I-A* products have been shown to be stimulatory for clones of T cells reactive with certain alloantigens (Fathman and Hengartner, 1978; Fathman and Kimoto, 1981). Additionally, recent studies have shown that such hybrid products restrict antigen recognition by clones of immune T cells (Kimoto and Fathman, 1980, 1981). Data obtained in our laboratory comparing the responses of such alloreactive and soluble antigen reactive T cell clones using normal B6A F_1 cells to those obtained using cells of bm12 × (B10.A)F_1 mice suggested that the mutation in bm12 not only affected $I\text{-}A^b$ subregion products but also affected trans-complementing (hybrid) I-A products. These hybrid I-A molecules are formed by free combinatorial association of A_α and A_β chains and consist of $A^b_\alpha A^k_\beta$ and $A^k_\alpha A^b_\beta$. Figure 10 is an artist's representation of such combinatorial association of alpha and beta chains to form four I-A molecules encoded within the I-A subregion of cells of heterozygote mice. Such hybrid I-A products have been shown to restrict antigen recognition by cloned T cells, stimulate MLR responses by cloned T cells, and react with monoclonal antibodies directed against *I-A* antigens (Fathman et al., 1981). Thus we have been able to suggest that determinants that are on the one hand recognized as alloantigens serve as functional antigen-presenting determinants in syngeneic systems and can be blocked at both levels by antibodies to Ia antigens. These studies suggest an identity between Ir genes (defined simply as the ability of immune T cells to recognize antigen), Ia antigens, and alloantigens stimulating MLR that are encoded within the *I-A* subregion.

11. Conclusions

Thus, despite the fact that we have no direct evidence that HLA-linked immune-response genes exist in humans, the correlative evidence is very strong; i.e., (1) antigen-specific helper factors exist, (2) there is HLA restriction on cytotoxic T effector cells, and (3) the *HLA-D* region that codes for Ia-antigen analogs in man also controls the expression of MLR-stimulating determinants. Finally, perhaps the most compelling reason for believing that there might be immune-response genes in man the very strong association of certain HLA phenotypes and an ever-widening circle of diseases. The explanation most frequently given for the associations of HLA and disease is that there exist closely linked loci ("disease" susceptibility loci) quite analogous to immune-response and/or immune-suppressor genes that have been demonstrated in experimental animals. Thus it is not of purely academic interest that we examine and categorize the phenomena of genetic control of immune responsiveness in experimental animals; there is the ultimate hope that the same mechanisms that are operative in our experimental states can be used to explain the mechanisms that allow HLA and disease association.

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Chapter 3

Immunosuppressive Agents

A Conceptual Overview of Their Action on Inductive and Regulatory Pathways

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1. Introduction

Surgery, radiotherapy, and chemotherapy are the principal modalities of treatment of cancer. Each is chosen in accordance with the type and stage of the disease, and each has its benefits and risks. Although radiotherapy and chemotherapy have the advantage of reaching tumors and cells that are inaccessible to surgery (or, because of their location or wide dissemination, that cannot be surgically removed), these forms of treatment may exert damaging ef-

fects on organ systems outside the intended targets. The lymphoreticular system is usually affected and as a consequence the immune mechanism may become impaired. In the past, immunologically compromised patients have often succumbed to the onslaught of severe infections even though their tumors were being restrained or eradicated by the therapeutic intervention. Fortunately, information about toxic and immunosuppressive effects of various therapeutic agents, the introduction of new agents in recent years, and the development of combinations of drugs have made possible more prudent utilization of these agents for maximal therapeutic benefits with relatively minimal undesirable effects. Nevertheless, chemotherapy and extensive radiotherapy continue to pose risks to the patient and further improvements in the management of cancer patients will in large measure depend on the acquisition of more knowledge about the effects of old and new therapeutic agents.

Immunosuppression is not always an undersirable byproduct of a therapeutic regimen. There are numerous clinical conditions that demand deliberate interposition of immunosuppressive treatment. Such treatment is essential for the prolongation of allograft survival and has become standard practice with organ transplantations. Immunosuppressive agents are also being used in the treatment of allergic disorders and autoimmune diseases that encompass some of the most debilitating or life-threatening afflictions, e.g., rheumatoid arthritis or lupus nephritis. Although the intention of such treatments is to attack an immunologic dyscrasia, this limited objective is not always achieved and the treatment may result in damage to normal components and functions of the immune system. Yet, as has been known for a long time, immunosuppressive agents may preferentially affect one or the other of the two major arms of the immune system: antibody production and cell-mediated immunity. Prior to the advent of modern cellular and molecular immunology, it was difficult to account for such predilection and there was no rational basis for predicting how and at what stages or sites of the immune mechanism an agent would work.

In earlier studies the investigators were guided mainly by levels of antibody and by the size and intensity of the delayed-type hypersensitivity (DTH). The treatment was administered to a host prior to or following immunization. The antibody levels or degree of DTH reaction was measured in the same host. Although such studies have brought forth useful information regarding the ability of various chemical and physical agents to suppress an immune re-

sponse in relation to dose, time of treatment, and nature of antigen, these variables could not be related to unique events or cells and molecules engaged in immune functions because these were not known. Technological and conceptual advances during the last two decades have led to the recognition of lymphocyte classes, sets, and subsets and to the identification of specialized functions performed by these cells. Dissective approaches utilizing cell isolation, enrichment, depletion, and reconstitution have furnished new means for scrutinizing the processes of initiation, propagation, and regulation of immune responses. These means have become available for investigations on the action of immunosuppressive agents. Moreover, discoveries that cells could perform primary or subsidiary functions and that a cell could lend a hand to another cell (by induction, facilitation, synergism, or compensatory action) or that a cell could stop another cell (by competition, blockade, masking, or killing) have provided a basis for explaining some actions of these agents. Relevant cells can now be removed from the body, placed in culture, and subjected to the action of various agents. This can be done with whole populations or with subpopulations enriched or depleted for a given set or subset. Adoptive transfer procedures have been developed that combine some of the dissective elements of cell culture and the integrative and interactive conditions of the intact host. In the adoptive transfer experiment, cells can be taken from an immunized host and transferred to a recipient, usually a syngeneic host that is either normal or immunologically deprived: nude mouse or B mouse (lethally irradiated and restored with bone marrow). Treatment may be applied to the original host or to the cells or to the recipient.

What has been learned from experiments utilizing the new concepts, approaches, and methods is that the action of various physical and chemical agents is much more complex than previously expected. It is complex because the workings of the immune mechanism are complex. We have devoted considerable space here to the discussion and analysis of these complexities. It is our belief that a better understanding of how and where immunosuppressive agents act will not only aid in better utilization of these agents in therapy, but will also provide new insight into the functions of the immune mechanism. Previous reviews relevant to this subject have been published by Schwartz (1965, 1968), Makinodan et al. (1970), Krueger (1972), Hersh (1974), and Heppner and Calabresi (1976).

Analysis of immunological modifications must address a large number of issues, some appreciated for a long time, others revised or put in different perspective by recent experiments and still oth-

ers raised by new discoveries (some cited below). The basic issues may be summarized as follows:

(1) One agent may be suppressive or potentiating depending on the conditions of dosage or time of administration.

(2) Some agents affect lymphoid cells after antigenic activation toward proliferation, while others are suppressive primarily before the immune system becomes activated by antigen. There are also agents that seem to suppress at all times.

(3) The action of several agents is predicated on the nature of the immunizing antigen.

(4) There are indications that some agents exert selective (or preferential) activity against a single cellular class, set, or subset, whereas others may be less discriminatory.

(5) Since an agent may affect more than one cellular subset, and since these subsets may interact in protagonistic or antagonistic ways, the end results may vary depending on: (a) what subset is performing the dominant function at a particular point in time; (b) the inherent susceptibility of a given subset to the agent in question; (c) the difference in susceptibility of the subset in resting or activated state; (d) the role of the subset in the genesis or regulation of the immune response; (e) the dependence of the function of the subset on other subsets of cells; and (f) the contribution of microenvironment to the functions of various subsets.

(6) The absence of an immune response has been attributed to either clonal deletion or inhibition by suppressor cells or factors that prevent maturation and differentiation of effector cells. The possibility remains, however, that effector cells may be generated, but that their functions may be restrained by other cells. Agents capable of exerting selective action may lift this restraint.

2. Inductive And Regulatory Aspects of Immune Responses: Current Concepts

Three developments have revolutionized the form and substance of immunology and have triggered an unprecedented torrent of conceptual and technological advances. Burnet's clonal selection

theory provided a rational basis for tackling the critical issues of antibody diversity and specificity. Elucidation of the structure of immunoglobulin molecules made possible the recognition of components that determine the function of antibodies in relation to antigens, cell membranes, and complement. The recognition of specialization among lymphocytes raised the curtain on a new world of immunobiology, exposing a previously unsuspected universe of heterogeneous lymphocytes. What followed was a series of ingenious experiments that, over the last twenty years, showed how:

(1) Lymphocytes could be distinguished by dozens of parameters and not merely by size.

(2) These cells, though endowed with unique membrane structures (markers), express on their surfaces certain structures that are either identical with or complimentary to structures on antibody molecules.

(3) Lymphocytes and other cells of the lymphoreticular system are responsive not only to antigens, but to other molecules of exogenous and endogenous origin.

(4) These cells are in constant communication generating, transmitting, and receiving signals.

The experiments of the last two decades have thus laid the cornerstone for the main theme of modern immunology, *cellular interaction in the operation of the immune mechanism*, an interaction that in its most fundamental form is comprised of two classes of lymphocytes: B cells (named after the bursa of Fabricius in the chicken, but also referring to their direct bone marrow derivation in mammals) and T cells (named for their differentiation in the thymus). In actuality, their interaction requires the participation of an ancillary partner, a macrophage or macrophage-like cell. Moreover, as will be developed later, these interactions enlist more than one kind of T or B cell.

Even though allowed to undergo early differentiation in discrete enclaves, these cells migrate to peripheral lymphoid organs in which they interact in a variety of ways essential to the functions of the immune mechanism. One type of interaction is the cooperation of T and B cells in the generation of antibody-secreting plasma cells. Under the aegis of T cells, referred to as T helpers (TH), B cells differentiate to plasma cells and produce antibodies of an Ig class, in part specified by TH cells. The discovery of two classes of lymphocytes served as an impetus for a further search of cellular identity. The magnitude of the search was accentuated by the observations

that T cells were not only providing helper functions to B cells, but were also involved in a variety of phenomena, such as delayed hypersensitivity, rejection of allografts, graft-vs-host reactions, cell-mediated cytotoxicity, mixed leukocyte reactions, and cell-mediated resistance to infection and to neoplasia. Studies linking lymphocyte functions with surface markers (membrane antigens and receptors) have made possible the recognition of subclasses or subsets of T cells with unique functions either performed terminally as effector cells (e.g., cytotoxic effects) or in concert with other cells as precursors, initiators, inducers, or modifiers of effector cell functions. *Help and suppression are considered to be the most crucial functions of T cells* because they constitute a system of checks and balances regulating immune responses and thereby providing for a type of physiological homeostasis.

Cellular interactions may occur through direct contact or by means of soluble factors delivered by the cells into their microenvironment. Cell membranes and soluble factors often contain molecules (antigens or receptors) produced by genes of the major histocompatibility complex (MHC). Antigens produced under the control of the *K* and *D* genes are involved in cytotoxic recognition and reactions, whereas products of the *I* region are concerned with certain aspects of cellular proliferation and regulatory functions. Other molecules on the cell membrane or produced by the cell for secretion are specified by genes outside of MHC. The recognition of cells and molecules with specialized functions has permitted the construction of a variety of models of pathways, networks, and circuits that would help to explain immune response induction and regulation. As already mentioned, the lymphocytic interactants require the participation of a third type of cell, an auxiliary cell that is most likely a macrophage or is macrophage-like, and that can serve in a helper capacity as well as in a suppressive role. In subsequent sections, we shall illustrate some of the current concepts relating to immunoinduction and immunoregulation.

3. Classes and Subclasses of Lymphocytes and Their Functions

In Table 1 we have collated certain of the properties and characteristics of T and B cells that provide a convenient means of identifying these cells. It should be noted that some of these attributes pertain to mouse cells, others to human cells, and that although there is a fair degree of analogy, the results cannot always be extrapolated

Table 1
Primary Characteristics of Markers of T and B Cells

Parameters	T	B
Immunofluorescent staining for		
Ig	−	++
Thy-1 or θ	++	−
Rosette formation with		
SRBC alone	++[a]	−
Fc coated RBC	+	++
C3 coated RBC	±	++
Virus receptors for		
Measles	+	−
EB	−	+
Ly 1, 2, 3	+[b]	−
LPS blastogenic response	−	+[b]
PHA/con A blastogenic response	+	−
Antibody production	−	+
Idiotype on membrane	+	+
Cooperation in response to TD Ags	Carrier recognition	Hapten recognition

[a]Human.
[b]Mouse.

from mouse to man. The Ly phenotype denotes a system of differentiation antigens of T cells distinct from theta (θ) antigen (Reif and Allen, 1964), more recently renamed Thy-1 antigen. All T cells express Thy-1 antigen, albeit in varying degrees, with cells of the thymus expressing the highest density and cells in peripheral lymphoid organs manifesting lower quantitites of antigen per T cell. The Ly antigens are glycoproteins first discovered by Boyse and colleagues (1968). The Ly123 system is restricted to T cells. The Ly1 glycoprotein is controlled by a gene on chromosome 19, whereas the Ly23 antigens are controlled by closely linked genes on chromosome 6 (Itakura et al., 1972). These antigens are expressed on the cell surface and are detected by means of alloantisera. The majority of thymocytes bear the $Ly1^+23^+$ phenotype and are thought to constitute a pool of precursors of $Ly1^+$ and $Ly23^+$ T cells (Cantor and Boyse, 1975; Huber et al., 1976a). Only two alleles seem to exist in Thy-1 and Ly systems among all strains of mice tested, i.e., Thy-1.1, Thy-1.2, Ly1.1, Ly1.2, Ly2.1, Ly2.2, etc., but their distribution is random so that it is possible to have a mouse strain with the combination Ly1.2, Ly2.1, Ly3.1, etc.

4. Antibody Responses to Thymus-Independent and Thymus-Dependent Antigens

4.1. T-Independent Responses

Antibody production and secretion is the exclusive function of B cells (especially their most differentiated form, the plasma cells). No other cells have ever been found to engage in this program. Even though T cells and macrophages possess receptors for the binding of antigenic determinants, they lack the machinery for producing immunoglobulin molecules. In fact, the presence of surface immunoglobulin (other than passively bound to membrane receptors) and the synthesis of cytoplasmic immunoglobulin provide the hallmarks of cells of the B lineage. Synthesis and secretion of immunoglobulin requires activation. The activating signals can be nonspecific or specific, and the latter may be thymus-independent (TI) or thymus-dependent (TD). Nonspecific activation, also termed polyclonal, can be achieved with B cell mitogens such as lipopolysaccharide (LPS). This type of activating process is said to be T-independent although this independence is not an absolute freedom from T cell influence as evidenced by recent reports (Ness et al., 1976; Goodman and Weigle, 1979, 1980; Jacobs, 1979). The fact that certain mitogenic substances can directly provoke a response in B cells has led Möller and Coutinho to postulate that TI antigens, in general, activate B cells to antibody production through the mitogenic event (Coutinho and Möller, 1975). According to this theory the directive to the B cell does not stem from action of the specific antigenic determinant that binds to the receptor (surface Ig), but rather emanates from a mitogenic configuration of the antigen. The antigen binding site is viewed simply as a means of focusing the antigen on the B cell surface and thus selecting for mitogenic induction and clonal expansion of those cells that possess a specific binding site for a given antigenic determinant. This theory allows for the existence of two different sites even though it presupposes the need for only one signal to activate the cell. The one signal notion had some attraction in view of the fact that certain TI antigens, notably polymers of repeating identical subunits, induce specific immune responses that appear to be relatively simple as they are characterized by the dominance of a single antibody class, IgM (Humphrey et al., 1964; Baker and Stashak, 1969; Katz and Benacerraf, 1972) and a lack of immunologic memory (Baker et al., 1971; Miranda, 1972; Klaus and Humphrey, 1974).

4.2. The Heterogeneity of B Cells

These limitations of TI responses are not universal, however, since IgG antibodies have been evoked to dinitrophenyl (DNP)-Ficoll (Sharon et al., 1975). Moreover, a recent report (Schott and Merchant, 1979) indicates that this TI antigen can activate memory B cells. It is intriguing that, in thymus-deprived nude mice, DNP-Ficoll can cause the generation of both Bμ and Bγ memory cells (producing IgM and IgG, respectively), whereas in euthymic mice of the same genetic stock they activate only Bγ memory cells. The memory response is carrier (Ficoll)-specific suggesting that Bγ memory cells can be triggered to provide a helper function upon recognition of the carrier, a function previously thought to be restricted to T cells (see below). Although it is not known whether the B cells that recognize the carrier are also able to recognize the hapten (dual recognition) and although it is quite possible that it is a third B cell that is carrier-specific and provides help, these findings intimate that B memory cells require *two* signals, one from the hapten and the other from the carrier either directly or via another B cell.

The possibility that B cells may provide a helper function not only to other B cells, but to T cells has been reinforced by recent studies with the lymphocyte-promoting factor (LPF) of *B. pertussis* (Ho et al, 1980). LPF is mitogenic for mouse T lymphocytes, but purified T cells are nonresponsive, as also are nylon-adherent B cells. Full responsiveness requires the interaction of T cells with B cells, and the studies performed by Ho et al. (1980) indicate that the B cell acts as a helper in this reaction. Since multiplicity of action implies a heterogeneity among the performing cells, one could conclude, therefore, that B cells represent heterogeneous populations, a conclusion that has been drawn from prior studies on B cells in primary TI and TD responses (Gershon and Kondo, 1970; Playfair and Purves, 1971a,b; Andersson and Blomgren, 1975; Jennings and Rittenberg, 1976; Quintans and Cosenza, 1976; Cambier et al., 1977; Lewis and Goodman, 1977). In fact, the TI response that involves principally B cells cannot be considered as a uniform type of response. Recently TI antigens were subdivided into two categories, TI-1 and TI-2 (Mosier et al., 1977). Trinitrophenyl (TNP)-LPS and TNP *Brucella abortus* belong to TI-1 and are able to induce responses in CBA/N mice that carry an X-linked B cell defect. These mice do not respond to TI-2 antigens that include TNP-Ficoll, TNP-dextran, and pneumococcal polysaccharide (SIII). The distinction in response to the two types of TI antigens has been attributed to

their action on cells in different stages of maturation, the TI-1 stimulating immature B cells (Mond et al., 1978). Direct proof for the existence of separate subsets of B cells, at least for the expression of memory, has recently come forth from the studies of Tittle and colleagues (Tittle and Rittenberg, 1980; Tittle et al., 1980). They describe two subsets of memory cell precursors in spleens of mice primed with the TD antigen, trinitrophenyl keyhole limpet hemocyanin (TNP-KLH): the B1γ that respond *in vitro* to TI-2 antigens, and the B2γ that respond to TD antigens (TNP-KLH). TI-1 antigens could stimulate memory precursors of both subsets. Reinforcing the distinctiveness of B1γ and B2γ precursors is the finding that the former are enhanced in their activity by LPS (nonmitogenic doses) or by allogeneic thymocytes, while the latter are diminished in their activity by the same agents (Tittle et al., 1980).

It should be emphasized that, even though B cells may respond to antigens that are thymus-independent in that they do not discernibly activate TH cells, the responses are regulated by other kinds of T cells, principally amplifier and suppressor cells (Baker et al., 1973; Braley-Mullen, 1976; Markham et al., 1977a,b; Tite et al., 1978; Baker and Prescott, 1979).

4.3. T-Dependent Responses

These responses differ from TI responses in that the antibody produced is predominantly IgG (a switch in Ig class under the control of T cells) and in that they lead to the development of strong immunologic memory. There is less controversy about TD responses requiring two signals. In the simplest version, one signal is provided by the antigen and the other by the helper cell. Proof of this type of arrangement was, in part, established by experiments with nude mice, which are congenitally athymic and which, therefore, lack fully competent T cells. When nude mouse B cells in culture were exposed to antigen, they responded by proliferating, but failed to differentiate into antibody-producing cells. When competent T cells were brought into the culture, the B cells gave rise to antibody-forming cells (Dutton, 1975). Thus, apparently the antigen served simply as a mitogen in the absence of T cells, providing the first signal, and the T cells or their products furnished the second signal required for the induction of antibody production and secretion. The term *signal* connotes a form of communication, a cell-to-cell transmission of information, but the nature of the information and the precise mode of delivery, acceptance, and mechanics of triggering of the response are not fully understood.

The above-cited division of labor is not absolute. The antigen undoubtedly plays a role in cellular differentiation and it is known that T cells can also issue proliferation-stimulating lymphokines (see below) in addition to providing "information" that is needed for efficient antibody synthesis or for the switch in antibody class (Davis and Paul, 1974; Romano and Thorbecke, 1975) or for the refinement of antibody quality, i.e., fine tuning of specificity (Campos-Neto et al., 1978) and affinity (Gershon and Paul, 1971).

4.4. Modes of Help and the Heterogeneity of TH Cells

The nature of physical arrangements of interacting cells remains a puzzle. The classical theory of T/B interaction encompassed TH cells recognizing the carrier portion of an antigen and the B cells recognizing the haptenic portion (Mitchison, 1969a; Rajewsky et al., 1969; Raff, 1970; Mitchison, 1971). Based on the prerequisite that the hapten and carrier be physically linked as a single molecule, one view pictured the interaction in the form of the two types of cells being bridged by the antigen (Mitchson et al., 1970) and thus required an accommodation for close proximity of the two types of cells. An alternative theory viewed the interaction as mediated by soluble products derived from carrier-activated TH cells and providing one signal to B cells (Lachmann and Amos, 1971; Feldmann, 1972; Feldmann, 1973). The other signal would result from the binding of the hapten to this B cell. This theory made it easier to circumvent the problem of distances between cells with appropriate recognition sites for the carrier and hapten determinants trying to find each other. Still, there was a need to explain how a TH cell with specificity for one determinant on an antigen and a B cell with specificity for another determinant on the same antigen would recognize that they were mutually suitable or essential. This dilemma seems to be reaching a partial resolution through new insights gained in several areas. One is new infomation about functions of antigen-presenting cells in conjunction with products of the MHC (see Section 5.4.5). Another bears on the distribution of antigen receptors (idiotypes) among various lymphocytes where similar idiotype structures are shared by T and B cells and their products (Binz and Wigzell, 1977a,b; Cosenza et al., 1977; Krawinkel et al., 1977; Rajewsky and Eichmann, 1977; Ward et al., 1977; Eichmann et al., 1978; Mozes, 1978; Adorini et al., 1979a,b; Germain et al., 1979) (for additional comments see Section 5.5.2.4 an 6.4.2.2). Moreover, there is a widening awareness that soluble mediators that activate B cells (or potentiate their activation) are sometimes produced by T cells

triggered by chance, or nonspecifically by mitogens, irrelevant antigens, or carriers unlinked to the hapten ("bystander" effect or noncognate recognition) (Schimpl and Wecker, 1972; Gorczynski et al., 1973; Armerding and Katz, 1974; Marrack and Kappler, 1975; Hoffeld et al., 1977; Waldmann, 1977).

We have been discussing TH cells as if they constituted a single entity. In point of fact there are indications to the contrary, namely that T cells with promotional or helper capabilities, even though bearing the same Ly1 markers, are heterogeneous, a point that will assume major significance in connection with the assessment of immunomodulatory agents. First of all, one must recognize the separate existence of precursors and mature (functional) TH cells. Second, it appears that different types of TH cells may determine the class (Kishimoto and Ishizaka, 1973) and allotype of antibody (Herzenberg et al., 1976). Third, amplifier cells that possess characteristics different from TH cells and that can act somewhat later in the course of the humoral immune response have been described (Muirhead and Cudkowicz, 1978). Fourth, splenocytes from primed mice have been shown to contain two distinct TH subsets that synergize in providing help to B cells (Janeway et al., 1977; Tada et al., 1978; Janeway, 1979; Lubet and Kettman, 1979; Keller et al., 1980). According to Janeway and colleagues (Janeway et al., 1977; Janeway, 1979), one member of the helper duet recognizes immunoglobulin on the B cell surface. This is of special relevance to the question of shared or complementary idiotypes. In another study (Tada et al., 1978), the two cells, although of the Ly1 phenotype, could be distinguished by several properties: TH1 was nylon wool-nonadherent and helped in a cognate interaction, whereas TH2 was nylon wool-adherent and provided help through a noncognate interaction. In Tada's (Tada et al., 1978) terminology, *cognate* refers to the carrier and hapten being covalently linked; *noncognate* refers to activation of TH by irrelevant carrier. This distinction was clearly demonstrated in experiments where KLH-primed TH1 cells provided help to DNP-primed B cells only when stimulated in culture with DNP-KLH; egg albumin (EA)-primed TH1 cells could help only when stimulated with DNP-EA. The TH1 cells did not respond to unlinked hapten and carrier. The noncognate interaction, on the other hand, was evidenced by EA-primed TH2 cells exerting a significant helper effect when the DNP-primed B cells and EA-primed TH2 cells were stimulated with separate molecules, i.e., DNP-KLH and uncoupled EA. The noncognate help, although nonspecific with respect to DNP-KLH, denotes specificity for the unlinked car-

rier, EA, since the presence of EA in the culture was essential for the stimulation of the B cells and DNP-KLH alone was not sufficient for this purpose.

The concept of synergistic interactions of two different TH cells has been extended by the investigations of Keller et al. (1980). These investigators had previously recognized the coexistence of antigen-specific and nonspecific T/B cell cooperative interactions (Marrack and Kappler, 1975; Hoffeld et al., 1977). In the current studies they describe the characteristics of the two TH cells present in spleens of immunized mice. One provides carrier-specific help and is required for *initiation* of the response to a hapten conjugated to the protein carrier, e.g., TNP-KLH. The other contributes carrier-independent amplification following nonspecific (polyclonal) activation. The second cell cannot act as a surrogate for the first in providing the initial signal. Instead, upon nonspecific activation [in the present work, by concanavalin A (con A)], the cell secretes a helper factor(s) (various factors are discussed in Sections 5.2 and 5.4.4) that furnishes supplemental help: 1. by sustaining B cell proliferation initiated by the specific TH cell in the presence of antigen, and 2. apparently by facilitating B cell differentiation. Thus, the second cell is providing the additional signal for the induction of the immune response. With TNP sheep red blood cells (SRBC) as the antigen, there appears to be no need for the first cell, and *antigen alone is capable of delivering the first signal.* There have been previous reports that B cells could respond to RBC without T cell help by going through at least one round of division (Dutton, 1975; Schimpl et al., 1974; Waldmann et al., 1976). The second signal from the carrier-independent TH cells is required apparently for the vigorous response as is the case with KLH-conjugated antigens. The carrier-specific and the carrier-independent TH cells were shown to differ with respect to expression of Ia antigen on their surfaces: the former being Ia^- and the latter Ia^+. These two cells appear to be analogous to the TH1 and TH2 cells described by Tada et al. (1978). In that study, TH1 with cognate KLH recognition was devoid of Ia antigen and the nonspecifically activated TH2 expressed it. We shall have more to say about these surface markers on T cells in Sections 5.4.2, 5.4.3, 5.4.5.3, 5.5.1, and 5.5.2. The findings that a strong TD response to an antigen on a cell membrane may occur with the help of a single type of TH cell, whereas a TD response to a protein carrier requires sequential help from two seemingly different TH cells, are significant on two accounts. They illustrate how the structure of an antigen determines the requirements for

launching of an immune response, a subject that will be discussed further in Section 5.3. They also provide a unique frame of reference for the analysis of selective effects of immunosuppressive agents.

Data on selective action of immunoregulatory agents are presented in Chapter 4. In the context of the foregoing discussion, two examples of selective action against different types of cells providing help are warranted. Lubet and Kettman (1979) demonstrated a differential susceptibility to radiation on the part of cells contributing specific and nonspecific help to B cells. In the studies of Muirhead and Cudkowicz (1978), amplifier T cells (TA) and TH cells could be distinguished by the effect of vinblastine, which inhibited the amplifying activity but did not alter the helper function. It should be noted that the two cells described by Muirhead and Cudkowicz (1978) resemble functionally the two types of helper cells in the report of Keller et al. (1980) in that one cell initiated B cell proliferation and the other amplified the B clonal expansion. However, there appeared to be differences in cells providing the secondary help. In contrast with the nonspecific TH cells of Keller et al. (1980), TA cells were reactive to the same carrier (RBC) as the TH cells and were only required in the primary but not the secondary response.

Before leaving this section it is pertinent to note that help-generating $Ly1^+$ cell pairs have been described where one of the partners is principally programmed for induction of T suppressor cells (see Section 5.5.1). The participation of two different T cells in an act of facilitation, when one can also launch a diametrically opposed process of interference, probably creates a fulcrum for the physiological equilibrium characterizing the functions of immune responses. Some of the intricacies of positive and negative regulation will be discussed in the next section and will provide the background for analysis of the effects of immunomodulating agents.

5. Inductive and Regulatory Functions: Antigens, Cells, Cognitive and Regulating Molecules, and Networks

5.1. Recapitulation and Projections

The fundamentals of the humoral immune response appear to be fairly straightforward. There is *recognition* of an antigenic determinant by a B lymphocyte bearing the appropriate receptor (specific combining site). This is followed by *proliferation* of this lymphocyte

(clonal expansion) and *differentiation* of the proliferating cells toward antibody *production* and *secretion* (B cells become transformed to plasma cells). This apparent simplicity is deceptive until one begins to examine more closely the operational details and their cellular and molecular underpinnings. The beginnings of complexity came into view by the entry of TH and TA cells as cited above. A solo performance may be a simple act but a duet, trio, or quartet need coordination. The complexity is further compounded by the requirement for another kind of cell, the macrophage. If each member of the combo were to play a constant role and make an invariant input there would be a predictable outcome and no surprises. As it happens, the roles may be modulated, reinforced, or subverted and the net result of interactions of immunologically relevant cells will range from strong responses to absent responses or even negative responses, i.e., evocation of cellular subsets that militate against *de novo* responses. Although there are gradations of intensity in a given response, there is also a discontinuity in the repertoire of responses. Thus, it is possible to obtain a good primary response with or without immunologic memory, and it is also possible to prime the system for memory without manifesting primary responses. Moreover, chemical agents, physical forces, and certain antigenic configurations can prevent, abort, or modify one response without affecting another. These variations arise from several sources. Although programmed to perform a major function, each cell class can be instructed to perform additional functions. For example, although the principal function of B cells is antibody synthesis, these cells can also be stimulated to produce lymphokines. Each class consists of heterogeneous populations composed of subsets of cells able to exert diverse effects. For example, certain subsets of T cells may provide help, others may exert suppression, still others may perpetuate immunologic memory. The end result depends on what subset and which functions are dominant at a given point. The same seems to apply to macrophage-like cells as some of these cells facilitate and others inhibit immune responses.

Negative regulation is an essential aspect of the immune response and, like positive regulation, requires the participation of cells either programmed to perform suppressive functions or receptive to instruction to exert these functions. Positive and negative regulations depend on communications between the various cellular participants. Recent developments indicate that there exist multiple systems of communication in which intercellular mediation is accomplished by molecules that are expressed on cell sur-

faces or are secreted. For some of these molecules, receptors have been demonstrated; for some others, receptors are assumed to exist. These communicational mediators may act directly or indirectly by first binding to an intermediary cell that transmits, transforms, or translates the signal of the primary molecule to a secondary signal for delivery to the ultimate target cell. Although current attention is focused on products under control of the MHC (see Sections 5.4.1 and 5.4.2) for years there has been an accumulation of reports about various kinds of soluble mediators produced by lymphocytes (lymphokines) and by macrophages (monokines) that exert positive or negative effects on cells of the immune system. Waksman (1979) has cataloged these substances and his list runs for three pages; an abbreviated sampling is given in Table 2. These are classified according to their function, origin, and, wherever possible, restriction by the MHC. Only a few have been purified and even fewer have been characterized physicochemically. We should like to consider a monokine and a T cell-derived lymphokine at this point, since they have been shown to cause significant polyclonal promotional effects on T cell proliferation and function. A third extensively studied soluble mediator, interferon, will be discussed in Section 5.5.2.3. Recently published information on lymphokines and monokines is included in Cohen et al., 1979; Friedman, 1979; Kaplan, 1979; and Hadden and Stewart, 1981.

5.2. LAF and TCGF Promote T Cell Proliferation and Function

One monokine that has been well-characterized is the lymphocyte activating factor (LAF), named *interleukin I* in the nomenclature recently proposed by a group of investigators (Aarden et al., 1979). This substance has been demonstrated in culture fluids of LPS or phytohemagglutinin (PHA)-stimulated human leukocytes and LPS-activated mouse peritoneal exudate cells (Gery et al., 1972; Gery and Waksman, 1972; Gery and Handschumacher, 1974). LAF activity has been associated with a peptide with a molecular weight of 10,000–20,000 daltons, as determined by Sephadex G-100 chromatography (Calderon et al., 1975; Blyden and Handschumacher, 1977; Koopman et al., 1978; Togawa et al., 1979). However, some activity has also been detected in fractions of higher mw (50,000–100,000 daltons) (Wood and Gaul, 1974; Blyden and Handschumacher, 1977; Katz et al., 1978; Togawa et al., 1979). This discrepancy may reflect, at least in part, a heterogeneity of cells producing LAF, as in pure macrophage cultures, P388D1, LAF with a mw of 16,000

Table 2
Soluble Mediators of Immune Responses[a]

Mediators	Monokine (MK) or Lymphokine (LK)
Specific Mediators, Restricted by MHC	
Helper factors	
GRF (genetically restricted factor)	MK
Helper factor for IgE	LK
Antigen-specific T cell factor	LK
Antigen-specific TRF	LK
TF (transfer factor)	LK
Suppressor factors	
Antigen-specific suppressive T cell factor	LK
Allotype suppression factor	LK
Immunosuppressive factor (GAT)	LK
Nonspecific Mediators, Restricted by MHC	
Helper factors	
AEF (allogeneic effect factor)	LK
Complement components	MK, other
Suppressor factors	
MLR suppressor factor	LK
Complement components	MK, other
Nonspecific Mediators, Unrestricted by MHC	
Helper factors	
LAF (lymphocyte-activating factor)	MK
BAF (B cell-activating factor)	MK
MF [mitogenic (blastogenic) factor]	LK
Suppressor factors	
Inhibitor(s) of DNA synthesis	MK
IDS (inhibitor of DNA synthesis)	LK
LIF (lymphoblastogenesis inhibition factor)	LK
FIF (feedback inhibition factor)	LK
MIFIF (MIF inhibition factor)	LK
SIRS (soluble immune response suppressor)	LK
Miscellaneous Factors	
MIF (migration inhibitory factor)	LK, other after viral infection
OAF (osteoclast-activating factor)	LK
LT (lymphotoxin)	LK
PIF (proliferation inhibitory factor)	LK

[a]From Waksman (1979).

daltons was obtained following stimulation with a variety of agents (Mizel et al., 1978a,b,c). LAF acts on T cells and, in vitro, augments the helper effect in antibody production as well as in T cell-mediated cytotoxicity (Farrar et al., 1979; Mizel, 1979). LAF is apparently able to provide a second signal to TD responses.

T cells also produce a proliferation stimulating factor, variously designated as thymocyte mitogenic factor (TMF), T cell growth factor (TCGF), and killer cell helper factor (KHF). This factor has recently been renamed *interleukin 2* (Aarden et al., 1979). Studies in the laboratories of Farrar and of Smith have brought forth information that provides a basis for explaining the modes of action of LAF and TCGF (Farrar and Koopman, 1979; Farrar et al., 1979; Smith et al., 1979a,b; Farrar and Fuller-Bonar, 1980; Farrar et al., 1980a,b; Smith, 1980a,b,c). Biochemical characterization of rat TCGF indicated that it had a molecular size of 15,000 daltons, as determined by gel filtration and polyacrylamide gel electrophoresis (Smith et al., 1980a). A similar size had been reported for human TCGF (Gillis et al., 1980), but a larger size, 30,000 daltons, was found in chromatographic determinations of mouse TCGF (Farrar et al., 1978; Shaw et al., 1978; Farrar et al., 1979; Watson et al., 1979).

One major distinction between LAF and TCGF is that the former is produced by macrophages whereas the latter is the product of T cells. However, macrophages are required for the production of TCGF and this requirement can be replaced by LAF. Although LAF and TCGF have been found to promote T cell helper and cytotoxic functions, the current view is that it is TCGF that enhances T cell activity, including proliferation, and LAF is required to render the T cell effective as the producer of TCGF. It therefore appears that a mitogen or an alloantigen sets off the chain of events by activating macrophages either directly or indirectly by first activating lymphocytes to release a lymphokine that stimulates the macrophages. These macrophages produce LAF which, in turn (and in the presence of antigen or mitogen), induces T cells to produce TCGF that is then available to enhance antibody production or lymphocyte cytotoxic reactions. Producers and acceptors of TCGF belong to different T subsets (Wagner and Rollinghoff, 1978; Smith et al., 1980a,b); the producer is an $Ly1^+$ cell (Wagner and Rollinghoff, 1978; Paetkau, 1980) and the targets of TCGF appear to be precursors of TS, TH, and possibly TS effector cells (Wagner and Rollinghoff 1978; Wagner et al., 1979; Watson, 1979; Watson et al., 1979a; Smith, 1980a,b). However, the targets have to be activated by antigen or mitogen before they can bind TCGF. According to Farrar and Fuller-Bonar (1980), one of the target T cells can also be stimu-

lated to produce interferon. Smith and associates (Baker et al., 1978, 1979; Smith, 1980c) have established permanent cytolytic T cell lines specific for a particular alloantigen. Continuous lines of T helper lymphocytes specific for sheep or horse RBC have been developed by Watson (1979). These have been sustained by the addition of TCGF in the absence of additional mitogen or antigen. It is this property of TCGF that separates it from LAF, which apparently cannot sustain cell culture lines. Long-term cultured and cloned specific TH cells have reconstituted nude mice in an antigen-specific manner (Tees and Schreier, 1980). Lymphokines and monokines are often thought to provide the second signal toward the activation or function of lymphocytes. In this instance TCGF may provide the third signal, with the first being the antigen or mitogen and the second being LAF.

These biochemical and functional studies have therefore made possible an understanding of sequential events caused by two separate soluble mediators, one of macrophage origin and the other of T cell origin. The functional linkage of these molecules envisioned by independent groups, Farrar and his associates (Farrar et al., 1979; Farrar and Fuller-Bonar, 1980; Farrar et al., 1980b), Smith and his colleagues (Smith et al., 1979b; Smith, 1980a,b; Smith et al., 1980b); and Oppenheim et al. (1980), provides a rational basis for explaining sequential interactions between macrophages and T cells that result in clonal expansion of the latter. The establishment of permanent antigen-specific cytolytic and helper T cell lines proves that, even though the proliferation stimulating molecules act in a polyclonal fashion, a single clone can be recovered and perpetuated with TCGF alone. The recognition that only T cells accept and respond to TCGF narrows the cellular target system and permits the inference that this molecule would act early in the induction of an immune response. These findings are therefore providing a basis for the analysis of mechanisms of action of immunomodulatory agents that exert their effects in early stages of immunization. One such agent is glucocorticosteroid which inhibits the production of TCGF (Gillis et al., 1979a,b). We shall return to this issue.

5.3. The Role of Antigens

Antigens provide the initial impulse and the driving force for virtually all specific immune responses. The impulse perturbs the cell membrane and triggers changes in its chemical and physical state. One of the most dramatic changes is the movement of antigen receptors manifested by their ultimate congregation, usually at one

pole of the cell (capping) (Taylor et al., 1971). The stimulating effect of the antigen depends on its chemical nature and physical state. We have already alluded to the importance of the carrier portion of the antigen in determining the nature of the immune response to a hapten bound to that carrier. Thus, TNP coupled to LPS acts as a TI-1 antigen; TNP conjugated to Ficoll is a TI-2 antigen; TNP bonded to KLH serves as a TD immunogen (Mosier et al., 1977; Mond et al., 1978; Tittle and Rittenberg, 1980; Tittle et al., 1980). What is particularly intriguing is that one region of a molecule can evoke a positive response, while another region on the same molecule can induce a negative response by activating TS cells. This was clearly demonstrated by the elegant experiments of Sercarz and his colleagues in studies with *E. coli* beta-galactosidase (GZ) (Eardley and Sercarz, 1976; Sercarz et al., 1978). Immunization of CBA/J mice with GZ in Freund's complete adjuvant induces a primary IgM antihapten response. Regulatory cells from these animals demonstrate successive waves of help and suppression. Suppression is most notable from about 5–10 days after priming and this interval is "sandwiched" between early and late helper periods in which an enhanced antihapten response prevails. In subsequent work, these investigators used cyanogen bromide-derived peptides isolated from GZ, representing the N terminus (CB-2), the middle region, and the C terminus (CB-C) of the molecule. CB-2, a peptide of 90 residues (amino acids 3–92) was the only one of the three peptides that caused suppression. Under the conditions examined, none of the peptide seemed to induce appreciable help.

Equally exciting was the discovery by Rosenthal and his colleagues (Barcinski and Rosenthal, 1977; Rosenthal et al., 1977) that the unique recognition of separate regions on a molecule of insulin is under the control of Ir genes (see Section 5.4.2). The study took advantage of a prior observation that different strains of guinea pigs responded preferentially to separate parts of the molecule (Arquilla et al., 1967), and the knowledge that pig, beef, and sheep insulins possess identical B chains and almost identical A chains except for amino acid residues 8, 9, and 10 in the loop of the A chain (Blundell et al., 1972). Strain 13 guinea pigs recognize insulin through a B chain determinant that is not recognized by strain 2 guinea pigs. Strain 2 guinea pigs, instead, respond well to the loop area of the A chain. The responses measured in terms of specificity of blastogenic transformation reactions to the appropriate antigen and the activation of TH cells were shown to be under the control of Ir genes (Barcinski and Rosenthal, 1977; Rosenthal et al., 1977). Interestingly, the genetic control was operational in T cells and macrophages, but not in B cells.

An identical genetic restriction was recently reported for the induction of suppression in guinea pigs using either whole insulin or the B chain in incomplete Freund's adjuvant (IFA) (Baskin et al., 1980). The B chain induced suppression only in strain 13 guinea pigs, while suppression owing to the A chain loop was manifested only in strain 2 guinea pigs (in strain 2, suppression could only be observed when the regimen consisted of pork insulin in IFA and the restimulation was with homologous pork insulin, but not with heterologous sheep insulin, which does not cross-react with pork insulin at the level of the A chain loop).

Additional insight into the association of genetic control and antigenic structure has come from the experiments of Sercarz and colleagues (Adorini et al., 1979a and b; Araneo et al., 1979) using hen's egg-white lysozyme (HEL) as the antigen. The molecule, consisting of 129 amino acids, is nonimmunogenic in C57BL/10Sn (B10.A) mice. A closely related lysozyme of the ringed-neck pheasant egg-white (REL) is immunogenic in both strains. Two peptide fragments have been identified that are relevant in the context of responsiveness vs nonresponsiveness: fragment N-C (residues 1–17:cys 6-cys 127:120–129), and the mixed disulfide peptide L_{II} (amino acids 13–105). Both peptides induce helper cells in B10.A mice, whereas in B10 mice, the N-C fragment activates suppressor cells and the L_{II} activates helper cells. The distinctive activity of N-C appears to be related to a single amino acid substitution in position 3, phenylalanine in HEL and tyrosine in REL. Thus, a change in one amino acid of a small peptide can alter its signal (presumably in combination with a product of an immunoregulatory gene), leading to suppression in one strain of animals and to helper activity in another. The suppressor cells induced by the N-C portion of the molecule render the B10 strain nonresponsive to the remaining immunogenic portion (L_{II}) of the same molecule. This is a most intriguing discovery (reminiscent of the findings with GZ, where a suppressor-inducing epitope within a restricted region of the antigenic molecule (CB-2) could suppress the response to the entire molecule (Sercarz et al., 1978), which the authors discuss in the light of several concepts of immune inductive and regulatory processes. They believe that their results can be explained by the phenomenon of antigen bridging, i.e., in B10 mice, N-C specific receptors on TS cells bind the native HEL molecule and these cells "switch off" TH cells that bind to and would otherwise be "switched on" by the L_{II} peptide. There is no idiotypic similarity or complementarity between N-C specific TS receptors in B10 mice and L_{II}-specific TH receptors. These receptors bear dissimilar idiotypes. However, these investigators have recently found a common

set of idiotypic determinants on HEL-specific suppressor cells and the large majority of anti-HEL antibodies, most of which have specificity for the N-C region (Harvey et al., 1979).

The antigen concentration is also relevant to the type of response that is elicited. As a concrete example, immunization with SRBC can lead to: (1) an efficient primary response: (2) an efficient secondary response in the absence of a primary response; or (3) immune suppression affecting either the primary and/or the secondary antibody response. As few as 10^8–10^9 cells stimulate a powerful primary response. Immunization with 5×10^6 cells fails to induce a detectable primary response, but sensitizes that host for maximal secondary response, in other words, activates memory cells for the anamnestic response (Playfair and Purves, 1971b). The injection of 4×10^9 cells leads mainly to induction of suppressor cells (Whisler and Stobo, 1976) (see Section 6.3 for additional discussion of the effects of antigen dose and route of injection).

These findings are important unto themselves because they demonstrate the regulatory contributions by the structure of antigen and antigen dose. They assume added importance at the practical level in the context of the action of immunomodulating agents. Since some of these agents will be shown to affect various cellular subsets in a differential manner (especially cells involved in helper and suppressor functions), it is possible that one would obtain entirely different end results depending on what antigenic determinant was activating the given cell set at a particular time in a given strain of animals. These observations must therefore serve as a caveat for investigators studying the effects of immunomodulating agents. They also emphasize the need for further research on what constitutes immunogenicity vs induction of nonresponsivenesss within the molecular structure of the antigen. Since antigen is usually processed prior to presentation to T cells, the manner of antigen handling, modification, and presentation also determines the nature of the immune response. In addition to dose, structure, and configuration, the mode of antigen administration is important in this context. These aspects will be discussed below (see Sections 5.4.5, 6.3, and 6.4.3).

5.4. Genes Regulating Immune Responses, Macrophages, and Antigen Presentation

5.4.1. Genes in K and D Regions Dictate T Cytotoxic Activities The MHC of the mouse located on chromosome 17 is bor-

dered on the left by the *K* region and on the right by the *D* region. Some products of these two regions are expressed as alloantigens on many kinds of cells, and others are expressed in the form of complementary structures, i.e., alloantigen receptors. This arrangement seems to establish a basis for cell-mediated reactions, e.g., allograft rejection, and for cytotoxicity in vitro. One type of cytotoxicity occurs when target cells with membranes modified by viral antigens or by chemicals (haptens) are confronted with T cells from animals sensitized to these nominal antigens. This type of T cell-mediated cytotoxicity is *K* or *D* region restricted in that the cytotoxic cell and the target must share determinants specified by either the *K* or *D* region. As conceptualized by Zinkernagel and Doherty (1975), and Shearer et al. (1975), the cytotoxic lymphocytes have to see two structures on the surface of the target cell. One is the nominal antigen, e.g., vaccinia virus; the other is the self-antigen specified by a gene in the *K* or *D* region. What follows is a two-pronged attack triggered by the recognition of "self" by the complementary *K* or *D* specified receptor and of the nominal antigen (vaccinia virus) by the second receptor, which in this example is specific for vaccinia virus. Different versions of this model have considered one large receptor capable of accommodating alloantigen complexed with nominal antigen ("altered self") in contrast with two separate receptors.

5.4.2. Ir Genes Specify Determinants Controlling Immune Induction The *I* region, situated between the *K* and *D* ends of the MHC of the mouse (McDevitt et al., 1972), is subdivided into subregions designated *A, B, J, E,* and *C*. The genes in these subregions (Ir genes) (studied in congenic mice) are primarily concerned with the regulation of immune responses, their role first being recognized in studies that demonstrated that TD immune responses to synthetic amino acid copolymers required the presence of responder alleles in these loci (McDevitt and Sela, 1965; Benacerraf and McDevitt, 1972; McDevitt et al., 1972). In the humoral response, these genes regulate the magnitude, class, and specificity of antibody. Ir genes also appear to be involved in regulating lymphocyte proliferation, helper and suppressor functions, and delayed-type hypersensitivity (DTH) reactions (reviewed or fully detailed in Katz and Benacerraf, 1976; Benacerraf et al., 1977; Katz, 1977; Sercarz et al., 1977; Benacerraf and Germain, 1978). Products of *I* subregions collectively termed Ia can be neutralized by specific anti-Ia sera that also inhibit certain immunologic functions, thus permitting an association between certain Ia antigens

and functions of the *I* subregions. I-a antigens are expressed on B cells and macrophages and, to a more limited extent, on T cells except for the product of the *I-J* subregion, which is found abundantly on TS cells (Murphy et al., 1976) (see Section 5.5.1 regarding Ia among T subsets). Some helper effects require the complementation of *I-A* and *I-E/C* with Ia molecules from each subregion being expressed on the antigen-presenting cell (Dorf et al., 1977; R. H. Schwartz et al., 1977; Cowing et al., 1978a; Schwartz et al., 1979). *I* region products have also been demonstrated in soluble mediators: the product of *I-A* in helper factors (HF) (Taussig et al., 1975; Tada, 1977) and the product of *I-J* in suppressor factors (SF) (Tada et al., 1976; Greene et al., 1977; Theze et al., 1977; Benacerraf and Germain, 1978, 1979; Germain and Benacerraf, 1980). Some of the factors are specific for the antigen used for the stimulation of T cells and there is evidence that they contain idiotypic structures identical or cross-reactive with those present on antibodies (see below).

Ir genes have been shown to regulate the responses to certain synthetic copolymers (Levine et al., 1963; Levine and Benacerraf, 1965; McDevitt and Sela, 1965; Campos-Neto et al., 1978; Schwartz et al., 1979) and protein antigens (Melchers and Rajewsky, 1978; Schwartz et al., 1978a,b). As discussed in Section 5.3, immunoregulatory genes can even determine which subregion of an antigenic molecule will be recognized by T cells (Barcinski and Rosenthal, 1977; Rosenthal et al., 1977; Sercarz et al., 1978; Baskin et al., 1980). Moreover, it appears that the decision whether TH or TS cells are to be activated by a given antigen is made by immune regulatory genes (Benacerraf and Germain, 1978, 1979).

5.4.3. Ir Gene Products Do Not Act Alone and Do Not Play an Exclusive Role in the Initiation or Maintenance of Immune Responses

All of the above notwithstanding, immune responses are not inexorably or exclusively MHC-restricted since restrictions can be bypassed by modifying the antigen, e.g., coupling a nonimmunogenic copolymer to a protein carrier (McDevitt and Benacerraf, 1969). Furthermore, the intensity of the immune response may be altered by various tactics and maneuvers: excess antigen or bypassing the antigen-presenting cells may switch a good response to a poor response; adjuvants (nonspecific activators) can enhance an otherwise weak response; antigen that is immunogenic by the subcutaneous (sc) route is often tolerogenic by the iv route; and lower responsiveness owing to suppressor cells can be overcome by treatment with chemical or physical agents that select-

ively eliminate these cells. Some antigens may initiate an immune response (provide the first signal) independently of specific TH_1 cells (see Section 4.4). As already discussed, help may be acquired through nonspecific stimulation of T cells (Tada et al., 1978; Lubet and Kettman, 1979; Keller et al., 1980) and the polyclonal or bystander antigen-related activation may be under the control of genes other than those regulating specific TH cells. It seems incongruous that the TH cells responding specifically to the cognate carrier are Ia^- whereas the TH cells providing nonspecific help are Ia^+ (Tada et al., 1978; Keller et al., 1980). This apparent incongruity may reflect the mode of antigen delivery where macrophage-derived Ia products complexed with antigen fragments are delivered to T cells. Theoretically, a T cell would have to be equipped with the proper receptor to receive this complex and need not express the Ia antigen (see Section 5.4.5.3).

The structure and disposition of the receptor is not known, but it is assumed to consist of molecules specified by the genes linked to the antibody variable region as well as genes of the MHC, since T cells and their products manifest idiotypes in common with B cells or antibodies as well as products of the *I* region (Cosenza et al., 1977; Julius et al., 1977; Krawinkel et al., 1977; Rajewsky and Eichmann, 1977; Ward et al., 1977; Eichmann et al., 1978; Mozes, 1978; Adorini et al., 1979b; Germain et al., 1979; Germain and Benacerraf, 1980). The receptor on T cells might be a single composite combining site able to accommodate antigen/Ia complexes presented by the macrophages or the receptor could be a functional cluster of separate binding sites, some specified by the MHC and others by VH-linked genes.

Indirect evidence for the presence of idiotypic determinants on T cells comes from the many experiments that document that anti-idiotype antibody can activate precursors to differentiate into functional TH cells (Eichmann et al., 1978), TS cells (Eichman, 1975; Eichmann et al., 1978; Sy et al., 1979c) or TDH (cells mediating delayed-type hypersensitivity; Julius et al., 1977; and Sy et al., 1980a). Thus anti-idiotype can stimulate the action of antigen by activating various precursors of T effector cells in the absence of antigen. There is at least one hypothesis (Schrader, 1979) that argues that the T cell receptors are constructed entirely of two products of Ig linked genes, one being the antigen-specific idiotype and the other an anti-Ia idiotype (hence the *I* region here would make only an indirect contribution rather than create part of the receptor). This controversial contention represents one aspect for future studies on the nature of the T cell receptor that will require the use

of monoclonal antibody in order to clearly discern the various elements present on cell surface structures and interactive molecules. We shall return to these questions in Section 6.4.2.3. One thing that we shall be addressing is that there exist subsets of T cells, for example some TDH cells, that apparently lack idiotypic structures (Sy et al., 1980a).

Whatever the nature of interaction of idiotypic structures with molecules specified by the MHC, the fact that these two channels of communication exist on some T cells calls to mind several analogies, for example, that between the two functions of separate regions of the immunoglobulin molecule where antigen recognition is performed by the hypervariable region (the idiotype) and biologic function, i.e., binding of complement, through the Fc portion. It has been proposed that the product of the MHC may do for the idiotype on T cells or in their soluble factors what Fc does for the idiotype on the antibody molecule (Rajewsky and Eichmann, 1977; Germain et al., 1979). Dual recognition by TH or TS cells of an antigen and of Ia molecules presented by macrophages also seems to resemble the requirement for dual recognition of nominal antigen and a product of the *K/D* regions in the evocation of the cytotoxic reactions against virally or chemically modified self-antigens in cytotoxic reactions. In both instances these dual recognitions attest to the existence of multiple signals (at least two, possibly more) for activation of a given subset of cells for the exertion of a particular function. Thus, control of immune responsiveness is in part outside of the *I* region and the response would depend on the balance between the regulations imposed by Ir genes and VH-linked genes. Other genes outside the MHC have been shown to regulate immune responses. For example, Munro et al. (1978) obtained data indicating that of the two genes complementing for the specification of the receptor for a T helper factor in the response to a synthetic antigen, one gene is located inside and the other outside the MHC. In their studies on the response to lysozymes, Kipp et al. (1979) demonstrated a function for a non-MHC gene regulating late and secondary antibody responses. It is interesting that the primary PFC response is exempt from control by this gene. There are other examples of non-H-2 linked gene regulation of levels of antibody responses (Dorf et al., 1974; Maurer and Merryman, 1974; Pisetsky et al., 1978). Non-H-2 genes may also be important in controlling the efficiency of cell traffic (Suzuki et al., 1979).

The existence of multiple centers of genetic control, coupled with the uneven distribution of products of these genes expressed on different classes and subsets of lymphoreticular cells (Ia mole-

cules being expressed more frequently on macrophages than on specific TH cells), provides a basis for alteration of immune responsiveness (either reinforcement or attenuation). Thus, random mutations may cause variable effects depending on whether they affect primarily the macrophage delivery system or the T cell receptor system. Likewise, endogenous factors such as LAF, TCGF, other hormones and mediators, as well as exogenous substances such as immunoregulatory drugs, may cause different effects depending on which type of cell in the communicational system is being affected in a positive or negative manner.

Some deviations from stringent Ir gene control are conditional since specifically or nonspecifically induced suppressor cells, which occur in genetically responsive animals, may obscure the presence of activated TH cells and, in the converse, nonspecifically induced TH cells may supersede TS cells in nonresponsive animals. What must be kept firmly in mind is that some discrepancies surrounding the question of Ia antigens on T cells may reflect variations in methods of analysis. For example, as disclosed by the experiments of Tada et al. (1978), the I-a^+ TH2 cell, although expressing the $Ly1^+$ phenotype, differed from the TH1 Ia^- $Ly1^+$ cell by being nylon wool-adherent. Thus, the presence of this cell or an appreciation of its role may have been missed in experiments employing purification by nylon wool filtration. One additional point deserves mention and that is, according to Lee and Paraskevas (1979), that T cells may acquire I-a molecules released by macrophages. In their experiments, Ia antigens were detected on 10–20% of T cells after nylon wool filtration. After 4 h in culture, the T cells became I-a^-, but the addition of a macrophage supernatant caused the reappearance of Ia in about 7–10% of T cells.

5.4.4. Problematic Areas Underlying THF and TSF

Soluble factors present additional complexities and invite wider controversies (Kapp et al., 1978; Rich et al., 1978; Cohen et al., 1979; Friedman, 1979; Kaplan, 1979). This is not surprising since the factors are either extracted from cells or are harvested from cell culture supernatants and therefore contain an assortment of cellular constituents: cytoplasmic and membrane structures, enzymes, and a variety of lymphokines and monokines, some of which are specified by genes outside the MHC. It would not be at all surprising to find that a given supernatant contained toxic and nontoxic substances and that the latter consisted of components with variable modes of action, i.e., stimulating or inhibiting proliferative or biosynthetic processes of lymphocyte subsets. Ambiguities with re-

spect to products of the MHC remain because only certain Ia antigens have been characterized (Cullen et al., 1976; Jones, 1977; Cook et al., 1978; Delovitch and McDevitt, 1975). Adding to the problem is the multiplicity of factors with similar functions, e.g., antigen-specific and *I* region related THF vs polyclonal helper or growth factors, TCGF, or diverse suppressor factors that are encountered in the same supernatant. Some are antigen-specific, others are not. In several instances, antigen-specific TSF have been shown to contain products of the *I-J* subregion (Tada and Taniguchi, 1976; Tada, 1977; Theze et al., 1977; Waltenbaugh et al., 1977; Benacerraf and Germain, 1978, 1979). In Section 5.5.1, we shall discuss several aspects of immune suppression, including models encompassing products of *I-J* genes. For the present, suffice it to say that certain nonspecifically induced TSF, for example SIRS (Pierce et al., 1979), lack Ia structures while others apparently contain them (Teodorczyk-Injeyan et al., 1980) and there are also specifically induced TSF that lack the Ia molecule specified by the *I-J* genes. In fact, a different Ia (controlled by the *I-C* subregion) has been found in an SF generated by mixed leukocyte reactions (Rich and David, 1979; Rich et al., 1979). The question of MHC restriction has been clouded by observations that even those factors that are produced under the control of the *I* region may lack *I* region restriction in their function (Kapp, 1978; Pierce et al., 1979; Germain and Benacerraf, 1980). It should be kept in mind that suppressor factors produced by TS cells may act on different cellular targets, subsets of T cells, B lymphocytes, or macrophages. For example, the suppressive substance(s) of Tada (model C, Section 5.5.1) *extracted* from TS is $I\text{-}J^-$ and acts on nylon wool-adherent $Ly1^+23^+$ T cells, whereas the suppressive factor of Kontiainen and Feldmann (1978) *secreted* by TS cells is $I\text{-}J^-$ and acts on nylon wool-nonadherent $Ly1^+$ TH cells (see also Section 5.5.1).

Nonspecific induction of helper or suppressor factors may be regulated by genes outside the MHC; yet Ia structures are apparently involved in certain aspects of nonspecific activation, e.g., by con A, toward helper function (Swain and Dutton, 1980) or toward suppression (Teodorczyk-Injeyan et al., 1980).

Despite this welter of diverse constituents of T cell products, there is substantial evidence that antigen-binding structures (idiotypes) and determinants encoded by the MHC are present in THF and TSF, and that they may influence T/T and T/B interactions (Taussig and Munro, 1974; Munro et al., 1974; Munro and Taussig, 1975; Taussig et al., 1975; Mozes, 1976; Taniguchi et al., 1976; Isac et

al., 1977; Tada, 1977; Mozes and Haimovich, 1979; Munro et al., 1978; Germain and Benacerraf, 1980).

Since cell functions are guided by the interactions of molecules on cellular surfaces and in their environment, we have given considerable attention to this area and to related problems discussed below in order to set the stage for the development of subsequent analyses of effects caused by immunosuppressive agents.

5.4.5. Macrophages (Accessory Cells) Play Key Integrative Roles in Cellular and Molecular Interactions

The existence of a multitude of signals that may emanate from the structures on the cell's surface, from the THF and TSF, and from the array of other lymphokines and monokines is mindboggling to the investigator and would probably place the cell in a state of exhaustion if not confusion (the mixed signal syndrome). As with other energizing circuits, one would suspect that an orderly function of an immunologic circuit or even a linear movement of signal-generating molecules would require some form of integration. One would therefore want to look for the equivalent of a capacitor. It appears that the macrophage or a macrophage-like accessory cell may serve this type of function.

During the past 50 years the esteem for the role of macrophages in the immune system has switched from high to low and back to high again. Although macrophages were once thought to play the key role in immune responses as cells actually involved in antibody synthesis, they lost this status with the recognition of lymphocytes and plasma cells as the actual producers of antibodies. For a while macrophages were relegated to an inferior position in the hierarchy of immunologically relevant cells since their main function was believed to be limited to degradation and disposal of antigen. The new ascendency of the macrophage to a more prestigious role was in large measure a result of the experiments of Fishman and Adler (Fishman, 1961; Fishman and Adler, 1963a,b; Adler et al., 1968). These investigators demonstrated that under certain conditions the processing of an antigen by macrophages was a prerequisite of immunogenicity. They inferred from their data that two immunologic principles were being released from the macrophages, RNA and antigenic fragments bound to RNA. The function of immune RNA remains not entirely clear, but it has excited the imagination of many investigators and has been pursued along several lines of research in basic immunology and immunotherapy. A separate line of investigations has emanated from the discovery

that weak antigens could be converted to strong antigens by the expedient of binding to macrophages. For example, macrophage-bound albumins were 1000- to 10,000-fold more immunogenic than soluble albumins (Mitchison, 1969b; Spitznagel and Allison, 1970; Schmidtke and Unanue, 1971). These experiments were performed in vivo, transferring antigen bound to mouse macrophages to recipient mice. The increased immunogenicity of macrophage-bound antigen has also been demonstrated in vitro (Katz and Unanue, 1973).

5.4.5.1. Macrophages regulate T cell responses under Ir gene control. Studies on cellular interactions that attempt to establish the requirements for induction of a positive immune response are often performed in vitro where it is possible to obtain quantitative measurements of the contributions by each cell type. The crucial role of macrophages in antibody production in vitro had been established in earlier studies (Mosier, 1967). More recently this role was also demonstrated for the proliferative response of immune T cells to specific antigens. Following the discovery that the presence of macrophages was obligatory for the efficient activation of immune T cells toward proliferation by antigen as measured by increased DNA synthesis (Rosenstreich and Rosenthal, 1973; Waldron et al., 1973), it was found that the cooperation between macrophages and T cells is genetically restricted (Rosenthal and Shevach, 1973; Farr et al., 1977). Thus, macrophages from strain 2 guinea pigs presented antigen to the homologous T cells more efficiently than they did to T cells from strain 13 guinea pigs and vice versa. The same type of restriction was found later with T cell blastogenic responses to insulin (Rosenthal et al., 1977). As was discussed earlier, strain 2 guinea pigs recognize the A chain loop of the molecule and strain 13 recognize the B chain. Macrophages from both strains were charged with insulin, added in culture to T cells from F_1 (2 × 13) guinea pigs immune to insulin, and were found to present antigen in accordance with the genetic constraints (macrophages of strain 2 presenting the determinants of the A chain loop and macrophages of strain 13 presenting the B chain antigen).

Working with the synthetic copolymer $Glu^{53}Lys^{36}Phe^{11}$ (GLϕ) in CFA_1 mice, Schwartz et al. (1979) showed that products of the *I-A* and *I-E/I-C* subregions of the mouse MHC were expressed on macrophages cooperating with immune T cells in the evocation of a proliferative response. In fact, both products had to be expressed on the same antigen-presenting cell. These results suggest that

complementation of genes from two subregions was required. This report is a culmination of many investigations following the lead of Dorf and Benacerraf (1975) and collaborators (Dorf et al., 1975, 1977; Schwartz et al., 1978a and b), demonstrating complementation of Ir genes in the regulation of immune responses. This requirement for gene complementation regulating macrophage participation is by no means limited to synthetic antigens since it has also been demonstrated with protein antigens (Cowing et al., 1978a).

5.4.5.2. Macrophages regulate antibody responses. Studies on the response of mice to burro erythrocytes further demonstrated that macrophages expressing Ia determinants of *I-A, I-J, I-E, I-C* subregions were required for in vitro antibody responses (Neiderhuber et al., 1979). Co-expression of Ia of subregions *I-A* and *I-E/C* on antigen-presenting cells was clearly demonstrated in immune responses to TNP-KLH and TNP-(T,G)-A-L (see Glossary) (Hodes et al., 1978). This paper also calls attention to the fact that the antigen-presenting cell, while resembling the macrophage in being radioresistant and glass-adherent, appears to lack one of the attributes of the macrophage, strong phagocytic activity. In fact, cells enriched for phagocytic activity were devoid of Ia antigen on the surface. It is therefore possible that the antigen-presenting cell is not a macrophage; alternatively, it is becoming increasingly evident that macrophages comprise several subgroups.

5.4.5.3. Heterogeneity of macrophages with respect to function, Ia expression, and antigen presentation. The existence of macrophage subgroups should not be surprising since macrophages are known to perform numerous functions in the induction and regulation of immune responses as well as in their expression. In addition to their positive contribution to the induction of immune responses, discussed in this section, they play a role(s) in negative regulation (discussed in Section 5.5.2.2). Their effector functions will not be reviewed, but should be cited. Current interest is focused on macrophage activity against tumor cells and here several modes of this activity have been described. Thus, macrophages may serve as effectors in antibody-dependent cytotoxicity (Miller and Feldman, 1977) as well as in collaboration with lympho cytes (Evans and Alexander, 1972; Evans and Grant, 1972). Activation ha s been achieved by nonspecific inducers (Hibbs, 1974). The effects have been measured by either target cell lysis (Keller, 1973) or by inhibition of growth (Evans and Grant, 1972). Functional differenc es related to the source of macrophages have been noted,

e.g., in the contribution of splenic and peritoneal macrophages to the augmentation and suppression of cytotoxicity of T lymphocytes against tumor cells (Ting and Rodrigues, 1980). Functionally distinct macrophages have been separated by various centrifugational methods (Walker, 1971; Rice and Fishman, 1974; Mill er et al., 1980). Tabulations of characteristics, including membrane markers of macrophages, have recently been published under the auspices of the Reticuloendothelial Society (Morahan, 1980).

The expression of Ia molecules varies among macrophages in accordance with their anatomical origin or distribution. Thus, in peritoneal exudates 5–15% of the macrophages bear Ia determinants (Cow ing et al., 1978b), whereas in the spleen and thymus 40–60% of the macrophages are Ia positive (Cowing et al., 1978a; Beller and Unanue, 1980). In a recent publication, Beller et al., (1980) found that the expression of Ia determinants on macrophages can be altered by immunologic reactions involving T cells. Thus, immunizati on with KLH or *Listeria monocytogenes* prepared animals for a heightened production of Ia bearing peritoneal macrophages and a similar effect was obtained with the ip transfer of immune T cells plus antigen. The latter findings suggest that T cells may be involved in the induction of Ia^+ macrophages or with the expression of these molecules, adding a new aspect to the macrophage–T cell interaction via Ia molecules. It should be noted that Ia molecules are newly synthesized by macrophages (Cowing et al., 1978b). According to Lee et al. (1979), Ia antigen occurs predominantly on small (immature) macrophages that are able to present antigen to cells. Large (presumably mature) macrophages lack Ia and are incapable of presenting antigen, but retain their capacity to produce factors promoting T cell proliferation (presumably LAF). Hence, maturation of macrophages seems to be associated with a shift from Ia-regulated (specific) to Ia-independent (nonspecific) immunostimulation.

The actual nature of the macrophage–T cell interaction under Ir gene control has been conceptualized by Benacerraf (1978). After being interiorized and degraded, fragments of the antigenic molecule are released onto the macrophage surface where they combine with the Ia determinants. These molecules must recognize a portion of the antigenic fragment, presumably equivalent to 3 or 4 amino acids (which accords with the findings on the loop on the A chain of insulin where amino acids A8, A9, and A10 are critically important in the determination of specificity). The antigen–Ia complex activates a T cell presumably endowed with an appropriate receptor. Since B cells also express Ia molecules on their surfaces

[and there are indications that B cells can also degrade antigen (Schreiner and Unanue, 1976)], the antigenic fragment may bind to their surfaces forming another complex. Such an arrangement may permit the selection of appropriate clones of B cells through a product of the Ir gene. Moreover, the B and T cells may be brought into interactive proximity by the antigenic fragment binding the Ia molecules on the respective cells. This is a very interesting hypothesis, one that provides an integrative view of cellular interactions under the control of Ir genes. Although another channel of intercellular communication is provided by idiotype or structures complementary to idiotype, these structures are believed to interact with MHC-specified receptors to form antigen-binding sites.

5.5. Inductive and Regulatory Networks and Circuits

5.5.1. Model Building The various pathways leading to the maturation of effector T cells are depicted in Fig. 1 prepared by Ahmed et al. (1979). It collates the findings, hypotheses, and concepts from several laboratories that will be presented in more detail below. The effector cells performing diverse functions are derived from a common pool of precursors bearing phenotype $Ly1^+23^+$. We want to call attention to the subdivision of effector functions into specific and nonspecific, which creates an additional diversification. In order to construct models of cells and intercellular means of communication (soluble factors or mediators), it is often necessary to perform experiments in vitro with separated cellular populations and with factors either physically extracted from cells or harvested from cell cultures. In some situations, the results of in vitro studies have been shown to correlate with in vivo events, but one must maintain the awareness that not every in vitro activity precisely reflects a process occurring in vivo.

One major aspect absent from the model prepared by Ahmed et al. (1979) is that $Ly1^+$ cells are not only capable of providing helper functions, but are apparently involved in inducing other functions (Cantor and Boyse, 1975, 1977). $Ly1^+$ T cells promote or amplify the function of $Ly23^+$ T cells toward cytotoxic activity (Cantor and Boyse, 1977), induce or potentiate macrophage activity in DTH reactions (Huber et al., 1976b) and induce $Ly1^+23^+$ T cells to become $Ly23^+$ TS cells (Cantor et al., 1976; McDougal et al., 1979). Since helper and suppressor functions are extremes in the regulation of immunologic activities providing the "on" and "off" signals, it is difficult to accept the notion that these two functions are served by the same cell, the $Ly1^+$ inducer cell. This apparent para-

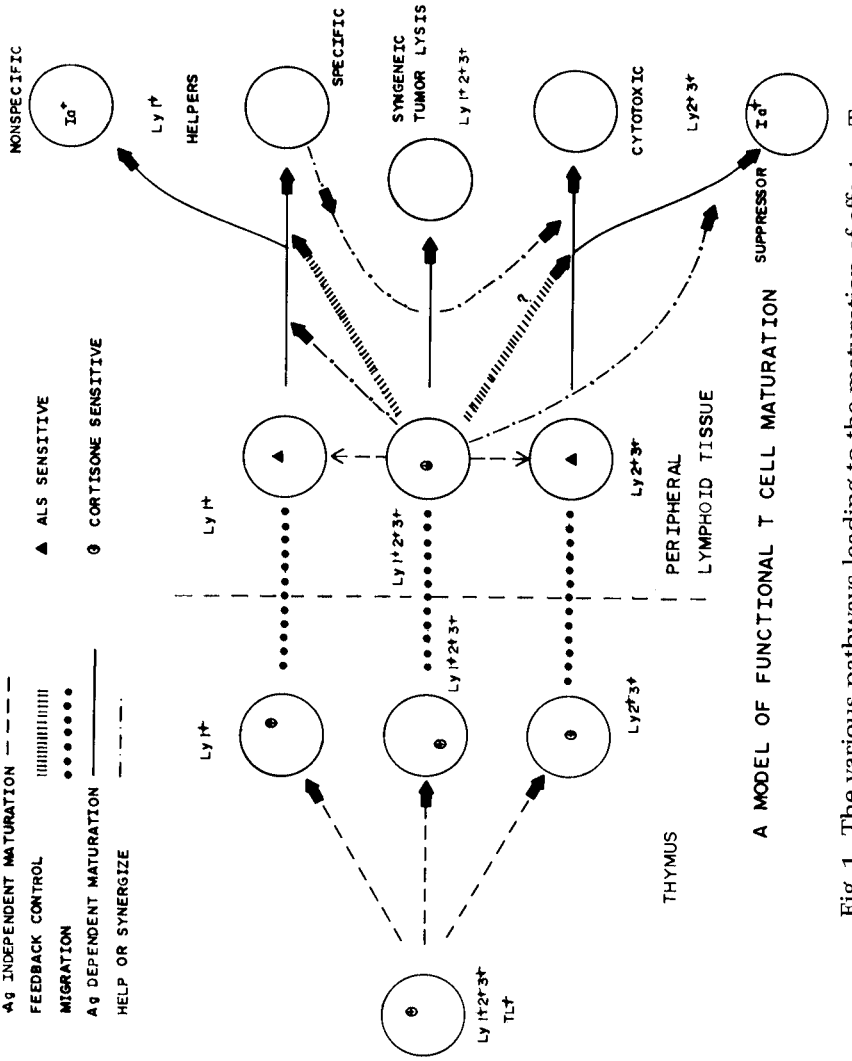

Fig. 1. The various pathways leading to the maturation of effector T cells (from Ahmed et al., 1979).

dox is but one of many as yet unresolved complexities of immune induction and regulation.

A significant clue for resolving the mystery of the $Ly1^+$ oxymoron was obtained from the elegant and persistent investigations in the laboratories of Cantor and Gershon. Their findings indicate that the $Ly1^+$ phenotype represents two separate T cells (Cantor et al., 1978a,b; Eardley et al., 1978; Cantor and Gershon, 1979) which can be identified by means of an alloantiserum directed against still another surface antigen, Qa1 (Flaherty, 1976; Stanton and Boyse, 1976). Although both $Ly1^+Qa1^+$ and $Ly1^+Qa1^-$ cells may be required for optimal induction of antibody formation by B cells, the $Ly1^+Qa1^-$ cell appears to provide the major helper signal, whereas the $Ly1^+Qa1^+$ cell induces $Ly1^+23^+Qa1^+$ cells to become suppressor cells ($Ly23^+$) that not only inhibit antibody production, but also suppress further activity of the inducer $Ly1^+Qa1^+$ cell (feedback inhibition). The net result is a decrease in both antibody secretion and TS formation (model A, Fig. 2).

This model combines several interesting features. It shows specialized functions performed by different subsets of T cells. It illustrates that suppressor activity originates from the interaction of cells and events, i.e., requires several steps. It assigns the principal role in the process to the $Ly1^+$ cell and places the $Ly1^+23^+$ cell in a precursor pool (from these precursors arise both $Ly1^+$ and $Ly23^+$ cells). The $Ly1^+Qa1^+$ cell appears to be capable of performing two functions, the induction of suppression and the cooperation with the $Ly1^+Qa1^-$ cell in the induction of the positive humoral response. This is another example of a helper pair of cells, although in this instance the pair may be viewed as strange bedfellows. In more recent studies, the proponents of this model have demonstrated a short circuit independent of the $Ly1^+23^+$

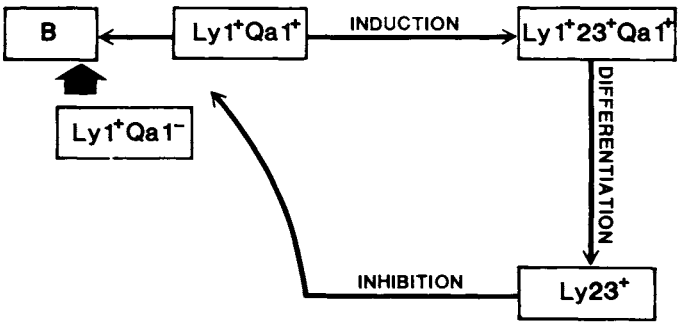

Fig. 2. Feedback inhibition model A (based on Cantor and Gershon, 1979).

cells. According to the findings of Eardley et al. (1980 and personal communication), the $Ly1^+$ cells can act directly on $Ly23^+$ cells to activate them toward suppressor activity. This would imply that a cell with the $Ly23^+$ phenotype may be a precursor of the fully functional $Ly23^+$ suppressor cell. The interaction represents a mini-regulatory circuit between the two cells since the $Ly1^+$ activates the $Ly23^+$ cells, which then turn on its benefactor and shuts it off. Additional developments that imply the existence of a point–counterpoint situation related to model A are dealt with later in this Section.

The view that the $Ly23^+$ cell can be both a precursor and an effector of suppression accommodates model A with prior models proposed by Feldmann et al. (1977) and Tada (1977). According to Feldmann et al. (1977; model B, Fig. 3) the $Ly23^+$ phenotype may denote either the precursor or effector suppressor cells and the $Ly1^+$ antigen marks precursors and effector TH cells with activation in both directions being amplified by $Ly1^+23^+$ cells.

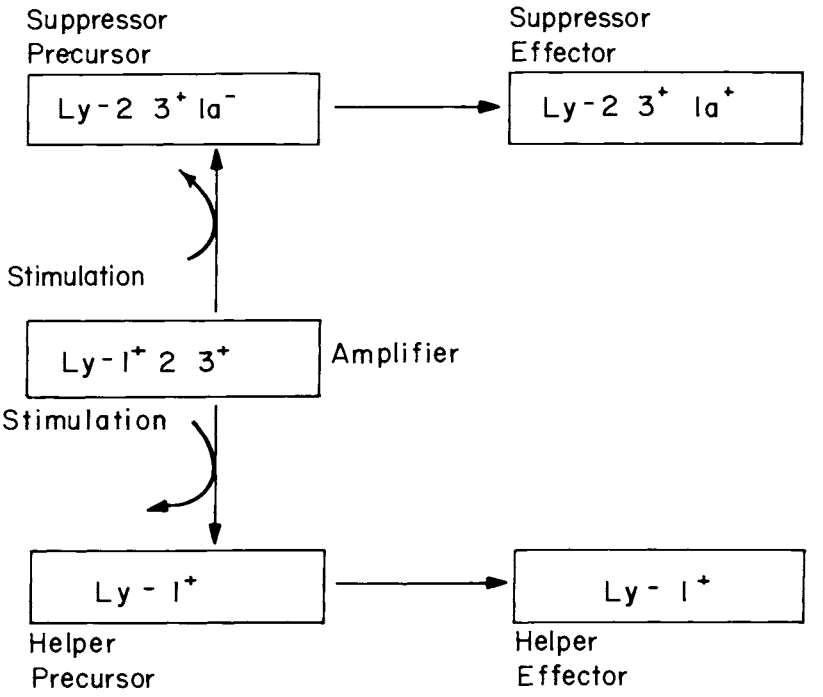

Fig. 3. Model B. Scheme of cell interactions and Ly phenotypes for helper and suppressor cell induction (from Feldmann et al., 1977).

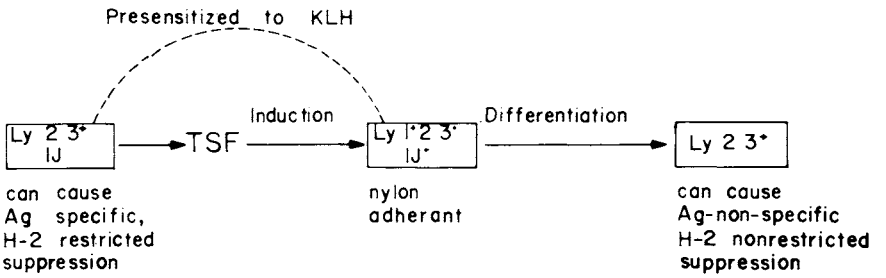

Fig. 4. Model C. Suppressor cell induction by antigen and TSF (based on Tada, 1977).

Model C, Fig. 4, illustrates some of the concepts proposed by Tada (Takemori and Tada, 1975; Tada, 1977) for the regulatory network. The $Ly23^+$ cell is activated by antigen, e.g., KLH produces a suppressor factor (TSF) that is KLH antigen-specific and which in the presence of KLH acts on KLH-activated $Ly1^+23^+$ cells causing them to differentiate to a new set of TS cells with the $Ly23^+$ phenotype. In contrast with the primary TS (and the initial TSF in Tada's network), the secondary cells are nonspecific in their function since they can inhibit antibody responses to ovalbumin or KLH as they are not under H-2 restriction.

Model D (Benacerraf and Germain, 1979), Fig. 5, is a composite of concepts which have emanated from the authors' laboratory and from the laboratories of Tada, Cantor, and Gershon. It integrates several salient aspects such as the important role of the antigen-presenting cell, the divergent pathways leading to helper or suppressor activities, and the co-*existence of distinct but sequentially ordered subsets* of suppressor cells, TS1 and TS2. Heavy lines indicate the dominant pathways, light lines the subordinate routes, and broken lines the possible but as yet unsubstantiated pathways. The synthetic copolymer L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT) that is under Ir gene control induces predominantly TH cells in responder (R) mice and TS cells in nonresponder (NR) mice (Germain and Benacerref, 1978). Inductive pathways toward TH and TS are available to either strain and the difference in response to GAT depends on which pathway is activated. Thus, macrophage-bound GAT triggers TH in preference to TS in R mice, but an excess of antigen or depletion of macrophages can alter the choice in favor of TS activation. Herein lies one explanation of supraoptimal antigen dose leading to immune suppression. In NR mice a defect in the Ir gene apparently impairs macrophage presentation of GAT to TH cells, thereby allowing unbound antigen to

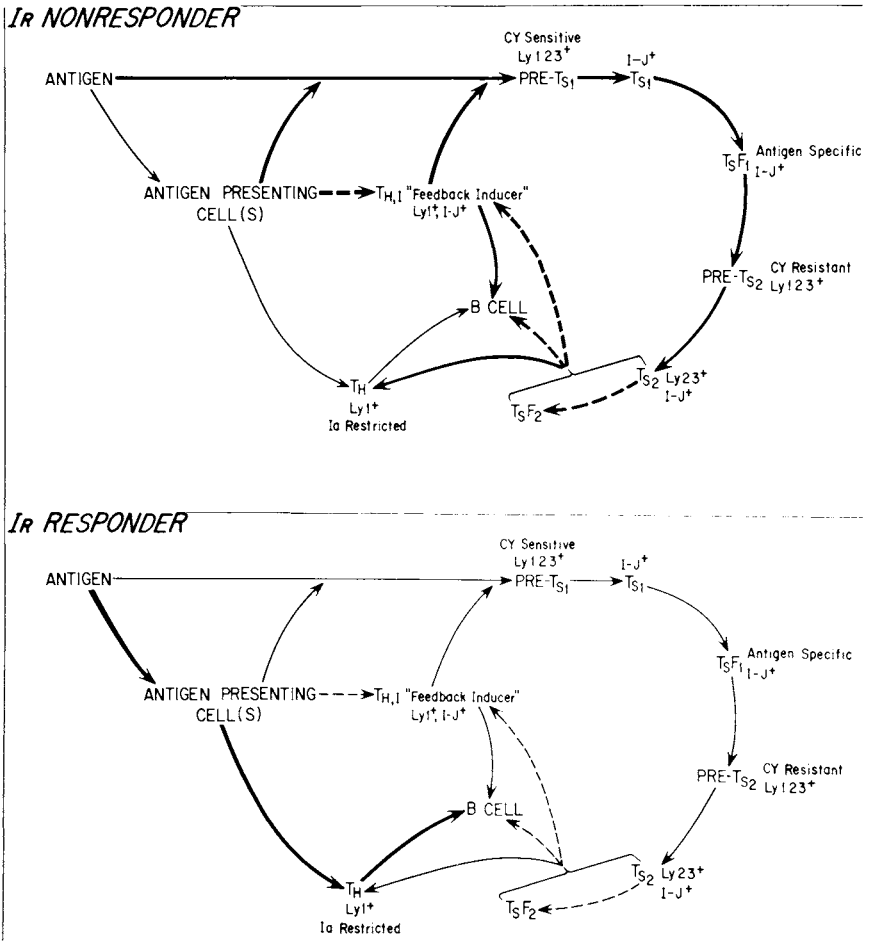

Fig. 5. Model D. A model for the stimulation of specific Ts responses in nonresponder and responder mouse strains. The heavy lines indicate predominant pathways. Broken lines indicate possible, as yet unsubstantiated pathways (from Benacerraf and Germain, 1979).

activate the suppressor pathway. Here again the outcome can be reversed by interventive procedures that interfere with the generation or function of TS, e.g., treatment with cyclophosphamide (Cy) (Debre et al., 1976). *The interposition of two subsets of TS precursors differing in susceptibility to Cy provides a frame of reference for discussion of selective action of alkylating agents* (see Chapter 4). An important feature of models D and C is the action of TS1 suppressor factor on the precursors of TS2, resulting in the induction of the effector suppressor cell, TS2.

A highly intriguing aspect of T cell subset interaction was recently added to model D. Investigations by Benacerraf and his colleagues on the generation of suppressor activity in immune responses to various antigens (GAT, GT, ABA, APA, and phosphoryl choline: see Glossary) have clearly established that the chain of events encompasses principally a communication between subsets of T cells bearing idiotype structures, and those expressing receptors for the idiotype (anti-idiotype structures) (Owen et al., 1977; Germain and Benacerraf, 1978; Germain et al., 1978; Bottomley et al., 1978; Greene et al., 1979; Pierres et al., 1979; Dohi and Nisonoff, 1979; Dietz et al., 1980; Germain, 1980; Sy et al., 1980c; Weinberger et al., 1979). As conceptualized by Germain and Benacerraf (1980; Fig. 6), the TS1 cells and their factor, TSF1, display the antigen-specific idiotype as well as the I-J$^+$ determinant, whereas precursors of TS2 I-J$^+$ cells express an idiotype-specific receptor with which they presumably accept the idiotypic determinant of TSF1 that activates the TS2 precursors toward function as effector suppressor cells capable of inhibiting TH and TDH cells with idiotypic and MHC restriction. Thus, although the antigen is the initial impetus responsible for activation of TS1 cells, the idiotype on the TS1 cells and in their product is the second driving force activating the second effector suppressor cell, TS2. These observations provide new insight into the mechanisms controlling humoral and cellular immune responses and add a new dimension to the idiotype/anti-idiotype theory of immune regulation proposed by Jerne (1974).

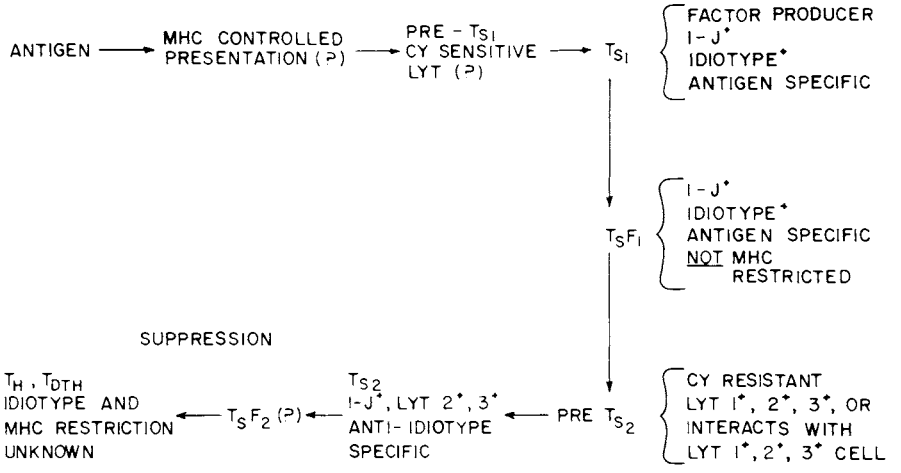

Fig. 6. Model D *continued.* Generation of suppressor cell activity by communication between subsets of T cells bearing idiotype and anti-idiotype structures (from Germain and Benacerraf, 1980).

Idiotypes may also be involved in the communication between $Ly1^+$ inducers and $Ly1^+23^+$ acceptors in model A. Eardley et al. (1979) found that the inducer/acceptor interaction occurred only when the two cells shared an identical IgV locus. This T/T interaction was independent of IgCH gene control.

Certain aspects of model A have recently been clarified and this model has been juxtaposed with a novel regulatory circuit referred to as *contrasuppression*. According to Eardley et al. (1980 and personal communication) the $Ly1^+$ cell of model A, which is the inducer of suppression, and the $Ly1^+23^+$ cell, which is induced by the $Ly1^+$ cell to differentiate into $Ly23^+$ cells are $I\text{-}J^+$ whereas the $Ly23^+$ suppressor cells, are $I\text{-}J^-$. Thus the I-J determinant distribution among cells in model A is:

$$\underset{(I\text{-}J^+)}{Ly1^+} \rightarrow \underset{(I\text{-}J^+)}{Ly1^+23^+} \rightarrow \underset{(I\text{-}J^-)}{Ly23^+}$$
(suppressor T cell)

The contrasuppressive circuit is described in companion papers (Durum et al., 1980; Green et al., 1980; Yamaguchi et al., 1980). The central issue is that the $Ly23^+$ cells derived by sensitization with SRBC in vitro are heterogeneous and are comprised of $I\text{-}J^-$ and $I\text{-}J^+$ subsets. The former are the TS of model A and the latter are the inducers of contrasuppression. These $Ly23^+$ $I\text{-}J^+$ cells produce an $I\text{-}J^+$ factor that acts on the $Ly1^+23^+$ $I\text{-}J^+$ acceptors causing them to mature to $Ly1^+$ $I\text{-}J^+$ contrasuppressors (TCS). Thus, it appears that the acceptor population contains precursors that can be induced to become either $Ly23^+$ $I\text{-}J^-$ TS or $Ly1^+$ $I\text{-}J^+$ TCS cells. TCS cells appear to perform their function by acting in some positive manner on $Ly1^+$ $I\text{-}J^-$ T helper cells thereby antagonizing the negative action of $Ly23^+$ $I\text{-}J^-$ T suppressor cells that also act on the same helper cells. Although the contrasuppressor cell differs from the helper cell, one may view the effect of the contrasuppressor cell as providing promotional action and the fact that this cell expresses an I-J determinant suggests that it may be the cell described by Tada et al. (1978) as the second helper that is $I\text{-}J^+$. If the contrasuppressor cell is identified as the $Ly1^+Qa1^+$ cell that is the inducer of suppression as well as the collaborator with the T helper cell in launching the B cell response, there would be a link between suppression and contrasuppression.

5.5.2. Heterogeneity of TS, Other Cells Involved in Immune Suppression, and Their Modes or Sites of Action and Interaction

5.5.2.1. TS effectors are heterogeneous cells and act at different stages of immunogenic induction and immune reactions. The existence of the various models differing in detail and in the cast of characters does not vitiate the basic concept that immunoregulation is a function of networks or circuits assigned specific roles in the drama of shifting equilibria. All models point to the complexity of induction and regulation and all reinforce the notion that suppressor activity depends on the interaction of two or more subsets of T cells. Although all models contain T $Ly23^+$ cells as the effectors of suppression, this phenotype does not define a single or homogeneous set of cells, nor does it necessarily represent the exclusive effector of suppression. Cytotoxic cells express the same phenotype; they do, however, lack the Ia product of the *I-J* subregion that is expressed on *some* $Ly23^+$ suppressor cells (the $Ly23^+$ are $I\text{-}J^+$ in model D and $I\text{-}J^-$ in model A). Models C and D feature two distinct subsets of TS cells, and in model D they are clearly distinguished by the different mechanisms of recognition (antigen-specific receptor on TS1 and a complementary anti-idiotype receptor on TS2) and by the difference in susceptibility of their precursors to Cy. Moreover, the different types of TS cells appear to act at different stages of the immune response. This has been best illustrated in the cell-mediated immune responses. Studies with DTH (see Section 6.4.2.1) indicate that at least two kinds of TS cells can be induced: one limits the effector phase of the reaction (Zembala and Asherson, 1976; Miller et al., 1978) and the other interferes with the initial stage of sensitization (Moorhead, 1976). Studies by Weinberger et al. (1979, 1980) indicate that these phase-related functions may correspond to activities of TS2 and TS1 cells (see Section 6.4.2.3). Multiple types of suppressor cells may also occur in other forms of cell-mediated responses. Thus, Gorczynski and MacRae (1979a) have found two types of TS cells distinguished by sedimentation velocity that are involved in the regulation of production of cytotoxic T lymphocytes (CTL). One (Supp A) inhibits the production of CTL from CTL precursors while the other (Supp B) appears to cause a decrease in production of CTL precursors from stem cells. Although apparently operating at different levels of differentiation of CTL, it is not clear whether the two TS cells repre-

sent different stages of maturation of TS cells or distinctive entities. The authors believe Supp A is associated with the induction of tolerance in newborn mice, whereas Supp B may be more concerned with tolerance maintenance (and possibly clonal deletion). Supp B cells (without added antigen) could transfer nonresponsiveness to newborn syngeneic hosts, but Supp A cells could not (Gorczynski and MacRae, 1979b). This is an important issue because adoptive transfer of suppressor activity is often reported as a *sine qua non* of the suppressor cell. Supp A cells apparently require the presence of antigen and therefore may not be detected in the absence of antigen, thus making *a case for caution in interpreting the negative adoptive transfer data.*

In most studies on humoral responses, the regulatory input is believed to take place early in the inductive process and to consist of T/T cell interactions. This temporal and structural order is the essence of the various networks, where T cells of one subset act on T cells of another, thus forming a regulatory constellation that generates the TS effector cells that in turn interface with TH or B cells. Work in our laboratory has shown that adoptively transferred TS effector cells are able not only to prevent the initiation, but also to interrupt the continuation of an ongoing humoral response, suggesting that they may act on activated B cells. This holds for the primary immune response. In the secondary response it appears that suppressor cells prevent the genesis of memory cells, but do not interfere with their expression or function (Sigel et al., 1980).

Adding to the problem of the heterogeneity of TS cells is the question of specificity of their action. Mitogens such as con A and other immunomodulatory substances, e.g., extracts of *Ecteinascidia turbinata* (see Chapter 4, Section 4.2) can induce TS cells that manifest nonspecific suppressor activities. What is more surprising is that the specificity of antigen-induced suppressor cells degenerates in the course of induction of different subsets of TS cells (and/or production of different TSF) (model C). The dose of antigen again comes into the picture, as evidenced by the findings of Pierres et al. (1980), which demonstrated that under certain conditions high-dose antigen (hapten on allogeneic cells used to induce tolerance) leads to activation of MHC nonrestricted TS cells, whereas low-dose antigen activates MHC-restricted TS cells.

5.5.2.2. Macrophages play multiple and major roles in immune suppression. Although T cells have been featured in all models as the cells responsible for suppressor activity and have constituted the principal characters in the various circuits, assum-

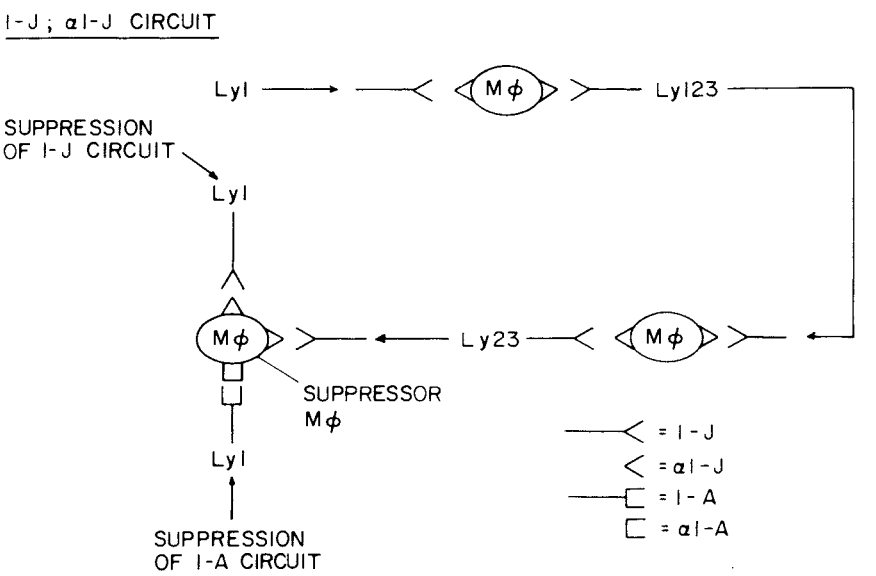

Fig. 7. Model of macrophages functioning as messengers between different types of T cells (based on Ptak et al., 1978; Gershon et al., 1980).

ing the roles of precursors, inducers, and effectors, macrophages have also been very much involved as active or passive participants in immune regulation. We have already referred to the importance of Ia-bearing macrophages in the presentation of antigen to T cells for the evocation of a positive response and we have related observations suggesting that antigens bypassing the macrophages are likely to switch off the response or induce TS. There is also strong evidence that macrophages may provide a messenger service between different types of T cells (Ptak et al., 1978; Gershon et al., 1980) (Fig. 7). Moreover, macrophages may be the effectors of suppression (Kirchner et al., 1975; Miller and Mishell, 1975; Veit and Feldman, 1976). In fact, in addition to the T. . .T cascade of factors, where one set of T cells makes a factor that acts on another set, that in turn may produce another factor (models C and D), there exist T. . .Mϕ cascades. This has been demonstrated for the SIRS (soluble immune response suppressor), which is the product of con A activated $Ly23^+$ T cells and inhibits TD and TI antibody responses (Rich and Pierce, 1973; Pierce et al., 1979). SIRS induces macrophages to release a second factor(s) that inhibits PFC responses (Tadakuma and Pierce, 1978). Interestingly, the mode of action appears to be the limitation of B cell proliferation (Tadakuma and Pierce, 1978). The T. . .Mϕ form of interaction may also be the basis

for suppression of blastogenic reactions (Yoshinaga et al., 1972; Folch and Waksman, 1973). A cascading sequence of events has been recently described by Kennard and Zolla-Pazner (1980) for immunosuppression in plasmacytoma-bearing mice. In their system, one factor produced by the malignant cells induces macrophages to produce a second factor that is capable of suppressing primary antibody responses. This factor has no effect on proliferative responses of normal cells to PHA, i.e., no suppression of T cell proliferation.

In the human there is considerable evidence that immunosuppression is associated with Tγ cells and helper activity is associated with Tμ cells (T cells with receptors for IgG and IgM, respectively) (Moretta et al., 1977). Other regulatory systems have also been described in man. Using inhibition of lymphoproliferative responses to alloantigens or PHA, Rice and colleagues (Rice et al., 1979) found that in addition to induced suppression exerted by con A-activated T cells, monocytes could also suppress the blastogenic responses by virtue of either excessive numbers or through prostaglandin production. *The three systems appear to differ in resistance to radiation and cortisone, and alteration with age of the host.*

Regulation of immune responses by macrophages may occur through an array of substances with potentiating or with suppressive activities (Bach et al., 1970; Nelson, 1973; Wood and Gaul, 1974; Calderon and Unanue, 1975; Rosenstreich et al., 1976; Mookerjee, 1977; Tadekuma and Pierce, 1978; Metzger et al., 1980; Kennard and Zolla-Pazner, 1980). Prostaglandins (Gordon et al., 1976; Metzger et al., 1980) and hydrogen peroxide (Metzger et al., 1980) have been shown to inhibit lymphocyte proliferation. We have already discussed the potentiator, LAF, in Section 5.2, and will discuss another monokine with a bidirectional effect immediately below. It is of interest that a soluble factor derived from monocytes and with an affinity for a subpopulation of T cells (cells characterized by low density) has been found in the sera of some patients suffering from recurrent disseminated fungal infections with an associated deficit in T cell functions. This factor apparently induces these cells to become suppressor cells as determined by in vitro assays (Stobo, 1977). These findings give significant status in the "real world" to monokines.

5.5.2.3. Interferon—the double agent. Among the various soluble factors that are continuously arousing interest among immunologists there is one, interferon, that surfaced about

a quarter of a century ago as an antiviral substance (Isaacs and Lindenmann, 1957). Although initially produced by chick embryo allantoic membranes exposed to heat-inactivated influenza virus, this glycoprotein has since been derived from a large array of sources including serum, fibroblast cell cultures, and lymphoreticular cells. We are focusing on interferon because in addition to antiviral effects, it has manifested immunoregulatory activities and because several immunomodulatory agents (discussed in Chapter 4) either induce interferon production or exert effects analogous to those caused by interferon. In actuality, the term interferon represents a family of molecules differing in physical, chemical, and immunologic characteristics, but sharing the common property of antiviral activity. [For excellent updated information, see Baron and Dianzani (1977), Stewart (1979) and Hadden and Stewart (1981).] This activity does not directly inhere in interferon, but is exerted by a protein that is induced in cells by interferon. For convenience, interferons have been subdivided into types I and II, the former representing classical interferon produced by exposure of intact animals or cells in culture to infectious or noninfectious viruses, double-stranded RNA, or synthetic polynucleotides. Type II designates "immune" interferon resulting from activation of lymphocytes by antigens or mitogens. The three major criteria used to characterize types I and II interferons are susceptibility to inactivation by low pH (pH 2), temperature (56°C), and antibody specific for type I. However, interferons produced in different species under different conditions do not always conform to a unique pattern of criteria and there are discrepancies and overlaps among their characteristics. There is therefore considerable heterogeneity among interferons and this is especially noteworthy when one compares type I interferon produced in fibroblasts with type I produced in lymphoreticular cells. Human leukocytes have now become a major source of the production of interferon used in the treatment of cancer patients. Both T and B cells have been implicated in interferon synthesis (Klimpel et al., 1977) but further fractionational studies by Yamaguchi et al. (1977) have indicated that viral interferon (induced by Sendai virus) originates from non-T lymphocytes, presumably B cells, null, or natural killer (NK) cells, whereas monocytes are essential for the induction of interferon by poly I:C. In contrast, type II interferon is produced by T cells (Epstein et al., 1974).

Both inhibitory and potentiating effects of interferon on the immune mechanism have been reported. There are accounts that interferon increases various cytotoxic activities (Svet-Moldavsky

and Chernyakhovskaya, 1967; Lindahl-Magnusson et al., 1972; Heron et al., 1976; Trinchieri et al., 1978). Some of the increased activities owe to stimulation of NK cells but, in part, the increase may have been caused by the effects on T cells and macrophages. Interferon can cause macrophage activation, as judged by several criteria including increased cytotoxicity for tumor cells. This was demonstrated by Schultz et al. (1977) using interferon inducers as well as a partially purified interferon. These enhancing effects of interferon are counterbalanced by suppressive effects observed in certain cell-mediated immune reactions (reviewed by Johnson, 1977). Thus interferon has been found to inhibit blastogenic responses of lymphocytes to mitogens and allogeneic cells (Lindahl-Magnusson et al., 1972; Johnson et al., 1975; Kadish et al., 1980). Interferon has also been demonstrated to suppress DTH reactions (deMaeyer and deMaeyer-Guignard, 1977). Both the afferent and efferent arms of this reaction were susceptible to interferon and maximal suppression was obtained when interferon was administered 24 h prior to sensitization.

The positive and negative effects of interferon have also been noted in humoral responses, but here the see-saw action seems to be shifted towards the suppressive effect. Moderate and high doses of type I interferon suppress in vivo PFC responses to SRBC as first reported by Braun and Levy (1972) and confirmed by Chester et al. (1973) and Brodeur and Merigan (1974). This suppressive activity occurred when interferon was administered prior to antigen. However, when injected 2–3 days after antigen, interferon caused a slight enhancement of antibody production (Brodeur and Merigan, 1975). This type of time-dependence accords with the findings of Virelizier et al. (1977), wherein a correlation was observed between time of appearance of interferon induced in mice by infection with murine hepatitis virus and the animals' ability to mount a response to SRBC. Presence of interferon before immunization reduced the antibody response whereas appearance of interferon after immunization enhanced the response. Not only is the time of administration important, but so is the dose of interferon, since low doses (250 units) enhanced antibody production whereas higher doses (5000–10,000 units) inhibited PFC responses (Braun and Levy, 1972). Antibody responses to LPS have also been inhibited by type I interferon (Brodeur and Merigan, 1975). Most of the studies on the effects of interferon on antibody production have been performed in vitro and in most of these, interferon caused suppression of the response. (Slight enhancement was noted when interferon was introduced into the spleen cultures 2–3 days after antigen; Johnson et

al., 1975.) Experiments conducted by Booth and associates (1976) indicated that type I interferon affected primarily nonactivated B cell precursors and that after entry into proliferation B cells were refractory to such suppression. The greater susceptibility of nonactivated B cells may explain why interferon is more effective in suppressing antibody induction when administered prior to antigen. This aspect is of major significance in relation to other immunological modulators that, in our experiments and in the work of others, have proven more suppressive prior to activation of the immune system by antigen (see Chapter 4).

Type II interferon also suppresses in vitro PFC responses when a small amount is added to cultures 24 h before SRBC (Sonnenfeld et al., 1977), but enhances when added after the antigen (Sonnenfeld et al., 1978; Sonnenfeld and Merigan, 1979).

Several investigators have demonstrated that interferon causes an increase in the expression of lymphocyte surface antigens (Lindahl et al., 1973, 1974; Sonnenfeld and Merigan, 1979). Increased amounts of antigen specified by the *K* and *D* regions have been detected by Vignaux and Gresser (1977) and by Lonai and Steinman (1977). No increase has been found in Ia expression. However, in contrast with type I interferon, type II was found to cause an increase in Ia, $Ly1^+$ and $Ly23^+$ antigens (Sonnenfeld and Merigan, 1979). Results obtained with interferon type II are still open to question because the stimuli that evoke the production of this substance (antigens and mitogens) also cause the release of other immunologically active lymphokines and monokines. Thus, a given cell culture supernatant may contain interferon type II, LAF, TCGF, SIRS, MIF, etc. Some of these substances are co-purified with interferon and may therefore be present in preparations of type II interferon and contribute to immunologic modulations. Interferon type I, on the other hand, which is produced by virus infection or by polynucleotides, is less likely to be contaminated with lymphokines or monokines.

The studies of Lonai and Steinman (1977) have also shown that interferon type I acts on $Ly23^+$ cells and increases their antigen-binding capacity (or their frequency within the lymphocyte population). The positive effect of interferon on the expression of products of *H-2K* and *H-2D* on $Ly23^+$ cells may explain the reason for the increased cytotoxic effects induced by interferon. It is also of considerable interest that interferon has been implicated as a product of suppressor cells activated by con A (Kadish et al., 1980). There are indications that $Ly23^+$ cells are involved in the production of interferon (Sonnenfeld and Merigan, 1979; Farrar and Fuller-Bonar,

1980). Since this phenotype denotes both cytotoxic and suppressor cells, the interaction of interferon with subsets of $Ly23^+$ cells may be the basis for its ability to enhance cytotoxicity on the one hand and lower humoral immune responses on the other.

The subject of physiologically active products of macrophages has received extensive attention (Unanue, 1978; Cohen et al., 1979; Friedman, 1979). Production of monokines is but one side of the contribution of macrophages to positive and negative regulation. It must be emphasized that the other side, the unique role of macrophages in presenting antigen to lymphocytes, is equally important and perhaps crucial. These two roles imply that a macrophage may be involved in providing two signals necessary for induction of a positive response. This notion is in part derived from the opposite results obtained with immunization by the sc and iv routes. In the former, the antigen is most likely acquired by the macrophages (Langerhans cells) of the skin and the end result is a positive response. In the latter, the antigen probably reaches the spleen in free form and can engage lymphoid cells as well as macrophages. The effects of route of immunization are discussed in Section 6.4.3, and for a further concise analysis, see Claman (1979).

5.5.2.4. B cells and their products also contribute to immune regulation. Surprising results were obtained recently in experiments based on feedback suppression, which in previous studies (Cantor et al., 1978a,b; Eardley et al., 1978; Cantor and Gershon, 1979) demonstrated that suppression was effected by $Ly2^+$ T cells. The new results disclosed that the suppressor cell was a B cell (Zubler et al., 1980a, b). Whole spleen cells were incubated with SRBC in vitro and the suppressor activity was measured against primary anti-SRBC PFC responses in fresh spleen cell culture. The whole spleen cell suspension exerted strong suppression, whereas separately sensitized T cells or B cells failed to generate suppressor cells. Combination of T and B cells allowed for generation of suppressor cells and the cell transferring suppression was found to be a B cell. Thus, the B cell was the key element in this type of feedback suppression, but the induction of this effector cell required the presence of T cells. The mediator of suppression was apparently antibody to SRBC and its activity was restricted to B cell targets sharing VH genes with B cells producing the suppressive antibody. These data are explainable on the basis of suppression being mediated by anti-SRBC antibody either by the blockade of different SRBC epitopes recognized by a limited set of B cell clones in each mouse strain or by the triggering of an anti-idiotype

response, again with the same VH restrictions, or by interference by preformed antibody with T cell help directed at idiotype-bearing B cells. The element of surprise is that the B effectors of suppression were generated under conditions that in previous work (model A) have led to the genesis of TS cells. Suppression by B cells was of itself not surprising since it had been reported by numerous investigators (Gorczynski et al., 1973; Katz et al., 1974b; LaGrange et al., 1974b; Mackaness et al., 1974; Neta and Salvin, 1976; Zembala et al., 1976; Stockinger et al., 1979). In part this suppression may reflect a negative feedback by antibody that is believed to mask antigenic determinants. In part the suppression may owe to immune complexes (Lee and Sigel, 1974; Mackaness et al., 1974), but there are also strong indications for regulation by anti-idiotype. Such regulation was predicted by Jerne (1974). Immunoregulation has been obtained in experiments employing immunization with idiotypes (Eichmann, 1972, 1974, 1975; Ramseir and Lindenmann, 1972; Rowley et al., 1973; Nisonoff et al., 1977; Pierce and Klinman, 1977; Sy et al., 1979b). In some of these studies, as well as in the work of Schrater et al. (1979) and Goidl et al. (1979), auto-anti-idiotypic antibodies were found to occur spontaneously in the course of immunization. Injection of anti-idiotypic antibodies can activate various types of T cells including T suppressor cells leading to suppression (Eichmann, 1975; Eichmann et al., 1978; Sy et al., 1979c). We have already discussed in Sections 5.4.3 and 5.5.1 the interplay of idiotypes and anti-idiotypes as they related to T cell receptors and models of immunoregulation involving T cells, and more will be said in connection with DTH (Section 6.4.2.3), but anti-idiotype may also work independently of T cells. Thus the TI response to levan was suppressed with anti-idiotype (Bona and Paul, 1979). This was achieved in both conventional mice and nude mice indicating that suppression was T cell independent (Bona et al., 1979).

Suppression has also been achieved (in miniature swine) by T cells with specific antibody bound to their Fc receptors (Setcavage and Kim, 1980). The role of Fc receptors on B cells in regulation of immune responses has been described (LaVia and LaVia, 1978).

5.5.3. TS Independent Humoral Nonresponsiveness

Although regulation by suppressor cells is in part under genetic control, genetically determined nonresponsiveness is not invariably tied to suppressor cells. In studies of the functions of genes in the *I* region, Benacerraf and associates have found that some forms of nonresponsiveness occur in the absence of demonstrable suppressor cells (Benacerraf and Dorf, 1977; Benacerraf and Germain,

1979). Several important aspects have emerged. Among 20 strains of mice that were nonresponsive to GT, some manifested suppressor activity and some did not (nonsuppressor mice). The control of suppressor potential in GT nonresponder mice is mediated by *Is* genes that map on either side of the *I-J* subregion (not *K/D*) and that can complement each other in such a way that F_1 or recombinant mice of nonsuppressor parents are able to generate GT-specific suppressor cells.

Nonsuppressor mice could be further subdivided into a group lacking the ability to produce a GT-specific TSF, but possessing the capacity to be suppressed by GT-TSF (exemplified by the A/J strain) (Waltenbaugh et al., 1977), and a group unable to respond to TSF (e.g., C57BL/6). Thus, nonresponsiveness in some strains is associated with production of and susceptibility to TSF; in others there seems to be a deficit in TS1 required to produce the primary TSF, but a presence of TS2 precursors; and in still others there may be a deficiency in the responsiveness of TS2 precursors to the signals contained in the primary TSF.

All this notwithstanding, nonresponsiveness independent of suppressor cells can be attained in animals in which TS-associated suppression can also be induced under altered physiological conditions or by modifications of immunizing procedures. Thus, according to Cinader and Nakano (1979) tolerance to rabbit IgG can be induced in strain A mice with equal ease early and late in life, but suppressor cells are detected only in older animals. In SJL mice that were partially resistant to tolerance induction, no transmissible suppressor cells could be demonstrated. A highly effective way to induce tolerance to contact sensitivity is through the injection of antigen coupled to splenocytes (see Section 6.4.2). A similar method of tolerization has also been applied in studies with humoral responses. A recent paper on tolerance to fowl gamma globulin (FγG) (Sherr et al., 1979) presents data that clearly distinguish T cell tolerance independent of detectable TS cells from nonresponsiveness mediated by TS. T cell tolerance was present 3 days after iv injection with 10^6 syngeneic spleen cells coupled with palmitoyl-FγG and *could not be forestalled by Cy pretreatment.* TS-related nonresponsiveness, on the other hand, could be induced with as few as 10^3 p-FγG coupled cells given iv, but required 7 days for expression and *was susceptible to Cy pretreatment.*

In Section 6 we are citing some examples of co-existence of suppressor cell dependent and independent nonresponsiveness in the same host following a single inductive procedure. Sometimes the two forms of nonresponsiveness are separated in time of appearance or persistence. This can be readily demonstrated when

TS cells become undetectable by adoptive transfer after a relatively short period of time even though the donor of the cells remains nonresponsive. However, when suppressor cells persist for a long time it is difficult to distinguish the two types of nonresponsiveness except through the use of selective cytotoxic agents. Thus, supraoptimal immunization (SOI) employing 4×10^9 SRBC leads to diminished responsiveness and the generation of suppressor cells that can be demonstrated by adoptive transfer to naive recipients. Treatment with Cy 2 days after SOI inhibits the induction of TS cells (Sigel et al., 1979), but leaves the animals unable to mount a secondary response (Ghaffar and Sigel, unpublished). Thus SOI appears to induce two forms of nonresponsiveness, one associated with Cy sensitive suppressor cells and the other which surfaces when the suppressor cells are deleted by Cy (or possibly resulting from combined action of SOI and Cy). Both forms of nonresponsiveness are antigen-specific. There are reports in earlier literature demonstrating that Cy facilitates the induction of tolerance to SRBC (Aisenberg, 1967; Aisenberg and Wilkes, 1967; Many and Schwartz, 1970) and, in view of the current knowledge that Cy removes certain constituents of the T suppressor paradigm, it would appear that this form of tolerance may also operate in the absence of Cy susceptible suppressor cells. In the 60s the existence of TS was not yet realized and this aspect was not addressed. Clonal deletion has been suggested and probably accounts for certain types of tolerance, but it should be kept in mind that in some of the earlier experiments the maintenance of tolerance required repeated injection of antigen for periods long after treatment with Cy, thus possibly allowing for the regeneration of TS. However, even when experiments to detect TS by adoptive transfer fail there is a need for caution in interpreting the negative results as absolutely excluding the presence of suppressor cells.

There are interesting situations where SOI in combination with Cy render animals immunologically nonresponsive and where TS cells cannot be demonstrated by adoptive transfer, *but where the nonresponsiveness appears to be associated with an active process of in situ suppression.* Thus, using the adoptive transfer procedure of Santos and Owens (1966) where Cy is administered to both donor and recipient animals, Marbrook and Baguley (1976) found no transferable TS in SOI mice treated with CY 24 h later (SOI-Cy mice), yet these mice could inhibit the response of normal lymphocytes administered to them. This form of nonresponsiveness has also been demonstrated by reverse adoptive transfer of normal or immune splenocytes to SOI-Cy mice depleted of TS by Cy in experiments in our laboratory (Ghaffar and Sigel, unpub-

lished). A similar pattern was observed by Kaufmann et al. (unpublished) with the additional facet that pretreatment with Cy would prevent this form of nonresponsiveness. The results have been interpreted to mean that this form of nonresponsiveness may result from depletion of B cells or alteration by SOI and Cy of the host's microenvironment and/or occurrence of TS capable of exerting their effect *in situ*, but not on transfer to another host. Although the possiblity of alteration of the microenvironment is an attractive explanation it does not explain the fact that this nonresponsiveness is antigen-specific.

6. A Closer Look at Induction and Regulation of Cell-Mediated Immunity

6.1. General Considerations

Cell mediated immunity (CMI) covers a multitude of reactions: DTH reactions, graft rejection, graft-vs-host (GVH) reactions and several types of cytotoxic reactions. Elicitation of lymphocyte proliferation by alloantigens, other cellular antigens, and mitogens also comes with the realm of CMI. Because T cells and macrophages can kill infected or transformed target cells, CMI has long been thought to contribute to resistance of the host to infectious agents and to neoplasia. Other aspects of CMI are considered highly important clinically in at least two settings: tissue transplantation and diseases caused by hypersensitivity, notably type IV and partially type II. Immunotherapeutic approaches in the management of cancer patients have been applied on the premise that they would potentiate the action of CMI against the tumor. The rationale for these approaches was based on findings that CMI was often diminished in cancer patients, partly because of chemotherapy and partly because of the cancer itself (Eilber et al., 1975; Lee et al., 1975; Mullins et al., 1975; Sigel, 1978). It is this consideration that has prompted us to undertake studies on the effects of various chemotherapeutic agents on inductive and regulatory pathways of immune responses.

6.2. CMI, Though Sovereign, Is Not Entirely Free of Antibody Influence. The Inverse Relationship of DTH and Antibody

CMI was so designated because the reactions associated with it are characterized by cellular infiltrations in which plasma cells and antibodies are either absent or present in low concentrations.

Antigen-presenting cells, helper cells, and suppressor cells are required for CMI, just as they are required for humoral responses, and there are still lingering questions about whether the same cells subserve in CMI and antibody production. There is less ambiguity about the effector cells since the task of antibody synthesis is assigned to cells of the B lineage and the ultimate effector function in CMI is vested in cells of thymic origin and macrophages. CMI effector functions include DTH, graft rejection, GVH reactions, and cytotoxicity. Although the induction of CMI and the elicitation of all these reactions can be achieved under conditions that exclude antibody production, the antibody responses that may accompany CMI induction can influence the end result. Blocking antibodies have been implicated in the inhibition of cytotoxic reactions and incriminated in interfering with the full expression of cell-mediated destruction of tumors (Kaliss, 1958; Sjogren et al., 1971; Baldwin et al., 1973; Hellstrom and Hellstrom, 1974; Robins and Baldwin, 1974). Conversely, antibodies plus complement can exert cytolytic effects and antibodies can also facilitate or actively initiate cell-mediated cytotoxic effects, i.e., the ADCC reaction. What constitutes an area of special interest is the frequently observed inverse relationship between humoral and cellular immunity where the strength of one is linked to the weakness of the other. The term *linked* is used descriptively since it may or may not denote a cause-and-effect relationship.

Nevertheless, in line with the reasoning that antibodies and antibody/antigen complexes may block CMI, the inverse relationship has been invoked as an explanation of the failure of tumor immunity.

6.3. DTH—Induction and Regulation As An Example of CMI/Antibody Interplay Under Control of Dose and Route of Antigen

In this section we have focused on DTH as one expression of CMI, not only because it may be relevant to resistance to infection and cancer, but also because recent investigations on mechanisms of DTH tolerance have brought forth an immense volume of information that provides new insights for the understanding of immune regulation by endogenous factors and exogenous agents (e.g., immunomodulatory drugs). Moreover, hypersensitivity is a perennially important problem because the obverse of its beneficial effect is its role in the etiology of immunologically inflicted tissue damage.

DTH reactions are induced by T cells bearing the $Ly1^+$ phenotype (Huber et al., 1976b; Vadas et al., 1976; Hahn et al., 1979b) (often

referred to as TDH cells) and require the participation of accessory cells, macrophages, including Langerhans cells (Toews et al., 1980). The latter are apparently also involved in antigen presentation to T cells, as previously discussed, for the generation of antibody responses. There is still an uncertainty regarding the question of TDH cells being identical with TH cells (Huber et al., 1976b; Vadas et al., 1976; Hahn et al., 1979b), but this is probably a moot question in view of the mounting evidence that TH cells represent a heterogeneous population.

Immune TDH cells can transfer specific sensitivity to naive recipients in which a DTH reaction can then be elicited by applying the relevant antigen either subcutaneously or epicutaneously (footpads of guinea pigs and mice and ears of mice are often used).

In recent years there has been a surge of interest in the mechanisms regulating the induction of expression of DTH. From earlier studies with protein antigens, it was known that the dose and route of antigen administration could determine the success of sensitization (Uhr et al., 1957; Salvin, 1958). Using SRBC as the antigen, Mackaness and colleagues demonstrated a difference in the dose and route requirements for maximal DTH antibody responses (LaGrange et al., 1974a; Mackaness et al., 1974). Although with a small dose (10^5 SRBC) approximately equally strong sensitizations were obtained by the iv or the intra-footpad routes, with a high dose (10^8SRBC) a high degree of sensitization was obtained by the footpad injection, but virtually no sensitization by the iv route. In sharp contrast, the higher dose, but not the lower dose given iv, stimulated a high antibody response. The dose and route-related dichotomy constitutes one form of evidence for the reciprocal relationship of DTH and antibody production and has been thought to represent a negative feedback by antibody on DTH induction. The inhibition of DTH by high-dose antigen is apparently not caused by antibody *per se,* but may arise from an antigen/antibody complex (Mackaness et al., 1974). There are indications from several studies that immune complexes can suppress various lymphocyte functions (Diener and Feldmann, 1972; Lee and Sigel, 1974).

The question still remaining is why the same dose of antigen (10^8 SRBC) prepared the animal for DTH when given sc and failed to do so when given iv. One could argue that this may owe to differences in the distribution and fate of the injected antigen. One could also suggest that the difference may be the result of the balance between TH and TS cells activated by the respective modes of immunization. Before touching on suppressor cells in this context, we should like to refer to the work of Sherr et al. (1979) in which FγG

coupled lymphocytes were used to induce nonresponsiveness. When injected iv, 10^6 or 10^7 cells induced DTH tolerance (without demonstrable presence of TS cells), but when injected sc the same dose of cells sensitized for DTH while simultaneously causing antibody nonresponsiveness. This split tolerance is another example of the reciprocal relationship between humoral and CMI responses.

Nowadays the most popular explanation for antigen dose effects would be based on the action of TS cells, which can interfere with the induction and/or expression of DTH. The induction of TS cells that inhibit antibody responses is favored with high dose antigen (Gershon, 1975; Whisler and Stobo, 1976; Sigel et al., 1979) and this may occur in DTH. Hahn and associates (1979a,b) induced peritoneal exudate cells in mice 4 days after immunization with low dose (10^6 iv) or high dose (10^9 iv) SRBC. Cells capable of transferring DTH (TDH cells) were found in the spleens of both groups and in exudates of mice receiving low, but not high, dose antigen, suggesting that the high dose prevented the release of these cells from the spleen to the peritoneum. In a subsequent paper from this group (Kaufmann et al., 1979), the mechanism of retention of TDH cells was analyzed. The high-dose antigen induces TS cells in the spleen (not demonstrable in the peritoneal exudate) and these cells apparently prevent the migration of TDH cells from the spleen. Such local arrest of TDH cells may therefore provide one explanation for the absence of DTH following immunization with high dose SRBC.

High-dose antigen (5×10^9 SRBC) apparently activates a heterogeneous population of TS cells capable of suppressing antibody and DTH. Whisler and Stobo (1978) were able to separate two subsets of TS cells by discontinuous BSA gradients. One subset appeared to influence both the humoral and cell-mediated responses, whereas the other subset inhibited the DTH, but not the PFC, response. *These two populations of TS cells registered a difference in susceptibility to Cy* and we shall return to them in Chapter 4, Section 5.2 as part of the discussion on the *selectivity of Cy action.*

Studies on regulation of systems in which both antibody production and CMI co-occur are complicated by the compounding of ambiguities inherent in each form of immunity and the various elements that determine whether the two responses are coordinately or inversely related, e.g., some antibodies may facilitate CMI, while antigen/antibody complexes may block CMI. Hence, there is a need to obtain additional information from systems that are relatively free of antibody production. Such systems exist in the form of DTH reactions derived from contact sensitivity and a large amount of

new and exciting information concerning regulation of DTH has come from investigations on contact sensitivity.

6.4. Contact Sensitivity (CS) Provides New Clues to Immune Induction and Regulation

6.4.1. General Considerations of Induction CS is an important problem in human medicine and its importance is bound to escalate with the increased production of industrial chemicals capable of sensitizing human beings. CS can be induced in mice by simple chemicals (haptens) such as 1-fluoro-2,4-dinitrobenzene (DNFB) or 2,4,6-trinitrocholorobenzene (TNCB, picryl chloride) painted on the skin. After a few days, a DTH reaction can be elicited by applying a dilute solution of the hapten on the ears and assayed 24–48 h later by measuring the degree of swelling. The sensitivity can be transferred by T cells, but not by serum (there are no detectable antibodies to the hapten). Based on experiments by Eisen et al. (1952), the ability of the chemical to sensitize by contact is dependent on its propensity to bind covalently to protein. The working hypothesis underlying CS is that the "ultimate" antigen recognized by the immune system is a *complex formed by the hapten and a "self" antigen*. Specific nonresponsiveness to contact sensitization can be obtained by prior treatment with the hapten intended for subsequent sensitization or closely related compounds (Chase, 1946; de Weck and Frey, 1966; Asherson and Ptak, 1970; Claman, 1976). Claman et al. (1977) have collated the findings of several investigators that demonstrate a correlation among the attributes of a given hapten: the ability to couple the proteins, to sensitize epicutaneously, to tolerize parenterally, and to stimulate sensitized lymphocytes to blastogenic reactions in vitro (Table 3). Direct proof that attachment of hapten to cell membranes constitutes the prelude to nonresponsiveness has been obtained through a series of elegant experiments by Claman and his associates utilizing hapten-derivatized cell membranes (Claman, 1976; Claman and Miller, 1976; Miller and Claman, 1976; Miller et al., 1978). The initial observation that cells with covalently bonded hapten could render animals nonresponsive to DTH with the same hapten was made by Battisto and Bloom (1966).

6.4.2. CS Negative Regulation Comes from Separate Mechanisms Claman and his associates have been using DNP-modified syngeneic and allogeneic lymphoid cells (DNP-Lc) for the

Table 3
Relative Efficiency of DNP Compounds[a]

Compound	Couple covalently to proteins	Sensitize epicutaneously	Tolerize parenterally	Stimulate DFNB-sensitized cells in vitro
DNFB	+++	+++	+++	+++
$DNBSO_3$	+	+	+	+
DNP-lysine	0	0	0	0

[a]From Claman et al., 1977.

induction of nonresponsiveness to DNFB. Tolerance that follows iv injection with DNP-Lc appears to be determined by two separate mechanisms: one is rapidly induced, long-lasting, and apparently independent of suppressor cells (nontransmissible by adoptive transfer). The other is slower in developing, transient in duration, and associated with TS cells (Claman et al., 1977; Miller et al., 1977).

6.4.2.1. Nonresponsiveness mediated by suppressor cells. Tolerance induced by iv injection of free hapten is also associated with the appearance of TS cells. This was described by Zembala and Asherson using picryl sulfonic acid (PSA) to induce nonresponsiveness to picryl chloride (PCL) (Zembala and Asherson, 1973; Asherson and Zembala, 1974). TS-related tolerance to DNFB was also induced with iv injection of $DNBSO_3Na$ (Phanuphak et al., 1974). Furthermore, suppressor cells "arise spontaneously" following painting of the skin with PCL, which is intended to establish DTH (Zembala and Asherson, 1976). Whereas at 4 days after application of PCL, lymph node and spleen cells could passively transfer sensitivity to DTH, at 8 days they could not do so and the failure of transfer occurred because of suppressor cells that were first detected at 6 days and persisted through day 11.

The suppressor cells detected under these various conditions differed in certain characteristics. Those that were induced by iv injection with PSA and those that made their appearance after injection with DNP-Lc were T cells capable of inhibiting the efferent phase of DTH, but had no effect on the afferent stage (Zembala and Asherson, 1973; Miller et al., 1978). In contrast, TS cells induced by $DNBSO_3Na$ were inhibitory of the afferent arm of the DTH response (Moorhead, 1976). TS cells acting on the efferent (TSeff) and afferent (TSaff) limbs of anti-DNFB DTH are distinguished by the dependence of TSeff, but not TSaff on a T auxiliary cell (TSaux) for media-

tion of suppression (Sy et al., 1979c). A new perspective on the nature of cells causing afferent or efferent suppression has emerged from studies by Weinberger et al. (1979, 1980) on idiotype and anti-idiotype-bearing TS cells in another (NP) DTH system (see Section 6.4.2.3).

Despite their common thymic ancestry, the cells differed in that the precursors of the PSA TS were Cy resistant, whereas the precursors of the DNP-Lc TS were Cy susceptible. The precursors of suppressor cells that appeared "spontaneously" after application of PCL were sensitive to Cy (in contrast with the precursors of cells induced by PSA) and because of this property Zembala and Asherson (1976) considered them B cells. This assumption was formally confirmed in additional studies that established the B cell nature of the suppressors (Zembala et al., 1976). There are other reports attesting to this role of B cells. Suppression by B cells of DTH induced by ovalbumin was previously reported by Katz et al. (1974a) and B cells have been also linked to suppression of other T cell functions (Gorczynski, 1974; LaGrange et al., 1974b; Mackaness et al., 1974; Neta and Salvin, 1976). Nevertheless, suppressor cells arising "spontaneously" following the percutaneous application of supraoptimal DNFB whose precursors are also Cy sensitive have been shown to be T cells (Sy et al., 1977). However, in view of the recent findings that the TSaux cells required for TSeff-mediated suppression are sensitive to Cy, the question remains whether the targets of Cy action are the precursors of TSeff or TSaux cells.

We shall consider sensitivity to Cy in the discussion of differential action of this drug against various immunologic functions and cellular sets (see Chapter 4, Section 5). However, the findings cited above are pertinent at this juncture *because Cy has helped to characterize the diverse forms of non-responsiveness in DTH.* Thus, whereas in the majority of instances *the slowly developing, evanescent nonresponsiveness associated with suppressor cells can be prevented by Cy, the immediate suppressor cell-independent host tolerance cannot be forestalled by pretreatment with Cy.* Moreover, there is selectivity with regard to certain types of suppressor as well as effector cells.

6.4.2.2. Tolerance in the absence of TS; clonal deletion; anti-idiotype (receptor blockade). We have already alluded to this type of tolerance in the citations of results obtained with humoral responses by Cinader and Nakano (1979) and Sherr et al. (1979). Other investigators have demonstrated TS-independent nonresponsiveness in DTH to SRBC (Kaufmann et al., unpub-

lished), to lysozyme (Kojima and Egashira, 1979) and to horse gamma globulin and cytochrome C conjugated to lymphoid cells (Miller et al., 1979). This nonresponsiveness cannot be transferred to recipients and is believed to owe to clonal deletion. As will be discussed subsequently, the mechanism of TS-independent nonresponsiveness may be rooted in idiotype/anti-idiotype interactions or clonal inhibition rather than deletion. Moreover, in the studies of Kaufmann and colleagues on anti-SRBC DTH (unpublished) evidence was provided that, even though transferable TS cells could not be detected in the pathway that these investigators termed "intrinsic unresponsiveness," experiments with Cy in reverse adoptive transfer intimated that this form of nonresponsiveness in hosts receiving supraoptimal antigen dose was associated with T precursor cells sensitive to Cy.

Of special interest are the recent findings of Miller et al. (1980) that the two pathways to nonresponsiveness initiated by the injection of DNP-Lc differ in their requirements of MHC control. The pathway leading to the induction of TS required that the nominal antigen (e.g., DNP) be presented in association with products of the MHC, apparently of the *H-2D* region. On the other hand, based on the results presented in that paper and other data (Conlon et al., 1979), the pathway to tolerance without TS activation appeared to depend on input from the *I* region. There is a report (Li and Scott, 1980) indicating that some forms of TS-independent tolerance may not require MHC control. Moreover, as already cited, a high concentration of antigen may overcome MHC restrictions, since a high hapten density on lymphocytes used to induce tolerance generated specificity for hapten alone, whereas low hapten density evoked a specificity for hapten plus self-antigen (Pierres et al., 1980).

A third type of nonresponsiveness occurs late after sensitization. Studies in Claman's laboratory (Sy et al., 1979d) have demonstrated that this form of suppression is caused by anti-idiotype antibodies. The antibodies were detected in the sera of animals 9–15 days after sensitization and blocked the ability of DNFB immune lymph node cells to transfer immunity. The sera did not react with DNP, but could be adsorbed by DNFB immune lymph node cells (and not by normal lymph node cells). The suppressor activity of the sera could also be adsorbed to and eluted from affinity columns conjugated with purified mouse DNP antibody. This nonresponsiveness owing to receptor blockade constitutes an additional regulatory pathway in the mechanism controlling DTH and provides further proof for the role of anti-idiotype affecting T cell functions (Binz and Wigzell, 1977a, b). It is possible that the serum factor

blocking DTH reactions in the experiments of Mackaness et al. (1974) may have also contained the anti-idiotype.

6.4.2.3. Regulation by idiotype/anti-idiotype interaction. We have previously made reference to the idiotype/anti-idiotype interaction in regulatory networks. A considerable amount of new information concerning this aspect of regulation of DTH has come from investigations in Benacerraf's laboratory utilizing *p*-azobenzenearsonate (ABA) as antigen. Intravenous injection of ABA-coupled syngeneic spleen cells stimulates hapten-specific suppressor T cells (Bach et al., 1978). These cells produce an ABA-specific TSF that suppresses ABA-specific DTH. These factors contain determinants of the MHC as well as an ABA-binding structure identified as the cross-reactive idiotype (CRI) found in 20–70% of anti-ABA antibodies in the strain of mice immunized with ABA-KLH (Bach et al., 1979; Greene et al., 1979). It appears that the two products, although coded by genes on separate chromosomes (*H-2* genes on chromosome 17 and genes controlling the idiotype) are expressed on the same TS cells and possibly assembled as an antigen binding complex (see Section 5.5.1). Taking advantage of the fact that ABA-coupled syngeneic lymphocytes can induce DTH when administered subcutaneously, Sy et al. (1979b) were able to show that this response could be diminished or abrogated by treating the animals with anti-idiotype antibodies (antibody raised in rabbits against ABA-CRI). It was further demonstrated through a series of elegant experiments that, in order for anti-idiotype to be suppressive, it had to be administered as the entire molecule by the iv route (Sy et al., 1980a). The emerging TS cells associated with the inhibition of DTH were shown to display ABA-specific idiotypes. When anti-idiotype F(ab')2 fragments were injected iv they caused induction of DTH rather than suppression. Furthermore, whole anti-idiotype injected sc also primed the animals for DTH that was associated with TDH cells bearing ABA-specific idiotype. Positive DTH induction also resulted when anti-idiotype antibody was injected subsequent to treatment with 50 mg/kg of Cy (Cy presumably eliminating precursors of TS cells). Thus, anti-idiotype could activate T effector cells for DTH or T cells capable of suppressing DTH, suggesting that anti-idiotype antibody could simulate functionally the action of antigen and that it presumably acted through the recognition of CRI on the relevant precursor cells. There is a counterpart for the action of anti-idiotype antibody and that is the action of idiotype on the induction of TS cells. Sy et al. (1979a) have demonstrated that ABA-CRI coupled with syngeneic spleen cells can in-

duce TS cells. In the most recent paper of the series, Sy et al. (1980c) presented evidence linking the actions of idiotype and anti-idiotype. TS1 cells generated by iv injection of mice with ABA-coupled syngeneic splenocytes displayed the CRI and their TSF contained CRI and *I-J* determinants. This TSF when injected into normal mice induced TS2 cells that lacked the idiotype, but displayed a receptor for the idiotype. These data from the ABA series of experiments constitute in part the basis for the updated model D.

Investigations on regulation of DTH to 4-hydroxy-3-nitrophenyl acetyl have charaterized two subsets of suppressor cells by their site of action: one acts in the induction (afferent) phase of DTH and the other in the effector (efferent) phase (Weinberger et al., 1979, 1980). The first subset appears to be analogous with TS1 of other systems and bears the antigen-specific idiotype, whereas the second appears to be analogous with TS2 and expresses a receptor for the idiotype of the first set. These findings provide further proof for the existence of an idiotype/anti-idiotype regulatory network originally envisioned by Jerne in 1974. Although no MHC restriction was demonstrated for the first set, the second set of suppressor cells is under restriction of genes in the *I-A* subregion and genes regulating VH. In the context of selective action by immunosuppressive agents, it is of interest to note that *Cy could eliminate precursors of the induction phase suppressor cells, but was ineffective against precursors of effector suppressor cells.*

6.4.3. The Effects of the Routes of Immunization Seem to be Determined by Delicately Poised Mechanism(s) Sensitive to Diverse Signals

It may be helpful to collate the instances where the route of sensitization has been shown to determine the end result: a positive DTH reaction, suppression of DTH, or a split tolerance. Mackaness and his colleagues provided one such example in which 10^8 SRBC cells administered by the sc route sensitized for DTH, but by the iv route failed to induce DTH, though immunizing effectively for antibody production (LaGrange et al., 1974a; Mackaness et al., 1974). As already reviewed, hapten-derivatized syngeneic spleen cells administered by the iv route lead to tolerance. Greene et al. (1978) not only confirmed this phenomenon, but in addition demonstrated that the same conjugated cells administered by the sc route evoked a positive CS that could be transferred with T cells. Moreover, they found that TNP-derivatized syngeneic macrophages were superior to syngeneic lymphocytes in the induction of CS, presumably because of a more efficient manner for presenting antigen to T cells. A similar antigen route dependence

was demonstrated for protein antigens. Using horse gamma globulin and cytochrome C conjugated to lymphoid cells, Miller et al. (1979) found that DTH follows sc immunization, whereas nonresponsiveness, both TS-associated and TS-independent tolerance, results from iv injection. Abbas et al. (1980) have coupled syngeneic splenocytes to isologous myeloma proteins and have shown that such cells could activate TDH and induce DTH if injected sc. When injected iv these cells induced nonresponsiveness associated with TS. These suppressor cells that could inhibit both the development of DTH and antibody production were characterized as nylon wool-nonadherent $I\text{-}J^+$ T cells whose precursors were sensitive to Cy. The TDH cells induced by sc immunization and the TS activated by iv immunization were idiotype-specific, recognizing the appropriate idiotype of the immunizing myeloma protein and not that of the other myelomas.

The shift in the immunologic balance dictated by the route of immunization is not absolute, however, since there are states of split responsiveness. Thus, the sc route of injection *can induce a state of PFC nonresponsiveness and simultaneously prime for DTH* (Sherr et al., 1979).

Another interesting form of split tolerance dependent on the physical nature of the immunizing carrier was previously described by Ptak and Rozycka (1977). TNP-labeled syngeneic RBC rendered mice tolerant to picryl chloride CS, but did not affect their anti-TNP humoral response. An inverted effect (inhibition of antibody production without suppression of CS) followed immunization with TNP-labeled isologous IgG. Both responses were suppressed by TNP-coupled macrophages.

The co-occurrence of positive and negative responses intimates that the balance is delicately poised and probably subject to tipping by endogenous or exogenous factors. A report by Cleveland and Claman (1980) shows that the balance may be shifted so that a mode of immunization that normally leads to nonresponsiveness in DTH can be overridden by a maneuver that apparently provides an additional signal for the immune mechanism. In their experiments, animals were given the DNP-Lc tolerogen and simultaneously or soon thereafter injected with con A. The result was development of DTH. The authors theorize that two signals are required to activate TDH cells: one provided by antigen complexed to a product of the MHC; the other by the nonspecifically activated T cell or a product from such a cell. These results suggest that the relevant TDH cells are still present and are probably blocked (receptor blockade), thus justifying the term *clonal inhibition,* a concept ad-

vanced by Claman, as opposed to *clonal deletion*. The second signal may originate from a variety of sources including macrophages and their monokines. Hence, sc injection into a macrophage-rich area is more likely to generate the two signals than an iv injection that bypasses an immediate encounter with macrophages. An hypothesis on the two signals is presented by Claman (1979).

The existence of split tolerance may represent a dichotomy at one of two (or more) levels. It may be that the second positive signal for TDH cells is read as a negative signal for TH cells. Alternatively, some cells providing help or amplification for one response may perform an opposite function in another. One such example is provided by the work of Ramshaw and colleagues (1976) in which it was found that T cells from mice expressing a positive antibody response to HRBC specifically suppressed the development of DTH. The same group earlier reported that a combination of SOI-Cy that, under their experimental conditions, led to TS-linked PFC unresponsiveness to HRBC permitted full-scale development of anti-HRBC DTH (Ramshaw et al., 1977).

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Glossary

ABA	*p*-Azobenzene-arsonate
ADCC	Antibody-dependent cellular cytotoxicity
APA	Azophenylarsonate
B	B cell
CMI	Cell-mediated immunity
con A	Concanavalin A
CRI	Cross-reactive idiotype
CS	Contact sensitivity
CTL	Cytotoxic T lymphocyte
Cy	Cyclophosphamide
DNF B	1-Fluoro-2,4-dinitrobenzene
DNP	Dinitrophenyl
DTH	Delayed-type hypersensitivity
Fc	Fraction crystallizable, C terminal half of heavy chain

FγG	Fowl gamma globulin
GAT	LGlutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}
GLϕ	L-Glutamic acid^{53}-L-lysine^{36}-L -phenylalanine^{11}
GT	L-Glutamic acid^{50}-L- tyrosine^{50}
GVH	Graft-vs-host
GZ	*E. coli* beta-galactosidase
HF	Helper factor(s)
IFA	Incomplete Freund's adjuvant
IgCH	Constant part of heavy chain of Ig
Ir	Immune response gene
KLH	Keyhole limpet hemocyanin
LAF	Lymphocyte activating factor
LPF	Lymphocyte promoting factor
LPS	Lipopolysaccharide
Mϕ	Macrophage
MHC	Major histocompatibility complex
MIF	Migration inhibition factor
NP	4-Hydroxy-3-nitrophenyl acetyl
NR	Nonresponder
PCL	Picryl chloride
PFC	Plaque-forming cell
PHA	Phytohemagglutinin
PSA	Picryl sulfonic acid
R	Responder
SIII	Pneumococcal polysaccharide
SF	Suppressor factor
SIRS	Soluble immune response suppressor
SOI	Supraoptimal immunization
SRBC	Sheep red blood cells
T	T cell
TA	T amplifier
TCGF	T cell growth factor
TCS	T contrasuppressor
TD	Thymus-dependent
TDH	T cells responsible for DTH
(TG)-A . . . L	Poly-L-(tyr,glu)-poly-D,-L-ala . . . poly-L-lys
TH	T helper cell
THF	T helper factor
TI	Thymus-independent
TNCB	Trinitrochlorobenzene
TNP	Trinitrophenol
TS	T suppressor cell
TS_{aff}	T suppressor of the afferent arm

TS_{aux}	T suppressor of the auxiliary cell
TS_{eff}	T suppressor of the efferent arm
TSF	T suppressor factor
VH	Variable heavy

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Chapter 4

Immunosuppressive Agents—Their Action on Inductive and Regulatory Pathways

The Differential Effects of Agents Used Clinically or Experimentally in the Treatment of Cancer

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1. Variables That Determine the Effect of Cancer Therapeutic Agents on Antibody Production

1.1. Suppression of Antibody Production in the Primary Response: Some Agents Preferentially Affect Stimulated (Proliferating) Cells, Others Are More Effective Against Resting Systems. TD and TI Responses Are Affected Differently

Although a considerable amount of information has been gathered regarding the effect of chemotherapeutic agents on the human immune response, the data are sometimes difficult to interpret because humans, especially adults, have usually experienced many exposures to bacterial, viral, and chemical antigens that may have sensitized their immune systems. Thus, a given vaccine may not represent a truly primary immunization. Furthermore, ethical considerations preclude manipulative approaches designed to probe the operations of the immune mechanism in vivo. For this reason, experimental animals of relatively young age (thus shorter exposure to environmental antigens) of known genetic background are preferable.

A comparison of the action of several immunosuppressive agents administered to mice was recently published by Berenbaum (1979). As summarized in Table 1 for Class I agents, the results demonstrate the importance of such variables as the nature of the agent, the nature of the antigen, and the temporal relationships between the two.

The Class II agents, 6-mercaptopurine, 6-thioguanine, 5-fluorouracil, methotrexate, vincaleukoblastine, and vincristine have little or no effect on antibody production when given prior to immunization, but cause suppression when administered 1 or 2 days after immunization. Agents that Berenbaum categorizes as Class I exert suppressive effects on antibody production when given either before or after antigen. The definition of Class I has been altered from the one proposed initially by the author in the early 1960s (Berenbaum, 1961, 1962) when on the basis of antibody titers the agents were found to be suppressive only when given prior to antigen. By adopting PFC assays in his recent work, Berenbaum was able to demonstrate more precisely, and more quantitatively, the effects of drugs at various states of immunization.

Table 1
Relative Effects of Immunosuppressive Agents (Class I) on Primary Anti-SRBC and Anti-LPS Responses: PFC Assay in Balb/c Mice[a,b]

	Prior to antigen		After antigen	
Agent	SRBC	LPS	SRBC	LPS
Radiation, 300 R (Ag ip)	↓ ↓ ↓	↓ ↓	↓	↓ ↓
Cholera toxin, 1μ iv, (Ag ip)	↓ ↓ ↓	– or ↓	↓	–
Cortisone acetate, 500 mg/kg sc, (Ag ip)	↓ ↓ ↓	↓	↓ ↓ – ↓	↓
Busul 100 mg/kg sc, (Ag ip)	↓ ↓	↓ ↓	– or ↓	– or ↓
Cyclophosphamide, 300 mg/kg sc (Ag iv)	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓
Aniline mustard, 45 mg/kg sc, (Ag iv)	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓ – ↓ ↓	↓ ↓ ↓ – ↓ ↓
Melphalan 12 mg/kg sc, (Ag iv)	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓
Nitrogen mustard 12 mg/kg sc, (Ag iv)	– or ↓	↓	↓ ↓ ↓	↓ ↓ ↓
Mitoclomine, 200 mg/kg ip, (Ag iv)	↓ ↓	↓ ↓ ↓	↓	–
Mitoclomine analogs:				
WB 4247, 5 mg/kg ip, (Ag iv)	–	↓ ↓	–	–
WB 4291, 100 mg/kg ip, (Ag iv)	↓	↓ ↓	↓ ↓	↓ ↓
WB 4325, 5 mg/kg ip, (Ag iv)	–	↓ ↓	– or ↓	↓

[a]From Berenbaum, 1979.
[b]– = no suppression; ↓ = approximately 60–90% suppression (fraction of control); ↓ ↓ = 90–99% suppression (fraction of control).

Even though, as newly defined by Berenbaum, Class I denotes agents that inhibit antibody responses when applied "at any time over a fairly broad period from before the administration of antigen to 1–2 days afterward," perusal of Table 1 indicates that there is a need for further subdivision within Class I based on consideration of comparative effects before and after antigen and selectivity with regard to the nature of antigen, i.e., thymus-dependent (TD) or thymus-independent (TI). Thus cyclophosphamide (Cy), aniline mustard, and melphalan (Mel) appears to be equally suppressive of antibody responses to sheep red blood cells (SRBC) and lipopolysaccharide (LPS) when given either prior to or subsequent to anti-

gen. These drugs would therefore appear to qualify as hard-core Class I with grossly analogous activity against resting or proliferating cells involved in TI or TD responses, at least with LPS and SRBC as representative antigens. In contrast, cholera toxin, cortisone acetate, busulfan, and irradiation appear to be more suppressive of the TD response when given before antigen than after antigen. As regards the TI response, radiation and cortisone have approximately the same moderate effect prior to and after antigen, cholera toxin has no effect at either time, and busulfan is more suppressive before antigenic stimulation.

A few years ago Lin (1973) compared the effects of cancer therapeutic agents on anti-SRBC PFC. The findings are summarized in Table 2. Our laboratory has been investigating the effects of various agents on (1) primary and (2) secondary immune responses to SRBC, (3) primary responses to pneumococcal and polysaccharide (SIII) as a thymus-independent antigen, (4) induction and expression of suppressor cells, and (5) various types of cell-mediated reactions. The findings in (1) and (3) are given in Table 3. The three laboratories involved in this work employed assays of antibody plaque-forming cells in mouse spleens, but the strains of mice differed: Balb/c in Berenbaum's work, AKR in Lin's, and BDF_1 in ours.

Table 2
Relative Effects of Immunosuppressive Agents on Primary Anti-SRBC Response: PFC Assays in AKR Mice.[a]

Agent	Prior to antigen, day −1	After antigen, day +1
Radiation, 300 R	↓ ↓ ↓	↓ ↓
Nitrogen mustard, 2.5 mg/kg	↓ ↓	↓ ↓
Methotrexate, 50 mg/kg	−	↓ ↓ ↓
5-Fluorouracil, 100 mg/kg	−	↓ ↓
Ara-C, 100 mg/kg	−	↓
Vincristine, 1 mg/kg	−	↓ ↓
Actinomycin D, 0.5 mg/kg	↓ ↓	↓ ↓ ↓
Cyclophosphamide, 100 mg/kg	↓	↓ ↓ ↓

[a]From Lin, 1973.
[b]− = no suppression; ↓ = approximately 60–90% suppression (fraction of control); ↓ ↓ = 90–99% suppression (fraction of control). Antigen and drugs injected ip.

Table 3
Effect of Cancer Chemotherapeutic Agents on Primary Immune Responses to SRBC and *Pneumococcus polysaccharide* (SIII)[a,b]

	Percent inhibition of PFC			
	Anti-SRBC		Anti-SIII	
Agent	Pre	Post	Pre	Post
Actinomycin D, 0.4 mg/kg	70	94	−195*	−103
Melphalan, 8 mg/kg	99	98	V	99
5-FU, 100 mg/kg	NT	97	NT	60
Cyclophosphamide, 100 mg/kg	99	99	26	98
Ara-C, 1600 mg/kg	42	99	NT	NT
Ara-C, 100 mg/kg	NT	95	NT	NT
Anguidine, 32 mg/kg	NC	96	NT	NT
Bruceantin, 4 mg/kg	13	51	NT	NT
Adriamycin, 8 mg/kg	−15	80	NT	NT
Daunorubicin, 8 mg/kg	29	42	NT	NT
BCNU, 30 mg/kg	98	97	86	97
MeCCNU, 30 mg/kg	13	NC	83	78
CCNU, 30 mg/kg	84	96	NT	NT

[a]From the authors' laboratory.
[b](−)denotes percent increase in response; NT = not tested; NC = not changed; V = variable. Ag given ip; Drug given ip.

Drugs that exert their effect through inhibition of DNA synthesis in proliferating cells (after antigenic stimulation), i.e., Class II agents, gave highly concordant results (see vincristine results in the three laboratories; 5-FU, Lin and Berenbaum; methotrexate, Lin and Berenbaum; cytosine arabinoside, Lin and our data). Anguidine, Bruceantin, Adriamycin, and Daunorubicin tested in our laboratory also demonstrated suppression of the anti-SRBC response when given after antigen, but not before antigen. Although these drugs varied in the intensity of the suppression, they would appear to qualify for Berenbaum's Class II. It is generally assumed that these agents suppress immune responses by virtue of interfering with DNA synthesis in proliferating cells, presumably B and T cells. The fact that these drugs are inactive prior to immunization implies that they are not interfering with the function of macrophages in presenting antigen to T cells and that they are not damaging to resting lymphocytes. These experiments do not disclose whether the drugs are equally effective against activated B and T cells. Moreover, in line with the prior discussion of multiple

TH cell participation in providing help to B cells where different helper cells may interact in a sequential fashion, one providing initial help and another supplemental or amplifying help, it is possible that the drug may eliminate one member of the pair, but not the other. Alternatively, a drug may affect the B cell response that is supported by one T cell, but not by the other T cell. In fact, the findings from studies of Muirhead and Cudkowicz (1978) demonstrate such a selective activity for vinblastine. This drug inhibited the late-occurring amplifier T cell-associated event, but not the initial helper T cell-directed event. Functionally, the two types of T cells in this study resemble the two helper cells described by Tada et al. (1978) and by Keller et al. (1980), but it is not possible to decide whether the TA cell of Muirhead and Cudkowicz is the same as the cell providing nonspecific help in the work of the other two groups. The effects of several immunomodulatory agents have been assessed against specific cellular targets, as will be related in subsequent sections. There is an obvious need to submit agents of Class II to a similar dissective analysis.

When we compare the results of the three laboratories concerning action of Class I agents, we note the following: there appears to be a fairly good uniformity with respect to the effects of Cy and Mel on anti-SRBC responses, i.e., inhibition when the drugs are given either prior to or after antigen. Although nitrogen mustard (NM) is listed in Berenbaum's Class I, his tests show that 2.5 mg/kg has little suppressive activity when given prior to antigen, but considerable activity when given after antigen. On the basis of these findings, NM would more appropriately fit into Class II. It is interesting that this drug behaves more like a Class I agent in AKR mice judging by results obtained by Lin (1973). Although radiation (300 rads) was more suppressive prior to immunization with SRBC than afterwards, this difference was not as striking in Lin's study (AKR mice) as in Berenbaum's study (Balb/c mice). We do not know whether this reflects a difference in the genetic background of the mice. Actinomycin D tested by Lin and in our laboratory was found to be suppressive before and after immunization with SRBC, but the effect was greater when the drug was given after antigen. Before turning attention to a comparison of the effects on TI responses, it should be noted that the work in our laboratory also demonstrated that two nitrosoureas, BCNU and CCNU, caused equivalent suppression of anti-SRBC responses when administered prior to or after antigen. In contrast, MeCCNU was ineffective at either time.

1.2. Some Drugs Seem to Discriminate Among B Cell Subsets

A different picture emerges when the results in our laboratory using SIII and the results of Berenbaum's studies using LPS as TI antigens are compared. Both Cy and Mel had little or no effect on the anti-SIII response in most experiments when administered 2 days prior to antigen (although the effect of Mel was variable), but both were strongly suppressive when injected 2 days after antigen, suggesting that these drugs were less effective against resting B cells. In view of Berenbaum's results showing that the response to LPS was suppressed by Cy and Mel given prior to antigen, the conclusion seems warranted that Cy affects B cell responses to TI-1 and TI-2 antigens differently. (See Chapter 3 for a description of TI-1 and TI-2 antigens.) The former are apparently damaged both prior to and after entry into proliferation, while the latter are susceptible only when in the proliferative state. However, there is a need for caution in interpreting these results with respect to TI-2 antigens. According to Howard and Shand (1979), pretreatment with Cy does inhibit the response to dextran (De). It should be recalled that when serving as a carrier for TNP, De is also classified as a TI-2 antigen (Mosier et al., 1977). Nevertheless, differences in immunogenic and tolerogenic properties of haptenized dextran and SIII polysaccharide have been noted (Desaymard and Howard, 1975; Howard and Hale, 1976). Moreover the TI-2 classification may not apply to all forms of De, i.e., native dextran vs DNP- or TNP-dextran. Dextran sulfate behaves more like a TI-1 antigen (Gronowicz et al., 1974). It is possible that the SIII and De may activate different subsets of B cells. Certain forms of dextran are known to cause polyclonal antibody responses as well as mitogenic effects involving what appears to be a heterogeneity among B cells (Bick and Shreffler, 1979), which further complicates the interpretation of the action of suppressive agents.

The resting B cells with specificity for SIII antigen are not the only B cells resistant to Cy. Duclos et al. (1977) demonstrated that pretreatment of mice with 40 mg/kg of Cy not only failed to impair the response of splenocytes to TNP coupled to polyacrylamide carrier (a TI antigen), but in fact caused an increase in response in vitro presumably by virtue of eliminating suppressor cells. Nevertheless, the B cells in this system were not entirely resistant, as shown by experiments in nude mice where a decrease in antibody response

was observed. The authors conclude that the results indicate a shift in balance owing to the effect of Cy on the suppressor cells and B cells. Apparently the elimination of suppressor cells in euthymic mice allows for an increased response, whereas in nude mice lacking T suppressor cells, the effect is more noticeable against B cells. Inhibition of responses to TI antigens will be discussed in the context of the effect of Cy on B cell receptors (see Section 5).

Our experiments with SRBC did not distinguish between effects of alkylating agents on T or B cells in the primary response, since the suppression may have been the result of action on either or both classes of cells. We do, however, have some preliminary indications from adoptive transfers of cells from euthymic to nude mice that resting as well as antigen-activated helper T cells involved in the primary SRBC response are resistant to Cy (Ghaffar et al., 1981). More will be said about the effects of Cy in Section 5.

In general, our data and those of Berenbaum agree in the overall greater vulnerability of the TD responses compared to TI responses. In his work radiation, cortisone acetate, cholera toxin, and L-asparaginase had a profound effect on the SRBC response and little if any effect on the LPS response, while in our work actinomycin D, melphalan, Cy, 5-FU, and cytosine arabinoside showed this kind of differential activity with SIII being the TI antigen. Actinomycin D, 5-FU, and cytosine arabinoside actually enhanced the TI response. Mitoclomine and its analogs and MeCCNU appear to represent exceptions to this trend. These were more effective against TI responses. Berenbaum considers three possible explanations for this type of selectivity of the naphthyl-substituted mustards: they may be relatively more selective for B cells, or they may be relatively more selective for the subsets of B cells that mediate T-independent responses, or they may interfere with the macrophage presentation of antigen to B cells. The last point needs some amplification. Although macrophages or macrophage-like cells are required for antigen presentation to T cells, their role in TI antigen presentation to B cells is not entirely clear. According to Kirkland et al. (1980), B cells are the primary presentors of TNP-Ficoll to other B cells (the two types of B cells being of apparently different subsets) and macrophage-like cells appear to play a secondary role. This diversity of function, i.e., antigen presentation vs response to antigen provides additional evidence for the heterogeneity of B cells.

These considerations outlined for mitoclomine may also apply to MeCCNU, which in our studies showed a similar preferential immunosuppression of the anti-SIII response and had no suppressive effect on anti-SRBC PFC production.

1.3. Differential Effects on Memory Cell Precursors and Effectors

In Table 4 are presented data on the effects of several drugs against the induction and expression of immunologic memory. The drugs were given at one of the four points in time in relation to primary immunization and secondary challenge: 2 days prior to priming (pre 1^0); 2 days post priming (post 1^0); 2 days prior to challenge (pre 2^0); and 2 days post challenge (post 2^0). The primary and secondary immunizations were made with 1×10^8 SRBC ip with a time interval of 16 days between the two injections. It can be seen that compared to their effects against primary responses the drugs had negligible to moderate activity against induction of memory, suggesting that some of these drugs could discriminate between precursors of cells involved in the primary response and precursors of memory. This distinction held only prior to activation by antigen. When the drugs were applied to the system 2 days after activation, the secondary response was impaired in all cases tested and impairment was especially pronounced with Mel and Cy. Memory was also vulnerable to the drugs administered 2 days prior to or after the secondary challenge. The resistance of the secondary response was not the result of insensitivity of the plasma cells to Cy because, at the time of secondary challenge, there would have been

Table 4
Effect of Cancer Chemotherapeutic Agents on Secondary Immune to SRBC[a,b]

Agent	Percent inhibition of PFC[c]			
	Pre 1^0	Post 1^0	Pre 2^0	Post 2^0
Actinomycin D, 0.4 mg/kg	42	NT	88	NT
Melphalan, 8 mg/kg	19	68	82	86
5-FU, 100 mg/kg	26	NT	52	NT
Cyclophosphamide, 100 mg/kg	NC	85	86	99
Ara-C, 1600 mg/kg	−84	NT	92	NT
Bruceantin, 4 mg/kg	33	NT	55	NT
Adriamycin, 8 mg/kg	NC	NT	61	NT
BCNU, 30 mg/kg	NC	59	88	76
MeCCNU, 30 mg/kg	−20	37	74	NC
CCNU, 30 mg/kg	NC	34	66	99

[a]Pre 1^0 = 2 days before priming; post 1^0 = 2 days after priming; pre 2^0 = 2 days before secondary; post 2^0 = 2 days after secondary. NT not tested; NC not changed. (−) denotes percent increase in response. Ag given ip: Drug given ip.

relatively few SRBC plasma cells since the peak of the primary response had already subsided. It thus appears that the precursors of memory cells differ from actively committed memory cells as well as from resting or activated cells involved in generating the primary immune response *by being resistant* to several immunosuppressive drugs, including the alkylating agents. As discussed in Section 5, Cy facilitates the establishment of immunologic tolerance when administered near the time of immunization. This effect probably results at least in part from action on activated memory cells since, unlike memory precursors, activated memory cells are susceptible to Cy. Long-term persistance of tolerance requires continual administration of antigen without Cy, but the precise mechanism underlying this requirement in relation to initial action of antigen plus Cy remains unknown. Future experiments employing separated cellular populations and adoptive transfer may disclose the individual vulnerability of each cell type and subset.

The results of the studies on the effects of immunosuppressive agents on antibody responses may be summarized as follows. Although it is still true that some agents are suppressive only when administered after the fact (antigenic stimulation) and others are equally suppressive before or after, this form of selectivity is only one aspect of their modes of action. The findings that some of these agents manifest differences in their effects against TD and TI antigens, as well as against cells participating in primary as opposed to secondary responses, suggest that these agents exert selective effects against individual cellular classes or subsets. These observations, as well as findings to be reported in subsequent sections, intimate that some of these agents may be useful probes for studies of the nature and function of such subsets.

2. Diversity of Effects of Immunosuppressive Drugs on CMI

We compared the effects of several chemotherapeutic agents on reactions associated with CMI. BDF mice were used for mitogen-induced blastogenic transformation, delayed type hypersensitivity (DTH) and antibody dependent cellular cytotoxicity (ADCC). C3H mice were used for macrophage cytotoxicity assays and Balb/c mice for particle clearance from circulation. Drugs were injected ip in the same concentrations as used for suppression of antibody responses and the tests were performed at stated intervals.

Con A and LPS were the principal stimulators of blastogenic transformation reactions; SRBC were used for immunization to DTH; *C. parvum* served as the activator of cytotoxic macrophages; chicken RBC coated with specific IgG antibody were the targets for ADCC reactions. The blastogenic responses, ADCC, and macrophage cytotoxicity effects were measured as previously described (Ghaffar and Cullen, 1976; Ghaffar et al., 1976; Ghaffar et al., 1978). To measure particle clearance, 0.2 mL of ^{51}Cr-labeled SRBC were injected iv into a lateral tail vein of mice. The mice were then bled from the eye at various time intervals with capillary tubes and the drawn blood was counted in a gamma counter (Sljivic and Warr, 1981). For DTH, mice were immunized with 1×10^7 SRBC sc and after 6 days were challenged with 1×10^8 SRBC injected into the footpad. Swelling of the footpad was measured 24 h later.

2.1. Dissociation of Antiproliferative, Anticytotoxic, and Anti-PFC Effects

In a preliminary experiment (Fig. 1), treatment with BCNU resulted in the impairment of lymphocyte proliferation assayed by ^{3}H-TdR incorporation of splenocytes cultured 2 days after treatment. Sig-

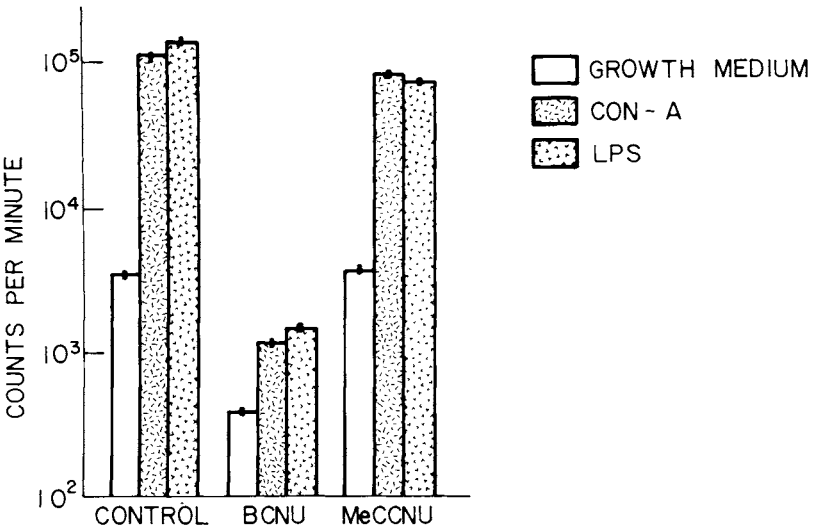

Fig. 1. Effect of BCNU and MeCCNU on the mitogenic response to LPS and con A in BDF_1 mice. BCNU or MeCCNU (30 mg/kg) was injected ip; splenocytes tested 2 days later.

nificantly reduced DNA synthesis was found in unstimulated cultures as well as in cultures activated by con A (T cells) and LPS (B cells). In contrast, treatment with the same dose of MeCCNU caused only a slight suppression in the responses to the T and B mitogens and no suppression of the spontaneous proliferation. These results appear to be consistent with the effects exerted by these drugs on humoral responses to SRBC (Table 3). In subsequent experiments mice were treated with each of the alkylating agents and blastogenic responses of their splenocytes to con A were assayed at several intervals (Fig. 2). BCNU was the most suppressive of the alkylating agents, causing a precipitous ablation of the response, which failed to recover over a period of 9 days of observation. CCNU, MeCCNU, Cy, and Mel caused relatively little impairment in the proliferative response by day 2 but the degree of suppression increased to a maximum between day 4 and day 6. Recovery from inhibition by CCNU and MeCCNU was fully visible by day 9 (restoration of response following treatment with Cy and Mel

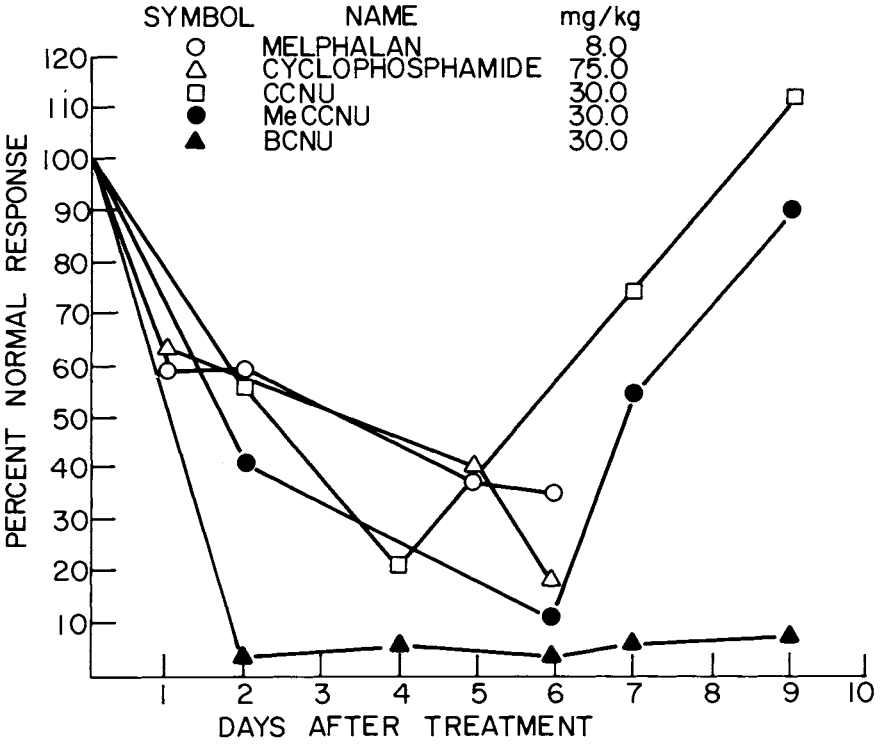

Fig. 2. Con A mitogenic responses of splenocytes obtained at several intervals from mice treated with alkylating agents.

was not ascertained). These findings point to a quantitative disparity in the action of alkylating agents on the blastogenic and antibody responses, reinforcing the premise that the two processes, though related, are separate and distinct. Thus, though there was a good correlation in the total suppression of both responses by BCNU, there was a lesser correlation with the others as Mel, Cy, and CCNU abrogated the humoral response, but *caused relatively milder or more gradual suppression* of the T cell proliferative response. Moreover, MeCCNU, which was ineffective against the SRBC antibody response, was comparable to the other alkylating agents in its suppressive effect on the blastogenic response.

We want to call special attention to the effect of Cy. As shown in Fig. 2, this drug caused approximately 40% inhibition of response to con A on day 2 after treatment and there was a further loss in response with time. Suppression of proliferative response to PHA and alloantigens by in vivo treatment with Cy has been previously reported by Milton et al. (1976). In another study, Lopez et al. (1980) measured the effect of Cy on blastogenic responses of splenocytes of normal and tumor-bearing mice using con A, PHA, and LPS as mitogens and mammary tumor extract as a specific antigen. On day 2 after treatment the con A response was inhibited by 44–54%, the PHA response by 63–75%, the LPS response was diminished by 84%, and the antitumor antigen response was decreased by 66%. These authors also measured the effect of Cy on the induction of cytotoxicity by PHA and found that Cy caused no decrease in this response. The drug therefore suppressed the proliferative capacity of T and B cells, including cells sensitized to a tumor antigen, but did not reduce the T cells' capability to perform a cytotoxic function triggered by PHA. These results imply that certain functions of T cells escape the damaging effects of Cy even though DNA synthesis may be interrupted. The cytotoxic activity of the nonproliferating cells is probably exerted through a soluble mediator (Lopez et al., 1980). In similar experiments, MacDonald and Lees (1979) have demonstrated that cytosine arabinoside (ARA-C) does not significantly inhibit the generation of cytotoxic activity by con A, even when used at concentrations sufficient to inhibit DNA synthesis.

What is of special interest is that Cy may also affect the proliferative response indirectly. There is a report by Milton et al. (1976) that lymph node cells from Cy-treated mice can depress the PHA response of normal lymphocytes when the former are harvested 1 or 2 weeks after treatment, but not earlier. This suggests that the effect probably did not owe to transferred Cy or its metabolites, but

may have been caused by altered cells (or induced suppressor cells). Although Cy is known to deplete certain cellular subsets in the T suppressor pathway, there are indications (discussed in Sections 3 and 5) that Cy may induce or contribute to the induction of suppressor cells. One must, therefore, be constantly reminded of the necessity to view an immunomodulatory result against the multiplicity of direct and indirect effects on inductive and regulatory functions of specialized cellular classes and subsets.

Collectively the results show that: (1) some alkylating agents affect lymphocyte proliferative responses to mitogenic stimulation differently from the way they affect antibody responses; (2) mitotic stasis or mitotic death does not preclude survival of other functions, e.g., lymphokine production or cytotoxicity. (3) there is a diversity of action of these agents that in part may represent a selective attack on a particular cell type or function.

2.2. Selective Action Against Macrophage Cytotoxicity

The effect of various drugs on macrophage-mediated cytotoxicity was assessed by ip treatment 2 days before or in some cases 2 days after ip injection of 1.4 mg of *C. parvum*. Control values for drugs were obtained by injecting the vehicle used for the preparation of the drug solution and control values for *C. parvum* were obtained by injecting saline. Peritoneal macrophages freed of nonadherent cells were tested against P388 target cells 4 days after treatment with the drug. The plastic-adherent macrophages (Pam) were overlaid with target cells at a ratio of 20 : 1 and after incubation, the surviving target cells were quantified on the basis of incorporation of pulsed ^{3}H-TdR. The results are presented as net effect (last column, table 5), which was obtained from the formula: NE = [(CIV-CID)/CIV] × 100 where CID and CIV stand for cytotoxicity indexes (CI) of PAM from drug- (CID) and vehicle-(CIV) treated animals. The respective cytotoxicity indexes were derived from the formula: CI = (N − T)/N where N equals CPM in cultures of target cells with PAM from control animals and T equals CPM in cultures of target cells with PAM from *C. parvum*-treated animals.

It can be seen that pretreatment with Mel and 5-Fu caused severe impairment of cytotoxic activity. BCNU was slightly inhibitory. MeCCNU gave variable results and the other drugs including Cy did not affect cytotoxicity. When drugs were given after activation by *C. parvum*, BCNU and Adriamycin were more inhibitory than prior to activation of macrophages, but the action of MeCCNU was not different.

Table 5
Induction of Cytotoxic Macrophages by *C. parvum* and Its Modification by Pre-treatment or Post-treatment with Drugs

Drug	Dose, mg/kg	Cytotoxic index (CI)[a]		
		Vehicle CIV	Drug CID	Net effect NE[b]
		Pre-treatment		
Mel	8	73	neg	100
5-FU	100	51	14	72
Cy	100	96	93	3
Ara-C	1600	96	99	neg
Bruc	4	88	91	neg
Adr	6	85	96	neg
BCNU	30	79	58	27
		95	69	27
		83	90	neg
MeCCNU	30	96	84	12
		61	25	59
		95	28	70
		Post-treatment		
BCNU	30	79	12	85
MeCCNU	30	96	56	42
		61	42	31
Adr	6	85	34	60

[a]CI = [(N − T)/N] × 100. Drug given ip; neg = negative value.
[b]NE = [(CIV − CID)/CIV × 100 (see text for further explanation).

Another form of cytoxicity has been investigated. ADCC using antibody coated ^{51}Cr-labeled chicken RBC as target cells with various drugs administered 3 days prior to testing. None of the substances tested (Cy, Mel, BCNU, 5-FU, Bruceantin, and Adriamycin) impaired this activity.

2.3. DTH Reactions Can Be Enhanced or Inhibited By Alkylating Agents

DTH reactions were studied in mice that were in most instances treated 2 days after antigen. In some experiments the treatment was administered 2 days before immunization. As shown in Table 6, Cy suppressed this reaction when administered after antigen, a finding that is concordant with those of others (Zembala and Asherson, 1976; Sy et al., 1977). It should be recalled that resting TDH precursors are resistant to Cy, as evidenced by the enhancement of DTH by pretreatment with Cy, which apparently depletes

TS precursors and spares TDH precursors (Zembala and Asherson, 1976; Sy et al., 1977). However, these issues are not entirely settled in view of some contradictory reports on the effects of Cy on DTH. For example, Kerckhaert and colleagues (1977) found that a high dose of Cy (300 mg/kg) caused enhancement of DTH to SRBC when administered between 7 days prior to and 15 days after ic injection of antigen in complete Freund's adjuvant. This implies that activated TDH cells were resistant to the drug. In contrast, there is a report by Jokipii and Jokipii (1973) that relates experiments indicating that in guinea pigs pretreated with 300 mg/kg of Cy there was suppression of DTH to azobenzenearsonate-*N*-acetyl-1-tyrosine in complete Freund's adjuvant, suggesting that precursors of these cells were sensitive to Cy. However, the role of adjuvant in relation to activation of TS and TDH cells and participation of other cells in inductive and regulatory pathways is not clear and requires considerably more investigation. Speculative thoughts on some of these apparent discrepancies will be considered under Section 5.

It is of considerable interest that, as shown in Table 6, Mel and BCNU, in contrast with Cy, caused an enhancement of the reaction when given after antigen. A slight enhancement was also noted with BCNU and MeCCNU injected prior to immunization. The fact that Mel and BCNU fail to affect activated TDH cells is surprising since these agents were shown to damage activated T cells responding to con A. These findings emphasize the difference in the ability of alkylating agents to attack proliferating cells of certain T

Table 6
Effect of Various Drugs on
Delayed Hypersensitivity Responses

Drug	Dose, mg/kg	Time in relation to Ag	Delayed reaction[a] Control	Delayed reaction[a] Treated
Mel	8	2 days before	29.89 ± 2.92	39.90 ± 6.27
5-FU	100	2 days before	34.81 ± 4.80	15.20 ± 4.74
Cy	100	2 days before	33.40 ± 3.40	8.50 ± 4.33
Adr	6	2 days before	45.30 ± 5.90	29.40 ± 3.71
BCNU	30	2 days before	35.27 ± 4.72	64.50 ± 3.53
MeCCNU	30	2 days before	35.27 ± 4.72	38.66 ± 4.87
BCNU	30	2 days after	35.27 ± 4.72	42.25 ± 5.11
MeCCNU	30	2 days after	35.27 ± 4.72	42.58 ± 4.66

[a]Mean of difference in thickness of injected and control foot × (100) mm. Drug given ip.

subsets. We cannot explain the enhancement by Mel, BCNU, or MeCCNU at this time. It is of course possible that the enhancement by pretreatment with BCNU and MeCCNU may represent the same phenomenon as that described for Cy, i.e., elimination of TS precursors, a point that needs to be tested. However, this explanation may not hold for the enhancement of DTH by treatment with Mel following sensitization with antigen since we have data (to be shown under Section 3) that this substance, as well as certain other alkylating agents, have no effect on activated TS inducers or activated TS cells. We have gathered the impression that several elements in the suppressor pathway are resistant to Mel and the nitrosoureas. Nevertheless, our data pertain to assays measuring suppression of humoral responses and, as has already been pointed out, there seems to exist an array of diverse suppressor cells in DTH (see Chapter 3, Section 6), some apparently unique for DTH (Whisler and Stobo, 1978), and it is possible that these TS cells or their inducers or precursors may be susceptible to these drugs.

A composite of the results is presented in Table 7, which shows that there was no uniformity in the action of a given agent on different types of CMI. Included for summation are the results on the diverse effects on the humoral response. We would like to call special attention to Cy and Mel. These two alkylating agents appear to act reciprocally on DTH and macrophage cytotoxicity. Cy suppressed DTH after immunization, but had no untoward effect on induction of macrophage cytotoxicity. Mel, on the other hand, severely impaired the macrophage cytotoxicity but did not suppress, and in fact enhanced, DTH (after immunization). In Chapter 3, Section 5.4.5, we have discussed the heterogeneity of macrophages and raised the issue that the diverse functions of macrophages may be performed by different subpopulations or sets. In the present study, peritoneal macrophages were eliminated or inhibited from expressing cytotoxicity by pretreatment of the host with Mel, yet Mel caused an enhancement of the DTH reaction. Since macrophages are also involved in that reaction it would appear that macrophages associated with the expression of DTH are not impaired by Mel.

Several drugs were tested to determine their effect on the ability of the RES to clear particles from the circulation. The results are not included in Table 7, but by way of brief summary, Cy, Mel, BCNU, MeCCNU, and CCNU did not alter the kinetics of clearance of SRBC, suggesting that these drugs did not impair this particular function of macrophages. The resistance of the phagocytic function to Mel and BCNU, two drugs that were found to diminish the

Table 7
Comparison of Selected Drugs: Effects on Humoral and Cellular Immunity[a]

Drug	Blast	Mϕ CTX	ADCC	DTH	1° α-SRBC		2° α-SRBC		1° α-SIII	
					D pr A	D af A	D pr P	D pr Chal	D pr A	D af A
5-FU	—	↓ ↓	—	↓ ↓	↓ ↓	↓ ↓ ↓	—	↓	↑	↓ ↓
Mel	↓ ↓	↓ ↓ ↓	—	↑	↓ ↓ ↓	↓ ↓ ↓	↓	↓ ↓ ↓	V	↓ ↓
BCNU	↓ ↓ ↓	↓ ↓	—	↑	↓ ↓ ↓	↓ ↓ ↓	—	↓ ↓ ↓	↓ ↓	↓ ↓ ↓
MeCCNU	↓ ↓	−/↓	—	—	—	−/↑	−/↑	↓ ↓ ↓	↓ ↓	↓ ↓
Cy	↓ ↓	—	—	↓ ↓	↓ ↓ ↓	↓ ↓ ↓	↑	↓ ↓ ↓	↓	↓ ↓ ↓
Ara-C	↓	—	nd	nd	↓	↓ ↓ ↓	↑	↓ ↓ ↓	↑	−/↓
Dominant antibody					IgM	IgM	IgG	IgG	IgM	IgM

[a]Mϕ CTX = macrophage cytotoxicity; ADCC = antibody-dependent cell-mediated cytotoxicity; DTH = delayed hypersensitivity; D pr A = drug prior to antigen; D af A = drug after antigen; D pr P = drug prior to priming; D pr Chal = drug prior to secondary challenge; (−) = no change; arrows down (↓) approximate intensity of depression ; arrows up (↑) expressed as a single arrow until limits of enhancement or amplification are established; V = variable; nd = no data; blast = blastogenesis.

ability of macrophages to respond to *C. parvum* in the cytotoxic reaction attest further to the utility of alkylating agents for dissociating separate functions (and possibly subsets) of macrophages.

3. Alkylating Agents Exert Selective Action Against Cells in the Suppressor Pathway

Injection of mice with supraoptimal antigen (SOI), i.e., 4×10^9 SRBC, leads to the induction of TS cells. Expression of suppressor cell activity can be ascertained by adoptive transfer of splenocytes from SOI donors into normal syngeneic recipients that are simultaneously immunized with an optimal dose of 1×10^8 SRBC. IgM PFC responses are measured 4 days later (Whisler and Stobo, 1976).

The T lineage of suppressor cells has been established by appropriate experiments using depletion of T cells by anti-Thy plus complement, while the specificity of suppression has been proven by the failure of the SRBC-SOI-induced suppressor cells to inhibit the response to an unrelated antigen, i.e., horse RBC. The generation of suppressor activity is a slow process. As shown in Fig. 3, 2 days after SOI there is minimal suppressor activity as measured by adoptive transfer. This increases progressively to a maximum suppressor activity by day 10 and persists at about the same level until day 49 after SOI.

We tested the effects of several alkyating agents on the induction and expression of suppresssor cells. The choice of times of treatment was dictated by several considerations. There are reports in the literature that indicate that resting precursors of suppressor

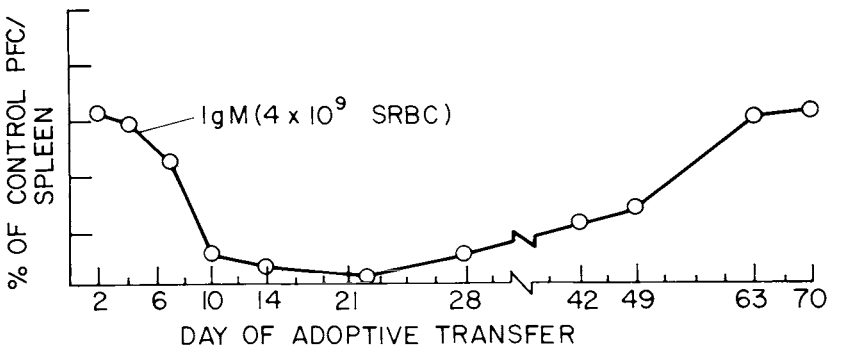

Fig. 3. Appearance and persistence of suppressor cells. Donor mice injected with 4×10^9 SRBC and their splenocytes adoptively transferred at various intervals thereafter.

cells are susceptible to Cy (Zembala and Asherson, 1976; Debre et al., 1976; Sy et al., 1977; Schwartz et al., 1978; Cantor et al., 1978; Benacerraf and Germain, 1979). On the other hand, there are also reports that Cy may actually induce suppressor cells (Milton et al., 1976; L'Age-Stehr and Diamantstein, 1978). We have shown that Cy exerts selective effects against different types of lymphocytes (e.g., B cells responding to SIII vs cells responding to primary SRBC challenge) and that Cy seems to be able to discriminate between separate stages of differentiation (e.g., precursors vs effectors of memory) (see Section 1). We therefore asked whether Cy and other alkylating agents would exert differential effects on the induction of TS cells. The results are summarized below.

The basic protocol for these experiments called for administration of SRBC-SOI to Balb/c or BDF_1 mice, treatment with alkylating agent (prior to or at intervals after SOI), and adoptive transfer of splenocytes on day 14 after SOI when T suppressor effectors (TSe) would be at their peak of activity. The data in Table 8 indicate that none of the alkylating agents injected 2 days prior to SOI prevented the induction of TSe capable of exerting suppression in the secondary host. When injected two days following SOI, both Cy and BCNU, but not the other drugs, caused a dramatic reduction in the capability of donor splenocytes to induce suppression

Table 8
Effects of Alkylating Agents
on Induction of Suppressor Cells
by Supraoptimal Immunization

		Percent suppression[b]	
Treatment[a]	Dose, mg/kg	2d Pre SOI	2d Post SOI
Saline	—	86	88
Cy	100	92	2
BCNU	30	82	1
CCNU	30	84	92
MeCCNU	30	65	89
Mel	8	91	81

[a]Mice were injected ip with drugs either 2 days before or 2 days after immunization with 4×10^9 SRBC; 14 days later, splenocytes from these mice were injected iv into syngeneic recipients which were simultaneously immunized with 1×10^8 SRBC. PFC in recipients' spleens were examined 4 days post cell transfer.

[b] $1 - \left(\frac{\text{PFC/spleen in mice receiving SOI splenocytes}}{\text{PFC/spleen in mice receiving nonimmune splenocytes}} \right) 100$

Table 9
Differential Sensitivity of Suppressor Cells to Cy and BCNU with Maturation

Treatment[a]	Dose, mg/kg	Percent suppression[b] when treated on day post SOI +2	+12	+26
Saline	—	88	89	83
Cy	50	2[c]	23[c]	53[c]
BCNU	30	1[c]	77	nt[d]

[a]Mice were injected ip with 4×10^9 SRBC or saline and treated with drugs 2, 12, or 26 days later. Ten days following drug treatment on day +2 or 2 days following drug treatment on days +12 or +26, splenocytes from SOI and control mice were transferred to syngeneic recipients that were simultaneously immunized with 1×10^8 SRBC. PFC in recipients' spleens were examined 4 days post cell transfer.

[b] $1 - \left(\frac{\text{PFC/spleen in mice receiving SOI splenocytes}}{\text{PFC/spleen in mice receiving nonimmune splenocytes}} \right) 100$

[c]Significantly different from saline treated groups. All other differences were statistically not significant.

[d]nt = not tested.

in the secondary host. The two active agents manifested a disparity in their effect on suppressor cell induction when administered at later intervals. It is apparent from Table 9 that on day 12 post SOI, Cy still caused significant reduction in the suppression, while BCNU had no such effect. It is also apparent that, with the passing of time after SOI as the suppressor cells matured, their sensitivity to CY gradually diminished. Thus, while on day 2 after SOI 50 mg/kg of Cy could totally alleviate suppressor activity, the same dose of Cy reduced suppression by only 74 and 36% on days 12 and 26, respectively.

It is noteworthy that, of all the alkylating agents tested, only Cy was effective over a long span of time after the activation of the suppressor network. Mel, MeCCNU, and CCNU were ineffective at any time and BCNU was able to relieve suppression only when administered soon after SOI.

We hypothesize that the cell that is sensitive to Cy and BCNU after activation by SOI is a TS inducer cell (TSi) that by itself does not cause suppression, but that induces another cell to become the suppressor. In the terminology of feedback inhibition model A depicted in Fig. 4, this would correspond to the Lyl^+Qal^+ cell. Further, we believe that the resting precursor of TSi is resistant to Cy

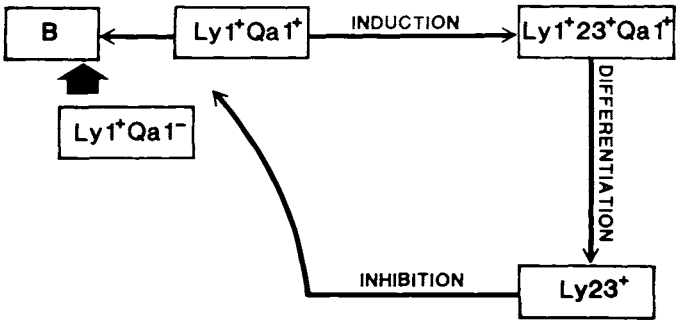

Fig. 4. Feedback inhibition model A (based on Cantor and Gershon, 1979).

and BCNU as well as to the other alkylating agents. At this point it is pertinent to state that our experimental design did not permit determination of sensitivity to alkylating agents of the precursor of TSe, the Lyl^+23^+ cell that, according to Cantor et al. (1978a), is sensitive at least to Cy. In our design the test for suppressor function is performed by adoptive transfer of splenocytes from SOI donors treated with drug into normal secondary hosts endowed with a full complement of Lyl^+23^+ precursors that would restore the missing precursors. Our data, therefore, do not argue against the sensitivity of TSe precursors to Cy, but simply imply that another precursor, that of TSi, is drug-resistant, while the activated TSi is sensitive to some alkylating agents, Cy and BCNU, but not others. The TSi cells were also studied in adult thymectomized mice. Seven months after thymectomy, animals given SOI possessed splenocytes capable of transferring inducers of suppression to normal recipients. These findings indicate that TSi cells were long-lived (Sigel et al., 1979).

Additional evidence supporting the role of the TSi cell as an inducer has come from preliminary experiments in which splenocytes were transferred 2 days after SOI and the challenge of recipients was delayed for 10 days to permit the acquisition of mature TSe. Some of the recipients were pretreated with 10 mg/kg of Cy 2 days prior to adoptive transfer. The latter animals were not suppressed whereas Cy-untreated recipients were significantly suppressed by the transfer of the 2-day SOI splenocytes. These findings suggest that what was being transferred were TSi inducer cells and not TSe, and that the TSi cells did not encounter a sufficient number of TSe precursors in the recipient because those were reduced by Cy.

Our findings with the alkylating agents used are summarized in Table 10, in which we have assigned presumed Ly and Qal status to the T subsets believed to be present at the various time intervals after SOI. This table should serve as a frame of reference for further experiments with alkylating agents as probes in the analysis of regulatory circuits. The experiments have raised several questions. The central question is what is the mechanism of selective action of the respective agents? Why is Cy effective in various degrees across the spectrum of cellular subsets involved in suppression, whereas Mel is totally devoid of any discernible activity even against early

Table 10
Comparison of Effects of Alkylating Agents on TSi and TSe

	Time of treatment in relation to SOI, days			
	−2	+2	+12	+26
Presumed status (subset of T cell)	Resting TSi ($Ly1^+Qa1^+$)	Activated TSi ($Ly1^+Qa1^+$)	Mature TSi and/or TSe ($Ly1^+Qa1^+$ and/or Ly2,3)	Mature TSe (Ly2,3)
Cy	—	+++	++	+
BCNU	—	+++	—	nd
Mel	—	—	—	nd
MeCCNU	—	—	—	nd

[a]nd = no data.

activated TSi? The answers to these questions must be sought in two areas, the mechanism(s) of action of alkylating agents and the metabolism and functions of T cell subsets. In Section 2 we discuss certain aspects of action of alkylating agents with particular emphasis on Cy (as there is relatively little knowledge of the actions of the other agents). Little is known about metabolic features of T cell subsets or about their modes of action except that ceratain T cells elaborate suppressor factors that have been discussed in Chapter 3, Section 5.4.4.

Judging from the achievements of recent years, solutions to these questions may come from methods of cellular separation, purification, and characterization that permit analysis of single sets, as well as reconstruction experiments.

4. A Closer Look at Agents That Are More Suppressive Before Immunization

4.1. General Considerations

Since the lymphocyte proliferative event is a major step in the immune response, it follows that any agent that interferes with replication of DNA would stop cell division and thereby cause immunosuppression. It is therefore intriguing that some agents appear to be more suppressive for "resting" cells and less effective against dividing cells. Such agents may provide new insight into a modulation of immune responses at steps and sites other than cellular proliferation. These events and sites (or targets of drug action) may include: (1) antigen presentation; (2) recognition of antigen by various relevant cells, i.e., presence or expression of primary receptors (for antigen) and secondary receptors (for Ia); (3) generation of signal molecules (lymphokines or other factors); (4) signal delivery; (5) signal recognition; (6) cellular interactions (as discussed in Chapter 3, section 5.5); and (7) antibody synthesis and release. Drugs and other agents that permit the dissociations of immunologic induction and regulation from cellular proliferation should therefore prove of great interest in future research.

In Berenbaum's studies, cholera toxin, busulfan, and cortisone acetate appear to mimic the effect of radiation by causing more suppression when administered prior to antigen compared to after antigen. In explaining the results with busulfan, Berenbaum (1979) refers to his work and the work of Dunn (1974), which indicated that this drug is more damaging for cells at early stages of differentiation or slowly proliferating cells and that rapidly proliferating cells are relatively resistant to it. Although this type of preference may also be the basis of selective action of other agents that are more suppressive prior to initiation of the proliferative events, the interpretation does not explain why nonproliferating or slowly proliferating cells are more vulnerable.

We would like to examine several agents of immune modulatory action prior to immunization based on our work and the studies of others. We shall first consider some natural biological immunomodifiers and subsquently discuss the effects and modes of action of radiation, briefly touching on cholera toxin and cortisone. The four agents that manifested preferential suppression prior to immunization were: (1) Extracts of *Exteinascidia turbinata* (Ete), a marine tunicate studied extensively by W. Lichter, L. Wellham, and M. M. Sigel for antitumor and immunosuppressive

capabilities. Extracts were prepared in 50% alcohol–water from specimens collected off the coast of Florida (Sigel et al., 1970; Lichter et al., 1972, 1974, 1975, 1978, 1979). (2) *Corynebacterium parvum* (Cp), a well recognized immunomodulator studied by several investigators (Neveu et al., 1964; Biozzi et al., 1965; Howard et al., 1973a; O'Neill et al., 1973) and by A. Ghaffar who initiated his work at the University of Edinburgh and continued in the United States (Woodruff et al., 1973; Ghaffar and Sigel, 1978); Cp was kindly provided by Dr. J. Whisnant of Burroughs Wellcome. (3) *Semecarpus anacardium* (Sa); preparations of partially purified fractions were provided by the Division of Cancer Treatment, National Cancer Institute. (4) Poison ivy antigen (*Rhus Toxicodendron,* Rt), provided by the Division of Cancer Treatment, National Cancer Institute.

4.2. *The Multifaceted Effects of* Ecteinascidia turbinata

Our initial findings demonstrated that Ete possesses antitumor activity when measured in vitro (KB cells) or in vivo (P388 leukemia in mice) (Sigel et al., 1970). Despite extensive efforts by Weinheimer and colleagues at the University of Oklahoma, the active principle(s) has not been defined although certain fractions provided by Weinheimer, especially one consisting of a low molecular weight fatty acid, appeared to display activity. Ete exerts a number of immunosuppressive effects; in earlier work it was found to inhibit antibody production as well as certain cell-mediated activities, notably graft rejection and GVH reaction (Lichter et al., 1972).

4.2.1. Inhibition of Antibody Production is Determined by Route of Administration of Ete and/or Antigen

It was noted at the time of the earlier studies that suppression of antibody production required treatment with Ete prior to immunization and that treatment after immunization had little effect. In contrast, a cell-mediated reaction (rejection of skin allografts) was more strongly impaired when the Ete was given after transplantation (Lichter et al., 1972). This suggested that Ete could differentiate between a humoral and cell-mediated response by selectively acting on nonactivated cells involved in the former and on the activated cells involved in the latter. Preferential suppression of antibody production by Ete prior to activation of the cellular components in the TD humoral response was confirmed in more recent experiments employing PFC assays. Ete administered ip prior to SRBC inhibited anti-SRBC PFC responses. This was demonstrated in DBA/2,

Table 11
Effect of Ete[a] on the Magnitude of the PFC Response to SRBC and SIII

Immunizing antigen	Dose of Ete, mg/kg	Log_{10} PFC/spleen[b]
SRBC	0	5.179 ± 0.100(150,860)
	1.25	5.392 ± 0.077(246,507)
	12.50	5.243 ± 0.044(174,883)
	125.00	4.460 ± 0.130(28,840)
SIII[c]	0	4.420 ± 0.072(26,304)
	1.25	4.039 ± 0.094(10,939)
	12.50	3.584 ± 0.090(3,833)
	125.00	2.934 ± 0.128(860)

[a]Single injection of Ete given ip 1 day prior to immunization.
[b]Mean ± se for 5 mice; geometric means are in parentheses.
[c]Anti-SIII responses were measured by Dr. P.J. Baker of the NIAID, NIH.

BDF_1, and C3H mice. The suppressive activity appeared to increase when the time between treatment and immunization was increased to 5–9 days. This substance was also highly effective against TI responses to SIII when administered prior to antigen; in fact, as shown in Table 11, on a per dose basis Ete was more effective in inhibiting anti-SIII PFC than anti-SRBC PFC. The suppressive activity of Ete was definitely *route- and time-dependent.* It was regularly demonstrable when both Ete and antigen were given by the ip route with Ete preceding the antigen by at least a day or prefereably a longer time interval. In most experiments Ete administered iv caused no suppression and in some instances it appeared to potentiate the response. When Ete was given on the day of immunization or after, it caused variable results, a point that needs further scrutiny.

The effect of Ete on secondary response was especially intriguing; one experiment is illustrated in Table 12. Some mice were treated with 250 mg/kg Ete, whereas others were given saline 1 day prior to immunization with SRBC. The primary response and the effect of Ete on this response was determined at 5 days, measuring IgM and IgG PFC. Sixteen days after the initial immunization, the remaining mice were reimmunized with SRBC and the secondary response determined 6 days later. As in previous experiments, pretreatment with Ete caused a significant decrease in PFC of the primary response, *but what is more important is that this treatment prevented the development of immunological memory,* as illustrated by a deficit in IgG PFC production. These results indi-

Table 12
Suppression of Anti-SRBC Responses by Ete[a]

Type of response	Treatment with Ete	PFC per spleen	
		IgM	IgG
Primary	None	23,269	63,147
	1 day before SRBC	6,805	15,880
Secondary	None	3,481	96,582
	1 day before priming	20,218	50,882
	1 day before 2° challenge	966	10,550

[a]BDF_1 mice injected ip with 250 mg/kg.

cate that Ete is capable of eliminating resting precursors of memory cells. Although the results of the experiment with the TI antigen indicate that Ete can suppress or eliminate resting B cells responsive to SIII, the experiments with SRBC, a TD antigen, do not discriminate between effects on resting B vs T cells, and no conclusion is possible regarding the respective susceptibilities of the two types of cells. Such distinction must await experiments utilizing adoptive transfer of separated cell populations. We have obtained, however, considerable information regarding the effects of Ete on certain parameters of T cells and macrophages.

4.2.2. Ete Inhibits Mitogenic Lymphocyte Responses and Induces T Suppressor Cells In a large series of experiments it was demonstrated that Ete can inhibit mitogenic responses of splenocytes to PHA and con A (Lichter et al., 1974, 1975). This was demonstrated by the administration of Ete to animals or by addition in vitro. In the in vitro experiments these mitogenic responses could be prevented by the addition of Ete prior to mitogen or could be inhibited by the addition of this substance as late as 48 h after stimulation by mitogen (Table 13). Thus Ete could prevent initiation of DNA synthesis in T cells and it could stop ongoing DNA synthesis. Ete was also found to reduce the rate of DNA synthesis following activation with con A (not shown). The in vivo effects are illustrated in Fig. 5. Injections of Ete (250 mg/kg) invariably caused spleen enlargement (hypertrophy and hypercellularity) and it was thought that the decrease in blastogenic response following treatment with Ete could be explained by dilution of responsive cells by other cells in the hypertrophic spleen or by the activation of suppressor cells. Subsequent experiments demonstrated that Ete induced, in mouse spleens, suppressor cells that could inhibit

Table 13
Effect of Ete on Human Peripheral Blood Lymphocytes in Blastogenesis[a]

	PHA[b]		Con A[c]	
Cultivated in	cpm	SI	cpm	SI
Growth medium (control)	410	1.00	2,564	1.00
Mitogen	123,794	302.00	21,299	8.30
Mitogen + Ete[d]	1,002	2.44	2,557	0.99
Ete 1 h, wash, then mitogen	38,184	93.00	2,362	0.92
Mitogen, 24 h later Ete	8,904	21.70	3,645	1.42
Mitogen, 48 h later Ete	56,430	137.60	8,307	3.23

[a]Comparison of pre-treatment with post-treatment stimulation with PHA and con A.
[b]PHA = 1:200 final concentration.
[c]Con A = 5 μg/mL.
[d]Ete = 100 μg/mL final concentration.

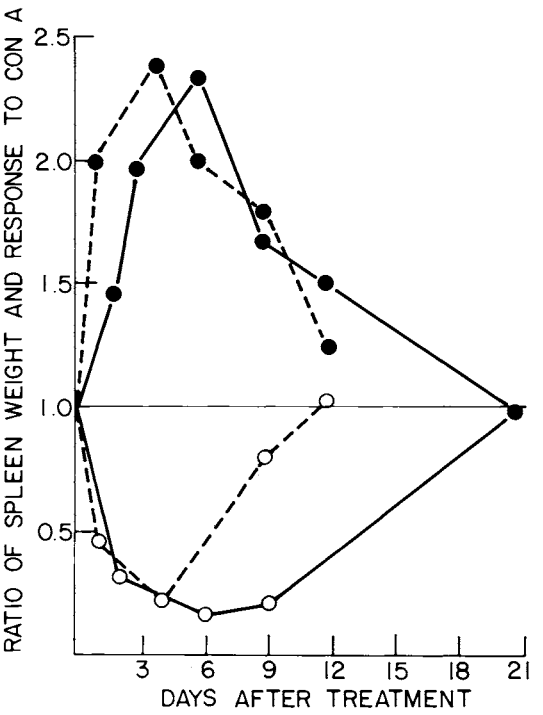

Fig. 5. Suppression of blastogenic responses in splenocytes from Balb/c (broken lines) and DBA/2 (solid lines) mice treated with ETe; 250 mg/kg Ete injected i.p. Note the inverse relationship between blastogenic response (open symbols) and spleen weight (closed symbols).

blastogenic responses of normal splenocytes to PHA or con A and that these nonspecific suppressor cells belonged to the T lineage (Lichter et al., 1979). In separate experiments it was also shown that cells from enlarged spleens of animals treated with Ete were able to suppress humoral responses to SRBC, but it has not yet been ascertained whether the same cells are involved in the suppression of blastogenic responses to con A and PFC responses to SRBC.

4.2.3. Ete Activates Macrophages Since macrophages are involved in the regulation of immune responses the effect of Ete on macrophage function was determined. This agent was found capable of stimulating the function and perhaps the proliferation of these cells. This was demonstrated by three independent parameters: increased phagocytic activity as measured by particle clearance from the circulation; increased ADCC, shown in Table 14; and increased cytotoxic activity against tumor cells, presented in Fig. 6.

4.2.4. Summary The results of studies with Ete may be summarized as follows:

(1) The substance has caused both inhibitory and stimulatory effects. Among the inhibited functions are primary and secondary antibody responses when Ete is administered prior to immunization and both Ete and the immunogen are given by the ip route.

(2) Inhibition was also noted in lymphocyte proliferative responses.

(3) Suppressive effects were observed in allograft rejection and GVH reactions.

Table 14
Effect of Ete[a] Treatment on the ADCC of Splenocytes in Balb/c Mice

	Percent lysis[b]			
	Experiment 1		Experiment 2	
Cell treatment	Control	Ete	Control	Ete
Unseparated	40	74	56	81
Glass nonadherent	44	73	56	86
Glass adherent	38	69	66	83
Glass and nylon nonadherent	15	28	25	57
Glass nonadherent and nylon adherent	20	23	25	24

[a]250 mg/kg injected ip 5 days before test.
[b]A pool of four animals was used.

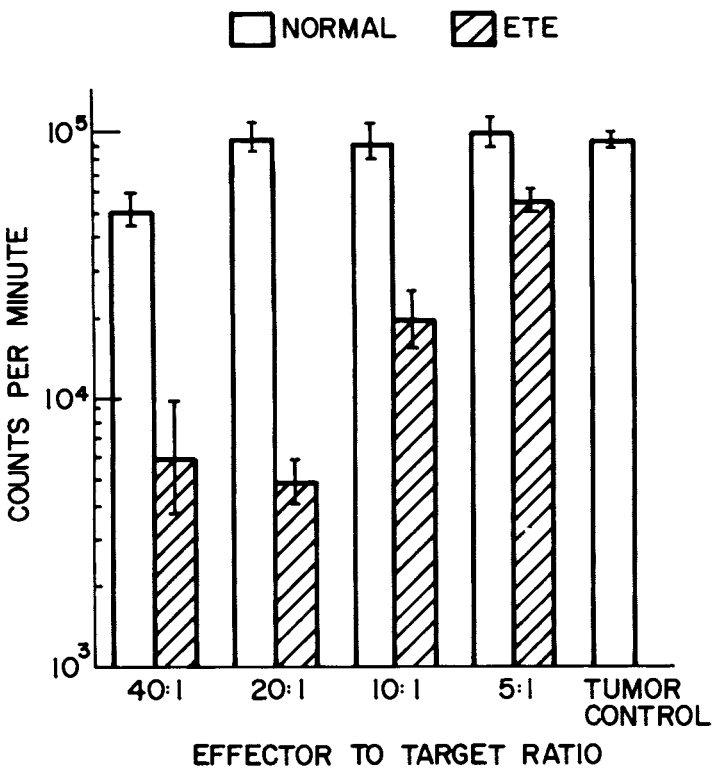

Fig. 6. Increase in macrophage cytotoxic activity against tumor cells induced by Ete.

(4) Activation by Ete was demonstrated with TS cells and with macrophages.

(5) Stimulation of TS cells and macrophages may in part explain the suppression of other functions.

(6) Since the stimulatory actions also potentiated phagocytosis, ADCC, and tumor cell killing, such actions may represent the basis of Ete's antitumor activity.

In some respects these activities resemble those of *C. parvum,* which will be discussed in the next section.

Another immunosuppressive substance derived from a marine invertebrate has been described by Willenborg and Prendergast (1973). Coelomocytes of the sea star *Asterias forbesi* produce a substance, designated by Prendergast as sea star factor (SSF), that interferes with immunization of mice to *Listeria monocytogenes* and that diminishes immune (T cell) cytotoxicity to tumor cells

(Prendergast et al., 1974). Like Ete, SSF inhibits blastogenic reactions in vitro (Prendergast et al., 1974) and, in addition, shares the capacity to activate macrophages. Prendergast (1971) demonstrated the activation by the increase in cell size and the increase in uptake of euchrysine in lysosomes. SSF was found to stimulate the reticuloendothelial system as evidenced by increased clearance of carbon from the circulation as well as increased capacity of nonimmune peritoneal exudate cells to kill tumor cells. There are, however, significant differences between the two substances. The data show that one mode of action of Ete is through the induction of suppressor T cells. According to Prendergast (personal communication), SSF does not induce suppressor cells. Furthermore, Ete not only inhibited responses to TD antigens, but was also found to suppress production of PFC to SIII. This contrasts with SSF, which failed to suppress the response to SIII (Prendergast et al., 1974). One can only speculate at this time that this difference may be a reflection of the ability of Ete to induce suppressor cells. Certainly Ete and SSF have the potential to be valuable tools for further studies of immunosuppression and immunoregulation in mammalian systems in vivo and in vitro.

4.3. C. parvum *Potentiates and Suppresses Immune Responses*

4.3.1. Brief Overview of the Literature *C. parvum* (Cp) was initially recognized for its ability to stimulate the reticuloendothelial system (RES) as manifested by increased clearance of particulate material from the circulation and increased spleen and liver weights (Halpern et al., 1964; Adlam and Scott, 1973; O'Neill et al., 1973; McBride et al., 1975). Since then the ability of this organism to modify various immune functions has been extensively studied. Among the prominent properties of Cp are its tumor inhibitory effects in experimental hosts (Halpern et al., 1966; Woodruff and Boak, 1966) and also in cancer patients (Reed et al., 1975; Rao et al., 1977). In mice injected systemically, this effect owes at least in part to RES activation and appears to be independent of T cells (Woodruff et al., 1973). However, when Cp is administered into the tumor the response is T cell dependent (Scott, 1975; Woodruff and Dunbar, 1975). It would appear from previously published data and our own observations that Cp can also modulate resistance to a secondary tumor challenge when Cp is administered in association with tumor-specific antigens, either as nonviable tumor cells or as live tumors that subsequently are treated chemothera-

peutically. Aside from enhancing tumor resistance, Cp has also been shown to increase resistance to bacterial, viral, and parasitic infections (Nussenzweig, 1967; Adlam et al., 1972; Morahan et al., 1977).

In addition, Cp also modulates phagocytic and cytotoxic functions of macrophages, their lysozomal enzyme contents, and their histochemical characteristics. Generally, Cp treatment in vivo results in increased phagocytic activity, cytotoxic potential, lysozomal enzyme, and prostaglandin content, and adherence of macrophages (McBride et al., 1974; White, 1975; Farzad et al., 1977). A number of authors have also demonstrated increased natural killer (NK) cell activity in lymphoid organs of animals treated with Cp (Herberman et al., 1977; Gangemi et al., 1980). Some of the altered macrophage and NK cell activities have been attributed to induction of interferon by this agent (Kirchner et al., 1977).

Warr and Sljivic (1974) studied the effect of Cp injected at different time intervals in relation to antigen (SRBC). Their results indicated that Cp injected iv influenced immune responses in a variable manner depending on the time of injection of Cp in relation to the antigen and also on the thymus dependence of the antigen. Enhancement of the anti-SRBC response by Cp injected 1, 4, or 7 days before, on the day of, or 1 day after an optimal dose of the antigen (1×10^8 SRBC) was consistent with previous findings using the same or other TD antigens (Neveu et al., 1964; Howard et al., 1973a; O'Neill et al., 1973; Warr and Sljivic, 1974; Greenberg and Dimitrov, 1976). With a suboptimal dose of SRBC (1×10^6), Cp, under the above conditions of route and time, caused significant suppression of the response. Warr and Sljivic (1974) also noted that Cp injected iv 0–7 days before iv priming with 1×10^8 SRBC enhanced the secondary response to the same dose of SRBC injected iv 90 days after the priming. In contrast, Cp injected one day after priming had a suppressive effect. The immune response to a T I antigen (SIII) was augmented by Cp injected 4 days before, but not at the same time of, the antigen (Howard et al., 1973a, 1973b; Warr and Sljivic, 1974). There are indications that the effect of Cp on anti-SIII response might also be dependent on the dose of SIII (Howard et al., 1973b).

Cp administration has been reported to cause suppression of certain parameters of the CMI function. Thus, splenocytes from Cp-treated mice failed to respond to various nonspecific mitogenic stimuli in vitro (Scott, 1974). This failure appeared to be mediated by suppressor macrophages as removal of macrophages restored normal responses of T and B cells to nonspecific mitogens. Furthermore, addition of macrophages from Cp-treated animals inhib-

ited mitogenic responses of fresh splenocytes. In vivo, certain doses of Cp caused suppression of CMI responses (Allwood and Asherson, 1972).

4.3.2. Experiments Establish the Overriding Importance of Route of Injection Among the Many Variables in the Interaction of *C. parvum* and Antigen in Determining Suppression or Augmentation

We have studied the effects of Cp on anti-SRBC responses with regard to the variables of mouse strain, dose, time, and especially the route of injection. We have also determined the effect of Cp on resistance (immunity) to certain tumors. These data will be summarized in the following paragraphs.

In Balb/c mice, when Cp was injected ip 10 days before 1×10^8 SRBC, there was over 90% suppression of anti-SRBC response. Similar suppression was observed when the effect of Cp on the immune response of BDF_1 mice was examined. Thus, Cp injected 1–16 days before SRBC caused significant suppression of anti-SRBC IgM and IgG PFC responses (Ghaffar and Sigel, 1978). Based on kinetic studies, this was a true suppression and not simply a delay in PFC response. By contrast, when injected on the day of immunization Cp significantly augmented IgM and IgG responses. When Cp injection was delayed to 1 day post antigen, it had no significant effect. Our previous observations also indicated that the suppressive effect was not caused by overstimulation of the RES by Cp overload since a small amount (56 μg) caused a significant suppression (Ghaffar and Sigel, 1978). The immunomodulating effect of Cp was, however, dependent on the route of treatment. Thus, Cp injected ip caused a suppression when SRBC were injected ip, but augmented the response to iv administered SRBC (Ghaffar and Sigel, 1978). Furthermore, iv administration of Cp always augmented the response to SRBC whether injected ip or iv (Ghaffar and Sigel, 1978). This interrelationship may explain the apparent discrepancy between our findings and those of Warr and Sljivic (1974) using optimal immunizing antigen doses. They used the iv route and obtained enhancement; we used the ip route and noted suppression.

Table 15 shows suppression of secondary responses when Cp was injected 1 day before priming or 1 day before the secondary challenge given 16 days after priming. This indicates that Cp injected before SRBC was capable not only of diminishing the primary response, but also of preventing the education, by antigen, of resting precursors of memory cells. Moreover, injection of Cp prior to secondary challenge also suppressed the occurrence of a sec-

Table 15
Suppression of Anti-SRBC Responses by *C. parvum*[a]

Type of response	Treatment with *C. parvum*	PFC per spleen	
		IgM	IgG
Primary	None	104,116	63,282
	1 day before SRBC	2,004	134
Secondary	None	23,383	270,628
	1 day before priming	1,312	14,317
	1 day before 2° challenge	1,475	27,607

[a]BDF_1 mice injected ip with 70 mg/kg Cp before ip immunization with SRBC. From Ghaffar and Sigel, 1978.

ondary response, suggesting that this agent interfered with the expression of memory cells.

4.3.3. Investigations of Mechanisms of Modulatory Actions by Cp Since Cp is one of the most powerful stimulants of the RES, the thought was entertained that modulations of antibody response might be related to its ability to alter the clearance rate and localization of the antigen. Figure 7 demonstrates that in mice injected with Cp by the ip route, relatively smaller amounts of antigen localized in the spleen. In contrast, iv injection of Cp leads to a several-fold increase in the splenic localization of SRBC. Although the altered distribution of the antigen in the spleen parallels the suppression or augmentation of the immune response associated with respective (ip or iv) route of administration of Cp, the magnitudes of the effects do not coincide. Thus, the striking increase in splenic uptake by mice injected with Cp by the iv route is disproportionately high in comparison to the modest augmentation of PFC responses in similarly treated mice. Conversely, the profound suppression by ip injection of Cp is incompatible with the small reduction of splenic uptake of SRBC in such animals.

In search of another mechanism for the reduced response in Cp-injected mice, we investigated the possibility that Cp might shift the balance of regulatory cells towards suppressor cells in response to optimal doses of SRBC. Two groups of BDF_1 mice were injected ip with either saline or Cp and 1 day later each group was subdivided into two groups, one receiving 1×10^8 SRBC and the other being treated with saline. Fourteen days after the SRBC injection, mice were sacrificed and their splenocytes (4×10^7 cells) were transferred into normal recipients together with 1×10^8 SRBC. The response of recipients measured 4 days later is summarized in Ta-

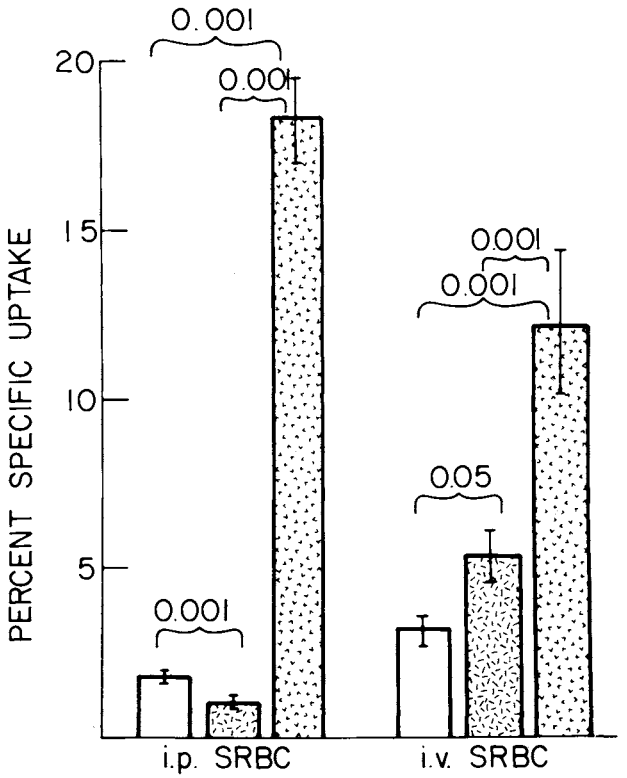

Fig. 7. Effect of *C. parvum* on the splenic uptake of ^{51}CR-SRBC in BDF_1 mice. Numbers above the bars indicate P values. Open column, control; hatched column, *C. parvum* ip; cross-hatched column, *C. parvum* iv.

ble 16. PFC production by mice receiving splenocytes from donors treated with Cp was similar to that in mice receiving splenocytes from untreated controls. Splenocytes from donors previously immunized with 1×10^8 SRBC were not suppressive in one experiment and slightly suppressive in another experiment (statistically significant). The striking observation was that when donor mice were injected with 1.4 mg of Cp 1 day before injection of 1×10^8 SRBC, their splenocytes caused a significant suppression in recipient mice in both experiments. These data indicate that Cp, when combined with a normally optimal immunizing dose of antigen, leads to the induction of suppressor cells. We also obtained preliminary data indicating that Cp in combination with an SOI dose of SRBC, which consistently induced suppressor cells, alleviated suppression (data not shown). Thus, Cp could play a dual role: in combination with optimal antigen dose it led to induction of suppres-

Table 16
Induction of Suppressor Cells by *C. parvum*[a]

Donor treatment	Mean[b] PFC per 10^6	
	Exp. I, IgM	Exp. II, IgM
Saline	1,061	649
C. parvum	1,466	846
1×10^8 SRBC	874	317[c]
C. parvum + 10×10^8 SRBC	424	88[d]

[a]1.4 mg *C. parvum* injected ip 1 day before immunization of spleen cell donors with 1×10^8 SRBC. Fourteen days after immunization, 40×10^6 splenocytes were transferred iv into normal syngeneic BFD_1 mice. Recipients also received 1×10^8 SRBC ip at the time of cell transfer. PFC measured 4 days later.

[b]Geometric mean of PFC per 10^6 nucleated spleen cells for groups containing a minimum of 5 mice.

[c]Significantly lower ($P < 0.0$) than group injected with splenocytes from donors treated with saline or *C. parvum*.

[d]Significantly lower ($P < 0.01$) than group injected with splenocytes from donors treated with SRBC alone, saline, or *C. parvum*.

sor cells whereas in association with SOI antigen dose it caused relief from suppression.

Although Cp has been shown to exert a large variety of changes including splenomegaly, hepatomegaly, altered distribution of antigen, altered phagocytic activity, modified immune responses, etc., it would appear that most if not all of these changes may be associated with the action of Cp on the RES. We would like to propose two hypotheses that may explain the route-dependent up and down regulation of anti-SRBC responses by Cp. One invokes the local effect of Cp. When injected ip, the agent activates macrophages in the peritoneal cavity. The inflammatory changes in the peritoneum may cause retention of antigen, i.e., deprivation of antigen from the antibody forming sites; hence a diminution of response when both Cp and antigen are given ip. When injected iv, Cp activates monocytes and macrophages in the spleen and may lead to antigen retention in that organ, hence increasing the opportunity for a good response.

The second hypothesis is based on the emerging concept of heterogeneity of macrophages. We postulate the existence of two functionally distinct subsets of macrophages being involved in the regulation of anti-SRBC responses, one being facilitating (F) and the other nonfacilitating (NF; a phagocytic cell not involved with facili-

tation). We can picture the F as involved in antigen presentation and NF in capture and degradation; one could even imagine that the NF cell exerts suppressive action. After ip inoculation of Cp, there is a local induction of a preponderance of NF macrophages in the peritoneal cavity. Thus SRBC injected ip are captured and trapped in the peritoneal cavity and few reach the spleen for the induction of an immune response. When the dose of SRBC is raised, the local macrophages are saturated and the overflow of the antigen reaches the spleen to induce a response. Apparently the correct balance is struck between the acquisition and handling of antigen by F and NF cells so that an otherwise suppressive dose of 5×10^9 SRBC is more immunogenic than the usually optimal dose of 1×10^8 or 1×10^9. By contrast, antigen injected by the iv route directly localizes in the spleen and induces a positive immune response. This hypothesis has been summarized in Table 17.

Both hypotheses are in accord with the data presented in Fig. 7. Moreover, evidence exists for one aspect of the second hypothesis, i.e., the improved function of Cp-activated cells in the presentation of antigen. Sljivic and Watson (1977) have demonstrated that adherent cells taken from spleens and peritoneums of mice treated

Table 17
Hypothetical Model of Facilitating (F) and Nonfacilitating (NF) Macrophage Functions

Route of injection			Peritoneal cells		Spleen cells		Response observed
CP[a]	Antigen		F	NF	F	NF	
ip	ip	Opt[b]	o[c] *[d]	oooo ****	oo *	oo *	↓
		Sub opt	o *	oooo ****	oo *	oo *	↓
		Supra opt	o *	oooo **	oo **	oo **	↑
	iv	Opt	o *?	oooo *?	oo ***	oo **	↑
iv	ip	Opt	ooo ***	oo *	ooo **	o *	↑
	iv	Opt	ooo *?	oo *?	ooo ****	o *	↑

[a]CP = *C. parvum*
[b]opt = optimal.
[c]o = predicted activation of macrophages by CP.
[d]* = antigen partitioning between subsets of macrophages.

with Cy by the iv route enhance SRBC responses of normal splenocytes. However, other points need to be clarified, and especially the nature of F and NF cells defined. There is need to determine the mechanism by which Cp, in combination with an optimal dose of antigen, induces suppressor cells. There is also need to invest more effort in studies on the role of the route of injection, and the iv/ip relation to enhancement and suppression described for Cp since it does not appear to hold universally. For example: using live BCG, the iv route tends to lead to suppression (Brown et al., 1980). It is interesting that enhancement occurs when 25% or more dead BCG organisms are included in the inoculum. The authors have actually demonstrated that the iv treatment with live BCG leads to an enhanced response or at least enhanced capability to respond to antigen, but this is masked by the induction of suppressor cells which, in this case, are apparently macrophages.

4.3.4. *C. parvum* Effects on Tumor Growth It is well established that *C. parvum* injected systemically can moderately retard the growth of various types of subcutaneous tumors. The antitumor effect of this organism injected systematically (ip or iv) is equally apparent in immunologically intact and thymus-deprived mice (Woodruff et al., 1973). By contrast, the antitumor effect of Cp injected into the tumor is dependent on an intact T cell function (Scott, 1975; Woodruff and Dunbar, 1975). Thus Cp appears to exert its antitumor effect by nonspecifically modulating the host RES as well as by acting as an adjuvant and augmenting immunity to tumor-specific antigens. Both effects make positive contributions to host survival, providing experimental justification for clinical use of Cp. Yet, closer scrutiny reveals that Cp effects are not invariably beneficial to the host since they may tip the balance in favor of the tumor with some types of tumors.

The effects of Cp on hosts implanted with two different lymphocytic tumors were compared. The tumors studied were the P388 lymphocytic leukemia of DBA/2 origin and the Gardner lymphosarcoma (GL) of C3H origin. Results indicated that Cp caused a significant prolongation of life of mice bearing the P388 tumor, and in contrast, a small but significant reduction in the survival of mice bearing the GL tumor (Table 18).

It has been previously noted that the immunomodulating effect of Cp on antitumor resistance was dependent on the dose of this agent and, while larger doses reduced immunity to the tumor, smaller doses actually potentiated resistance (Woodruff et al., 1976). This dose-dependent effect was clearly demonstrated with a

Table 18
Effect of *C. parvum*[a] on Survival of Mice with Two Different Tumors[b]

Tumor	Mean survival time (MST) ± 1 se[c]		
	Control	*C. parvum*	*p* values[d]
P388	16.6 ± 0.05 (16)	25.7 ± 1.0 (15)	<0.001
Gardner	15.1 ± 0.3 (36)	13.8 ± 0.3 (39)	<0.005

[a]1.4 mg *C. parvum* injected ip 4 days after tumor.

[b]BDF_1 mice injected with 1×10^4 viable P388 lymphocytic leukemia cells and C3H/HeN mice injected with 5×10^4 viable Gardner lymphosarcoma cells.

[c]Figures in parentheses are number of mice in each group; no survivors beyond 28 days.

[d]*C. parvum* caused significant prolongation of MST in mice bearing P388 tumor, but caused a small although significant reduction in the MST of mice bearing the GL tumor.

fibrosarcoma but, when graded doses of the agent were administered to animals with GL, no such dose-dependence could be seen and Cp caused either a deleterious effect in doses of 1.4 mg to 14.0 μg or was inert at lower concentrations.

The effect of Cp was also studied in tumor-bearing mice that received chemotherapy with the nitrosourea, MeCCNU. Mice were injected with 30 mg/kg MeCCNU 2 or 6 days following tumor inoculation. Some mice also received 1.4 mg Cp 4 days after tumor injection. Results summarized in Table 19 indicate that chemotherapy with MeCCNU administered 2 days after P388 or GL tumor completely cured over 90% of mice (survival beyond 42 days for P388 and beyond 52 days for GL) whereas treatment delayed until day 6 was less effective and only 25–30% of mice survived beyond 52 days. Combination of Cp with delayed chemotherapy on day 6 in mice bearing P388 tumor had a synergistic effect and resulted in 100% cure. By contrast, when Cp was combined with MeCCNU in C3H mice bearing the GL tumor, the vaccine produced an antagonistic effect and reduced the survival time from 35 to 17.5 days. Thus Cp, in the same dose (1.4 mg) administered at the same time and by the same route, could aggravate the course of some tumors and ameliorate that of others. It is clear that combination of Cp with chemotherapy on day 2 did not have any effect on the survival time of mice implanted with P388 or GL tumors, presumably because the treatment on day 2 eradicated the tumor.

Table 19
Modulation Chemotherapeutic Effect of MeCCNU by *C. parvum*[a]

	P388[c]			Gardner[c]		
MeCCNU[b] day	Control	*C. parvum*	*P*	Control	*C. parvum*	*P*
None	16.6 (0/16)	25.7 (0/16)	— 0.001	15.1 (0/8)	13.2 (0/8)	— 0.025
+2	39.5 (11/12)	39.5 (6/8)	ns	49.9 (7/8)	49.7 (7/8)	ns
+6	34.1 (2/7)	42.0 (8/8)	— 0.001	35.0 (2/8)	17.5 (0/7)	— 0.001

[a]1.4 mg *C. parvum* injected ip 4 days after 1×10^4 P388 or 5×10^4 Gardner lymphosarcoma cells.

[b]30 mg/kg MeCCNU injected ip 2 and 6 days after tumor.

[c]Mean survival time in days. Figures in parentheses are number of survivors/number of mice injected. *P* values were calculated by the Student's *t*-test; ns not significant (*P*, 0.05). Data obtained on day 42 after the initial injection of P388 and day 52 after the injection of GL tumor.

Mice surviving the primary tumor following chemotherapy (MeCCNU on day 2) or chemoimmunotherapy (MeCCNU on day 2 and Cp on day 4) were rechallenged with a second load of the respective tumor and the resistance of these mice to this challenge was assessed. It is clear from the data in Table 20 that mice cured of P388 leukemia by treatment with MeCCNU alone, 2 days following the primary tumor implantation, were not resistant to the second challenge. However, if chemotherapy on day 2 was followed by Cp treatment on day 4 after P388 inoculation, the survivors were significantly more resistant to the second tumor challenge than mice that received chemotherapy alone. By contrast, combination of immunotherapy with chemotherapy did not improve the resistance of mice to the second challenge with GL. The suppression by Cp of resistance to a secondary tumor challenge described above is similar to observations reported by Woodruff et al. (1976).

One thing that remains certain is that Cp is an immunologic modifier. It has been shown to cause both potentiation and suppression of cellular as well as humoral immune responses. Another point remains well established, that Cp is a potent stimulator of macrophages. According to the studies cited, such stimulation may lead to a number of end results and the diversity of the results at-

Table 20
Resistance of Mice Against Tumor Following Chemotherapy of the Primary Tumor and its Modulation by *C. parvum*

Previous exposure to tumor	MeCCNU[a]	P388[b]			Gardner[b]		
		Control	*C. parvum*[c]	P^d	Control	*C. parvum*[c]	P^d
No	No	17.4 (0/8)	nt	14.2 (0/10)	nt		
Yes	Yes	15.7 (0/11)	23.2 (0/6)	— 0.001	14.0 (1/7)	14.0 (0/7)	ns

[a]30 mg/kg MeCCNU injected 2 days after the first tumor inoculation (see Table 18). All survivors were rechallenged with 1×10^4 P388 or 5×10^4 Gardner lymphosarcoma cells 42 days after the primary tumor inoculation.

[b]Mean survival time calculated 42 days after the second challenge. Figures in parentheses are survivors/numbers challenged.

[c]1.4 mg *C. parvum* injected 4 days after the primary tumor inoculation (see Table 18); nt not tested.

[d]Values calculated by the Student's *t*-test; ns not significant (*P*, 0.05).

tests to the probable heterogeneity of macrophage functions. We are especially impressed with alterations in the intensity of response determined by the route of Cp injection and attribute the differences to the distribution of Cp and antigen to given sites, i.e., spleen vs peritoneum, as well as to activation of macrophage subsets performing unique functions (facilitation, i.e., antigen presentation, or nonfacilitation, i.e., antigen degradation of immune suppression).

As complex as the effects of Cp are in modulating antibody responses, the complexity may be compounded in tumor systems where some tumors are strongly immunogenic, others are not, and where tumor alone can modify the immune response by the production of substances that can suppress immunologic functions. At present, we can not explain the dichotomy in the immunogenic GL and nonimmunogenic P388 tumors. Recently, however, we have obtained preliminary data that provide a new aspect of the tumor-host-Cp interaction. When GL tumor bearing mice are treated with a chemotherapeutic agent, i.e., BCNU 6 days after tumor implantation, they not only become resistant to subsequent tumor challenge, but their lymphocytes can inactivate tumor cells in a Winn assay. When such mice are given Cp 4 days after tumor transplantation (2 days prior to BCNU) they are not only less resistant to subse-

quent challenge, but they do not appear to possess immune lymphocytes as measured by the Winn assay. Parenthetically, these mice do not generate suppressor cells capable of inhibiting the immune cells of GL-bearing, BCNU-treated mice not given *C. parvum*. Thus, one additional aspect of Cp action, at least in the tumor bearing host, is the preclusion of generation of tumor-specific immune lymphocytes.

4.3.5. Summary The immunomodulatory activities of *C. parvum* may result from a multitude of effects such as alteration in antigen distribution, presentation, disposition, and activation of cellular populations and subpopulations performing specific functions at any particular point in time (protagonistic or antagonistic). There is ample evidence that *C. parvum* activates macrophages and that depending on the type of macrophage being activated, the result may be a down or up regulation of the response. Other cells, notably T cells as well as NK cells, can also be activated by *C. parvum*. Under certain circumstances, Cp can induce suppressor cells. Thus, activated early-acting macrophages of the NF variety described in our hypothesis and suppressor cells may explain the suppressive activity of Cp administered prior to antigen. One may wish to add to this the possibility that part of the suppressive activity may be associated with the induction of interferon. Kirchner et al. (1977) have demonstrated that Cp is an effective interferon inducer and in Chapter 3, Section 5.5.2.3., we have already alluded to the ability of interferon to diminish the immune response when administered prior to antigen.

In the tumor-bearing host, Cp contributes an additional facet to immune induction and regulation. Preliminary studies suggest that treatment with Cp may inhibit the production of tumor-specific immune lymphocytes.

4.4. Other Natural Products

In previous sections we have discussed two natural products, Ete and Cp, with regard to their immunomodulatory effects on the immune response. In this section we will address the immunomodulatory effects of two other natural products, Rhus tox antigen derived from *Rhus toxicodendron* (Rt) (poison ivy) and an extract derived from the nut *Semecarpus anacardium* (Sa).

Although the role of poison ivy in contact dermatitis is well-known, there is little or no published data available on the regulatory effect of Rt or Sa on the immune response. Studies by the

Table 21
Effects of *Rhus toxicodendron* Antigen
and *Semecarpus anacardium* Extract on Anti-SRBC IgM Response

Treatment and dose[b]	Percent suppression[a]	
	Pre antigen	Post antigen
S. anacardium, 50 mg/kg	98	19
R. toxicodendron, 100 mg/kg	97	NC

[a]Percent suppression compared to controls of saline.
[b]Antigen SRBC 1×10^9 in 0.2 mL ip on day 0; treatment at day −2 or day +2. PFC assayed on day 4 after antigen. NC = no change.

authors (Ghaffar and Sigel, in preparation) have demonstrated that these substances behave like Ete and Cp in that they cause suppression of primary anti-SRBC PFC responses when injected ip 2 days prior to antigen; moreover, they were ineffective in suppressing primary responses when given after antigen (Table 21).

No extensive studies of the modes of action of these natural products have as yet been undertaken except for an assessment of their effects on macrophage cytotoxicity. Sa administered 2 days after Cp (used as the activator of macrophage cytotoxicity) abolished the cytotoxic effect but, when given 2 days prior to Cp, it showed no effect, suggesting that this substance could damage activated macrophages, but not resting macrophages. It should be noted parenthetically that this substance, in the absence of Cp, exerted a growth-promoting effect for the tumor target cells used in the cytotoxicity assay. The poison ivy antigen had no deleterious effect on macrophages when administered prior to or after Cp and, in fact, it appeared to cause, by itself, a moderate activation. From these initial experiments, it is obvious that further studies must be conducted on not only the humoral response to SRBC, but the cellular component of immunity as well, if the mechanism of suppression by these natural products is to be understood. It is also clear that further studies on the influence of route and timing of treatment are essential for the dissection of the modes of action of these natural products.

4.5. Radiation Paradox

4.5.1. Background Review Radiation administered prior to immunization is more immunosuppressive than when administered after immunization (Taliaferro and Taliaferro, 1954) and, in

fact, there have been reports of enhancement of antibody production by ionizing radiation given after antigen (Taliaferro and Taliaferro, 1954; Gengozian and Makinodan, 1958; Morgan et al., 1960; Dixon and McConahey, 1963; Taliaferro et al., 1964; Hoffstein and Dixon, 1974). Hence the radiational paradox. The development of radioresistance in the course of immunization has received many explanations from the time it was first observed in the early 1950s. The various experiments, interpretations, and theories have been comprehensively reviewed by Anderson and Warner (1976). Radioresistance has been attributed to:

1. Disproportionate replacement of radiation-damaged lymphocytes by rapidly proliferating antigen-stimulated cells.
2. Release from the gut of LPS, which may promote immune responses.
3. Alleged greater propensity for self-repair on the part of the DNA of replicating lymphocytes.
4. Local cellular destruction, creating a milieu favorable to the proliferation of immunocompetent cells.

Some of these explanations were based on circumstantial evidence rather than on solid facts. It has been difficult to reconcile what has appeared to be paradoxical or contradictory findings because of the problems arising from the coexistence of multicompartment systems of interactive cells (with unique sensitivities to ionizing radiation) and the use of methods lacking dissective capability, for example: measuring antibody titers as opposed to PFC, or performing an entire experiment in the intact animal (*in situ*) as opposed to assessing effects on individual classes or subclasses of lymphocytes with the aid of adoptive transfer.

More recent experiments, based on cell separation techniques, in vitro immunization, and in vitro effects of radiation, adoptive transfer and other refinements, have made possible more precise delineation of the radiosensitivities of various cellular subpopulations and their functions. These have been elegantly summarized and analyzed by Anderson and Warner (1976). Some facts stand out clearly and decisively, others are more speculative and often controversial. Nonetheless, certain trends and patterns are beginning to emerge that may some day fully explain the differential effects of radiation on the immune system. In order to understand some of the discrepancies, one has to realize that the effects may register in the form of mitotic death or interphase death. The former occurs when the cell reaches the first or second mitosis fol-

lowing irradiation, whereas the latter may occur almost instantaneously, within an hour or less after irradiation, with no particular reference to entry into the mitotic cycle. The inability to divide does not preclude the capacity to synthesize mRNA or protein. Thus, functions of a cell may remain fairly intact despite their loss of ability to undergo DNA synthesis or cell division. This is of special importance in the immunological context where cell proliferation is but one of the steps in the immune response and its failure need not affect the other steps, such as recognition of antigen, presentation of antigen to other cells, cellular cooperation through various soluble substances (lymphokines), or antibody production. Some of the findings cited by Anderson and Warner (1976) indicated that one of the effects of radiation is altered cell traffic, which may cause a change in cell distribution leading to a disruption in the cellular interactions required for the initiation of propagation or the immune response.

4.5.2. Cellular Targets in the Lymphoreticular System and Indications of Selective Action In general it appears that B lymphocytes are more radiosensitive than T lymphocytes, particularly with respect to interphase death. However, there is a considerable amount of variation pointing to the existence of heterogeneity of B cells with respect to radiosensitivity. A change in radiosensitivity occurs in the process of B cell maturation that terminates in the formation of plasma cells characterized by extreme radioresistance. For example, exposure of spleen cells in diffusion chambers to 10,000 rads during the late phase of secondary antibody production resulted in a relative increase in the proportion of plasma cells (Makinodan and Albright, 1967). This same dose of radiation did not cause a diminution in the rate of antibody synthesis (Vann and Makinodan, 1969). Considerably more work has been done on the effects of radiation on T cells. This work has encompassed a dissective approach permitting the measurement of sensitivity under various conditions in vivo and in vitro, thereby allowing the determination of effects on such diverse aspects as cell type, cell traffic, cellular microenvironment, and status of the cell with respect to stimulation by antigen. The role of these variables can be detected by the use of such assays as GVH, allograft rejection, helper function, or proliferation (Anderson and Warner, 1976). Unprimed T cells (presumably precursors of T helper cells) are radiosensitive. With respect to primed T cells, the results of radiation seem to depend on the stage of cellular differentiation, activation, and/or function. We are referring here to such functions as

proliferation, migration, and the ultimate provision of helper activity. When assayed in vitro, activated T cells appear to be radioresistant. Since in this assay system cellular homing patterns are not critical, the radioresistance has been interpreted as indicating that the antigenically activated helper T cells are not sensitive to radiation-induced interphase death and that continuous cellular proliferation is not required for the expression of helper functions. One assumption has been made: that, although early proliferation may be vulnerable to mitotic death, the heightened metabolic activity of the activated cell permits repair of the DNA synthetic apparatus.

These findings appear to set a rational basis for the increased resistance of the activated immune system to radiation damage. However, this apparent correspondence disappears when one looks at some in vivo experiments (Anderson and Warner, 1976). Adoptive transfer experiments have shown that primed T cells are radiosensitive. To quote the reviewers, "If radiation has not caused interphase death in these cells and proliferation is not required, other factors must influence the ability of the helper cells to mediate their effects after transfer." Studies by Anderson et al. (1974) have demonstrated marked alteration in the homing patterns of irradiated lymphocytes, thereby suggesting that a failure of irradiated helper cells to home properly to lymphoid organs is the reason for the failure to satisfactorily interact with B cells.

The complexity of the problem is further exemplified by a comparison of sensitivity of different types of TH cells. Cells providing nonspecific help in vitro were resistant to 1000R whereas cells providing specific (cognate) help were sensitive (Lubet and Kettman, 1979).

Currently, almost every immunological seesaw is considered in the light of suppressor cell functions and it is only natural that the radiation paradox be viewed in this light. Since it has been thought that suppressor cells are more vulnerable to radiation than helper cells, it would follow that radiation after antigen should eliminate these cells preferentially and make for a better response. However, as cited by Anderson and Warner (1976), mature activated suppressor cells appear to be more resistant than precursors of suppressor cells (Dutton, 1972; Rich and Pierce, 1973), thereby providing a counterargument.

Earlier studies have demonstrated that radiation affected the localization of antigen in lymphoid follicles (Jaroslow and Nossal, 1966; Williams, 1966). In part, such alterations may reflect changes in macrophage functions. The different macrophage functions

have been found to manifest differences in radiosensitivity. Replication is radiosensitive, but migration and phagocytic activity are resistant. There is controversy regarding the post-phagocytic functions: catabolism, degradation, and retention of antigen. It is of interest that, under some conditions, radiation causes increases in DNA and RNA synthesis, as well as increases in the release of monokines (Geiger et al., 1973). Another controversial aspect concerns the efficiency of antigen presentation by macrophages to the lymphocytes. According to Anderson and Warner (1976), in some experiments irradiated macrophages appear to be more efficient, while in others they appear to be less efficient. One can therefore not find comfort in these macrophage-related findings as a guide to the explanation of the paradox of suppression by radiation prior to immunization and enhancement by radiation after immunization.

4.5.3. The Role of Receptors Linked to Adenylate Cyclase, Consideration of cAMP, Hormones, and Suppressor Factors

Other explanations will have to await the elucidation of changes caused in extranuclear cellular sites. Some of these changes are rather subtle or may not be readily perceived through immunological assessment. We are particularly intrigued by alterations in plasma membranes as manifested in expression and function of receptors. In the present context, we should like to focus on receptors linked to the activity of adenylate cyclase because of the findings of Kemp and Duquesnoy (1975) that lymphocytes surviving total body radiation (800R) manifested a greatly diminished response to epinephrin. In order to appreciate the potential significance of these findings it is necessary to examine the current status of information regarding cyclic AMP-stimulating substances in relation to cell membrane receptors and the consequences of increased formation of cAMP:

1. Lymphocytes possess receptors for: (a) epinephrin and other beta-catecholamines; (b) histamine, and (c) prostaglandins (Melmon et al., 1972; Weinstein et al., 1973).
2. Beta-catecholamine, histamine, and prostaglandins of series E stimulate increased formation of cAMP (Bourne et al., 1972).
3. The increased intracellular accumulation of cAMP is associated with a dampening of a variety of immunologic responses and reactions (Bourne et al., 1974; Melmon et al., 1974a).

4. The population distribution of the receptors on lymphocytes is not random, but appears to be in part related to their stage of differentiation and function (Bourne et al., 1974).

Thus, receptors have been demonstrated on antibody-forming cells (PFC), but not on their precursors (Melmon et al., 1974a,b). They also exist on suppressor cells, but on these they seem to be expressed prior to immunization. One could therefore construct the following hypothesis: (1) Under normal conditions, an endogenous hormone (epinephrin) would not interfere with induction of a positive immune response because the precursors of antibody-forming cells would not bind the hormone as efficiently and would therefore function normally, whereas suppressor cells may be kept in check owing to the presence of appropriate receptors. (2) After immunization an opposite situation may abound owing to the expression of receptors on activated antibody-forming cells. Hence histamine or beta-adrenergic hormones may now play a role in reducing or regulating the ongoing response (Rocklin et al., 1978). (3) Any action that removes receptors or makes them inactive may reverse the pattern cited under #2 thereby deregulating the system and enhancing the response.

Thus, action of radiation on the catecholamine receptors may be a clue to the lesser suppression and/or the enhanced response caused by radiation when administered after antigen. Similarly, radiation administered before antigen may act on the receptors of suppressor cells, diminish their hormone responsiveness and thereby release the suppressor cells from the quiescent state and prepare them for their suppressive action.

Results similar to those obtained with radiation were recorded by Kemp and Duquesnoy (1975) *with treatment with cortisone.* There was a relative sparing of the basal cyclase activity, but diminution of responsiveness to epinephrin, suggesting that cortisone altered the receptors for beta-catecholamines. It is of interest that, in mice, radiation and cortisone have similar time dependence for the exertion of optimal immunosuppression; they have to be administered prior to immunization (Berenbaum, 1979). It should also be noted that it is possible to prevent impairment of immunologic functions by agents that block the binding of beta-adrenergic agonists. In connection with this it is pertinent to cite the action by lymphocytosis-promoting factor of *B. pertussis;* this agent inhibits the increase in cAMP in human lymphocytes caused by the beta-adrenergic agent, isoproterenol. It also blocks the effects of prostaglandin E1, which binds to a different receptor

(Morse, 1974). The in vivo production of beta-adrenergic blockade by *B. pertussis* may, in fact, owe to the action of this factor.

We should point out in discussing the radiational paradox that, although we have slanted our thoughts toward cAMP as the responsible mediator of regulatory function, it now appears from the studies of Rocklin et al. (1978, 1979) that a more direct mediation of suppression may also be involved, a suppressor factor produced by TS cells. These cells possess receptors for 4-methyl histamine and produce histamine-induced suppressor factor (HSF) in response to this agent. This process appears to be independent of the production of cAMP. Since other agonists, including cholera toxin, that raise cAMP fail to induce HSF, these recent findings emphasize the need to keep a clear distinction between causal effects and parallel effects, since they seem to indicate that accumulation of cAMP and production of suppressor factors are parallel effects, but that cAMP need not be the critical mediator of suppression. Thus, although lymphocytes possess receptors for various hormone agonists and their functions can be suppressed, especially in vitro in parallel with induced synthesis of cAMP, there are also reports that cAMP can actually potentiate immune responses. Ishizuka et al. (1970) demonstrated that cAMP enhanced the anti-SRBC PFC responses in mice when antigen and this nucleotide were injected simultaneously or cAMP was given 24 h after antigen. More recently, Northrop and Fauci (1972) demonstrated significant potentiation of anti-SRBC response by simultaneous iv injection of SRBC and cholera enterotoxin, which was an excellent cAMP inducer but which, according to Rocklin et al. (1979), does not evoke induction of suppressor factor. We emphasize simultaneous injection because the timing of treatment and immunization determines the outcome of response. For example, cholera toxin is highly suppressive when given 2 days prior to antigen (Berenbaum, 1979). These results taken as a whole undoubtedly reflect the interaction of multiple receptors involved in perturbing the dynamic equilibrium during an immune response. Future studies on the role of cyclic nucleotides in the regulation of protein synthesis and specialized functions of T and B subsets may clarify some of the controversial findings, but then again, the controversy may be built into the system. As already discussed throughout both chapters, the effect of an agent may depend on time and dose. This applies to antigens and may also apply to endogenous modulators such as interferon and regulatory cells. In the present context we suggest that this type of up and down regulation by the same molecule may occur with cyclic nucleotides and catecholamines or other agonists including PGE. In fact, a recent report (Prosser et al., 1980) intimates that PGE in

low concentrations can amplify early responses of macrophages to activational stimuli, but that higher concentrations appear to suppress subsequent expression of activation.

4.6. Ultraviolet Radiation and Immunosuppression

A uniquely intriguing example of suppression by radiation prior to antigenic challenge is the effect of ultraviolet (UV) irradiation on tumor rejection. Fisher and Kripke (1977) demonstrated that chronic irradiation of mice with UV light results in failure to reject highly antigenic, transplanted UV-induced tumors that are rejected by unirradiated syngeneic recipients. An immunological basis of this alteration was demonstrated by transferring lymphoid cells from UV-irradiated mice to lethally X-irradiated recipients. These recipients were unable to resist a later challenge with a syngeneic UV-induced tumor, whereas recipients given lymphoid cells from normal donors were resistant to tumor growth. Studies by Spellman and Daynes (1977) demonstrated that the UV-mediated suppression of the antitumor response can be adoptively transferred to normal syngeneic mice with lymphoid cells derived from UV-irradiated donors. Transfer of the suppressive effect was found to be dose-dependent and appeared to require viable T lymphocytes, suggesting the UV light increased suppressor cell activity. The results of Fortner and Kripke (1977) using an in vitro microcytotoxicity test suggest that the end result of this suppression is a decrease in the level of cytotoxicity produced at the effector stage of the immune response to UV-induced tumors.

The impairment of tumor resistance by UV light may have multiple causes. In one study, Lill and Fortner (1978) compared the cellular populations associated with regressor tumors in normal mice and progressor tumors in UV-treated mice and demonstrated a significant deficit in T cells in the latter, even though there were no major differences in the number of macrophages, granulocytes, or B cells in the two types of tumors. The studies of Spellman and Daynes (1978) have brought forth evidence for the induction by UV treatment of a suppressor lymphocyte that is capable of specifically inhibiting effective responses directed against antigens of UV-induced tumors. Studies by Fisher and Kripke (1978) have established that T lineage of the suppressor cell.

The cellular perturbations produced by UV-irradiation apparently include impairment of an antigen-presenting cell (Fox et al., 1980). The T suppressor cell that interferes with rejection of tumors may be activated as a consequence of inappropriate antigen pres-

entation by the antigen presenting cell, presumably the macrophage.

Clearly there is a multitude of potential targets that may be altered by radiation, leading to suppression or enhancement of the immune response, depending upon which cellular target is hit and what stage or state of response that particular cell has reached.

To summarize, modulation of immune responses by radiation would appear to be the result of the interaction of a panoply of factors and events that can synergize with each other or can antagonize each other. In the present context it is possible to view the effects of radiation (and probably other immunosuppressive agents) as exerting direct action and setting the stage for contributory action by certain endogenous components including hormones. If the accessory activities add further suppressive effects, the result will be suppression of the immune mechanism, but if the secondary effects would tend to augment certain immunological functions, they may impart a rescue or compensatory effect thereby eliminating the overall impact of radiation. Moreover, if there is selectivity in the direct action, the balance may be shifted in the other direction, i.e., potentiation instead of suppression. The hormones and the presence of appropriate receptors on lymphocytes provide an example that may thus help to explain some of the paradoxical effects of radiation.

We have been fascinated by another immunoregulatory paradox, one presented by corticosteroids. Unfortunately, we shall not review the subject, but refer the reader to recent publications on glucocorticoid-mediated suppressive action due to inhibition of TCGF production (Gillis et al., 1979a,b) and the potentiating action that may in part owe to a loss of responsiveness to epinephrin cited in this section (Kemp and Duquesnoy, 1975) or to some modulatory effect on the triggering of B cells (Fauci et al., 1977). Elimination of suppressor cells by corticosteroids has also been intimated (Waldmann et al., 1976). See also a recent review by Parrillo and Fauci (1979).

5. The Many Faces and Interfaces of Cyclophosphamide Action

No other drug, except possibly the corticosteroids, has received as much attention as Cy in experimental and clinical situations in which immunosuppression was either a deliberate or incidental occurrence. The end result of treatment with Cy may be suppres-

sion, enhancement, or no discernible change, depending not only on the dose of the drug, but also on the regimen of treatment, the nature of the antigen, and the type of response measured. The seemingly paradoxical ability of a drug to exert opposing effects, suppression and potentiation, may go undetected unless the effects are assessed under a variety of conditions with various antigens and in relation to an array of immunologic functions. Thus, in the studies of Berenbaum (1979) utilizing SRBC and LPS (TD and TI antigens, respectively), Cy manifested no selectivity and no paradoxes in comparison to radiation, which was substantially more suppressive prior to immunization. In contrast are the results from our laboratory indicating a selectivity in the action of Cy against another TI antigen, SIII. Based on data from many sources, Cy and other alkylating agents appear to be capable of discriminatory actions against specialized immunologic functions of cellular subsets. In this section we shall summarize some of the information regarding metabolism of Cy, briefly review the published reports on the multifaceted effects of Cy, and focus on what we consider to be particularly interesting aspects of Cy activity relating to modifications of membrane structures and selective activity on regulatory cells.

5.1. General Considerations of Cyclophosphamide Action

The purpose of chemotherapy is to attack biochemical pathways essential to the replication and survival of tumor cells. Unfortunately cytotoxic and cytostatic agents, as a rule, lack selectivity for cancer cells and often cause damage to normal tissue. Among the various strategies considered in the development of selective action against tumor cells was the construction of molecules that are inert in their original form, but that possessed latent activity that came to the fore when the molecule was metabolized in vivo. The synthesis of Cy by Arnold and his associates (1958) was inspired by the belief that this phosphorus-containing alkylating agent would be activated efficiently by tumors thought to possess high levels of phosphamidase activity (Ichihara, 1933; Gomori, 1948). This ingenious strategic approach had been attempted earlier by Seligman and his associates (1949) and by Friedman and Seligman (1954). They prepared phosphoric esters that could be converted by acid phosphatase to hemisulfur mustard and they also produced a variety of phosphamide alkylating agents to be activated by phosphamidase.

The activity (including cytotoxicity) of alkylating agents of the

nitrogen mustard-type depends on the basicity of the nitrogen atom (Ross, 1962; Bardos et al., 1969). The basicity of the nitrogen is increased by the addition of electron-releasing groups or the removal of electron-attracting groups. The strategy of preparing latent alkylating agents calls for addition of electron-attracting groups that can be removed enzymatically in vivo by hydrolysis or reduction, thus rendering the inert substance chemically active. Activation can be achieved by reduction, by esterases, by changes in pH, by hydrolytic enzymes, and by microsomal oxidation.

The initial intent was to have a latent alkylating agent that would release nitrogen mustard upon activation by enzymes present in high concentration in the tumors. However, it soon became obvious that the primary activation of Cy occurred in the liver (Foley et al., 1961). Liver homogenates as well as isolated liver microsomes incubated with an NADPH-generating system can activate Cy in vitro to form metabolites possessing antitumor and immunosuppressive activities (Colvin et al., 1973; Shand, 1978). The basic nitrogen acts as an electrophile and combines with atoms of oxygen, nitrogen, or sulfur. Thus, although strongly reactive with DNA, alkylating agents, including Cy, can react with other macromolecules including proteins, RNA and low molecular weight moieties (Ludlum, 1977). This must be kept in mind in order to appreciate the effects of alkylating agents on cells in various stages of growth and differentiation. The importance of attack on DNA is demonstrated by the multiplicity of effects such as mutation, carcinogenesis, and cytostasis. Some of these effects are summarized in Table 22, reproduced from the review by Ludlum (1977).

Connors (1976) has reviewed some of the metabolic events associated with the activation of Cy and a diagram from the review is given in Fig. 8. The various metabolites have been isolated from urine after Cy treatment (Montgomery and Struck, 1973) or from

Table 22
Reactions of Alkylating Agents with DNA[a]

1.	Substitution reactions
	a. Alkylation of bases
	b. Esterification of phosphate groups
2.	Cross-linking reactions
	a. Intrastrand cross-linking reactions
	b. Interstrand cross-linking reactions
3.	Strand-breaking reactions
	a. Single-strand breaks
	b. Double-strand breaks

[a]From Ludlum, 1977.

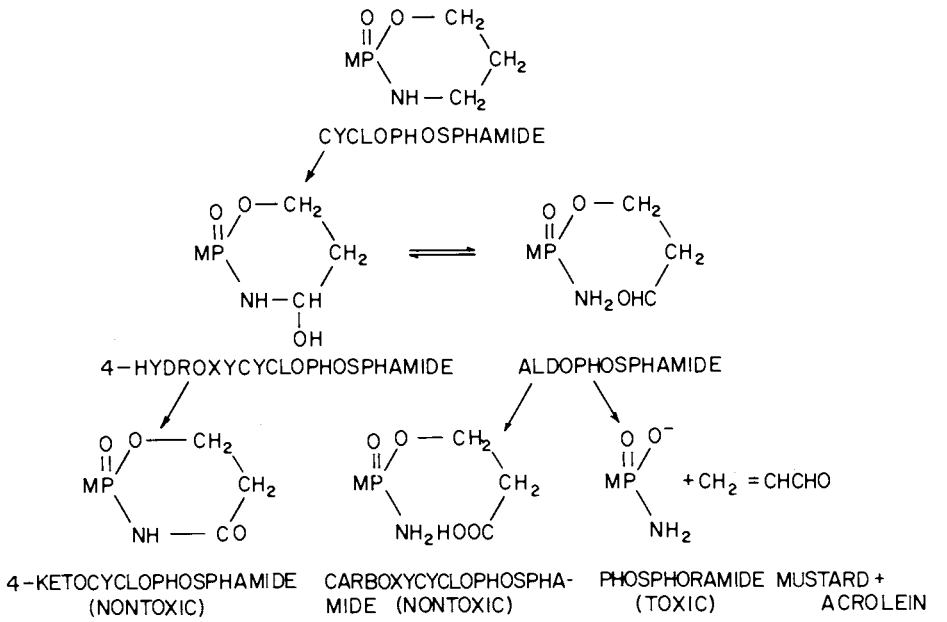

Fig. 8. Metabolism of cyclophosphamide in vivo after Connors (1976). See text for description M=—N $(CH_2CH_2\ cell)_2$.

microsomal incubations (Colvin et al., 1973; Shand, 1978). Carboxycyclophosphamide is the major urinary metabolite, but it, as well as the 4-ketocyclophosphamide derivative, are not tumor static or tumoricidal in vivo, suggesting that the active component is produced at an earlier stage in the metabolic pathway. Based on the scheme proposed by Connors (1976), 4-hydroxycyclophosphamide is the primary metabolite of microsomal oxidation. This product and its tautomer, aldophosphamide, become converted enzymatically to stable and nontoxic 4-ketocyclophosphamide and carboxycyclophosphamide. On the other hand, in the absence of enzymes, aldophosphamide can break down spontaneously into phosphoramide mustard and acrolein, both of which are toxic.

5.2. A Brief Review of Reports on the Variable Action of Cyclophosphamide on the Immune Response

With regard to humoral immunity, there is general agreement that Cy suppresses primary antibody responses to TD antigens when given either before or after antigen. However, although this drug belongs to Berenbaum's Class I (effective before and after immunization; see Berenbaum, 1979), its effect is more profound when the

drug is administered after antigen, presumably because of its greater toxicity against proliferating cells. The effect of Cy is also dose-dependent and when administered prior to antigen is reversible. Thus, in the work of Shand there was complete functional recovery of the immune system in CBA mice 6–7 days after drug administration (Shand and Howard, 1979) whereas, in the studies of Berenbaum (1979) using Balb/c mice, there was partial recovery in 7 days. The secondary response (immunologic memory) appears to be more resistant to Cy than the primary response. As described in Section 1.3 in our studies Cy given prior to priming with antigen had no untoward effect on the secondary response. When given two days after the priming antigen or two days prior to secondary challenge, the drug had a substantial suppressive effect (but below that demonstrated for the primary response). The most significant suppression was obtained when Cy was administered two days after secondary challenge. These results suggest that although activated memory cells are more sensitive to Cy than memory cell precursors, these precursors of memory cells (resting state) are more resistant to Cy than are resting cells involved in generating the primary antibody response.

Cy has also been shown to inhibit TI antibody responses to LPS, levan, and dextran when administered prior to immunization. It failed to inhibit the TI response to SIII under the same conditions, but inhibited this response (and the LPS response) when administered after immunization. The difference in susceptibility of the separate TI responses to Cy reinforces the probability that there is selectivity with respect to subsets of B cells responsive to different antigens.

There is obviously a need for more work with B cell subsets, but more urgent is the need for investigations on the relative susceptibility of B and T cells to Cy. Such work has already begun as exemplified by the investigations of Shand (1978) applying microsomally activated Cy (50 μg/mL) to mouse splenocytes in vitro, transferring these to lethally irradiated animals, and attempting to restore immune responses with purified B or T cells. Neither T nor B cells alone could *fully* restore the response, suggesting that both classes of lymphocytes were damaged by the Cy derivatives. A different approach was taken in our laboratory aimed at a determination of whether T helper cells would be eliminated by Cy treatment making use of nude mice as indicators for adoptive acquisition of T helper function. Normal Balb/c or Balb/c mice immunized with SRBC were treated with 25 or 100 mg/kg of Cy dosages capable of inhibiting primary immune responses. Splenocytes of these ani-

mals were transferred to Balb/c nude mice that were immunized simultaneously with 1×10^8 SRBC. The PFC responses of nude mice receiving splenocytes from Cy treated animals were essentially on a par with nude mice receiving corresponding splenocytes from Cy untreated donors. We interpret the data to mean that the donors' *T cells,* both resting and activated, *were resistant to Cy* as they were capable of providing help to the nude recipients' B cells and that the loss of response in the donor owed to damage to B cells (Ghaffar et al., 1981). This is a provisional interpretation since nude mice are known to possess precursors of T cells that may have been brought into play by some cellular elements contained in the transfer population. This point needs considerably more attention and study. Our data contrasts sharply with those of Shand and the difference may owe to any of the factors inherent in the in vivo vs in vitro Cy activational processes and modes of treatment, i.e., number of metabolites released, detoxification, dilution, diffusion, and proximity of cells to the drug.

Although helper functions of certain T cells appear to be preserved in Cy-treated animals, other functions of T cells are altered. In the remainder of this section, we shall attempt to integrate some thoughts based on results that have demonstrated selective activity of Cy against individual forms of cell-mediated immunity and take another look at the effect of Cy on regulatory cells.

Although Cy causes suppression of blastogenic transformation (Milton et al., 1976; Lopez et al., 1980), this suppression may result from the direct action of Cy or the indirect action of a suppressor cell induced by Cy (Milton et al., 1976). In addition, it would appear that spleen cells that are inhibited from proliferation by Cy are still able to carry out cytotoxic functions and that the cytotoxic activity of the nonproliferating cells is apparently mediated through a soluble factor(s) (Lopez et al., 1980). It is interesting to note that Cy also had no effect on either ADCC or macrophage cytotoxicity induced by *C. parvum.*

With regard to DTH, the observations that Cy suppresses DTH when given after antigen (Table 6) (LaGrange et al., 1974b; Sy et al., 1977) and has no effect or enhances DTH with pretreatment (LaGrange et al., 1974b; Zembala and Asherson, 1976; Sy et al., 1977), suggest the generalization that precursors of TDH cells are Cy-resistant and activated TDH cells are Cy-sensitive. There is at least one contradictory report (Kerckhaert et al., 1977) in which it was claimed that a high dose of Cy (300 mg/kg) enhanced DTH to SRBC when administered to mice from 7 days before to 15 days after antigen. The reason for enhancement by Cy after immunization in this

study is not clear, but it should be pointed out that SRBC were administered together with complete Freund's adjuvant (CFA). This combination introduces major variables, including delay in antigen distribution and presentation, recruitment of inflammatory cells, and granuloma formation. The use of CFA may also explain another type of discrepant finding, that of Jokipii and Jokipii (1973), who demonstrated that treatment of guinea pigs with 300 mg/kg of Cy prior to sensitization with azobenzene-arsonate-*N*-acetyl-1-tyrosine caused suppression of DTH. It is possible that Freund's adjuvant creates a shift in antigenic dose available for immediate sensitization. The interrelationship of antigen dose and effect of Cy on DTH has been considered by Schwartz et al. (1978)(see below). It would appear therefore that Cy enhances DTH reaction when injected before antigen and suppresses when given after antigen, but results may depart from this "rule" when antigens are incorporated in CFA or antigens themselves are granuloma forming.

The diversity of effects of Cy is determined not only by the dose of Cy used and the use of CFA, but also by the dose of antigen as well. This is exemplified by the results of Askenase et al. (1975) who reported that pretreatment of mice with Cy (20 mg/kg) augmented the DTH response to SRBC at doses of antigen higher than required to induce optimal DTH. Likewise, Sy et al. (1977) demonstrated that pretreatment with Cy reversed the suppression in mice SOI sensitized with DNFB, but Cy had no effect on the optimal sensitization regimen. These findings may, in fact, reflect potentiation by Cy owing to elimination of suppresor cells, a possibility alluded to by others showing *enhancement* of DTH by pretreatment with Cy (Maguire and Ettore, 1967; Turk et al., 1972; Ramshaw et al., 1977).

Potentiation by Cy owing to elimination of suppressor cells is compatible with the hypothesis of Schwartz et al. (1978), who propose that at high antigen doses, Cy-sensitive regulatory cells suppress DTH. Thus, pretreatment with Cy would eliminate the suppressor cell produced by a high antigen dose, giving enhancement of the DTH response. These investigators also propose that, at optimal doses, regulatory cells would become unnecessary whereas, at low doses, a Cy-sensitive regulatory helper cell would come into play. Thus, Cy pretreatment would lead to a lowered DTH response to low antigen doses, but would not modify the response to optimal antigen doses. This hypothesis allows one to begin focusing on the interplay of helper and suppressor cells involved in the DTH response, but must be looked upon as only a guide since examination of the hypothesizers' own work (Askenase et al., 1975) indicates they obtained augmentation of DTH in mice

immunized with a suboptimal (low) antigen dose of SRBC following Cy pretreatment.

It is generally agreed that precursors of TDH cells are resistant to Cy. From our data and those of others (LaGrange et al.,1974b; Sy et al., 1977), it could be intimated that differentiating pre-effector TDH cells are sensitive to Cy because Cy administered 2 days after sensitization diminished the TDH response. More recent studies of Kaufmann et al. (1980) have shown tht mature effector TDH cells are relatively resistant to at least one derivative of Cy tested in vitro, 4-hydroxycyclophosphamide. This would seem to indicate that TDH cells acquire resistance to Cy with maturation, but there is a caveat in order: the 4-hydroxycyclophosphamide in vitro need not reflect fully the potential of Cy in vivo, as the whole molecule yields other metabolites that may have selectivity.

The suppressor cells involved in regulating both the cellular and humoral immune responses are undoubtedly a quite heterogeneous group of cell subsets. The result of Ramshaw et al. (1977) would suggest that the TS cell regulating the antibody response to horse RBC is different from the suppressor cell regulating the DTH response. The authors observed that high levels of DTH could be maintained in mice simultaneously with specific antibody unresponsiveness induced by Cy injected 24 h post-HRBC. In addition, the T cells from unresponsive mice specifically repressed the anti-HRBC response of normal spleen cells when they were mixed and injected into irradiated recipients.

We have cited other examples of *split* tolerance, where one limb of the immune mechanism is turned on to a positive response while the other is suppressed (see Chapter 3, Section 6.4.3).

Cy has proven highly useful both in induction of nonresponsiveness and in analysis of its diverse mechanisms. We have reviewed the elegant studies by Claman and his associates (Chapter 3, Section 6.4.2) in which Cy made possible the recognition of the two unique forms of nonresponsiveness, TS-independent and TS-dependent. This drug has also proved valuable in the characterization of DTH regulation in the studies of Zembala and Asherson (1976). Cy has also been instrumental in clarifying many of the important interactions observed by Benacerraf and his associates. Yet the precise mode of action of Cy remains unclear and the targets of Cy action are confounding. We have related the numerous findings concerning differences when nonresponsiveness was achieved by a variety of means and when investigators looked at cells involved in the afferent vs efferent phase of DTH (Chapter 3, Section 6). We also cited findings on Taux cells. T cells were not the only targets; B

cells and macrophages have also been implicated in regulation. Then there was the system described by Hahn and associates (1979) of active suppression in which transferable TS cells could not be demonstrated, but where Cy seemed to abrogate this suppression, presumably by eliminating a putative suppressor cell. An even more spectacular complication arises from the findings that Cy may in fact activate suppressor cells. This appeared to be the case in the report of Milton et al. (1976) and in the report of L'Age-Stehr and Diamantstein (1978). In the last instance the suppressors may be B cells. Although complicated, the situation is not hopeless, since identification of the regulatory targets of Cy action has already succeeded to some extent.

Thus, Whisler and Stobo (1978) have demonstrated suppressor cell heterogeneity in SOI mice by density on discontinuous BSA gradients and sensitivity to Cy. The low density cells that suppressed DTH but not PFC responses were resistant to 20 mg/kg of Cy. By contrast, the high density cells that suppressed both responses were sensitive to this dose of Cy. In vitro studies of Kaufman et al.(1980) have shown the variable sensitivity of precursor vs effector TS cells in DTH reactions: the latter being relatively less sensitive. The relative resistance of mature suppressor cells is similar to our findings on the TS cell regulating the humoral response (Ghaffar et al., 1980). The precursors of TSI cells functional in the humoral response were found resistant to Cy in our experiments whereas the precursors of TSe ($Ly1^+23^+$) were found sensitive by Cantor et al. (1978). But even at this stage there is heterogeneity among precursors of TSe cells as displayed in model E (Chapter 3, Fig. 6), which indicates that antigen-activated precursors of idiotype-bearing TSe are Cy-sensitive whereas the anti-idiotype bearing precursors of the second generation TSe cells are Cy-resistant.

5.3. The Effects of Cyclophosphamide on Cell Membranes

At the present time there is no explanation for the inhibition by Cy of functions of cells displaying idiotype structures (antigen receptors) and lack of inhibition of a function of cells displaying anti-idiotype structures. These observations lead to a recall of previous findings on the action of Cy on membrane receptors. Although the main action of Cy appears to be on cellular DNA, resulting in the cessation of cell proliferation, there are indications that this agent may modify the immune response by altering other constituents of

the cell. Guttmann (1974) found that Cy affected the expression of surface antigens on lymphocytes, making them nonstimulatory and nonresponsive in mixed leukocyte cultures. Shand and Howard (1978) have recently reported evidence for the impairment of surface immunoglobulin receptor regenerative capability in B cells following treatment with Cy. This is an important discovery since the surface Ig receptor is the primary site for binding antigen to the cell membrane. Following injection of Cy (150 mg/kg), B cells from the spleen were unable to regenerate surface Ig receptors subsequent to capping with an anti-Ig serum and culture in vitro. This loss of regenerative capacity in the absence of antigen was reversible with time, with normal capacity being restored 7 days after treatment with Cy. Other immunosuppressive agents (melphalan, chlorambucil, and 6-mercaptopurine) did not exert this effect (Shand and Howard, 1979).

The authors believe that this action of Cy may constitute the basis for increased vulnerability of Cy-treated animals to the induction of tolerance to TI antigens. Data reported by Shand and Howard (1979) support this contention. Normal spleen cells were treated with 50 μg/mL of microsomally activated Cy and transferred into lethally irradiated recipients that were *then immunized* with varying doses of levan the next day. The recipients, along with control mice, were challenged 10 days later with 10 μg/mL of levan and their spleen PFCs were measured after 5 days. The results clearly showed that a treatment with Cy (which impairs in B cells the capacity to regenerate receptors) renders B cells hypersusceptible to tolerance induction. They discuss the inability to regenerate surface Ig (SIg) *with persistance of tolerance* (in the presence of antigen) by pointing to the fact that anti-Ig treatment of immature B cells in fetal liver, neonatal spleen, and adult bone marrow causes an irreversible disappearance of SIg, and the generally accepted concept that immature B cells become easily tolerized. They also suggest that Cy induces a temporary reversion of mature B cells to a state of functional immaturity.

The effects of Cy expressed in the loss of the ability to regenerate SIg may be the result of drug action on DNA or on RNA or protein since this alkylating agent can interact with all of these cellular macromolecules. It would be of interest to determine whether other receptors are altered in their presentation or regenerative capacity. It would also be of interest to study Fc and complement receptors in view of the fact that there is a modulation in the expression of these receptors on lymphocytes in the presence of the growing tumor and by treatment with immunomodulators. Any

substance or condition that can modify receptors may set the stage for alteration of responsiveness or regulatory functions of the cell because such modifications can affect the binding of antigens or the formation of idiotype/anti-idiotype networks and proper recognition of signals.

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Glossary

ADCC	Antibody-dependent cellular cytotoxicity
Ara-C	Cytosine arabinoside
B	B Cell
BCG	Bacille Calmette Guerin
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CCNU	1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
CFA	Complete Freund's adjuvant
CMI	Cell-mediated immunity
con A	Concanavalin A
Cp	*Corynebacterium parvum*
Cy	Cyclophosphamide
De	Dextran
DNP	Dinitrophenyl
DTH	Delayed-type hypersensitivity
Ete	*Ecteinascidia turbinata* extract
F	Facilitating
5-FU	5-Fluorouracil
GVH	Graft-*vs*-host

HSF	Histamine-induced suppressor factor
LPS	Lipopolysaccharide
MeCCNU	1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea
Mel	Melphalan
NF	Nonfacilitating
NK	Natural killer cell
NM	Nitrogen mustard
PAM	Plastic-adherent macrophage
PFC	Plaque-forming cell
PGE	Prostaglandin E
PHA	Phytohemagglutinin
Rt	*Rhus toxicodendron*
RES	Reticuloendothelial system
SIII	Pneumococcal polysaccharide
Sa	*Semecarpus anacardium*
sIg	Surface immunoglobulin
SOI	Supraoptimal immunization
SRBC	Sheep red blood cells
SSF	Sea star factor
T	T cell
TCGF	T cell growth factor
TD	Thymus-dependent
TDH	T cells responsible for DTH
TI	Thymus-independent
TNP	Trinitrophenol
TS	T suppressor cell
TSe	T suppressor effector cell
TSi	T suppressor inducer cell
UV	Ultraviolet

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Chapter 5

The Immunobiology of Human Non-Hodgkin's Lymphomas

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1. Introduction

The non-Hodgkin's lymphomas represent an important interface between basic immunobiology and lymphocyte pathophysiology. In the last decade, the rapidly expanding knowledge of immunocytes has been applied to this spectrum of diseases and has resulted in more scientifically accurate classifications. Of perhaps greater import, however, is the nascent realization that these dis-

eases represent malignant counterparts of normal lymphocyte development, and that the concept of a maturational block originally applied to Chronic Lymphatic Leukemia (CLL) (Preud'homme and Seligmann, 1972) may be operative in other compartments of the spectrum. If this proves correct, these diseases may afford an opportunity to study relatively homogenous populations of cells at different stages of development and thus gain knowledge of the developmental processes of normal lymphocyte subpopulations. In addition, this spectrum may permit a dissection of those elements that define pathophysiology and determine the clinical presentation of disease. These must include the cell type affected (T, B, null) and the roles of lymphocyte homing patterns and immunoregulatory influences.

2. Classification Schema

Adult non-Hodgkin's lymphomas (NHL) are a group of disorders involving the malignant transformation of a single clone of lymphocytes that may arise from any of the estimated 10^{12} lymphocytes (Douglas, 1976) in the human body. It is a heterogenous group of disorders in terms of morphologic appearance, patterns of dissemination, and response to therapy. As a result of this variability, pathologic classification has played a critical role in the initial evaluation, staging, and therapy in these diseases. The classification in NHL against which any new schema must be judged is that of Rappaport (1966). In addition to introducing order into the chaos that preceded his classification schema (Table 1) and correcting misleading nomenclature, he called attention to the patterns of dissemination, their relationship to prognosis, and effectively defined some clinically homogenous groups. In addition, this schema recognized the prognostic import of nodular versus diffuse disease and began to classify the tumors in their presumed order of dedifferentiation. Nonetheless, any schema is only useful if it defines homogenous groups of patients and thereby creates standards that can be applied therapeutically. In this regard, the Rappaport classification and others that depend on a morphologic interpretation of a dynamic process at any given instant are subject to error.

This is readily apparent if one views Well-Differentiated Lymphocytic Lymphoma—Diffuse (WDLL-D). Although the diffuse nature of the disease should place it in the poorer risk groups, it is actually one of the favorable categories in the Rappaport system.

Table 1
Classification of Malignant Lymphomas[a]

Nodular lymphomas	Diffuse lymphomas
Lymphocytic, poorly differentiated	Lymphocytic, well-differentiated
Mixed lymphocytic–"histiocytic"	Lymphocytic, intermediate differentiation
"Histiocytic"	Lymphocytic, poorly differentiated
	Mixed lymphocytic–"histiocytic"
	"Histiocytic"
	Undifferentiated, Burkitt's
	Undifferentiated, pleomorphic (non-Burkitt's)
	Lymphoblastic
	Unclassified

[a]The Rappaport histopathologic classification schema for NHL.

Conversely, Diffuse Histiocytic Lymphoma (DHL), composed of the most dedifferentiated cells, encompasses a group or subgroup in which some, but not all, patients are curable even at advanced stages with current chemotherapeutic regimens (Anderson et al., 1977; Cadman, 1977). Finally, the rigidity of the system precludes accurate classification of composite lymphomas (Van den Tweel et al., 1979). It is clear, therefore, that this schema, which is based on morphology alone, does not delineate all biologically homogenous groups or explain the transformations from one stage to another. Additional criteria are needed to explain the discrepancies and identify the patients whose prognosis differ so strikingly from the predictions.

This added dimension was provided by an immunologic approach to lymphomas. This in no way, however, detracts from the important contributions of Rappaport and his colleagues, since when his schema was introduced in 1966 (Rappaport, 1966) little of the complex heterogeneity of the immune system was appreciated and T (Raff, 1971) and B (Basten et al., 1972) cells were yet to be described. Nonetheless, cell surface marker analysis has begun to detect heterogeneity within groups that had previously been considered homogenous in the Rappaport classification. It became apparent that the favorable histologies including WDLL (D), PDL

(N), MC (N) were primarily B cell tumors (Jaffe et al., 1974) in various stages of differentiation and that the curable subset of histiocytic lymphomas were, in reality, not histiocytes, but differentiated B lymphocytes (Bloomfield et al., 1977). In addition, many of the tumors in each category (5% CLL, WDLL) (Brouet et al., 1975) and histiocytic lymphomas (Berard et al., 1978) were either T or null cells by newer cell surface marker criteria and could not be distinguished in the Rappaport histiopathologic classification. In fact some investigators (Bloomfield et al., 1977; Bloomfield et al., 1976) have shown that marker studies may predict survival better than histopathology in the PDL and DHL categories, although the series remain small and further confirmation is needed.

An attempt to incorporate both immunologic and morphologic criteria into the diagnosis and classification of non-Hodgkin's lymphoma was introduced in 1974 (Lukes and Collins, 1974) (Table 2). It is an unique classification system in that major subdivisions are listed according to immunologic parameters and are based on the premise that these diseases can be identified as T or B on the

Table 2
Immunologic Classification of NHL[a]

U cell (undefined)	
T cell	B cell
Small lymphocyte	Small lymphocyte
Convoluted lymphocyte	Plasmacytoid lymphocyte
Cerebriform cell of Sezary's syndrome and mycosis fungoides	Follicular center cell (FCC) types
Lymphoepithelioid cell	Follicular, follicular and diffuse, diffuse and with and without sclerosis
	Small cleaved
	Large cleaved
	Small noncleaved
	Large noncleaved
Immunoblastic sarcoma	Immunoblastic sarcoma
Histiocytic	
Cell of uncertain origin	
Hodgkin's disease	
Unclassifiable (for technical reasons)	

[a]The Lukes and Collins Histopathologic Classification schema of malignant lymphomas.

basis of morphologic criteria. In addition, it recognizes that the different classifications in NHL reflect malignant alterations of the normal ontogenic development of lymphocytes (Lukes, 1979) and that different areas of the lymph node give rise to different malignant transformations with the follicular center generating B cell tumors and interfollicular and paracortical areas T cell lymphomas. Finally, it recognizes that many of the morphologic criteria that define various categories in NHL can be generated by in vitro stimulation of normal lymphocytes with a variety of agents.

In the B cell category, Lukes and Collins postulate that the follicular center cell has a distinctive morphology and can be divided into large and small, cleaved and uncleaved compartments. In addition to these tumors, which comprise 75% of B cell NHL malignancies, they incorporate a category of lymphoma composed of large lymphoid cells with distinct plasmacytoid features. It is designated as a B-immunoblastic sarcoma and is presumably not of follicular center cell origin.

In the T cell category, they include both mycosis fungoides and Sezary syndromes, which are malignant proliferations of regulatory populations of lymphocytes. This, in itself, represents a departure since these entities, which are probably the same disease process pathophysiologically (Lutzner, et al., 1975), are excluded in the Rappaport schema. Another major contribution is the recognition of a distinct subcategory of patients who present with mediastinal masses composed of convoluted T cells (convoluted lymphocytic lymphoma) (Barcos and Lukes, 1975). This has proven to be a biologically homogeneous subgroup of patients with a distinct clinical presentation and clinical course (Rosen et al., 1978) and represents a major advantage of the Lukes and Collins system. Finally, they include a T cell immunoblastic sarcoma with distinct morphologic features (Lukes et al., 1978b).

Despite these advances, however, it must be recognized that this classification still contains markedly heterogenous groups from a biologic and clinical standpoint. The increasingly recognized heterogeneity of T cell subsets as both regulators and effector cells in the murine (Cantor and Boyse, 1977; Cantor et al., 1978, Cantor and Boyse, 1975a) and now the human system (Schlossman et al., 1978; Rice et al., 1979) suggests that other T cell malignant transformations have yet to be recognized. In fact, recent reports (Broder et al., 1976; Hauptman, 1979) that Sezary's syndrome may represent a malignant proliferation of either helper or suppressor T cells suggest that heterogeneity exists within this syndrome and that more homogenous biologic and clinical subgroups will be forthcoming.

Another problem in the Lukes and Collins system is the undefined classification. As has been pointed out (Nathwani, 1979), these remain difficult to comprehend since they are recognized morphologically, but defined immunologically and as stated (Lukes and Collins, 1974): "At the present time the U cell group is theoretic and provides a grouping for those proliferations of lymphocytes that prove to have no discriminating surface markers." Whether this represents a technical problem (i.e., membrane turnover occurring too rapidly to allow surface marker studies) or is the result of cells that are too primitive to display definitive surface markers awaits further study. Nonetheless, if cell surface marker criteria are used to define this group, the undefined category becomes a morphologically heterogenous group with minimum predictive value (Nathwani et al., 1978a). If, on the other hand, it includes only neoplasms composed of primitive-appearing round cells without distinguishable surface markers (nonconvoluted lymphoblastic lymphoma (Nathwani et al., 1976) and nonconvoluted ALL (Pangalis et al., 1979) some of these have been shown to have T cell markers (Koziner et al., 1978).

This schema, then represents a significant advance since it recognizes the fact that these disorders are malignancies of immunocytes and that immunologic techniques are important in these diagnoses. Problems still exist, however, since recent reports (Frizzera et al., 1979; Bloomfield et al., 1979) suggest that as many as 20% of cases in some subgroups are misclassified immunologically if cell typing is based on morphology alone. In addition, the classification and biologic behavior of tumors in the undefined group remain less than precise. Finally, in our opinion, the heterogeneity, complexity, and numbers of subgroups of cells contained within the immune system and the fact that stimulation of these cells results in similar morphologic changes (Yeckley et al., 1975) may well preclude delineating subgroups of cells on morphologic criteria alone. It is clear, however, that only when we can recognize and identify the plethora of normal lymphocyte subgroups and their malignant counterparts will homogenous biologic subgroups of tumors be delineated.

A number of other classification schema have been proposed that rely primarily on histology (Bennett et al., 1974; Mathé et al., 1976), or immunologic concepts (Lennert and Mohri, 1978) similar to Rappaport and Lukes and Collins. In an attempt to resolve the discrepancies, compromise schemas have also been devised (Dorfman, 1974; Nathwani, 1979). These have been reviewed recently (Nathwani, 1979). They attempt to incorporate the best

points of various schema and may represent further advances. Unfortunately, their clinical utility has yet to be proven and a definitive classification of NHL has not emerged.

If the histopathologic schema fail in their ability to recognize the immunocyte subpopulations that represent NHL, then the current immunologic systems may also be criticized for their attempts to define broad immunologic characteristics (T, B, null cell) and functional properties on the basis of marker studies alone. Since neither histology nor primitive marker studies that fail to define subsets are adequate alone, it appears obvious that a sophisticated approach involving histology, histochemistry, and a battery of cell surface marker tests is needed. Histology will still define the proliferation as malignant, while histochemistry may define broad subgroups (Taylor, 1978; Yam et al., 1971; Donlon et al., 1977) and functional analysis involving subset cell surface marker analysis, in vitro cell reactivity, and estimates of immunoregulatory influences both in the involved node and peripheral blood may better define the biologic behavior and thus the clinical course of disease. This combined approach and an increased knowledge of normal ontogenic development of immunocyte subtypes (Cooper and Lawton, 1978; Grossi et al., 1978) should allow a more precise understanding of the pathophysiology of malignant lymphoproliferation and allow selection of more appropriate therapy.

3. Functional Studies

The analysis of biologic behavior in human lymphoproliferative disease requires attention to the clinical features, modes of presentation, patterns of spread, and the ultimate prognosis. Several excellent reviews (Mann et al., 1979; Bloomfield et al., 1974; Jones et al., 1973) have appeared recently and will not be reviewed here. Studies of the functional properties of the lymphomas, however, have awaited the development of modern immunologic techniques and are just beginning to achieve their place of importance beside the clinical investigations.

The earliest and most extensively studied of the human non-Hodgkin's lymphomas is Chronic Lymphatic Leukemia (CLL) that may be considered as peripheral blood and bone marrow involvement with the same cell type as WDLL-D (Pangalis et al., 1977). A B cell neoplasm in 95% of cases (Aisenberg et al., 1974), peripheral blood involvement makes CLL an ideal candidate for in vitro investigations. In addition, the rare but reported transformation of this

tumor to other forms of NHL (Richter, 1928; Brouet et al., 1973; Zacharski and Linman, 1969) and the common transformation (20%) (Dick and Maca, 1978) of the morphology of lymph nodes in the terminal phase of CLL to DHL suggests that studies of CLL patients could have important implications for our understanding of the basic pathophysiology of the entire spectrum.

Cell-mediated immunity as assessed by delayed-hypersensitivity skin tests is normal in most patients (Block et al., 1969), but humoral immunity is compromised in 50–75% of patients and hypogammaglobulinemia affecting at least one class of immunoglobulin is a frequent finding (Boggs and Fahey, 1960). Studies evaluating the in vitro immunocompetence of peripheral blood in these patients have consistenly (Smith et al., 1972; Berard et al., 1964; Robbins, 1964; Oppenheim et al., 1965) demonstrated decreased response to a variety of plant mitogens known to stimulate predominantly T cells—concanavalin A (con A) (Greaves and Janossy, 1972b), Phytohemagglutinin (PHA) (Janossy and Greaves, 1971), or B cells Pokeweed Mitogen (PWM) (Greaves and Bauminger, 1972a). These defects (Han, 1973) however, are partially reversible when the patient is in clinical remission. Moreover, several groups (Bouroncle et al., 1969; Rubin et al., 1968; Smith et al., 1972; Eijvogel and Schweitzer, 1972) have reported that the maximal response to mitogens is delayed in CLL and that this defect persists when patients are in remission.

A variety of explanations have been postulated to explain these findings. Most authors (Dameshek, 1967; Schrek, 1967; Thompson et al., 1966) have suggested that the CLL lymphocyte is inherently abnormal and the minimal reactivity noted is due to residual normal T and B cells. This might be considered a dilutional hypothesis and would predict that the return towards normal reactivity noted in remitted patients is a result of a decrease in malignant cells and a corresponding increase in normal immunocytes. This explanation, however, fails to consider the observed delayed maximum response seen both in patients with active disease and remission. In addition, treatment with low-dose alkylation depresses reactivity (Han, 1973), which might be predicted if the therapy affected normal as well as diseased lymphocytes, but it does not explain a further temporal delay in maximum responsiveness during therapy (Han, 1973) whether the disease is active or remission has been achieved.

An alternative explanation, however, may be postulated. Low dose alkylating therapy has been shown (Poupon et al., 1977) to increase suppressor cell function in experimental animals and sup-

pressor cells may proliferate and die rapidly in humans (Bresnihan and Jasin, 1977; Fujimoto et al., 1975). The delayed maximum responses, therefore, could reflect alterations in suppressor cell pools. The return towards normal reactivity noted in patients who respond may reflect reestablishment of normal immunoregulation either coincidentally or causally related to remission. In this regard, one case of complete remission in CLL following smallpox vaccination (Hansen and Libnoch, 1978) adds credence to this possibility since viral infections are known to increase suppressor cells (Tosato et al., 1979). Furthermore, Catovsky et al. (1974) have suggested that those patients with a benign course in CLL have significantly more peripheral T lymphocytes than those with aggressive disease, which suggests that T cell immunoregulation could have significant effects on the in vitro mitogen reactivity and perhaps the clincial course of disease. Additional evidence for altered immunoregulation in CLL was suggested by co-culture experiments measuring plant lectin binding (Faguet 1979) and studies that demonstrated increased numbers (Kay et al., 1979) and function (Keller et al., 1979a) of T_γ cells that have been reported to define a T suppressor cell population (Moretta et al., 1974; Cooper et al., 1977; Fauci et al., 1978; Moretta et al., 1978). It would seem, therefore, that altered immunoregulation could have an important role in the pathogenesis and/or control of this disease process. In a speculative view, perhaps low-dose alkylation (Wiltshaw, 1977) is effective in controlling disease at least partially by increasing suppressor influences, although this remains unproven.

In contrast to CLL, functional analyses in other stages of NHL are uncommon, although many reports exist describing cell surface membrane markers and the correlation between histology and immunologic subtypes. Several excellent reviews on these correlations have recently appeared (Bom-VanNoorloos et al., 1978; Brubaker et al., 1979; Pinkus and Said, 1978; Berard et al., 1978; Bloomfield et al., 1977) and will not be reviewed here. Unlike CLL, however, cell-mediated immunity as assessed by skin tests is commonly depressed (Lamb et al., 1962; Sokal and Primikirios, 1961) in other stages of NHL and some authors have attempted to correlate the patterns of anergy and immunoglobulin defects with the histopathologic diagnosis (Jones et al., 1977). In our own experience (Orlowski et al., 1980), however, anergy is uncommon unless fever and/or infection are present. Humoral immunity, as assessed by serum immunoglobulin levels, has also been reported to be decreased (Lapes et al., 1977) in significant percentages of patients with advanced disease. Here, too, the series are small and the true

incidence of generalized or selective anergy as well as immunoglobulin defects awaits further study.

In vitro immunologic responses have been reported variously to be normal or decreased (Heier et al., 1977; Thatcher et al., 1977; Libansky et al., 1973) in NHL. Although cells from involved lymph nodes manifest a uniformly decreased response (Keller et al., 1979c) caution must be used in evaluating these studies. In CLL, in vitro lymph node responsiveness (Keller et al., unpublished) has been reported to be increased compared to peripheral blood. From our own studies (Keller et al., unpublished), it is clear that the reactivity markedly changes if one compares involved lymph nodes and peripheral blood in NHL and that the degree of reactivity in uninvolved lymph nodes (Keller and Tomasi, unpublished) is different still. Furthermore, the recent report (Ault, 1979) suggesting that 40% of patients with NHL in clinical remission had an abnormal population of lymphocytes in their peripheral blood bearing the heavy and light chain immunoglobulin isotype of the tumor further questions the import of these studies. Compared to Hodgkin's disease, where anergy and decreased in vitro mitogen reactivity correlate with tumor burden (Young et al., 1972; Eltringham and Kaplan, 1973) and the presence of suppressor macrophages (Twomey et al., 1975), similar data has not been forthcoming in NHL.

From the studies reported, however, some conclusions are possible. Both cell-mediated and humoral immune mechanisms are impaired, but the extent of these defects, their correlation with tumor burden and/or the category of disease within the NHL spectrum is less than clear. In vitro reactivity to both T and B cell mitogens is altered, but the degree of response depends on whether normal nodes, involved nodes, spleen, or peripheral blood are assayed and again correlations between tumor burden and category of disease await further study. Finally, peripheral blood cells from patients with NHL function normally as stimulators in one-way mixed leukocyte reactions, but their ability to react to histoincompatible cells (Ruhl et al., 1975) is impaired. In summary, therefore, scarce and contradictory data exist concerning the in vitro activity of normal and malignant lymphocytes in patients with NHL. In addition, these studies have not considered the variability of response between involved and uninvolved lymphocytes nor possible immunoregulatory abnormalities. A prospective, organized, and systematic study of all NHL subgroups analyzing in vivo cell-mediated and humoral immunity, in vitro responses to plant lectins, specific antigens, and histoincompatible donors, and an analysis of immunoregulatory influences in involved and

uninvolved tissue as well as peripheral blood is necessary before any definitive statements concerning immunocompetence can be made in this spectrum of disease.

4. The Ontogeny Of Lymphocytes

Given the problems inherent in the current classification schema and the contradictory data concerning the functional characteristics of lymphocytes in NHL, it would seem logical to reevaluate this spectrum of disease in terms of current immunologic concepts of the ontology of lymphocyte differentiation, the network theory of immune reactivity (Touraine, 1978; Williamson et al., 1976; Cantor and Gershon, 1979) and the role of immunoregulation in normal and pathologic lymphocyte reactivity (Waldmann and Broder, 1977). To achieve this, however, it is necessary to use the murine model, which is better defined than the human system. Nonetheless, there is increasing evidence (Touraine, 1974; Vogler et al., 1976; Moretta, 1976) that many of the processes occurring in the mouse are similar to the developmental and interaction schema in humans. In addition, this idea is by no means novel and has been suggested recently by a number of other investigators (Gathings et al., 1976; Greaves et al., 1974; Siegal and Good, 1977; Touraine et al., 1977).

A simplified schema of overall lymphoid development is presented in Fig. 1. Here, a multipotential stem cell that cannot be identified morphologically, although both bone marrow reticulum cells (Bessis, 1973) and small lymphoid-appearing cells (Yoffey, 1960) are candidates, generates a variety of cell types in both the lymphoid and hemopoietic cell series. These cells, then, under undefined influences, become progressively focused, differentiate along separate lines, and subsequently assume specific functions as a consequence of a succession of differentiation steps. As viewed by others (Cooper, 1966; Good, 1973), these steps involve: processes of cellular commitment; triggered influences on pre-committed cells effected by cell–cell interactions in the microenvironment; hormonal influences that result in intracellular changes and cause either further differentiation and/or selective gene activation; changes in cell surface determinants that may play a role in further cell–cell interactions; and a special form of cell division (Dienstman and Holtzer, 1974), called quantal proliferation, that may prepare cells for the next differentiation step. Of paramount importance, then, is that cells of a single progenitor undergo a succession of bi-

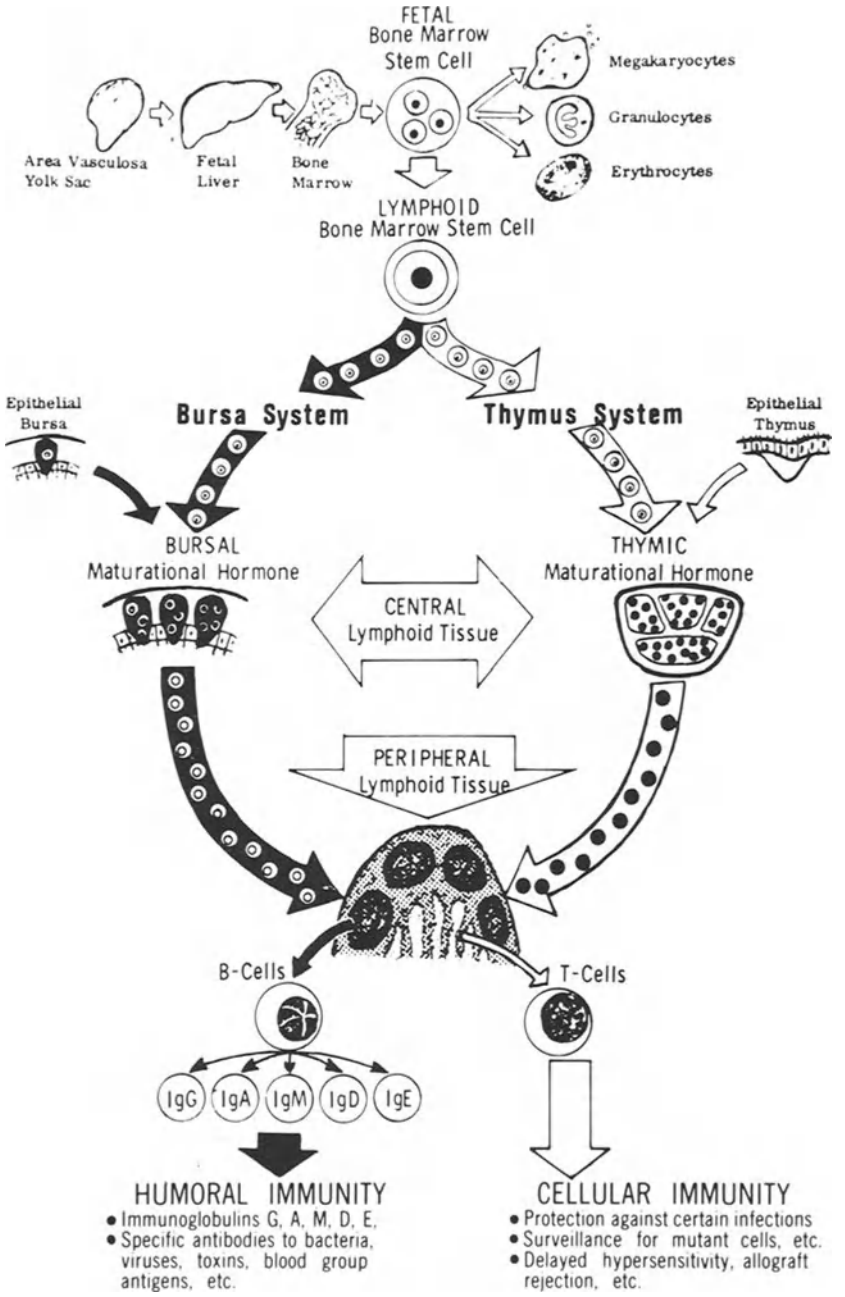

Fig. 1. A simplified schema of lymphocyte ontogeny (from Ritzmann, S.), Developmental Aspects of B and T cell Systems of Immunity, in *Serum Protein Abnormalities* by Ritzmann, S. E., and J. C. Daniels, with the kind permission of Little Brown and Co., publishers.

furcations in development that ultimately results in the plethora of cell types that constitute the mature lymphohemopoietic system. Viewed in this way, apparent paradoxes such as terminal deoxynucleotidyl transferase (TDT), positive chronic myelogenous leukemia blast crisis (McCaffrey et al., 1975) and others are far less difficult to understand.

4.1. B Cells

With a seeming geometric progression (although no formal proof for this assumption exists), it is clear that relatively few bifurcations must occur to explain the multitude of cell subtypes that constitute the human lymphoid system. In order to understand developmental abnormalities, therefore, it is essential to delineate the basic processes of commitment and begin to dissect the events that induce the bifurcations. Figure 2 represents a composite of the steps that occur in the development of the B cell line (Cooper and Lawton, 1974; Hammerling et al., 1976; Lifter et al., 1976; Melchers and Andersson, 1974). Although complex, this undoubtedly still represents an oversimplification of normal B cell ontogeny. In addition, many of these hypothetical compartments may involve transcriptional and/or divisional events that are not depicted. The B cells, however, do derive from primordial stem cells either in fetal liver or bone marrow and by a succession of differentiative steps that are under precise, albeit undefined, controls, commit, proliferate, mature, develop surface receptors or antigens, and eventually secrete immunoglobulins as mature plasma cells. Although classic dogma (Williamson et al., 1974) would suggest that plasma cells die rapidly and that memory B cells represent an earlier bifurcation, recent evidence (LaVia and Vater, 1969; Winklehaake, personal communication) from animal studies suggests that plasma cells may be capable to reverting to memory cells. If this proves correct, it might explain the transformation of myelomas to less-differentiated forms of NHL (Grano et al., 1970; Banks et al., 1978), where both tumors bear the same immunoglobulin isotype and presumably are derived from the same clone. In most cases, however, the inability to prove idiotype identity makes this presumption less than certain and more studies are needed.

4.2. T Cells

Similar events occur in the T cell system. Figure 3 depicts a schematic of cell surface changes seen in murine T cell development (Kisielow et al., 1975; Cantor and Boyse, 1975a; Cantor and Boyse, 1975b). When the precursors of the T system, which are already

HYPOTHETICAL SCHEMA FOR B CELL DEVELOPMENT AND ITS RELATIONSHIP TO B CELL NHL

DIFFERENTIATION STEP	STEM CELL	LARGE PRE B	SMALL PRE B	IMMATURE B CELL	VIRGIN B CELL	COMMITTED B CELL	PROLIFERATING B CELL	MATURING CELL	PLASMA CELL	MEMORY CELL
MORPHOLOGY	? 									
SURFACE MARKER	None	Cytoplasmic U Chain Only	Cytoplasmic U Chain Only	Surface IgM Ia+	Surface IgM, IgD Ia+ ?CR+	Surface IgM, IgD +/– IgG, A, C Ia+ CR+	Surface Ig+ Ia+	Surface Ig– Ia+ ? Secretion	Surface Negative Secretory +	Surface IgD+
RESIDENT ORGAN	Fetal Liver Bone Marrow	Fetal Liver Bone Marrow	Bone Marrow Peripheral Blood Spleen	Bone Marrow Peripheral Blood Spleen Lymph Node	Spleen Bone Marrow Peripheral Blood Lymph Node	Lymph Node Spleen Peripheral Blood	Lymph Node Spleen Bone Marrow ? Peripheral Blood	Bone Marrow Spleen Lymph Node	Bone Marrow	Lymph Node
INDUCTIVE INFLUENCE	Bone Marrow Microenvironment	?	?	?	?	Antigen Macrophage T Cell	? Antigen	?	?	Antigen T Cell
POSSIBLE NHL COUNTERPART	None Known	? Common Cell ALL	Common ALL	CLL	CLL ? Intermediate Cell NHL	? PDL	Diffuse Histocytic Lymphoma	Waldenstroms Macroglogulinemia	Multiple Myeloma	?

Fig. 2. A hypothetical shema for human B cell development and its relationship to B cell NHL.

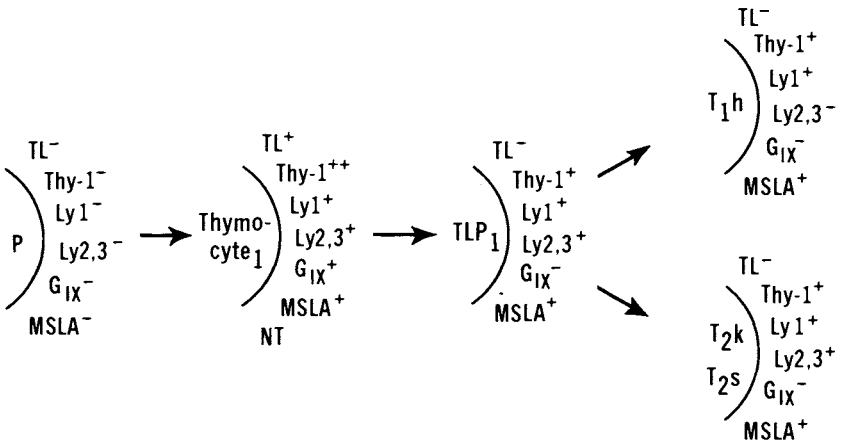

Fig. 3. An overview of murine T cell ontogeny. P = precursor cell, TLP = thymic lymphocyte, T1k an T2k, T2s represent mature T lymphocyte subsets.

precommitted by unknown inductive events, enter the thymus, they undergo rapid and dramatic changes and acquire an array of surface markers. These changes do not require DNA synthesis, but transcription and translational events do occur (Cantor and Boyse, 1975a). Although these cells are still immunoincompetent, the cell surface changes permit interaction with other elements of the immunologic network. Through another series of unknown influences, these cells change cell surface antigens, leave the thymus, alter their surface constituents again and, after a further series of inductive steps, develop new surface antigens and functional characteristics that constitute the multiple subsets of mature T cells subserving a number of different functions.

Although our understanding of the inductive events in the murine system is primitive, the data on the inductive events and influences in T cell ontogeny in humans is even less precise. Nonetheless, it has now become clear that in humans, as in mice, subsets of T cells exist that can be delineated by functional assays as well as cell surface markers (Touraine, 1974; Moretta, 1976; Greaves et al., 1974; Siegal and Good, 1977). These include suppressor cells (Schwartz et al., 1977; Shou et al., 1976; Siegal et al., 1976; Waldmann et al., 1974), helper or amplifer cells (Claman et al., 1966), T killer cells (Cerottini et al., 1972), natural killer cells (Haller et al., 1977; Herberman and Holden, 1978) T cells involved in antibody-dependent cell-mediated toxicity, T cells with histamine receptors (Shearer et al., 1972), cells responsive to plant lectins and/or allogeneic stimuli as well as other regulatory subsets (Strelkauskas

et al., 1978b). Thus, as in the B cell system, the numbers of subsets continue to proliferate and the problems of defining homogeneous groups of patients with malignant transformations of these subsets becomes increasingly difficult.

It is evident, however, that a limited number of bifurcations have spawned a plethora of T cell subtypes that are long or shortlived, subserve memory functions, and are clonally committed to specific antigens. In their fully differentiated form these cells defend the host against viral infections, fungi, and intracellular parasites (Good et al., 1970); react against foreign antigens; function in allograft rejection; and act as killer cells. In addition, however, since immunoglobulin from B cells can activate amplification systems such as complement, other cells, and the kinins (Day and Good, 1977), lymphokines secreted by activated T cells amplify their effects by interacting with other cells, inducing inflammation and promoting clotting mechanisms.

5. Overview of Immunoregulation

This, of course, is still an oversimplified view of modern immunology. Not only are there two separate and distinct arms, but through a variety of interactions (Alexander and Good, 1977; Miller, 1972) the cells communicate effectively and achieve precise control of the processes that recognize and react to foreign substances. This network, in essence, functions as a thermostat to either amplify or dampen generalized and/or specific immune reactivity, depending on the complex set of signals received at the given instant. It is schematically depicted in Fig. 4. It is important to recognize that, as the inductive signals change, so will the immune thermostat. Thus, evaluation of the system at any one instant may not reflect either antecedent or future immune reactivity. An appropriate example of these alterations is seen in infectious mononucleosis, where an early intense reaction of T cells against B cells infected with EBV virus (Virolainen et al., 1973) is associated later with the appearance of suppressor T cells that dampen the immune response (Tosato et al., 1979). Thus, any evaluation of immune competence must take into account that it is a dynamic, precisely controlled, rapidly changing, and often compartmentalized process (Katz and Benacerraf, 1972; Gershon, 1974).

Recently, however, the work of Cantor and Gershon has introduced even more complexity to the regulatory system in the mouse, and data is accruing that a similar regulatory network may

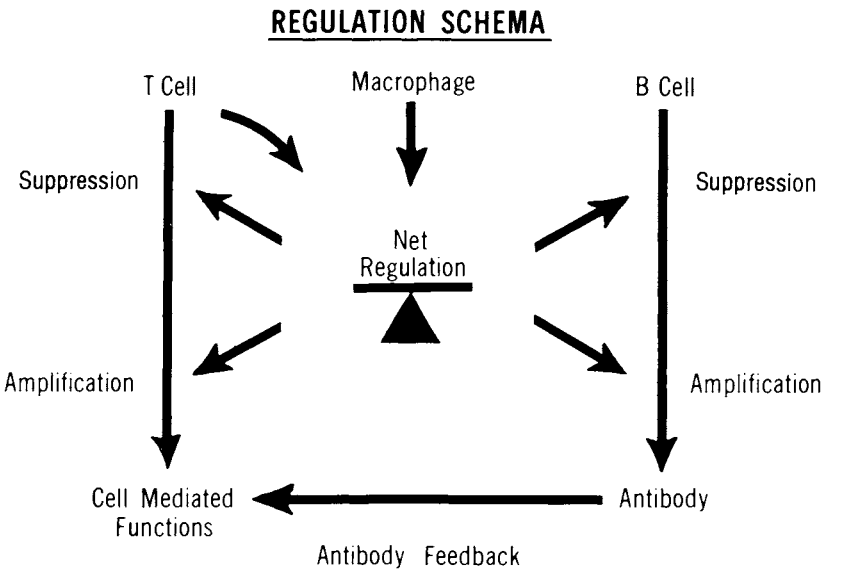

Fig. 4. A conceptual schema of the system of checks and balances regulating the immune response.

exist in humans (Strelkauskas et al., 1978a). In an elegant series of experiments, they have demonstrated that one subset of T cells with certain phenotypic markers $Ly1^+$, $Qa1^+$ functions to recognize foreign antigens. Once this recognition occurs, the clone of cells not only secrete lymphokines to activate amplification systems and promote B cell development to plasma cells, but they induce immature T cells to become suppressor and perhaps effector cells. In the murine schema, these suppressor elements then function to turn off the sentinel cell ($Ly1^+$, $Qa1^+$) responsible for their activation. In this system NZB and MRL mouse strains seem to represent important aberrations in this loop (Cantor and Gershon, 1979). There is a lack or malfunction of the immature T cells that are induced by the sentinel cells to become suppressor cells in NZB mice and an insensitivity of sentinel cells to the effect(s) of the suppressor cells in MRL mice. These experiments of nature, therefore, have permitted a more precise delineation of the cell types involved in the immunoregulatory network in the murine model.

Data in the human is less precise since appropriate reagents have not been available to phenotypically mark the relevant subgroups of cells. Recently, however, data has appeared that suggests that certain sera from JRA patients (Strelkauskas et al., 1978b) delineate an immature or prosuppressor cell subset and certain sera

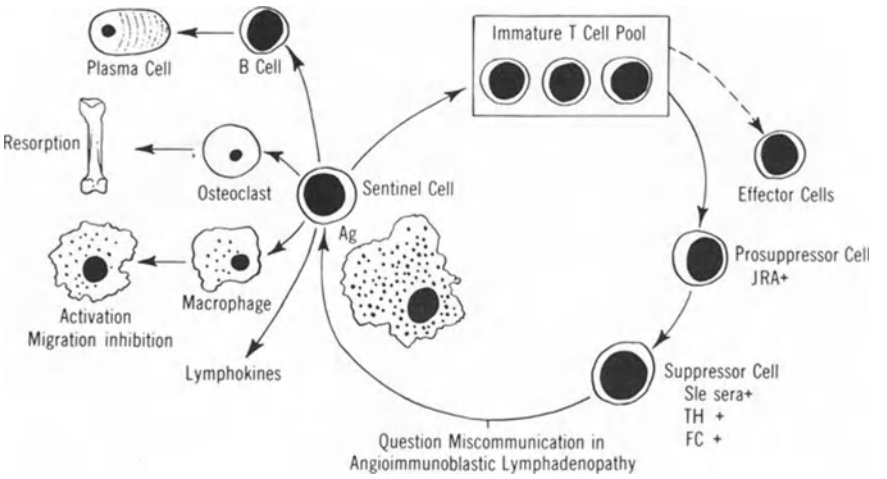

Fig. 5. A hypothetical schema for human T cell immunoregulation.

from patients with active systemic lupus erythematosis (Glinski et al., 1976) as well as Fcγ markers (Moretta et al., 1974) and TH_2 sera (Schlossman et al., 1978) delineate functionally mature suppressor cells. In our laboratory, preliminary evidence suggests that angioimmunoblastic lymphadenopathy (Frizzera et al., 1974) may represent an entity where sentinel cells are insensitive to the effects of suppressor cells and may be analagous to the MRL mouse. It is possible, therefore, that the immunoregulatory network is similar in both species, although this remains unproven. Nonetheless, a hypothetical schema for a human immune regulatory network based on available data is depicted in Fig. 5.

6. Lymphocyte Indentification

The fact that the current classification schema of NHL has generated clinically relevant data seems remarkable given the complexities, plethora of cell types, and network of interactions that constitute the immune system. It would seem appropriate, nonetheless, to utilize a multifaceted approach for further studies, as suggested by Lukes and Collins (1974). Of essential importance would be histopathology, but an important role would also be played by marker studies. In this area, however, there are many pitfalls in correctly interpreting the results. These have been reviewed recently (Parker, 1979) and only the more important ones will be reviewed here.

A number of markers have been utilized by various investigators to enumerate immunocytes. These are outlined in Table 3. For B cells they include surface immunoglobulin (Raff, 1970; Unanue et al., 1971) Fc receptors (Basten et al., 1972; Dickler and Kunkel, 1972)

Table 3
Surface Markers for Human Immunocytes[a]

Marker	Cell distribution	Assay procedure
Surface immunoglobulins IgM, IgD, IgG, IgA, K and λ	B cells	Immunofluorescence, immunobeads
Ia-like antigens	B cells	Immunofluorescence
Complement receptors		
Cr_1	B cells, K cells, and third-population cells	EAC14B or EAC1-3b rosette assay
Cr_2	B cells (lymphoma)	EAC1-3d rosette assay
IgG-Fc receptors (FcλR)	B cells, K cells, suppressor T cells, third population cells, macrophage	Fluorescence or rosette assay
IgM-Fc receptors (FcμR)	Helper T cells, some B cells	Rossette assay
IgE-Fc receptors (FcεR)	B cells	Rosette assay
Epstein-Barr virus receptors	B cells	Immunofluorescence
Erythrocyte receptors		Rosette assay
Sheep	T cells and K cells	
Mouse	B cells	
Monkey	B cells and third-population cells	

[a]Surface characteristics and assay procedures for the identification of various human lymphocyte subpopulations.

and C_3 complement receptors (Bianco et al., 1970). In addition, mouse rosettes have been used (Stathopaulos and Elliott, 1974). There are problems in interpreting these markers since many of them occur on other types of cells, such as Fc receptors on macrophages (Berken and Benacerraf, 1966), neutrophils (Messner and Jelinek, 1970), platelets (Henson, 1969), and certain T cell subsets (Moretta et al., 1974). In addition, as depicted in Fig. 3, B cells change their surface determinants during the maturational process so that only certain subgroups of B cells bear complement receptors, B cell antigens, or rosette with mouse erythrocytes. Furthermore, alterations in surface receptors occur during the tranformation to malignancy since a subgroup of normal B cells bear receptors for both b and d fragments of C_3, but lymphoma cells preferentially bind C_3d (Stein, 1978). It would seem, then, that the most definitive proof that a cell is of B cell lineage would be measurement of surface immunoglobulin. Even here, however, problems arise since antilymphocyte antibodies and/or absorbed immunoglobulin, which occur in lymphomas (Lukes et al., 1978a), may falsely elevate the number of cells enumerated as B cells and may also suggest polyclonality.

6.1. B Cell

B cell tumors in NHL, however, are monoclonal (Levy et al., 1977) since they arise from a single clone. Although it is possible to have a double heavy chain surface marker (see Fig. 2), both heavy chains have been shown to bear single light chain type and a single idiotype in the limited studies performed (Fu et al., 1975; Taylor, 1979). Thus, the few cases reported that suggest polyclonality may represent technical difficulties. One exception to this rule, however, may be found in that group of patients who develop lymphoma in association with immunodeficiency (Kersey et al., 1973), iatrongenic immunosuppression (Fosdick et al., 1969), renal transplant recipients (Matas et al., 1976), and angioimmunoblastic lymphadenopathy (Nathwani et al., 1978b). The lymphomas that evolve in these clinical settings are pleomorphic, polyclonal, and characterized by a high mitotic index (Fig. 6). They are analogous to the lymphomas that spontaneously occur in NZB mice (Gershon et al., 1978) and mice undergoing chronic graft-vs-host disease (Taylor, 1976). They have been termed immunosarcomas, but do not correlate with the immunoblastic sarcomas of Lukes and Collins. Whether they represent a malignant counterpart of angioimmunoblastic lymphadenopathy, a benign polyclonal proliferation, is speculative. In our laboratory, however, we have not seen a B cell tumor in the NHL

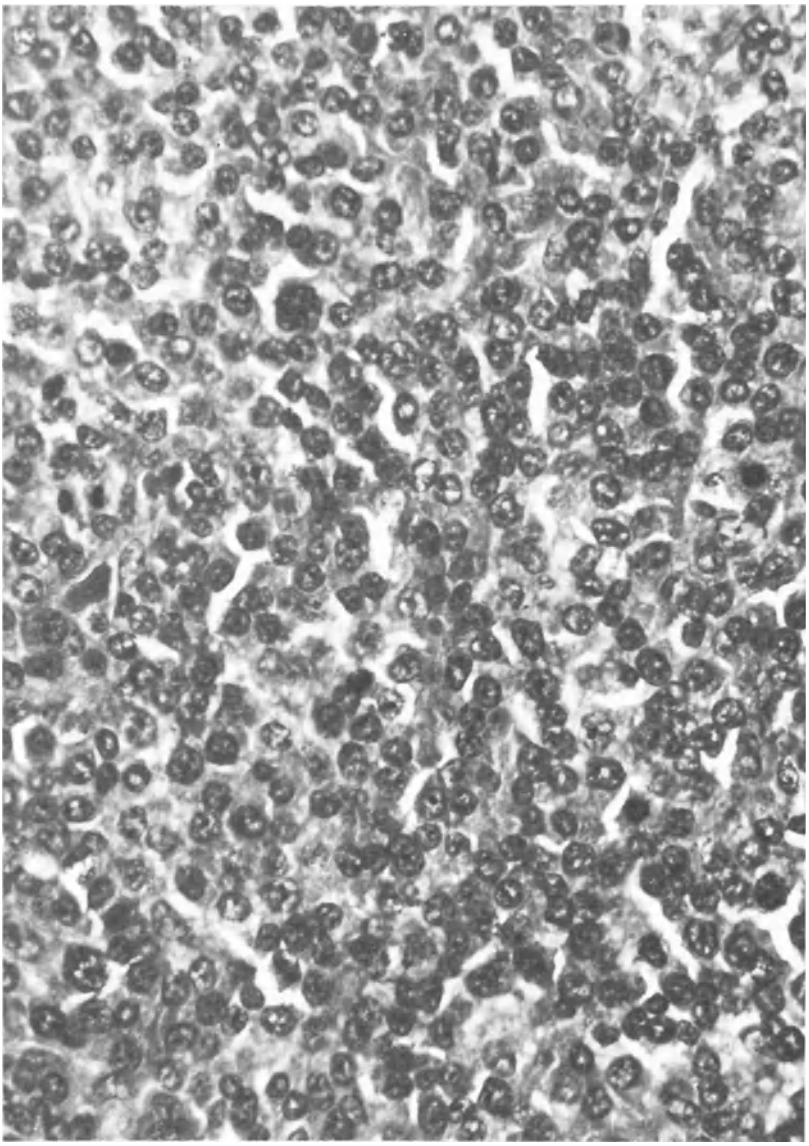

Fig. 6. An H & E Section from a lymph node diagnosed as polyclonal immunosarcoma (×360).

spectrum, with the exception of immunosarcomas, that were not monoclonal.

In order to prove monoclonality, however, heavy and light chain specific antisera must be utilized. In addition, cells must be incubated for more than 1 h at 37°C to ensure removal of absorbed

immunoglobulin or antilymphocyte antibodies. Furthermore only $(Fab')_2$ fragments of immunoglobulins can be used to prevent falsely elevated levels caused by binding of the Fc fragment (Winchester, 1974) to cells other than B cells. Finally, in some cases (Banks and Keller) radioimmunoassays for immunoglobulin or internal incorporation of radiolabel in cell cultures stimulated by PWM must be used to assess the B cell nature of the tumor. Using these techniques as well as cytoplasmic staining for Ig are time-consuming and labor intensive, but in our opinion necessary to determine whether the tumor is derived from a B cell.

6.2. T Cell

T cell marker analysis, at this time, is far more limited. Although some laboratories have developed heteroantisera (Owen and Fanger, 1974; Whiteside, 1977) that recognize all or certain subsets of T cells, these have not been standardized and may represent dedifferentiation antigens peculiar to certain subgroups or maturation compartments of T cells. Until these are further developed and characterized, therefore, spontaneous rosettes with sheep RBC (E rosette) will remain the standard to mark human T cells. Even here, however, problems exist. Factors such as incubation times, temperatures, pretreatment of the sheep RBCs with neuraminidase or aminoisoethylthiouronium (AET) (Hoffman and Kunkel, 1976) all contribute to the normal values and vary from laboratory to laboratory. Normal values for immunocytes from various tissues in our laboratory are presented in Table 4. In addition, anti-T cell antibodies (Parker, 1979) are also found in lymphomas, so that preincubation, as with B cells, is necessary to assure correct interpretation. Of greater concern, however, is that spontaneous T rosettes do not allow any assessment of monoclonality, and even with the development of subset antisera (including Fcγ, Fcμ, TH_2, JRA, etc.), we are

Table 4
Normal Immunocyte Percentages in Various Tissues[a]

Tissue	T cells	B cells	Monocytes
Peripheral blood	74 ± 3	12 ± 2	15 ± 4
Bone marrow	19 ± 5	31 ± 4	39 ± 3
Thymus	97 ± 2	3 ± 1	—
Spleen	46 ± 4	45 ± 6	11 ± 4
Lymph node	69 ± 3	20 ± 4	12 ± 3

[a]The percentages of T cells, B cells, and monocytes in various human tissues.

still unable to judge the clone of cells from which the tumor arose. As a result, numerical data and more recently functional studies (Broder et al., 1979) have been used to assess malignancy. It is obvious, however, that far more investigation is needed to allow further subtyping of T cell NHL. The further development and characterization of heteroantisera combined with fluorescence-activated cell sorting (FACS) should make this a fertile area of research in the next four years.

6.3. Enzyme Studies

Another area where additional data can be obtained is that of cellular enzymes. Nonspecific esterases (Loffler, 1961) already delineate macrophages and acid phosphatase (Stein et al., 1976) and terminal deoxyribonucleoside transferase (Coleman et al., 1976) are found in certain T cell tumors. In addition, enzyme deficiencies such as adenosine deaminase (Hirschhorn, 1977; Pollara and Meuwissen, 1973) are associated with combined immunologic defects, whereas purine nucleoside phosphorylase deficiences are associated with only T cell immune defects. Finally 5'-ectonucleotidase levels have been shown to change with increasing activity of disease in CLL (Kanter et al., 1979). It is possible, therefore, that the addition of enzyme studies to histology, histochemistry, and marker analysis employing an increased and standardized battery of reagents and the FACS, will finally allow us to categorize NHL into truly homogenous biologic and clinical groups. In addition, our increased understanding should allow more rational and sophisticated therapy and thus improve the prognosis and survival in this spectrum of disease.

7. New Directions

Present therapeutic effects in NHL are directed at killing the tumor cells by a variety of agents including irradiation, single agent chemotherapy, combinations of cell cycle specific and nonspecific cytotoxic drugs, and combined modality therapy. Although this has proven effective in some categories of the spectrum, such as DHL where 50% of patients achieve long-term remission and may be cured (Anderson et al., 1977; DeVita et al., 1975; McKelvey and Moon, 1977) and the nodular mixed cell compartment, where similar results may be occurring (Anderson et al., 1977), many problems remain. One of these is the propensity for patients to recur with more dedifferentiated tumors after intensive treatment (Berard et

al., 1978). Another is the historically low rate of remission induction in patients with Poorly Differentiated Lymphocytic Lymphoma Diffuse (PDLL-D) and the nondurability of these remissions (Schein et al., 1974; Ezdinli et al., 1976). Furthermore, there is the relatively favorable prognosis of nodular compared to diffuse tumors (Berard and Dorfman, 1974). In fact, one group (Portlock and Rosenberg, 1979) has suggested that (PDL-N) may remain asymptomatic for as long as 10 years. Finally, there is the data in CLL (Catovsky et al., 1974) that the subgroup of patients with a benign course and prolonged survival have significantly more peripheral T cells than those patients whose disease is aggressive. The question might be posed, then, are there any factors that could begin to explain these discrepancies before a definitive and homogenous classification schema emerges?

There are no definitive answers at present, but increasing evidence suggests that some of these findings might relate to the interaction of normal and/or compensatory immunoregulatory elements of the immune system with the malignant lymphocytes. One difficulty in assessing this possibility relates to the nodular lymphomas. Although B cells can be assessed *in situ* using an immunoperoxidase technique (Antoine et al., 1974), only one group (Jaffe et al., 1977) has been able to assess T cells *in situ*. They found that the cells surrounding the area of malignancy were T cells, but in addition found T cells within the tumor nodule containing malignant B cells. Unfortunately no other group has been able to achieve these results and no studies of regulatory T subsets have been performed *in situ*. In a speculative vein, however, if these T cells surrounding the tumor were T suppressor and/or T killer cells, it might explain why nodular NHL disease had a more favorable prognosis and why therapy, which would damage regulatory elements as well as lymphoma cells, could predispose to more diffuse and dedifferentiated disease if the tumor recurred.

Although this remains unproven, evidence in other categories of NHL suggests that further research in this area is warranted. In CLL, others have reported increased numbers (Kay et al., 1979) and function of suppressor cells (Faguet, 1979). Our data (Keller et al., 1979b), however, suggests that only asymptomatic CLL (Stage O) (Rai et al., 1975) is associated with near normal numbers of Fcγ bearing T suppressor cells. In fact, if one compares the ratio of T suppressor cells to the number of malignant B cells per milliliter of blood, symptomatic (Rai Stage III or IV) is characterized by a tenfold decrease in the ratio compared to asymptomatics. This is in agreement with Catovsky's data (1974) since asymptomatics also have a

significantly greater number of total T cells per millileter of blood than asymptomatics. It suggests, although it does not prove, however, that the relevant population is not total T cells, but rather T suppressor cells, and may explain why vaccination that could stimulate T suppressors has been associated with remission in CLL (Hansen and Libnoch, 1978). Of note in our studies was the finding that as disease activity in CLL increased and T suppressor cells decreased, adherent macrophage-like suppressors increased. This may explain why many studies have reported uniformly decreased mitogenic reactivity in both symptomatic and asymptomatic patients.

Can these findings be related to the data that suggests that 50% of advanced DHL is curable using current chemotherapeutic modalities? Again definitive answers are not possible, but some data has accrued. First, patients with DHL who present with mediastinal masses (Miller et al., 1978) are essentially incurable with standard therapeutic modalities. It is of note, however, that marker analysis on limited numbers of these patients suggests they are either T or null cell types (Weinstein et al., 1979; Keller et al., unpublished). This might suggest then that it is the B cell DHLs who are potentially curable. Although this remains unproven, it is interesting to note that approximately 50% of DHL (Berard et al., 1978) is of B cell type. This correlates closely with the percentage that is curable, but only large cooperative series using marker analysis and similar chemotherapeutic regimens will discern whether this numerical correlation is more than circumstantial. It is of note, however, that in ten cases of B cell histiocytic lymphoma (Keller et al., unpublished observation) we have found no Fcγ-bearing T suppressor cells. Whether the lack of T suppression is causally related to the percentage of cells in these tumors that are in cell cycle and/or whether this relates to the efficacy of chemotherapeutic regimens in DHL awaits further study. It is of interest, however, that increasing aggressiveness of disease in the NHL spectrum appears to correlate with decreasing T suppressor elements. In fact, based on our studies in these and other compartments of the NHL schema, we have constructed a hypothetical schema (Fig. 7) that equates decreasing T cell and increasing macrophage suppressor cell activity with the stages of the B cell NHL spectrum. A schema for the T and null cell groups awaits further delineation of the ontogenic compartments and immunoregulation of these cells. In addition, whether these are causally or circumstantially related to the histopathologic stage at presentation remains speculative. If immunoregulation is involved, however, it may have far-reaching

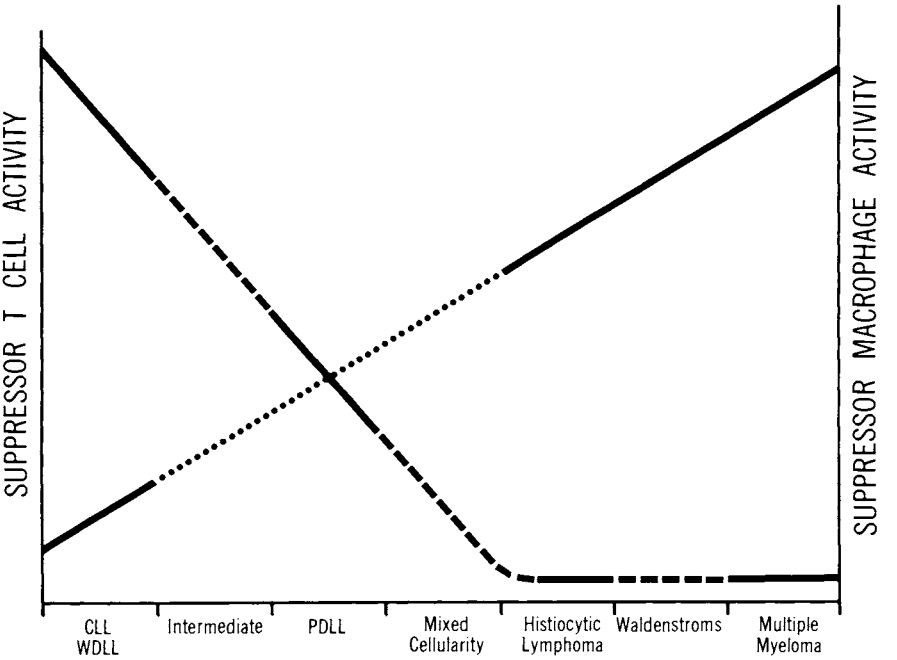

Fig. 7. The possible relationship between T cell and macrophage suppressor activity in human NHL. The dashed areas represent compartments of NHL where too few patients have been analyzed to warrant conclusions.

implications in regard to therapy since X-irradiation (Gupta and Good, 1977) and cytotoxic agents (Turk et al., 1972; Röllinghoff et al., 1977; Askenase et al., 1975) may preferentially kill T suppressor cells, rather than the malignant clone in the indolent compartments of NHL. If this concept is proven true, it would stand in sharp contrast to the role of suppressor cells in nonlymphoid malignancies as discussed in Chapter 7.

In summary, then, the non-Hodgkin's lymphomas simultaneously represent an exciting challenge and a significant enigma for both immunobiologists and practicing hematologists. They are malignancies of immunocytes, but our inadequate understanding of the inductive signals, ontogenic development, and control mechanisms of the lymphoid system have precluded a definitive classification system for these diseases. Nonetheless, the stages of disease that may represent maturational blocks and/or malignant transformations of normal maturational compartments and their increasingly recognized relationship to immunoregulatory ele-

ments offers many exciting challenges. To the immunobiologist, the ability to study relatively homogenous groups of cells in various maturational compartments should facilitate our understanding of the inductive events that lead to the bifurcations and eventual maturation of immunocytes. In addition, these tumors provide a unique opportunity to study cell–cell interactions and control mechanisms of immunocytes in the human system. To the hematologist, these studies should lead to a better understanding of the complexities of this spectrum of disease and more rational decisions concerning therapy, whether it involves irradiation, chemotherapy, or immunotherapy. The non-Hodgkin's lymphomas, therefore, truly represent an important, if not an unique, interface between basic immunobiology and clinical medicine.

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Chapter 6

Cell-Mediated Immunity in Autoimmune Disease

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1. Introduction

The history of cell-mediated immunity began before the turn of the century with the description by Koch of the delayed hypersensitivity reaction following infection by tubercle bacilli. Dienes many years later (1936) was the first to consider delayed hypersensitivity as an independent branch of the immunological response rather than a unique response to infectious microorganisms. He was successful in producing delayed hypersensitivity by injecting nonliving protein antigens into tuberculous foci.

It was only in 1942 that experiments by Landsteiner and Chase clearly demonstrated that the delayed hypersensitivity reaction was mediated by cells and not by antibody. They induced the delayed hypersensitivity type of skin test by transfer of viable lymphoid cells from a sensitized animal to a normal recipient,

demonstrating that the recipient developed a response to the particular antigen after receiving living lymphoid cells, but not after injection of immune serum.

Greatly influenced by Dienes, Freund found that he could produce experimental autoimmune lesions by injecting guinea pigs with guinea pig sperm mixed with an emulsion of aqueous antigen, mineral oil, and killed tubercle bacilli, now known as Freund's complete adjuvant (CFA). Freund and his associates (1953) demonstrated cell-mediated immunity as well as humoral antibody to sperm antigens and found a closer correlation between disease and the delayed hypersensitivity skin test reactions than the disease and sperm antibody levels.

A classical review by Waksman entitled "Experimental Autoallergic Diseases" (1959a) detailed the various autoimmune conditions produced by experimental immunization using CFA as well as describing the common features of the "autoallergic" lesion. The lesions generally began as perivascular infiltrates of mononuclear cells, thus resembling the histological changes characteristic of delayed hypersensitivity skin reactions. Waksman propounded the idea that these induced lesions could be attributed to delayed hypersensitivity. He, too, found a significant association of delayed hypersensitivity to brain antigen and the severity of experimentally induced encephalomyelitis. This disease could be transferred to syngeneic or tolerant recipients by means of viable lymphoid cell suspensions, but not by injections of immune serum (Stone, 1961; Paterson, 1960). A close association between delayed hypersensitivity and thyroid lesions was also shown in experimental thyroiditis in guinea pigs. McMaster et al. (1961) found that thyroiditis could be transferred from diseased to normal histocompatible guinea pigs using suspensions of lymph node cells. Similar observations were reported in rats by Twarog and Rose (1970). In further support of this viewpoint were experiments that demonstrated the association of delayed hypersensitivity with thyroid lesions when humoral responses had been blocked by the use of picrylated thyroglobulin (Miescher et al., 1961). Taken together, these experimental findings strongly suggest that cell-mediated immunity is an integral part of many autoimmune responses.

2. Self-Recognition and Tolerance

The primary function of the immune system as we envision it today is to distinguish between autologous and foreign antigens. The immune response is not usually triggered by the body's own antigens.

Recognition of self components from among the myriad of external antigens we encounter daily has long been one of nature's great mysteries. The subject still engenders considerable controversy, but gradually we are starting to understand the controlling mechanisms. It was originally proposed that precursor lymphocytes capable of reacting against self-antigens were inactivated or destroyed during embryonic or fetal development, presumably owing to the large quantities of antigen presented to the overly sensitive precursor cells (Burnet, 1959). This mechanism would ensure the absence of an immune response to self components later in life. The clonal elimination theory, as it was called, was developed by Burnet (1959) to help explain the considerable difficulties in accounting for self-recognition. Experimental support for the hypothesis that prenatal exposure to large amounts of antigen leads to elimination of the reactive clones was lent by the work of Billingham and his colleagues (1954) who induced immunologic tolerance to foreign antigens.

The original theory of clonal elimination has undergone revision because of recent information on cellular interactions in the immunological response. Unresponsiveness in only one population of lymphocytes (T cells) was found to be sufficient for tolerance to foreign antigens. Experiments reported by Weigle (1971) demonstrated that low concentrations of antigens can produce unresponsiveness of the T cells but not B lymphocytes, whereas large concentrations of antigen can produce both T cell and B cell unresponsiveness. During fetal development when large concentrations of many self-antigens are present, it is postulated that both T and B precursor cells are rendered unresponsive, hence for many circulating components autoimmune responses are virtually unknown. However, for the antigens relatively sequestered from the immune system during fetal life, e.g., spermatozoa and lens, autoimmune responses are not infrequent occurrences. For antigens present in low concentration, only T cell unresponsiveness is maintained while B cells remain potentially active. This concept presupposes that B cells capable of binding autoantigens and undergoing clonal expansion are present in the body. In support of this hypothesis, Bankhurst et al. (1973) demonstrated the presence of B lymphocytes capable of binding thyroglobulin in peripheral blood of humans. Subsequently, B cells capable of binding other self-antigens such as DNA have been found (Ada, 1970). Theoretically, any mechanism that would either bypass the requirement of T helper function for activation of B cells or induce nonspecific activation of B cells could evoke an autoimmune response.

As with other physiologic processes, mechanisms required to maintain homeostasis are incorporated into the immune system. Several regulatory features of the immune response have been well-characterized. These include both cellular and humoral elements. Uhr and Möller (1968) showed that antibody to foreign antigen itself may limit further production of antibody. Large amounts of antigen may be responsible for blockade of antibody production (Felton et al., 1955) as may antigen–antibody complexes (Diener and Feldman, 1972).

Immunoglobulin molecules have specific unique antigen-combining sites called idiotypes. According to the theory proposed by Jerne (1974), immunological control may be based on complementary idiotype anti-idiotype structures on lymphocytes. To control autoimmune responses, autoantibodies developed to idiotypic receptors on T or B cells may prevent continuing immune responses to self-antigens (Kohler and Rawley, 1977; Binz and Wigzell, 1978).

Other cellular mechanisms in the regulation of immunologic homeostasis involve the interaction of diverse lymphocyte populations. Not only is there a subset of T cells involved in the initiation and augmentation of antibody production (helper T cells), but there is another subset with different surface markers, T suppressor cells, that act in an inhibitory fashion (Gershon and Kondo, 1971). Recent evidence supports the view that suppressor T cell action plays a role in maintaining self-tolerance as well as in tolerance to foreign antigens (reviewed in Doyle et al., 1979).

T cell recognition of antigenic determinants seems to be quite distinct from that of B cells. T cells have recognition properties that include part of the major histocompatibility complex (MHC) of cell membranes (Zinkernagel and Doherty, 1974) as well as specificity for foreign antigens. Recognition of MHC appears to be a necessary requirement for their immune response to viral and chemically substituted cell surface antigens (Zinkernagel, 1977; Shearer, 1974). Receptors on cell membranes linked to the MHC antigens appear to regulate the cell–cell interaction that controls the immune response and may prevent the immunological response to self-components under normal circumstances. Thus, the body seems to have evolved several different mechanisms to prevent harmful autoimmune reactions.

3. Induction of Autoimmunity

Any event that interferes with normal immunological homeostasis could result in an autoimmune response. There are many possible

mechanisms, each of which may be the responsible event in individual cases of autoimmunity.

The antigens that incite autoimmmune responses may be exogenous or endogenous. Somatic mutation of lymphocytes is one mechanism that could theoretically replace precursors of deleted clones. Mutations of these rapidly dividing cells could result in competent lymphocytes now able to react to self-antigens.

In the case of unresponsiveness that is based on T cell tolerance, there are several possible events that could bypass the requirement for T cells and activate immunocompetent B cells. Experimental evidence for this event was provided by studies of induced tolerance of human gamma globulin (HGG) in mice. T cells from HGG-tolerant mice were injected into irradiated, bone-marrow-reconsituted hosts. Subsequent testing showed that the recipients were unresponsive to the tolerogen. Tolerance was present in T cells for 81 days following induction of tolerance. Agents that nonspecifically stimulate T cell functions or override T cells to activate B cells directly (such as certain adjuvants) were capable of abrogating tolerance under these experimental conditions. Allogeneic cells injected into experimental animals previously rendered unresponsive to foreign antigens also result in termination of T cell tolerance (McCullagh, 1970), but only when responsive B cells were present (Weigle et al., 1974). These results suggest that when T cells are nonspecifically activated by the allogeneic cells, they are capable of promoting B cell responses. A comparable circumstance may be provided by chronic graft-vs-host responses. Recently, Gleichman and his co-workers have discussed the development of autoantibodies during chronic graft-vs-host reactions. They believe that there is an abnormal cooperation of alloreactive donor T cells (presumably T helper cells) and H-2 incompatible recipient B cells providing a mechanism for breaking the immunological unresponsiveness to self-antigens (Gleichman and Gleichman, 1980; van Elven et al., 1981). Lipopolysaccharides (LPS) and other adjuvants have also been shown to override the T cell tolerance. LPS injection with tolerizing antigen abrogates unresponsiveness to antigen (Chiller and Weigle, 1973). Derived from gram-negative bacteria, LPS could be a natural agent that can circumvent tolerance to self-antigens.

Termination of self-tolerance can also be accomplished by injection of related antigen. Experiments with chemically altered self-antigen have resulted in development of antibodies that cross-react with the unaltered antigen. In some cases, only minor differences, such as accidental changes produced by protein purification, are all that is necessary to produce self-reactive antibodies.

Usually, however, the antigen requires deliberate alteration. One method, which may mimic a natural process, is the proteolytic digestion of the antigenic material. For example, treatment of thyroglobulin with proteolytic enzymes results in smaller molecular weight fragments that are antigenic in the same species, while the untreated thyroglobulin would elicit no response (Stylos and Rose, 1969). Intravenous injection of papain-treated thyroglobulin generates not only autoantibodies, but also lymphocytic infiltration of the thyroid (Anderson and Rose, 1971).

An alternative method for the induction of autoimmune disease is the introduction of antigens that cross-react with self-antigen (Terplan et al., 1960; Weigle, 1965). Injection of thyroglobulin of a foreign species, for instance, produces autoantibodies reactive with the recipient's own thyroglobulin. Repeated injection may result in lymphocytic infiltration of the host's thyroid. Analogous situations also occur under natural circumstances. Cross-reactive antigens have been implicated in the autoimmune response to heart muscle in rheumatic fever, as the surface antigens of B-hemolytic streptococci closely resemble components of the myocardium (Kaplan, 1965).

Self-antigens may also be altered through the action of viruses or other infectious agents. Whether or not the immune response is directed to viral antigens and to host antigens separately or in combination is not always clear (Zinkernagel and Doherty, 1975). In any case, virally infected host cells can induce immune cytotoxicity of the infected cells and subsequently development of specific autoantibodies to uninfected cells. This may explain why there is a transient production of auto-antibodies and/or tissue damage so often with certain viral infections. In addition the virally infected cells may be targets of cytotoxic T cells that have the capacity to destroy tissues infected with these agents. An example of this phenomenon was furnished by Wong et al. (1977) who showed that, in vitro, neonatal myocardial cells infected with Coxsackie virus were destroyed by immune spleen cells.

Abnormalities in the immunological regulatory mechanisms may also culminate in autoimmunity. The existence of a subset of T cells with specific membrane markers capable of dampening the responses of immunologically competent cells has previously been described. Defects in suppressor T cell activity has been demonstrated in humans with systemic lupus erythematosus (SLE) as well as in animal models of SLE such as (NZB $\times$ NZW) F_1 mice, (Talal, 1976; Palacios et al., 1981). In addition a recent report stated that, following thymectomy, a myasthenia gravis patient developed SLE

with subsequent defects of suppressor cell function (Calabrese et al., 1981). Treatment with thymic hormone appeared partially to reverse the in vitro suppressor cell function.

T suppressor activity in NZB mice was found to decline with age (Jacobs et al., 1971; Chused et al., 1973). A parallel observation has been noticed for some time in humans where the incidence of autoimmunity increases with age. Although there is considerable evidence associating depressed activity of suppressor T cells with autoimmunity, the cause–effect nature of the association has not yet been demonstrated.

Hormonal factors influence autoimmune disease. Autoimmunity in humans has long been recognized as being more prominent among females. The same observations have been made in some animal models. Female NZB mice are prone to earlier and more severe diseases than males. Experiments have demonstrated that the male hormone in mice exerts a protective effect on autoimmune susceptibility. Experimental work has suggested that the effect of hormones is exerted at the level of thymic regulation (Roubinian et al., 1977).

Genetic influences have been recognized as an important determinant of susceptibility to autoimmune disease in humans and in many induced or spontaneous animal models. Since the genetic control appears to be complex and polygenic, studies with inbred animals provide the most detailed information. In mice, it has been found that autoimmune responses to thyroglobulin can be linked to a gene located within the major histocompatibility locus on chromosome 17. The controlling gene has been called the immune response to thyroglobulin (Ir-Tg) gene. The production of antibody to thyroglobulin, however, did not always correlate with development of lesions. For example, in induced responses to thyroglobulin, Rose et al. (1973) found two strains of mice (BSVS and BRVR) that responded equally in the development of thyroid antibodies. However, although BSVS developed severe lesions, the BRVR animals exhibited only mild disease. It was found that additional Ir genes at the D-end of H-2 influence the mechanisms producing autoimmune tissue injury (Kong et al., 1979).

Further experiments were carried out to determine the cellular basis of genetic control (Vladutiu and Rose, 1975), in which it was shown that T lymphocytes primarily determined the response to thyroglobulin in the mouse since T cells both promoted the development of antibody and the production of lesions. T cells were also implicated in suppression of disease in susceptible strains of mice (Kojima et al., 1976).

Experimental organ-specific autoimmunity can be induced in animals by the use of adjuvants, but no such experimental regimen has been able to simulate the more generalized disorder that occurs in some human autoimmune conditions such as SLE, where multiple systems are affected. The genetics of NZB and its hybrid strains with other NZ mice has been extensively reviewed (Warner, 1977), with evidence indicating existence of a major gene acting in autoantibody development that can be modified by at least two other genes.

Immunological abnormalities that have been implicated as being under genetic control in the NZ mice strains include B cell hyperactivity, depression of suppressor T cells, functional deficiency of splenic adherent cells (proposed to be limited to a subpopulation of macrophages), and resistance to induced tolerance for certain antigens (reviewed by Warner, 1977). Individual NZ strains may not express all abnormalities, but in crossbreeding experiments some of the F_1 hybrids (i.e., NZB $\times$ NZW) demonstrated more severe lupus-like disease than either parental strain.

In humans, certain autoimmune diseases have been observed to have strong familial associations (Doniach et al., 1965). Furthermore, there is a variable but greater risk in the development of autoimmune disease in people who have certain major histocompatibility (HLA) antigens. An increase of frequency of HLA-B8 (a serologically defined antigen of the MHC) was found in myasthenia gravis, chronic active hepatitis (Mackay and Morris, 1972; Svejgaard et al., 1975), idiopathic Addison's disease, juvenile diabetes, and Graves' disease (Thomsen et al., 1975; Whittingham et al., 1975a), and SLE. Many of the diseases were even more closely associated with locus HLA-Dw3, a lymphocyte-defined allele that is in linkage disequilibrium with HLA-B8. The association of so many autoimmune diseases with a particular HLA haplotype, B8/Dw3, suggests that these alleles are linked to a gene that regulates the immune response or one of its effector mechanisms. More recently, the effect of gene interaction on the susceptibility to disease has been discussed (Whittingham et al., 1981). It appears that not only are HLA alleles important when considering autoimmune disease and high risk populations, but also the allotypes of immunoglobulin heavy chains (Gm allotypes).

Although there is a considerable speculation, immune response genes have not been clearly defined in man. There is no doubt that further evidence of complex genetic control over the immune system in the human will be discovered in the near future, primarily through the knowledge that comes with testing hypotheses in animal models.

4. Pathogenic Mechanisms in Autoimmune Disease

Autoantibodies, as markers of autoimmunization, may not necessarily be responsible for pathologic lesions. Autoantibodies by themselves are directly implicated in only a few disease states of man. Generally, they work with other components of the immune system. Although a great proportion of the normal population develops autoantibodies at one time or another with no apparent deleterious effect, there are some cases in which antibody-induced disease is present. The most clearly defined immune mechanisms of tissue injury attributable to antibody are those involving antibody alone, antibody-dependent complement-mediated cytotoxicity, immune complex deposition, antibody collaborating with mononuclear phagocytic cells, and antibody-dependent cell-mediated cytotoxicity. There are precedents and examples of each of these mechanisms, and more than one might be obtained in any given animal model. In human disease, it is not always clear which mechanism is responsible for pathology.

In autoimmune hemolytic anemias, antibodies to erythrocytes promote their sequestion and premature destruction by phagocytic monocytes mainly in the spleen. The mononuclear phagocyte has complement receptors that can bind erythrocytes with subsequent phagocytosis (Huber and Weiner, 1974; Holm and Hammarström, 1973). Antibodies to instrinsic factor may block its functional activities, as in the case of pernicious anemia, where antibodies bind to intrinsic factor and prevent one from binding and absorbing vitamin B_{12} (Roitt et al., 1969; Ashworth et al., 1967). Autoantibodies may also cause agglutination reactions of sperm and thus be responsible for some types of male infertility (Rumke and Hekman, 1975). Certain autoantibodies have specificity for thyrotropin (TSH) receptors on thyroid cells and mimic the action of TSH, resulting in hyperthyroidism (Adams, 1976; Adams, 1981).

In each of the above examples, complement does not seem to be required for activity. There are other disorders in which only compement-fixing autoantibodies are cytotoxic. They are seen primarily in hemotological disorders where leukocytes may be lysed, but may also be demonstrated to tissue cells such as spermatozoa or thyroid cells (Rumke and Hekman, 1975). Antibodies to the acetylcholine receptors are found in many cases of myasthenia gravis and, acting with complement, block neuromuscular transmission (Lennon et al., 1978).

Pathogenesis of disease by generation of immune complexes of autoantibodies and soluble antigens has been studied exten-

sively. Immune complex deposition is frequently found in the kidney and sometimes lung and skin of many patients with SLE. The immune complexes bind complement and initiate inflammatory reactions. Severe and often fatal glomerulonephritis may result from such a mechanism (Lambert and Dixon, 1968).

Complexes have been identified along the basement membrane of thyroid glands in cases of human chronic thyroiditis (Kalderon et al., 1975). They have also been detected in the extraocular muscles in patients with exophthalmus, suggesting a role for antigen–antibody complexes in these lesions (Kriss, 1970).

In other cases, normal lymphocytes can cooperate with antibody to produce cytotoxic effects (Perlmann et al., 1975a). Antibody-dependent cell-mediated cytotoxicity (ADCC) depends upon IgG antibody for specificity. The effector cell is an unsensitized lymphocyte-like cell bearing receptor for the Fc part of the immunoglobulin molecule. The effector cells, called killer cells (K cells), lack conventional markers for both mature T and B lymphocytes (Perlmann et al., 1975b; Cordier et al., 1976). Cells capable of mediating ADCC have been found in the blood of patients with autoimmune thyroiditis (Calder et al., 1973b; Wasserman et al., 1974; Calder et al., 1976) and in infiltrates of animals with experimental thyroiditis (Paget et al., 1976).

Direct cell-mediated damage is recognized as a significant mechanism of tissue destruction in rejection of allografts and in tumor immunity. Its role in autoimmune disease has never been clearly established. Lesions may be the result of one of several mechanisms brought about by the action of the cellular effector arm of the immune response. Sensitized lymphocytes themselves may bear receptors reactive with tissue surface antigens and produce direct cell damage (Cerottini and Brunner, 1974). Specific T lymphocytes have been found to attack allogeneic target cells both in vivo and in vitro without the cooperation of antibody, complement, or macrophages. Considerable effort has been made to demonstrate direct lymphocyte effects in autoimmune disease, but the results have not been clearcut, in part because stimulated lymphocytes nonspecifically injure susceptible epithelial target cells. In support of direct lymphocyte damage, peripheral blood lymphocytes from patients with autoimmune thyroiditis were found to lyse thyroglobulin-coated mastocytoma cells, although it was not clearly established whether T cells were the responsible effector cells (Podleski and Podleski, 1974; Calder et al., 1973a). Sensitized T lymphocytes may also produce pathology indirectly by the release of lymphokines subsequent to antigen-specific or nonspecific

stimulation. For example, in vitro correlates of delayed hypersensitivity such as lymphocyte blast transformation and migration inhibition factor were produced from peripheral blood lymphocytes of patients with autoimmune thyroid conditions after stimulation with thyroid antigens (Søborg and Halberg, 1968; Ehrenfeld and Klein, 1971; Brostoff, 1970; Calder et al., 1972; Wartenberg et al., 1973a).

In most autoimmune lesions the mechanism by which pathology occurs is as yet unclear, but probably more than one effector is involved. For instance, in autoimmune orchitis it was found that antibody produces initial damage allowing lymphocytes access to the specific antigen to which cells are sensitive (Brown et al., 1972). Subsequent work has indicated, at least in the mouse, that lymphoid cells, but not serum from immunized animals, transferred orchitis to unimmunized mice (Bernard et al., 1978). However, although the investigators were sure of an absolute requirement for T cells in the induction phase of autoimmune orchitis, they could not rule out the involvement of both T cells and antibody in the effector phase since the transfer of disease was only partly inhibited by anti-Thy 1.2 treatment of the lymphoid cells. Cell-mediated reactions are probably an important mechanism in the production of pathological lesions in autoimmune disease. The steps that culminate in the final destruction of body tissue are a series of specific and nonspecific immune reactions that are difficult to analyze, but are currently the topic of continuing investigation.

5. Cell-Mediated Immunity in Autoimmune Diseases of Animals

5.1. Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an immunologically mediated autoimmune central nervous system (CNS) disease. Accidental occurrence of EAE was first observed in patients given the Pasteur antirabies vaccine in which the virus had been grown in the rabbit nervous system tissue (Shiraki and Otani, 1959). Later investigations showed that the paralytogenic activity of the vaccine was a property of the nervous tissue and not the rabies virus. This disease is induced in laboratory animals by the injection of brain or spinal cord homogenates emulsified in CFA (Morgan, 1947; Kabat et al., 1947). Following sensitization to brain protein, within 2 to 3 weeks animals develop clinical signs of paralytic dis-

ease that include weight loss, paralysis of both hind legs, fecal impaction, and urinary retention owing to autonomic nervous system dysfunction from spinal cord injury (Paterson, 1976). These clinical manifestations are accompanied by histopathological changes within the brain and spinal cord consisting of focal areas of intense vascular and perivascular cellular infiltration of lymphocytes and macrophages with occasional involvement of plasma cells and polymorphonuclear leukocytes. Demyelination also occurs and is more pronounced in the lesions of higher primates such as monkey (Eylar, 1972). The histopathological similarities of EAE to multiple sclerosis, MS (Paterson et al., 1977) led to its widespread usage and an animal model system for MS.

Kies et al. (1958) and Einstein et al. (1958) together isolated myelin basic protein (BP) as the encephalitogenic antigen of the CNS that induces EAE. The molecular weight of myelin BP is 18,400 and consists of 170 amino acid residues (Eylar, 1970; Carnegie, 1971). Studies with defined peptides have suggested that there is a species-specific variability in the region of BP that induces EAE. In the guinea pig, a nonapeptide with an intact tryptophan residue defines the major encephalitogenic determinant (Eylar et al., 1970). Immunological recognition of antigenic sites is related to genetic factors that govern the susceptibility of various animals to EAE. In rat, Gasser et al. (1973) have shown that the susceptibility of rats to EAE is a polygenic trait, with non-MHC (major histocompatibility complex) genes being capable of strongly modifying the effects of MHC-linked responder or nonresponder genes. This concept has particular importance for understanding the disease process itself. In addition Lando et al. (1980) presented evidence corroborating the proposal that genes controlling susceptibility or resistance to the induction of EAE in mice may be influenced by naturally occurring suppressor cells. They found that cyclophosphamide treatment used to eliminate suppressor cells resulted in induction of EAE in usually resistant mouse strains.

In animals with EAE, sensitized lymphoid cells and circulating antibodies that are specifically reactive with whole CNS tissue, purified myelin, or myelin BP have been demonstrated in their circulation and peripheral lymphoid tissues (Paterson, 1969, Bornstein and Appel, 1961; Rauch et al., 1969). Several studies have implicated the involvement of cell-mediated immunity (CMI) in the pathogenesis of EAE. EAE has been passively transferred to normal histocompatible recipients with lymphoid cells from donors sensitized to BP (Paterson, 1960, Stone, 1961; Rauch and Griffen, 1969; Rauch and Griffen, 1970), but not with immune serum (Paterson, 1963).

Successful transfer of EAE was reported with rat thymus lymphocytes sensitized to brain antigens in vitro (Orgad and Cohen, 1974). EAE could be induced in bursectomized agammaglobulinemic chickens (Blaw et al., 1967), while neonatal thymectomy of chickens or of rats reduced the incidence of disease (Lennon and Byrd, 1973; Arnason et al., 1962). Furthermore, genetic resistance to EAE in BN rats was traced to their lack of T cells with receptor for BP (Ortiz-Ortiz and Weigle, 1976). Convincing evidence was provided by Gonatas and Howard (1974), who showed failure of EAE induction as well as antibody production in severely T cell-depleted rats. Reconstitution with syngeneic thymocytes restored the expected response to BP.

Delayed-type skin reactivity to CNS tissue or myelin BP can be demonstrated in animals developing EAE (Waksman and Morrison, 1951). Shaw et al. (1965) reported a strong positive correlation between delayed skin test reactions to BP and the development of EAE in guinea pigs actively sensitized to myelin BP. In vitro correlates of delayed hypersensitivity have been utilized with varying degrees of success to measure cellular immune response of animals with EAE. Lymphoid cells from rats and guinea pigs sensitized to human BP undergo proliferative-mitotic activity when placed in culture with myelin BP (Dau and Peterson, 1969). Production of macrophage migration inhibition factor (MIF) is a well established in vitro correlate of CMI. Sensitized lymphoid cells from guinea pigs injected with homologous spinal cord plus adjuvant produced MIF in the presence of rat brain (David and Paterson, 1965). Although in some situations development of MIF and lymphocyte transformation correlated with EAE, several studies suggested that development of EAE and occurrence of delayed-type hypersensitivity to nervous tissue may represent independent responses to CNS tissue sensitization. Spitler et al. (1972) could dissociate MIF production and EAE activity using synthetic encephalitogenic peptides modified to have different amino acid sequences from native BP. Furthermore, myelin BP with its tryptophan residue blocked, still could induce MIF production to unaltered myelin BP. These studies emphasized the importance of multiple antigenic determinants on myelin BP, some of which lack EAE activity and yet induce antibody or cell-mediated immune responses. Thus measurements of lymphocyte activity by such immunological testing may not reflect the pathologic mechanisms which cause clinical disease.

The role of antibody in the immunopathogenesis of EAE is controversial. During active induction of EAE, low levels of circulating antibodies to homologous BP were demonstrated (Hruby et al.,

1969). Gonatas et al. (1974) found no correlation between EAE and circulating antibody or the presence of BP antibody forming cells. Until recently, the encephalitogenic tryptophan peptide was thought incapable of inducing antibody (Coates et al., 1974). However, Hung and Rauch (1980) reported the production of antibody to the synthetic tryptophan peptide in the absence of disease. Simon and Simon (1975) reported a successful transfer with a single intraventricular injection of anti-CNS antisera, although it has not been confirmed. Recipient rabbits developed mild clinical neurological symptoms accompanied by histological lesions similar to those of EAE. However, the antigenic constituents against which these antibodies are directed may well not have been myelin *per se*. Seil et al. (1973) suggested that a central nervous system constituent, other than myelin BP, induced cytotoxic antibodies demyelinated organotypic brain culture targets. Such antibodies did not appear in animals sensitized to myelin BP plus adjuvant. The pattern of EAE in animals injected with myelin BP plus adjuvant does not have the same degree of demyelination characteristically associated with that form of EAE induced by whole nervous tissue. Although successful passive transfer of the clinical, neurological and histological signs by lymphocytes and the predominance of mononuclear cells in the inflammatory lesions of EAE argues in favor of a cell-mediated immune response, the presence of plasma cells in EAE lesions (Johnson et al., 1971) and local specific antibody production (Lennon et al., 1972) open the possibility that both humoral and cellular mechanisms may cooperate in the final disease syndrome.

The prevailing view of the immunopathogenesis of EAE is as follows (Paterson, 1977). Potential clones of lymphoid cells genetically programmed to respond to CNS-specific antigenic determinants are triggered by the CNS tissue or myelin BP plus adjuvant. Randomly, many of these sensitized lymphocytes gain access to the CNS "target" when they undergo proliferation. In the "target" tissue, a variety of effector products are released as a result of lymphocyte-target tissue interaction. These products of activated lymphocytes in turn generate an inflammatory response, and antibody-producing lymphocytes may then enter CNS injured areas. Once stimulated by tissue antigen, both T cells and B cells produce lymphokines or antibodies *in situ*. The net result is an inflammatory and demyelinating form of tissue damage and histopathology characteristic of EAE.

5.2. Experimental Allergic Orchitis

Experimental allergic orchitis (EAO), an autoimmune disease of the testis, is readily induced in guinea pigs (Voisin et al., 1951) and rabbits (Tung and Woodroffe, 1978) after immunization with sperm or testis suspension in Freund's Complete Adjuvant (CFA). The histopathology of EAO is complex and consists of at least three changes: (Tung and Alexander, 1976; Waksman, 1959b) (1) interstitial mononuclear infiltrate with invasion of macrophages into the seminiferous tubule (ST), (2) degeneration of the germinal epithelium in the absence of mononuclear infiltrate with resultant hypo- or aspermatogenesis, and (3) polymorphonuclear leukocyte-rich lesions in the ductus efferentes and the cauda epididymis. Many investigators agree that the primary lesions are an inflammatory orchitis followed by secondary hypo- or asperamatogenesis.

Evidence has been presented to suggest that allergic orchitis may be a sequel of vasoligation in some species such as the guinea pig (Tung and Alexander, 1977) and the rabbit (Bigazzi et al., 1976). In guinea pigs that have been vasoligated for over a year, testicular lesions that resemble those of allergic orchitis can be described. In rabbits, vasoligation has resulted in immune complex-associated orchitis that in many respects resembles the allergic orchitis induced in rabbits by immunization with testis antigens plus CFA. This model provides new insight into the autoimmune response to sperm in man following vasectomy or vasoligation.

Freund et al. (1953) established the immunological specificity of EAO by the demonstration of anti-sperm antibodies in guinea pigs injected with testicular suspension plus CFA. Although sperm possess multiple intracellular and surface antigens that are capable of inducing autoantibodies, only some antigens have the capacity to induce the disease EAO. In guinea pig, aspermatogenic antigens are associated with the surface and acrosome of sperm (Toullet el al., 1973; Jackson et al., 1975). These antigenic determinants elicit different types of immune responses and illustrate possible pitfalls in correlating serum antibodies and immunopathological events in the testicular disease.

Various types of humoral antibodies as well as the cell-mediated type of immunity as demonstrated by delayed hypersensitivity skin tests are observed in the immunized animals. The immune mechanisms responsible for tissue damage have been much debated. Positive delayed intradermal skin test is observed in ani-

mals developing EAO (Baum et al., 1961). Evidence that EAO is primarily cell-mediated disease was provided by successful passive transfer of EAO using immune lymphocytes. Lymph node cells (Tung et al., 1971a) or peritoneal exudate cells (Tung, 1978) from donors immunized with guinea pig testicular antigens transferred lesions indistinguishable from those of EAO when injected via subcapsular route (Kantor and Dixon, 1972). Cell preparations depleted of T cells fail to transfer EAO (Stadecker et al., 1973). Tung (1977) demonstrated that the incidence of disease correlated with the number of sensitized T lymphocytes locally transferred. T lymphocytes autosensitized in vitro against autologous testis cells produced severe progressive orchitis when injected into a normal recipient (Wekerle and Begemann, 1976). Hypothymic BALB/c nu/nu mice were not able to develop EAO upon adequate orchitogenic challenge, but reconstitution with syngeneic thymocytes completely restored the capacity of such mice to develop orchitis (Bernard et al., 1978). These results suggest that T lymphocytes are the cells primarily responsible for tissue damage in EAO.

Role of the antibody in the pathogenesis of EAO is much less clear. The levels of sperm antibodies did not always correlate with the degree of cellular damage in EAO (Freund et al., 1953). A protective role of antibodies has been demonstrated in guinea pigs pretreated with testicular antigen in physiological saline (Chutna and Rychlikova, 1964). These antibodies suppressed the development of EAO upon rechallenge with antigens in CFA. However, cytotoxic antibody correlating with testicular pathology were demonstrated in guinea pigs injected with testicular antigen plus CFA. Passive systemic transfer of antiserum alone generally failed to induce testicular lesions (Waksman, 1959a; Tung et al., 1971b), unless the recipient had been pretreated with CFA, presumably because of CFA-induced testicular permeability is a prerequisite to the induction of EAO (Voisin and Toullet, 1973; Wilson et al., 1972). Injection of antiserum directly into the testis also induced only minor reaction (Tung et al., 1971b). In general, although T lymphocytes from peritoneal cells of guinea pigs immunized with testicular antigens in CFA can adoptively transfer lesions of EAO to syngeneic recipients, lesions in the ductuli efferentes and rete testis, and hypospermatogenesis in the ST (seminiferous tubules) can be transferred passively with immune serum.

Very likely immune reactions involving humoral antibodies and T lymphocytes produce different histopathological lesions in EAO. T lymphocytes are responsible for macrophagic accumulation in EAO and function as mediators of delayed hypersensitivity.

It is conceivable that antibodies are responsible for degenerative changes in ST. In vitro evidence demonstrated the cytotoxic property of anti-sperm antibody in the presence of complement (LeBouteiller et al., 1975).

Other investigations showed that animals with only humoral or cellular immunity developed lesions after passive transfer of missing factor (Brown and Glynn, 1969; Brown et al., 1967). Thus several immunological mechanisms involving both branches of the immune response are implicated in the pathogenesis of EAO.

5.3. Spontaneous and Experimental Autoimmune Thyroiditis

Two animal models are currently available for study of autoimmune thyroiditis. The first is experimental autoimmune thyroiditis (EAT) induced by immunization with thyroglobulin. A second form of thyroiditis develops spontaneously in several animal species. EAT has often been cited as an analog of human chronic thyroiditis. EAT was first demonstrated in rabbits (Witebsky and Rose, 1956) followed by dogs (Terplan et al., 1960), rats (Jones and Roitt, 1961), mice (Metzgar and Grace, 1961), guinea pigs (McMaster et al., 1961), and chickens (Jankovic and Mitrovic, 1963). Induction of EAT was generally accomplished by concomitant use of CFA in an emulsion with crude thyroid extract or thyroglobulin deposited in an intradermal or subcutaneous site. In recent years, lipopolysaccharide has been found to serve as an effective adjuvant in mice (Esquivel et al., 1977). Pertussis vaccine has been used as an additional adjuvant in producing thyroiditis in rats (Paterson and Drobish, 1968; Twarog and Rose, 1969).

In most species of experimental animals tested thyroglobulin has been identified as an actual component of the thyroid extract that is responsible for inducing autoimmunity (Shulman and Witebsky, 1960; Rose et al., 1965). Thyroglobulin is a 19S iodoprotein with a molecular weight near 6.7×10^5 daltons and an isoelectric point of 4.58. Thyroglobulin migrates as an alpha-globulin in agar electrophoresis (Beutner et al., 1958). The complete amino acid composition of thyroglobulin shows 5500 amino acids per molecule with the remaining mass consisting of 300 monosaccharides (about 8–10%) and iodine (Rolland et al., 1966). Recent data indicate that thyroglobulin molecules consist of two symmetrical monomers presumably representing two 12S subunits of similar molecular weight (3.3×10^5 each), but with slightly different amino acid composition (Marriq et al., 1977; Van der Walt et al., 1978).

Induction of EAT generally features three manifestations of autoimmunity: (1) circulating antibody to thyroglobulin, (2) specific dermal hypersensitivity of the delayed type to thyroglobulin, (3) inflammatory changes in the thyroid gland of the immunized animal. The histological lesions are characterized by a reduction in size and colloid content of the thyroid follicles as well as inflammatory infiltrates made up mostly by lymphoid cells and macrophages.

Immune responses to thyroglobulin and susceptibility to EAT are genetically controlled. The importance of genetic constitution of the experimental animal in the mechanism of production of EAT was pointed out by McMaster and his colleagues (1965). These workers compared the results in two inbred strains of guinea pigs. The Hartley strain developed thyroiditis more readily in response to low doses and more severely in response to high doses compared to strain 13 guinea pigs. Using congenic and intra-MHC recombinant mouse strains, Vladutiu and Rose (1971a) demonstrated obvious correlation between responsiveness and MHC type. The major immune response (Ir) gene governing response of mice to mouse thyroglobulin has been tentatively designated Ir-Tg. However, additional genetic controls regulate production of thyroid lesions.

Evidence that responsiveness to thyroglobulin is dependent upon the presence of T lymphocytes was provided by Vladutiu and Rose (1975). Congenitally athymic (nu/nu) mice or "B" mice prepared by lethal irradiation and reconstitution with syngenic bone marrow cells treated with anti-theta antiserum plus complement failed to respond to murine thyroglobulin. Cell transfer experiments using T lymphocytes from good or poor responders to murine thyroglobulin showed that poor responder mice that had been thymectomized, lethally irradiated, and restored with high responder T cells developed high autoantibody titers and severe thyroiditis regardless of the source of transferred bone marrow cells. These results point out that it is the T lymphocyte that principally determines the vigor of response to thyroglobulin in the mouse. Alkan's recently developed T lymphoproliferation assay, used to assess T cell proliferation to mouse thyroglobulin, demonstrated that this response is under MHC-linked Ir gene control (Christadoss et al., 1978). This autoimmune response to mouse thyroglobulin maps at the *K* region or *I-A* subregion, as do many of the best-studied immune response genes.

In thyroiditis, the possible phenomena leading to tissue damage can be grouped into two major categories. One consists of the action of circulating autoantibody in the expression of thyroiditis as a tissue-specific lesion. It involves the function of a cytotoxic an-

tibody with appropriate specificity for the target tissue aided by interaction with complement and probably followed by chemotactic attraction of lymphoid cells. The second category includes the cell-mediated immune responses involving target tissue damage by mechanisms of delayed hypersensitivity. In this situation the circulating autoantibodies simply represent a side issue or at most a marker of the disease rather than a pathogenic factor.

Many studies have been performed to explore the mechanism responsible for the thyroid lesions. In animals developing experimentally induced thyroiditis, circulating antibodies to Tg could be demonstrated by a variety of techniques, including precipitation, complement fixation, indirect hemagglutination, and passive anaphylaxis (Rose et al., 1964). Although IgM antibodies may be found early after immunization, most often the antibodies are the IgG class. A fairly good correlation of autoantibody titers with the degree of thyroid damage was observed in guinea pigs (Metzgar and Buckley, 1967), rabbits (Nakamura and Weigle, 1969), mice (Vladutiu and Rose, 1971b), and chickens (Wick et al., 1970), while little correlation was found in rats (Rose, 1975; Twarog and Rose, 1970). Although these reports are suggestive, there is no convincing evidence of a causal relationship between antibodies and lesions. On the other hand, a good correlation between cellular immunity and the appearance of lesions in thyroiditis was reported in guinea pigs (McMaster et al., 1961; Wasserman and Packalen, 1965).

Rose and his colleagues (1962) clearly showed that one can dissociate the response of autoantibody production from the development of tissue lesions. They were able to stimulate a high titer of antibody production by means of immunization of rabbits with alum-precipitated antigen. Under these conditions, there was no development of thyroiditis. Antimetabolites such as 6-mercaptopurine and aminopterin were used to dissociate autoantibody response from the development of thyroiditis lesions (Spiegelberg and Miescher, 1963). Both compounds depressed the cell-mediated response and the development of thyroiditis. Aminopterin strongly depressed the formation of circulating autoantibodies. Furthermore, thyroiditis was developed in animals treated with aminopterin. The converse experiment was reported by Miescher et al. (1961), in which guinea pigs were injected with picrylated guinea pig thyroglobulin. Diminished circulating antibody response was observed in treated animals. Delayed hypersensitivity and thyroid lesions, however, were not decreased.

In other attempts, efforts were made to transfer thyroiditis to normal animals by serum or cells from diseased animals. EAT was successfully transferred by lymphoid cells from guinea pigs

(McMaster and Lerner, 1967), rabbits (Nakamura and Weigle, 1967), and rats (Twarog and Rose, 1970). In rats, severe disease could be passively transferred using rather large numbers of immune lymph node cells taken 7 days after active immunization of donors. Focal infiltration was observed as early as 18 h after transfer. Antibody did not seem to play a role in the development of lesions since circulating antibody was not detected at these early times either in donor or recipient animals. Furthermore, simultaneous transfer of antiserum with immune lymph node cells decreased severity of disease suggesting some protective role of antibody. Other workers were able to transfer EAT using immune serum from guinea pig (Sharp et al., 1967), rabbits (Nakamura and Weigle, 1969), monkeys (Rose and Kite, 1969), and mice (Vladutiu and Rose, 1971b). Lesions produced are generally less severe than those caused by active immunization. In rabbits, disease was produced using immune sera from thyroidectomized donors and taken within 15 days after active immunization. Rose and Kite (1969) could passively transfer disease in monkeys by injecting directly into thyroid gland using immune sera known to have cytotoxic antibodies to the thyroid cells.

Direct study of lymphocytes infiltrating the lesions in guinea pigs with experimental thyroiditis demonstrated that T lymphocytes were the predominant cells present (Paget et al., 1976). Recent observations of Lin and Salvin (1976a) demonstrated that sensitized lymphocytes from guinea pigs with EAT could directly lyse thyroid monolayers in vitro. This cytotoxic effect was further shown to be mediated by a soluble cell factor called thyroid cytotoxic factor (TCF) that was released from sensitized lymphocytes (Lin and Salvin, 1976b). Release of TCF and development of delayed skin responses occurred in parallel with thyroid lesions. Thoracic duct lymphocytes of rats immunized with rat thyroid extract in CFA destroyed monolayers of rat thyroid cultures (Bjorklund, 1964). These results suggest that T lymphocytes are primarily responsible for thyroid damage. Other findings suggest that antibody and cellular factors combine to produce the final effect of an organ-specific autoimmune disease. Spleen cells from immunized guinea pig lysed thyroglobulin-coated chicken red cells in the presence of IgG antibodies from the same animal (Wasserman et al., 1971).

A well-defined model of spontaneous autoimmune thyroiditis (SAT) has been extensively studied in the Obese Strain (OS) of chickens. Autoantibody specific for chicken thyroglobulin can be demonstrated in most OS chickens at 4 weeks of age (Kite et al., 1969a,b). These animals also demonstrate lymphocytic and monocytic infiltration of the thyroid parenchyma. Cole (1966) suggested that thy-

roiditis in OS chickens is inherited as a polygenic trait expressed with some degree of dominance. Autoantibody levels and thyroid pathology are influenced by one or more genes linked to the B locus, the MHC of the chicken (Bacon et al., 1974).

Spontaneous thyroiditis has also been found in the inbred BUF strain of rats. Spontaneous development of thyroiditis in this strain of rats was first recognized by Hajdu and Rona (1969) and its autoimmune as well as genetic nature was established by Silverman and Rose (1975). The incidence of SAT in BUF was at 30 weeks of age. Females were afflicted about three times more frequently than males. More severe disease occurred if the rats were fed methylcholanthrene (MCA) at an early age. There is a close correlation between circulating antibodies and thyroid lesions. Neonatal thymectomy enhances the severity and incidence of disease (Silverman and Rose, 1974).

Evidence has been provided that the bursa of Fabricius is necessary for the development of SAT in OS chickens (Cole et al., 1968; Wick et al., 1970). Antibody titers increase with age after hatching and in general seem to correlate well with thyroid pathology (Witebsky et al., 1969). Bursectomy prevents the development of SAT in OS chickens (Cole et al., 1968; Nilson and Rose, 1972). Although serum alone generally does not transfer the disease (Kite et al., 1969) to normal Cornell strain chickens, it increases the severity of lesions in partially susceptible sublines of OS (Jaroszewski et al., 1978).

Cell-mediated immunity has been demonstrated by the injection of chicken thyroid extract or thyroglobulin into the wattle (Welch et al., 1973). Positive delayed hypersensitivity was greatest only after thyroid pathology was well-established. Further evidence elucidating the role of B cells in the pathogenesis of SAT was provided by Polley et al. (1977). Chemical bursectomy by cyclophosphamide treatment reduced lymphoid infiltration and autoantibody production. When cyclophosphamide-treated OS birds were restored with either CS or OS bursa cells, severe thyroiditis and high antibody production resulted. Thus bursa cells of CS chickens were as effective as those from OS chickens in producing anti-thyroglobulin antibody or thyroid lesions if provided with proper T helper lymphocytes. This result suggests that the genetic resistance or susceptibility to thyroiditis does not solely depend upon the bursa cells. Immune mechanisms involving both antibody and lymphoid cells are implicated in the thyroid damage.

Nonspecific regulatory mechanisms may be genetically disordered in highly susceptible OS chickens. Recent observations have

suggested a functional difference in T lymphocyte immune responses of OS chickens compared to normal animals (Jakobisiak et al., 1976). Neonatal thymectomy of CS chickens allowed most allografts to survive over 50 days whereas such grafts were rejected in similarly treated OS chickens. This finding indicated that effector T lymphocytes migrate from the thymus to peripheral sites in OS birds. This suggests that the ratio of effector to suppressor T lymphocytes is greater in young OS compared to normal chickens. Additional indications of T lymphocyte abnormalities in OS chickens were found in the in vitro proliferative responses of peripheral blood lymphocytes to the T cell mitogens PHA and con A (Jones and Bacon, 1977). OS birds showed a lower response than CS chickens.

A genetic disturbance in self-recognition mediated by abnormalities of the central lymphoid tissue was shown in OS chickens. Neonatal thymectomy of OS chickens caused a significant increase in the severity of thyroiditis (Wick et al., 1970). Analogous results have been observed in BUF rats (Silverman and Rose, 1974). Neonatal thymectomy reduced thymic suppression as observed in aging rats so that thymus functions decreased as reflected by lowered circulating T lymphocyte levels and decreased suppression. It is probable that OS chickens and BUF rats are genetically endowed with diminished thymic suppressor function as well as elevated responsiveness to thyroglobulin. Certain strains of rats that have been deliberately depleted of T lymphocytes by neonatal thymectomy followed by sublethal irradiation developed chronic thyroiditis associated with antibodies to thyroglobulin in the serum (Penhale et al., 1973). The loss of circulating suppressor T lymphocytes enhanced thyroiditis in highly susceptible strains. The concept that organ-specific autoimmune diseases arise from a disorder in immunological regulation is compatible with these observations.

In spite of the fact that genes linked to the MHC greatly influence development of autoimmunity, other genes not linked to the MHC also bear an influence. A genetic influence on the target thyroid gland itself had been found in both OS chickens and BUF rats. Early investigations by Sundick and Wick (1974) suggested that some metabolic defects are present in the thyroid of OS chickens prior to lymphoid cell infiltration. When tested on the day of hatching, thyroid glands of OS chickens incorporated significantly more ^{125}I than normal chickens. Similar findings have been demonstrated in BUF rats (Kieffer et al., 1978). Decreased thyroid function

indicated by elevated plasma TSH and decreased plasma T_4 was found.

Work on OS and CS chickens was continued by Sundick et al. (1979), who reported additional evidence pointing to an abnormality of the thyroid gland in these birds. They found that the iodine uptake by thyroids of OS and CS progenitor birds was not completely suppressed by markedly elevated levels of serum thyroxine as in other chicken strains. These studies suggested that the thyroids from OS and CS birds may have a TSH-independent component of thyroid function. Subsequently, the incomplete suppression was found intrinsic to the thyroid gland itself and independent of other in vivo parameters, as demonstrated when the abnormal thyroid response was maintained after transplantation to normal birds (Livezey and Sundick, 1980).

6. Cell-Mediated Immunity in Autoimmune Disease of Humans

There are a considerable number of human autoimmune conditions in which cell-mediated immunity has been implicated. In many autoimmune diseases cellular infiltrates have been recognized as an important part of the pathological process and, in a few instances, research has suggested that a cellular immune response is actually responsible for the pathology. Most often human disease is probably an end product of the interplay between the cellular and humoral aspects of the immune response. As we previoulsy discussed in connection with autoimmune disease of animals, by the time human autoimmune disease is recognized it is often impossible to delineate the individual effector mechanism by which pathology has occurred. The involvement of particular elements has come from recognition of common components within lesions, observations in the progressive stages of disease, in vitro demonstrations of cell-mediated immunity that correlate with in vivo situations (i.e., skin tests) in addition to the evidence derived from animal models. The animal models of human disease, although not perfect replicas, have helped considerably in the understanding of the basis and pathogenesis of human autoimmunity.

Implications of cellular immune reactions in human autoimmune disease includes information about the role of T lymphocytes in initiation of the autoimmune process as well as evidence that lymphocyte cytotoxicity is involved in the pathological conse-

quences of autoimmune responses. The techniques used in studies of human disease to evaluate cell-mediated immunity include skin testing and histological examination and the in vitro correlates such as macrophage migration inhibition tests, lymphocyte blast transformation, and direct lymphocyte cytotoxicity. Quantitation of peripheral T lymphocyte populations has also been useful, although only in rare circumstances were the total number of T cells altered in autoimmune disease. Not all diseases with autoimmune features have evidence that cellular immunity contributes to the disease. As examples of autoimmune human disorders, we will discuss the generalized disorders such as in connective tissue diseases, and the organ-specific disorders such as the autoimmune endocrinopathies and non-endocrine associated organ specific diseases.

6.1. Connective Tissue Disorders

Systemic lupus erythematosus (SLE) is characterized by production of multiple autoantibodies and is the clearest example of an immune complex-mediated disease. Evidence has been produced suggesting the involvement of cell-mediated immunity in SLE in addition to the major defects in immunologic homeostasis responsible for the production of autoantibodies. Experimental work using techniques such as skin tests and in vitro exposure of lymphocytes to mitogens and nuclear antigens have elicited responses that are normal, increased, or decreased depending on the time of investigation (Tan and Rothfield, 1978). Direct binding of nucleotides to peripheral blood lymphocytes was demonstrated.

Rheumatoid arthritis (RA) is a condition in which there is more direct evidence implicating CMI in the initiation and progression of inflammatory events despite the known contribution of immune complexes. In fact, Good et al. (1975) reported that approximately one-third of patients with congenital agammaglobulinemia were found to suffer from a seronegative rheumatoid arthritis-like syndrome. Histological examination of the rheumatoid synovial membrane showed dense collections of lymphocytes. The predominant infiltrating cell was found to be a T cell (Van Boxel and Paget, 1975). Ziff (1974) proposed that antigen-stimulated T cells release lymphokines producing concomitant macrophage accumulation and subsequent tissue damage owing to release of toxic products of activated macrophages. Furthermore, it was shown that thoracic duct drainage to decrease the levels of circulating T cells led to significant clinical improvement in patients with RA. In addition, if the

drained lymphocytes were introduced into either peripheral blood or joints, there was an obvious increase in inflammation, thereby demonstrating that thoracic duct lymphocytes, which are primarily T cells, were involved in the production or maintenance of the inflammatory reaction and synovitis associated with RA (Pearson et al., 1974; Wegelius et al., 1970). Synovial fluid of patients with RA have been found also to contain soluble factors that, when tested in vitro, are hallmarks of CMI, such as macrophage migration inhibition factor, lymphocyte blastic factors, and immunoglobulin synthesis enhancing factor (Stastny et al., 1975).

The pathological features of RA are consistent with a cellular immune response to a persistent antigen. Possible candidate antigens include infectious agents, IgG in native or aggregated state, or components of synovial tissue, cartilage, fibrous tissue, or synovial fluid. The various agents have been used for in vivo and in vitro assays of CMI to determine the extent of T cell recognition in RA patients. The most consistent results have come from use of synovial membrane. Crude synovial membrane extracts from RA patients have been shown to induce positive intradermal delayed skin tests (Multz et al., 1968) and inhibition of peripheral blood leukocyte migration (Bacon et al., 1973) in RA patients. Eluted fractions of partially purified membrane suggested that the antigen responsible for the positive leukocyte migration inhibition is on the surface of the membrane of cells in RA synovium. Synovial fluid from rheumatoid patients has also been shown to be blastogenic for RA lymphocytes (Kinsella, 1970). Results of tests using native or aggregated IgG, either autologous or homologous, have been conflicting. While Kinsella (1974) found significant lymphocyte blast transformation responses to various IgG preparations, other have not (Runge and Mills, 1971). Although there appears to be cellular immune recognition of the various autologous agents in RA, it is not clear whether the cellular immune response functions to initiate or serves to perpetuate the pathological response.

Direct cytotoxicity experiments have also been performed using peripheral blood lymphocytes (Sukernick et al., 1968), synovial fluid mononuclear cells (MacLennan and Loewi, 1970), and lymph node cells (Braunsteiner et al., 1964) of patients with RA. Results showed a higher level of cytotoxic activity than with normal control lymphoid cells. However, the target cells were not synovial tissue (i.e., human connective tissue cells, Chang liver cells) and the authors concluded that the response was a nonspecific reaction of the lymphoid cells that had been previously stimulated by specific antigen—possibly of synovial origin. Later studies were done using

human RA synovial cell lines as targets. Peripheral blood lymphocytes from rheumatoid patients demonstrated preferential cytotoxicity for the target cells of RA subjects over non-RA targets (Person et al., 1974; Griffiths et al., 1975). It was concluded that RA lymphocytes may be presensitized to antigens present on RA synovial cells and therefore also react, to a lesser extent, on non-RA synovial cells.

Other related connective tissue disorders also have autoimmune elements. Myopathies, such as polymyositis and dermatomyositis, although the etiology is unknown, are suspected of being mediated through cellular hypersensitivity mechanisms. The chief clinical symptom is muscular weakness and the principal pathological lesions are degeneration of muscle fibers and infiltration by chronic inflammatory cells. Cell-mediated immune responses have been found to skeletal muscle extracts (Dawkins et al., 1972). Lymphocyte blast transformation using human muscle as antigen has also been demonstrated in patients with polymyositis (Currie et al., 1971; Esiri et al., 1973). In addition, cytotoxicity to muscle tissue by lymphocytes from patients with polymyositis has been shown by various techniques (Currie et al., 1971; Dawkins and Mastaglia, 1973). Although the mechanism of cytotoxicity was not clear, the evidence supports the suggestion that there was direct T cell killing of the muscle target cells.

Progressive systemic sclerosis (scleroderma) is another generalized disorder of connective tissue with uncertain etiology. The disease is characterized by inflammatory, fibrotic, and degenerative changes in skin, synovium, and certain internal organs including gastrointestinal tract, heart, lungs, kidneys. Often times progressive systemic sclerosis (PSS) will be found in "overlap" syndrome with mixed connective tissue disorders or Raynaud's phenomenon, systemic lupus erythematosus or polymyositis. Patients with PSS exhibit serological abnormalities such as antinuclear antibody directed primarily against ribonuclear protein. They also have abnormal cellular responses. These responses are thought to play a role in pathogenesis. Although no consistent alteration in the number of B cells in peripheral blood has been noted, a marked reduction in T cells has been found in some patients (Carapeto and Winkelmann, 1975). The large number of lymphoid cells in affected organs and in the dermis have been identified chiefly as T cells (Kondo et al., 1976). In vitro function tests of CMI demonstrated that lymphocytes from patients with PSS were reactive to a variety of autoantingens including autologous lymphocytes, liver microsomes, and mitochondria, and myelin basic protein (Hughes et al., 1974). It is not surprising that so many antigens would elicit CMI

responses considering the generalized nature of the disease. In addition to the other responses, chemotactic factor for monocytes was produced using human skin collagen as antigen (Stuart et al., 1976).

The above observations point out that lymphocytes from patients with PSS recognize and produce lymphokines in response to stimulation by components of human connective tissue. This finding is particularly important because of in vitro observations of the enhanced accumulation of collagen in fibroblast cultures owing to lymphokine-rich supernatant from mitogen-stimulated lymphoid cells from PSS patients (Johnson and Ziff, 1976). This chain of events in turn may explain the chronic inflammatory nature of PSS.

Sjögren's syndrome is another connective tissue disorder of unknown etiology consisting of keratoconjunctivitis sicca, xerostomia, and sometimes rheumatoid arthritis. Patients exhibit lymphoid infiltrations of salivary and lacrimal glands and sometimes other organs. Patients have several autoimmune serological markers such as antibodies to salivary gland duct, cell nuclei, and often to thyroid antigens. Frequently, they also have abnormalities of the cellular immune system. Some patients have a decrease in the number of peripheral blood T lymphocytes (Talal et al., 1973) and show functional deficiency when tested in vitro and in vivo, including an inability of T cells to undergo mitogenic stimulation and failure to develop delayed hypersensitivity to dinitrochlorobenzene (Leventhal et al., 1967). On the other hand, peripheral blood lymphocytes of some patients with Sjögren's syndrome were shown to produce migration inhibition factor (MIF) in response to extracts of parotid tissue (Berry et al., 1972). Although T cell abnormalities have been demonstrated, whether or not they are responsible for tissue damage is still uncertain.

6.2. Endocrine-Associated Organ-Specific Diseases

Unlike the generalized autoimmune disorders, some autoimmune conditions show immune responses that are directed primarily to a single target organ. The factors that may predispose a patient to organ-directed autoimmunity include genetic defects in immune regulation, enhanced specific immune responses to particular organ-specific antigens, and target organ defects. The thyroid is a common target organ for an autoimmune response. Both chronic lymphocytic thyroiditis (CLT), or Hashimoto's disease as it is also called, and Graves' disease are now believed to be immunologically mediated diseases. CLT is a chronic disease characterized usually

by a goiter exhibiting epithelial cell abnormalities and lymphocytic infiltration, occasionally progressing to hypothyroidism. Graves' disease, on the other hand, results in thyrotoxicosis, that is, overactivity of the thyroid gland because of the stimulatory activity of autoantibodies on the thyroid-stimulating hormone (TSH) receptor sites. In many cases, moreover, histological examination of the thyroid in Graves' disease patients shows pathologic characteristics similar to CLT. Although the two autoimmune thyroid conditions manifest in clinically distinct diseases, autoimmunization to thyroid antigens resulting in autoantibodies to thyroglobulin and the microsomal fraction of the thyroid epithelial cell and in cell-mediated immune responses are noted with regularity.

Cell-mediated immune reactions to thyroid antigens have been observed in many patients with autoimmune thyroiditis. A few observations have been made in vivo, but most of the data are from in vitro studies. Positive delayed hypersensitivity skin tests were noted by Buchanan et al. (1958) in nine out of eleven patients with Hashimoto's thyroiditis 24 h after interdermal injections of thyroid extract. These results were later corroborated when 29 out of 30 patients with goiter associated with mononuclear infiltration were also skin-test positive (Saarma, 1971). Early investigators using in vitro correlates of cell-mediated immunity were unable to demonstrate positive reactions in lymphocyte blast transformation tests using lymphocytes from Hashimoto's or Graves' patients when incubated with thyroid antigen (DeGroot and Jaksina, 1969). Other investigators, however, were later able to demonstrate lymphocyte transformation in chronic lymphocytic thyroiditis and Graves' disease patients when the peripheral blood lymphocytes were stimulated by thyroglobulin or thyroid extract (Ehrenfeld and Klein, 1971). Delespesse et al. (1972) even found that there was a correlation between lymphocyte transformation and the presence of thyroglobulin antibodies. Additonal evidence for the involvement of cell-mediated immune reactions in autoimmune thyroiditis was provided by experiments using leukocyte migration inhibition tests. Exposure of crude thyroid extract to capillary tubes containing peripheral blood leukocytes from patients with autoimmune thyroiditis inhibited migration of the leukocytes from the tubes. The leukocyte migration test (LMT), first demonstrated by Søborg and Halberg (1968), was later confirmed by additional investigators using a variety of thyroid-specific and other antigens in both CLT and Graves' disease (Brostoff, 1970; Calder et al., 1972; Lamki and Row, 1973; Wartenberg et al., 1973a; Wartenberg et al., 1973b; Whittingham et al., 1975). Reactivity to unrelated agents such

as rat liver mitochondria and human liver mitochondria was also noted in some cases of autoimmune thyroiditis, but no explanation was offered at that time. The source for the thyroid-specific antigen-positive leukocyte migration inhibition test reaction was also found to be important; for example, Calder et al. (1972) found that 60% of patients produced MIF when using crude thyroid extract, but only 44% were positive if thyroglobulin was used, and only 27% positive if the thyroid microsomal fraction was used. Another group of autoimmune thyroiditis patients studied by Wartenberg et al. (1973a) all produced leukocyte inhibition factor (LIF) when they used the microsomal antigen, but none when thyroglobulin was the antigen. The differences in result from the above two examples stress the need for caution in evaluating the role of cell-mediated immunity in autoimmune thyroiditis. A recent investigation (Aoki and DeGroot, 1979) studied the lymphocyte blastogenic response in Graves' disease and Hashimoto's thyroid patients to human thyroglobulin, and they found that the optimum dose level of thyroglobulin for maximal blastogenesis differed from patient to patient, which may account for some of the discrepancies in previous work.

The direct cytotoxic effect of lymphocytes from patients with autoimmune thyroiditis has also been observed. Podleski (1972) found that mouse mastocytoma cells coated with thyroglobulin or microsomal antigen were lysed by lymphocytes from over half of the patients with Hashimoto's thyroiditis that he tested. Though most cases reacted to both antigens, there was no apparent correlation between presence of antibody and cytotoxic activity of the lymphocyte. It was later reported that peripheral blood lymphocytes from patients with autoimmune thyroiditis could destroy human thyroid monolayer culture cells after incubation for 5–6 days, suggesting a direct lymphocyte–thyroid cell interaction (Laryea and Row, 1973). Lymphocyte-mediated cytotoxicity induced by thyroid antigens was also observed when mouse fibroblasts were incubated as nonspecific targets with supernatant from antigen-stimulated lymphocytes of patients with autoimmune thyroiditis (Amino and DeGroot, 1974), alternatively suggesting a role for a soluble factor produced by sensitive lymphocytes, rendering targets susceptible to lysis.

In addition, antibodies capable of inducing lymphocyte-mediated target cell lysis in the absence of complement in conjunction with killer cell activity (ADCC) have been observed in patients with autoimmune thyroiditis (Calder et al., 1973a; Calder et al., 1973b; Wasserman et al., 1974; Calder et al., 1976). The serum

from patients with autoimmune thyroiditis was incubated with target cells consisting of thyroglobulin-coated chicken erythrocytes labeled with radioactive chromium, and it promoted lysis of target cells by nonimmune lymphocytes. In addition, the ADCC activity of lymphoid cells was elevated above normal levels in patients with Hashimoto's disease, primary hypothyroidism and Graves' disease (Calder et al., 1976). Further interaction between lymphocyte antibody and thyroid tissue was observed with frozen sections of human thyroid tissue pre-incubated with sera from patients with autoimmune thyroiditis and then incubated with nonimmune lymphocytes. The lymphocytes adhered to the thyroid tissue (Pinedo and Mul, 1976).

Although both the relative and absolute numbers of peripheral blood T and B cells do not appear to be altered from normal levels in autoimmune thyroiditis patients, there are differences in the lymphocyte population of thyroid glands. Farid and Row (1973) reported an increase in T lymphocytes in thyroid tissue from biopsy specimens from patients with autoimmune thyroiditis. Urbaniak and Penhale (1973) reported at least one patient that had a 100-fold increase of thyroglobulin-binding lymphocytes in thyroid gland biopsies vs peripheral circulation. A more recent report by Tötterman et al. (1977) studied blood and thyroid-infiltrating lymphocyte subpopulations in juvenile autoimmune thyroiditis and later in adult autoimmune thyroid disorders. They found normal levels of T (about 70%) and B (about 20%) cells in peripheral circulation, but approximately equal numbers of T (about 40%) and B (about 48%) cells in the thyroid glands, indicating a relative increase in the number of B cells in relation to the peripheral blood population. Tötterman (1978) also evaluated thyroglobulin-binding cells in thyroid infiltrating lymphocytes. He found 4–67 times higher numbers of thyroglobulin-binding lymphocytes in the thyroid gland than in the blood in patients with Hashimoto's and Graves' disease. Also, of the lymphocytes binding thyroglobulin, from 6 to 15% were T lymphocytes, as measured by the ability to form rosettes with sheep red blood cells. Since there is previous evidence that some populations of T cells can bind soluble antigen (Basten et al., 1971; Kontiainen and Andersson, 1975), the T cells that bind thyroglobulin that are found in the thyroid gland could possibly function as T helper cells. Further exploration of the immune regulatory mechanisms was done by Aoki et al. (1979). Suppressor cell functions of peripheral mononuclear cells were examined in Graves' disease patients, Hashimoto's thyroiditis, thyroid cancer patients, and in normals. They found that suppressor cell activity was signif-

icantly reduced in the Graves' disease population; however, it was not changed in the other groups. They suggested that this reduction in suppressor cells may be the etiologic basis for induction of autoimmunity in Graves' disease.

Subacute thyroiditis (de Quervain's disease), another thyroid disorder usually resulting from a viral infection, is sometimes associated with at least a transient autoimmune response. Tötterman (1978) found a predominant T cell population (85%) in lymphocytic thyroid infiltrates in contrast to the equal numbers of T and B cells in the thyroid infiltrates of Hashimoto's and Graves' disease patients. He also demonstrated that thyroid-infiltrating lymphocytes were able to express cell-mediated immune responses against thyroid antigens in the leukocyte migration inhibition test, in comparison to the activity of peripheral blood leukocytes of the same patient, indicating that specific sensitized T cells were present in the thyroid gland in subacute thyroiditis. Previous work has also indicated at least a transient positive response to thyroglobulin in lymphocyte blast transformation tests and leukocyte migration inhibition tests (Delespesse et al., 1972; Wall et al., 1976). The in vitro experiments, along with the demonstration of T cell dominance in the thyroid infiltrates, suggest a possible role for antigen-sensitive T cells in the pathogenesis of subacute thyroiditis, possibly by cytotoxic T cells exhibiting killer activity on virally infected parenchymal cells (Tötterman et al., 1978).

Adrenal insufficiency owing to idiopathic Addison's disease, also called chronic autoimmune adrenalitis, is another endocrine organ-specific autoimmune disorder. The histopathology of the adrenal cortex shows the normal cortical cell architecture reduced to small areas of cells surrounded by fibrous tissue and lymphocytic infiltration (Irvine et al., 1967). The condition is associated with adrenal autoantibodies that by themselves do not appear detrimental to the adrenal. Nerup et al. (1969) studied cell-mediated immunity in patients with Addison's disease using the leukocyte migration tests with pooled fetal adrenal extracts with antigen. Positive inhibition tests were seen in eight out of 11 male and six out of 19 female patients with idiopathic Addison's disease. Reactivity was not seen in patients with Addison's disease of tuberculous origin. Further work using lymphocyte blast transformation tests was unable to confirm cell-mediated immune responses in these patients (Nerup and Bendixen, 1969). However, as in the in vitro tests for the thyroid autoimmune responses, positive cell-mediated immunity is very much dependent on the use of the proper antigen in the test. Presumably, if immune mechanisms are important in the

etiology of idiopathic Addison's disease, they operate through the mechanism of delayed hypersensitivity or ADCC (Irvine, 1978).

Pernicious anemia and atrophic gastritis are conditions often accompanied by an autoimmune response in the form of autoantibodies to parietal cell antigens. Both diseases are characterized histologically by diffuse lesions of the gastric mucosa, which includes atrophy of the gastric glands and infiltrations of the lamina propria with inflammatory cells. It is speculated that the leukocytes infiltrating the lamina propria area near gastric glands or the undifferentiated gland cells may be responsible for injury of the mucosal surfaces in pernicious anemia and atrophic gastritis (Jeffries, 1978). Cell-mediated immune responses to gastric antigens have also been identified. In vitro tests for cell-mediated immunity have demonstrated reactivity of lymphocytes to gastric components. Lymphocyte blast transformation was found in six out of 16 patients with pernicious anemia in response to the presence of concentrated gastric juice (Tai and McGuigan, 1969). Other investigators showed the presence of migration inhibition in nine out of 12 patients with pernicious anemia in response to gastric antigens (Finlayson et al., 1972). Even though cell-mediated immune reactions have been demonstrated, no effect of sensitized cells, however, on their ability to destroy gastric parietal cells in vivo or in vitro has yet been demonstrated.

Insulin-dependent diabetes mellitus (IDDM), usually but not exclusively found in juveniles, is now believed to belong to the group of autoimmune endocrinopathies (Nerup et al., 1978). This conclusion was made on the basis of pathologic appearance of the pancreas, the finding of cell-mediated immune responses to pancreatic antigens, and the demonstration of autoantibodies to pancreatic islet cells. The appearance of the pancreas in IDDM patients would best be described by the term insulitis, a descriptor first used by von Meyenburg (1940). Pathology consists primarily of mononuclear infiltration of islets of Langerhans with selective destruction of the beta cells (Egeberg et al., 1976). Insulitis, originally thought to be a rare condition in IDDM, is now found on a regular basis (Gepts, 1965; Egeberg et al., 1976). It does appear, however, to be of short duration, diminishing with increasing duration of disease. The insulitis can range from 10 to 75% of the islets involved. There is speculation that the lymphocytic infiltration causing beta cell destruction has been present before diabetes was clinically manifested (Nerup et al., 1978). Investigators have been searching for the presence of cell-mediated immunity against the endocrine pancreas in IDDM since the observation of cellular infiltration.

Nerup et al. (1971) was the first to demonstrate in vitro leukocyte migration-inhibition tests (LMT) against antigen specific for the endocrine pancreas. The antigen that he used was a homogenate prepared from pooled porcine pancreas, in which atrophy of the exocrine pancreas was induced by duct ligation. They found that 12 out of 22 patients with diabetes had substantial reduction of migration. All the LMT positive patients were insulin-dependent. Four out of the 12 positive patients were also delayed-hypersensitivity skin test positive, using the same antigen preparation. LMT studies were subsequently confirmed and extended by others (MacCuish et al., 1974; Nerup et al., 1974). The nature of the antigen responsible for migration inhibition, however, remained unknown. Population studies of patients with different duration of disease showed that the ability of the leukocytes to produce migration inhibition tended to fade with increasing duration of disease, coinciding with the disappearance of the lymphocytic insulitis (Nerup et al., 1973; Gepts, 1965).

The presence of autoantibodies to islet cells was not reported until some time later after the identification of cell-mediated immunity (Bottazzo, 1974). Although a marker of disease, the relevance of autoantibodies to the etiology and pathogenesis still remains to be elucidated.

6.3. Non-Endocrine Organ Diseases

Organ-specific autoimmune responses have also been recognized in non-endocrine associated diseases. The most common of these in which cell-mediated immune reactions have been identified include certain neurological disorders and inflammatory diseases of the liver.

Myasthenia gravis (MG) is a disease in which the transmission through the neuromuscular junction is impaired. There is evidence that immunological abnormalities contribute to the development of MG. Autoantibodies to skeletal muscle also cross-react with thymus myoid cells as well as antibodies that bind acetylcholine receptors in the neuromuscular junction have been described. Although autoantibodies are most prominent in patients with MG, cellular abnormalities have also been identified. Lymphocytes from MG patients, but not control subjects, showed positive stimulation to muscle extract (Alpert et al., 1972). A positive but weak lymphocyte stimulation to acetylcholine receptors was reported in five out of 11 patients with myasthenia gravis and not in controls (Abramsky et al., 1975). A similar finding was reported by Richman

et al. (1976) in 14 out of 21 MG patients. Evidence of thymic abnormalities in MG patients has been discussed frequently (Abdou et al., 1975; Richman et al., 1976; Smiley et al., 1968). An additional interesting finding has been the demonstration of an increased number of acetylcholine receptors in thymus epithelial cells in myasthenia gravis patients over that of normals (Engel et al., 1977).

The possibility that immunological mechanisms cause demyelinating disease of the central nervous system (CNS) of humans has been supported by various studies over the last few decades. Experimental animal models such as EAE, discussed earlier, support this idea. Demyelinating diseases represent a spectrum of CNS diseases from acute to chronic and remittant. Although they have distinct clinical presentations, both acute and chronic diseases seem to share important immunohistopathological features, suggesting common pathogenic mechanisms. Acute disseminated encephalomyelitis (ADE) occurs in some cases following vaccination to such agents as rabies or smallpox, and sometimes spontaneously occurring viral disorders, such as measles (either rubella or rubeola) and chickenpox. Pathological features are characterized early by infiltration of small vessel walls by mononuclear cells and occasionally polymorphonuclear cells, and possibly small hemorrhages. Later there is an intense perivascular infiltration, primarily by lymphocytic cells, accompanied by areas of perivascular demyelination. Immune responses to neural antigens are often found (Paterson, 1966; Paterson, 1978). Evidence for cell-mediated immunity include lymphocyte stimulation from patients with ADE in response to myelin-basic protein as well as production of migration inhibition factor. It has been suggested that persistence of virus in CNS tissue leads to immunological response to the host's own CNS antigenic components.

Multiple sclerosis (MS), classically a subacute or chronic disease covering a period of years, is characterized by a remitting and relapsing course. The etiologic agent or mechanism is as yet unknown, although viral agents are strongly suspected. Immunopathological features have been found in both cerebrospinal fluid (CSF) and CNS tissue. Approximately one-third of the patients with MS have been found to have an abnormal number of cells in the cerebrospinal fluid, the majority of cells being small lymphocytes. One investigator reported an increase in percentage of T cells in the CSF of a majority of MS patients with relapsing forms of the disease (Allen et al., 1976). These lymphocytes exhibited proliferation to a T cell mitogen phytohemagglutinin (PHA), and formed rosettes with

sheep red blood cells. There are also some B cells present, as noted by an increase of IgG in the cerebrospinal fluid (Cohen and Bannister, 1967). However, no direct correlation between concentration of IgG in the cerebrospinal fluid and severity of clinical course of MS was found.

Characteristic pathologic findings in the central nervous system tissue are the presence of well-defined, grayish plaques of various sizes (Greenfield et al., 1958). In addition, macrophages, lymphocytes, and plasma cells can be found around small vessels, associated with the development of the plaques. Microscopically, the plaques consist of disintegrating myelin and gliosis. In addition, immunoglobulin was found in association with the plaques and in the surrounding light matter in MS brain specimens (Tourtellotte and Parker, 1966; Tourtellotte and Parker, 1967).

The abnormal presence of both sensitized lymphocytes and antibodies has been found in the CNS of patients with demyelinating disease, as discussed above. However, the evidence implicating immunological mechanisms in the pathogenesis of demyelinating disease is indirect and involves three main areas of investigation. Much of the work has been done in parallel to the animal model of EAE. First, injections of nervous tissue into man or animals induced acute encephalomyelitis, characterized by perivascular infiltration and demyelination in association with immune responses directed against central nervous system tissue antigens (Paterson, 1966). Second, sera from animals with EAE and patients with MS (especially active disease) contained complement-dependent immunoglobulins that were cytotoxic for glial cells and myelinated nerve fibers in culture (Bornstein, 1973; Paterson, 1978). Finally, peripheral blood lymphocytes of animals with EAE and patients with MS (especially just before or after the onset of acute neurological disease) produced soluble mediators of delayed hypersensitivity following in vitro stimulation with nerve tissue or myelin basic protein (Bartfeld and Atoynatan, 1970; Rocklin et al., 1971; Sheremata et al., 1974; Sheremata et al., 1976). What is not clear, however, at least in humans, are the relative roles of antibody-secreting cells and sensitized lymphocytes in pathogenesis of MS.

The similarities between EAE in animals and MS in humans made the former the best animal model available, even though there is not perfect concordance between the two. As pointed out by Paterson (1978), both conditions exhibit elevated immunoglobulins in the cerebrospinal fluid. They both have circulating cytotoxic antibodies to brain cell cultures, and there is delayed hypersensitivity to basic protein as demonstrated by MIF production.

Discrepancies include a lack of demonstrable circulating antibodies to myelin basic protein in MS patients, an absence of delayed hypersensitivity skin tests to neural antigens in humans. Although there is evidence for immunoglobulin production within the central nervous system of MS patients, it has not been demonstrated in animal models. EAE, one must remember, is an experimentally induced condition in which there is a vigorous cell-mediated immune response by peripheral lymphocytes, resulting in delayed hypersensitivity reactions to neural antigens. The resulting immune response acts on antigen in a central location, and the ensuing inflammation causes EAE. MS, on the other hand, probably begins and remains a localized immune response within the central nervous system compartment, perhaps resulting in less intense systemic immunological responses.

Further work needs to be done to elucidate the etiologic factors in MS. Persistent neurotropic virus infections provide a likely explanation for the origin of the disease and the enhanced immunological activity associated with MS. The observed pathology may result from the action of cytotoxic T cells on virally infected central nervous system tissue. An important part of the disease may be in the manner individuals handle such viral attacks. There is questionable evidence for aberrant immunological response to measles virus in patients with MS and abnormal immunoglobulin synthesis against measles virus with an increased proportion of IgM antibody (Haire et al., 1973). MIF assays have demonstrated a suppressed cell-mediated response to measles virus in many MS patients (Utermohlen and Zabriskie, 1973). Human lymphocytes, probably T cells, exhibited surface receptors for measles virus, and the receptors appear to be abnormal in MS patients (Valdimarsson et al., 1975). One group of investigators found that lymphocytes from MS patients were agglutinated by virus less than lymphocytes of normal individuals (Utermohlen et al., 1975). Other investigators, however, were unable to confirm these results (Levy et al., 1976). The lack of consistency among experimental studies can be attributed to technical problems for the most part.

The cell-mediated immune response of MS patients and control subjects was recently studied with a leukocyte migration inhibition test in response to parainfluenza, measles, and vaccinia viral antigens, brain antigens, and mitogens (Rauch and Lewandowski, 1979). The investigators found that the group of MS patients were hyperreactive to the parainfluenza virus antigens compared to healthy controls. They found no significant differences between responses of MS patients and controls to human white matter sus-

pensions. The authors suggest that the results were consistent with the proposal that CNS pathology in MS reflects destruction of host cells expressing viral antigens rather than an autoimmune response elicited by antigenic determinants of normal white matter itself. They point out that if viruses are implicated in the pathology of MS, further longitudinal studies are needed on the association between clinical course and the cellular immune response of patients to selected viruses in human CNS demyelinating disease.

The term, "chronic active hepatitis," is used to delineate a condition of chronic inflammatory liver disease eventually culminating in cirrhosis. Classic chronic active hepatitis (CAH) is frequently associated with autoimmune responses. The autoimmune response seen most often in CAH is production of autoantibodies to smooth muscle (60–70% incidence), nuclei (60% incidence), and, less frequently, to mitochondria (11–29% incidence). These antibodies are not felt to participate in liver damage (Doniach et al., 1966; Whittingham et al., 1966; Smalley et al., 1968; Doniach, 1970). Histological features of the liver from patients with CAH include dense lymphoid infiltrations in portal tracts and occasional formation of lymphoid follicles, and infiltrations of plasma cells into hepatic lobules, along with the hepatic cellular necrosis and increasing fibrosis, leading to the appearance of cirrhosis (Mackay, 1978). The isolation of liver specific proteins separated from liver tissue by column chromatography has helped provide evidence for involvement of CMI responses in CAH. One protein (LP-I) is a macromolecular lipoprotein (mw $>$ 1,000,000) isolated from cell membrane. Another is a smaller cytoplasmic antigen (LP-II) with a molecular weight of 190,000 (Meyer Zum Buschenfelde and Miescher, 1972). These antigens were found to be organ-specific (Hopf et al., 1974). Since no cutaneous delayed hypersensitivity responses could be demonstrated in response to liver antigens, most of the work utilized the in vitro correlates of CMI, such as leukocyte migration inhibition tests and lymphocyte transformation. An early investigation used whole cell extract as antigen, and found inhibition in 24 out of 32 patients with either CAH or primary biliary cirrhosis (PBC) (Bacon et al., 1972). Subsequently, liver-specific protein was used as antigen and positive results were obtained in most of 34 cases of nontreated CAH and in some cases of cirrhosis, but not in other types of chronic liver disease nor in treated cases of CAH (Meyer Zum Buschenfelde et al., 1974). Another study showed that 11 of 16 cases of CAH and seven of 12 cases of primary biliary cirrhosis (PBC) demonstrated inhibition to liver-specific protein (Miller et al., 1972). Subsequent studies confirmed these findings (Meyer Zum Busch-

enfelde et al., 1975; Lee et al., 1975). Cell-mediated immunity was also demonstrated by lymphocyte transformation in patients with CAH or PBC, when either whole liver was used or liver-specific protein was used (Tobias et al., 1967; Warnatz, 1969; Thestrup-Pedersen et al., 1976).

These investigators provide evidence to show that autoimmunity is directed to liver cells. For additional support of the suggestion, in vitro cytotoxicity experiments were utilized in which peripheral blood lymphocytes brought in contact with appropriate target cells. Thomson et al. (1974), using cultured rabbit hepatocytes as targets, found significant cytotoxicity in 90% of 22 patients with CAH. In addition, they showed that the cytotoxic effects could be abolished when small quantities of human liver-specific protein was added to the culture, but was unaffected when similar quantities of a different macromolecular protein prepared from kidney in an identical manner was added. These results strongly show that the cytotoxicity reaction is specific for hepatocyte membrane protein. Autochthonous liver cells from biopsy specimens were used as target cells in experiments by Paronetto and Vernace (1975), who reported positive cytotoxicity in various types of chronic hepatitis. Additional investigators assaying cytotoxicity by chromium release from cultured Chang liver cells and autochthonous liver cells also reported positive reactions in CAH patients (Wands and Isselbacher, 1975; Geubel et al., 1976).

The above findings provide impressive evidence for the presence of damaging autoimmunity in chronic active hepatitis. However, it is not yet known which populations of lymphocytes are primarily responsible for this cytotoxic reaction. At least one group of investigators has suggested the involvement of K cells in liver cell cytotoxicity as they showed that the removal of T cells by rosette formation with sheep erythrocytes did not affect cytotoxicity, suggesting that the cytotoxicity is not T cell-dependent (Cochrane et al., 1976). The etiology and pathogenesis of CAH is still very uncertain. One recent investigation has also implicated the defect in immunoregulation, as they found decreases in suppressor cell activity in chronic active hepatitis patients (Hodgson et al., 1978). It is probable that the autoimmune response is secondary to antigen alteration, either viral or drug-induced, perhaps in genetically predisposed individuals.

Primary biliary cirrhosis patients also show cell-mediated immune responses to certain liver antigens. Histological features for PBC included a lymphoid accumulation and granuloma formation in portal tracts, sometimes with germinal centers, but fewer plasma

cells than CAH. The most characteristic immunological feature of PBC, however, is autoantibodies to mitochondria. Very little is actually known about the cell-mediated immune response in connection with primary biliary cirrhosis. One investigator, however, did find a general cell-mediated immune depression in relationship to these patients (Fox et al., 1973).

7. Summary

In summary, the cellular arm of the lymphoid system influences autoimmune responses at different levels. Firstly, T cells are involved in recognition of autoantigens and maintainence of the homeostatic mechanisms of self-tolerance. Secondly, the cells of CMI responses may be inducers of autoimmune responses owing to the breakdown of homeostasis brought on by genetic, hormonal or other environmental factors (i.e. drugs or viral infections). Finally, effector cells, alone and in combination with other immune elements, may be responsible for pathological lesions incurred during autoimmune responses either as a primary or a secondary event.

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Chapter 7

Cell-Mediated Immunity in Tumor Rejection

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It is now almost axiomatic that host immune reactivity is an important factor in resistance to tumors, regardless of the tumor initiating agent or event. Although much effort was initially expended concerning humoral immune mechanism in malignancy, many studies over the last decade or so have focused attention mainly on cell-mediated immune (CMI) interactions of the host with tumors. It is, however, quite apparent that such CMI reactions are highly complex phenomena. For example, it is now widely accepted that tumors may adversely affect the immune response mechanism not only to tumor-associated antigens *per se*, but also to unrelated antigens to which the host normally would be expected to have an effective immune response. Furthermore, cellular immunity is thought to be a major contributor to either progression or rejection of tumor cell growth. For example, the state of "normal" host immunity and the ability to control or prevent growth of tumors is believed to be under the regulatory control of cells involved in CMI. Among these are regulatory T lymphocytes, either helper or suppressor cells. The balance between these two

activities, either manifested by the same cell or by different cell lineages, may determine the fate of the host upon tumor challenge, either natural or experimental, in regard to progression or rejection of the malignancy. Enhancing the balance in the direction of helper-effector activity is thought to lead to tumor rejection and heightened anti-tumor immunity. Conversely, an imbalance in favor of suppressor activity is thought to lead to tumor progression. This aspect of tumor immunity, i.e., the role of cell-mediated immunity in tumor progression or regression, has been examined in great detail in many laboratories and in many reviews and reports. In this present review, an attempt will be made to examine the mechanisms of antitumor immunity from the viewpoint of "natural immunosurveillance" to specific immunity against established tumor cells. Also, the mechanism by which tumors escape the effects of host immunity will be examined. A number of tumor model systems will be discussed as examples in order to review several mechanisms whereby tumors may depress immune functions. Finally, a brief discussion concerning the current status of immunotherapy will be presented as an indicator of how CMI may be effectively manipulated in controlling and/or prohibiting established tumors.

1. Immune Surveillance— A Theory Under Scrutiny

The term "immune surveillance" was popularized by Sir MacFarlane Burnet (1970a,b; 1971), who formulated a general theory expanding upon Louis Thomas' suggestion (1959) that lymphoid cells of the immune system can recognize altered self cells that arise as a result of somatic mutation and destroy these cells before detectable neoplasia develops. Although some dispute the concept of surveillance as a general immunologic principle (Prehn, 1970; Andrews, 1974), it now appears well-accepted that a first line of defense against malignancy may involve some aspect of lymphoid cell-mediated immuno-surveillance. However, such surveillance is no longer considered a general phenomenon, but one that serves merely to involve specific reactions under well-defined situations. This changing concept occurred mainly because of experimental evidence indicating that, under certain well-defned situations, no antitumor immune response could be attributed to such a surveillance mechanism (Andrews, 1974; Lappe, 1971; Slemmer, 1972; Stutman, 1975). However, immune surveillance may be a relevant

phenomenon on those occasions when somatic mutations occur and upon interference of host defenses to tumor formation. However, other yet undefined mechanisms appear more likely to contribute to the general system whereby the host prevents or controls tumor cell development.

The ability of tumor cells to "escape" the postulated immunosurveillance mechanisms is not clearly defined, although several factors may be involved in such escape, some tumor initiated and others related to the host *per se* (Seigel, 1978). Expression of tumor antigens appears to be closely associated with the possible process of escape from the host immune defense system (Prehn, 1964, 1972). This may be related to (a) weakly antigenic tumors, either because of a lack of "foreign" antigens on the cell surface or because such antigens are masked or covered by other substances, e.g., sialic acid capsules, etc.; (b) the host may be tolerant to these antigens; (c) tumor cells may replicate too rapidly to be detected and/or effectively destroyed by host cells; or (d) an immune response associated with noncytotoxic antibody may develop and prevent or inhibit destruction of the cells bearing tumor-associated antigens. An additional important consideration is the possibility that suppression of immune responsiveness may result from the tumor *per se* or tumor-induced host suppressor cells perpetuating tumor growth. This aspect of interference with anti-tumor immunity will be discussed in detail below.

2. Tumor Antigens

Antigens to which the host might respond are varied, depending mostly on the etiology of the malignancy, such as the transforming or tumor-inducing agent. For example, virus-induced tumors express tumor-specific antigens that are related or identical in all tumors induced by the particular virus (Hellström and Sjögren, 1967; Klein et al., 1968). In contrast, chemical carcinogen-induced tumor-associated or -specific antigens are distinctive for each tumor induced and differ from animal to animal even when induced by the same carcinogen (Benacerraf and Unanue, 1979). One feature common to tumor antigens is that chemically they are proteins or glycoproteins. Expression of new antigens may also occur as a result of poor cellular differentiation or dedifferentiation. Many of these neoantigens either cross-react or are identical with embryonic tissue antigens (Ristow and McKhann, 1977). Two such fetal or neonatal antigens, carcinoembryonic antigen (CEA) and alpha-

fetoprotein (AFP), have been extensively studied (Abelev, 1974; Alpert et al., 1974; Terry et al., 1974). Tumor-specific or -associated transplantation antigens are expressed on the tumor cells and generally are thought to induce immunorejection in allogenic individuals by stimulating appropriate effector lymphocytes (Herberman, 1973).

Isolation and detection of tumor-associated antigens has been performed by methodologies generally adapted from the area of transplantation immunology (Ristow and McKhann, 1977). Extracted tumor antigens have been demonstrated to be reactive in assays as diverse as those based upon stimulation of tumor rejection, delayed cutaneous hypersensitivity reactions, lymphoblast stimulation reactions, cell migration inhibition, and cellular cytotoxicity; all of these are expressions of CMI (Ristow and McKhann, 1970). Such tumor-associated antigens also have been widely utilized to assess humoral immunity as determined by immunofluorescence, complement-fixation, immunoelectron-microscopy, complement-mediated and antibody-dependent cellular cytotoxicity, etc. (Ristow and McKhann, 1977).

3. Effector Mechanisms in Cell-Mediated Immunity

Control of neoplastic growth, either *in situ* (i.e., surveillance) or in response to established tumor masses, is thought to require a cytolytic interaction of appropriate lymphoid cells with the tumor cells (Green et al., 1977). Thus, cells of the immune system, either sensitized to the tumor or nonspecifically activated, are considered necessary to inhibit or kill tumor cells. At least four distinct systems of killer cell activity have been described. These include T lymphocytes, macrophages, natural killer (NK) cells, and antibody dependent "null" cells. Cytotoxic T lymphocytes are thought to be similar for both allograft rejection and tumor cell killing, except that killer lymphocytes in mice express the marker antigens Ly 1, 2, 3, whereas alloreactive cells are thought to express only antigens Ly 2, 3 (Benaceraff and Unanue, 1979; Green and Henney, 1981). Such cells are generated after initial contact with tumor antigens. However, the precise antigenic determinants which stimulate these cells have not yet been purified or analyzed in detail. Brunner et al. (1968) and Cerotinni and Brunner (1974) have provided much information depicting the mechanism of T cell-mediated killing. The cytotoxic destruction of tumor cells appears to be based on a "one hit" mechanism with one lymphocyte required to contact and kill

one target (i.e., tumor) cell. The effector killer cell is thought to be able to move from one cell to another, resulting in the killing of several cells, generally similar to the mechanism first postulated for "hemolytic" antibody in which one molecule of antibody, after resulting in the lysis of one target erythrocyte, "moves on" to another sensitized target cell in a sequential manner. The nature of lymphocyte receptors involved in triggering this type of killing remains undetermined and mirrors the lack of knowledge concerning the biochemical nature of the antigenic determinants that stimulate such lymphocytes (Golstein and Smith, 1977).

Macrophages have been recognized as a primary cell class important in host defenses since the early part of the century (Metchinkoff, 1907). However, the role of these cells as effectors in tumor cell killing has only recently been widely accepted (Evans and Alexander, 1970, 1972; Hibbs, 1973). Macrophages that are nonspecifically activated by bacteria (Alexander and Evans, 1971; Hibbs, 1974a; Likhite, 1974), parasites (Hibbs et al., 1971, 1972a,b; Krahenbuhl et al., 1973) or other stimulatory agents, including tumors *per se* (Keller, 1974; McIvor and Weiser, 1971) are thought to kill a broad spectrum of tumors. It is important to note that normal tissue cells in culture are thought to be resistant to killing by such activated macrophages (Hibbs, 1972). Resident monocytes or macrophages from unstimulated hosts are generally not considered effective target cell killers unless extremely large numbers of killer to target cell ratios are used. The manner by which macrophages recognize tumor cells as well as the mechanisms whereby they effect the destruction of tumor cells is not fully understood (Taffel and Russell, 1981). For example, the mechanism(s) of tumor cell killing may owe to direct cell contact, or release of soluble or particulate substances from the cells. For example, Hibbs (1974b) reported that macrophages can inject lysosomal enzymes directly into tumor cells and this ultimately leads to cell death. Phagocytosis of intact tumor cells, however, does not appear to be involved in such a killing process (Hibbs, 1974b).

Natural killer (NK) cells are the most recently described population of tumoricidal cells. Such NK cells are thought to be present even in the absence of a known exposure to tumor antigen and are capable of killing tumor cells in vitro. NK cells were initially described in young rats and shown to destroy syngeneic tumor cells (Benacerraf and Unanue, 1979). Subsequently mouse NK cells were identified and most studies performed to date use mouse model systems (Herberman et al., 1973). NK cells of unimmunized mice have been shown to lyse many tumor cell lines (Herberman, et al.,

1975; Kiessling et al., 1975; Kumar et al., 1979). Characterization studies have been generally inconclusive. At present the general consensus is that these cells do not exhibit any T cell markers and are not affected by anti-Ig serum plus complement. However, based on sedimentation velocity studies, these cells can be differentiated from macrophages and from cells involved in antibody-dependent cellular cytotoxicity (Benacerraf and Unanue, 1979; Herberman and Holden, 1979). Some NK cells (for example, anti-Yac-1 lymphoma reactive cells) have been reported to be bone marrow-dependent (Kiessling et al., 1977; Haller and Wigzell, 1977), although Kumar et al., (1979) reported a system (anti-EL-4 lymphoma NK cells) that does not involve bone marrow dependency. Those authors concluded that NK cells recognized a hybrid or hemopoietic histocompatability antigen (Hh-1) on lymphoma cells. Stutman et al. (1978) described killer cell activity for a methylcholanthrene-induced tumor that he designated a natural cytotoxic (NC) cell. This may be identical to the NK cell class, but a comparison of the cell type has not yet been reported. A close association of NK and K cells has also been established (Timonen et al., 1981).

Null or K cells constitute the fourth general cell type thought to be involved in tumor cell killing (Cerottini and Brunner, 1977; Kondo et al., 1981). These cells constitute an uncharacterized lymphoid cell class. They are unlike other effector cells described above since they depended upon antibody for mediation of cell lysis. Such ADCC is usually demonstrated in nontumor cell systems, but has been shown to be important in the latter stages of murine tumor virus infections (Haskill and Fett, 1976; Shin et al., 1975). The active cell involved in such reactions is characterized by the presence of Fc receptors, but no other lymphoid cell markers are present (Melewicz et al., 1977). Because of the requirement of specific antibody, B cells are also considered involved in this form of antitumor immunity (Bubenik et al., 1970).

4. Effector Molecules

Both lymphocytes (Gateley et al., 1975, 1976; Lawrence and Landy, 1969; Lee and Lucas, 1977; Mayer et al., 1979) and macrophages (Pincus, 1967) are now known to secrete soluble substances that have many activities, including antitumor abilities. These lymphokines or monokines now number several dozen "factors" and it certainly seems likely that factors described in various laboratories may be identical. Two general groups of factors, lymphocyte-

produced lymphotoxins and macrophage-derived cytotoxins, have been directly demonstrated to inhibit or kill tumor cells in vitro (Gateley et al., 1975, 1976; Pincus, 1967). Although these substances have been repeatedly demonstrated to be cytotoxic in vitro, their role in vivo is uncertain. Other lymphokines/monokines that alter immunocellular functions are also involved in modifying antitumor responses in vitro (David, 1971; Friedman, 1979; Lawrence and Landy, 1969).

Recently, interferon (IF) has been indicated as an antineoplastic agent (Strander, 1977). IF is a product of immune lymphocytes and thus some interferons may be classified as lymphokines (Baron, 1979). The mechanism of action of IF is poorly understood with regard to antitumor activity. Suggested mechanisms include a direct inhibition of tumor growth by IF or a modulation of immune reponsiveness to the tumor. More recently, interferon has been demonstrated to stimulate NK cell activity against tumor cells (Trinchieri and Santoli, 1977) and this could be the mechanism of its antineoplastic activity.

5. Depression of Immunity by Tumors: Suppressor Cells and Factors

The concept of suppressor cells as regulators of immune function was originally proposed in 1970 by Gershon and Kondo (1971). Subsequent studies from many laboratories have implicated T lymphocytes as suppressor cells in tumor-induced immunosuppression (Benacerraf, 1978; Janeway, 1978; Jerrells et al., 1978). Additionally, other host cells, including macrophages (Broder et al., 1975; Elgart and Farrar, 1978; Kolb et al., 1977; Krakauer et al., 1977) and possibly B lymphocytes (Eggars and Wunderlich, 1975; Gorczynski, 1974; Kilburn et al., 1974) as well as tumor cells *per se* (Cimprich et al., 1978a,b; Kamo et al., 1976; Katzmann, 1978), have been reported to induce immune suppression in tumor-bearing individuals. More recently, soluble factors derived from tumors or tumor-induced host cells have been reported to be similarly responsible for immunosuppression (Kamo and Friedman, 1978; Katzmann, 1978; Mohagheghpour et al., 1979; Rao and Bonavida, 1976).

5.1. Suppressor T Cells

Trainin and coworkers were among the earliest to describe tumor-induced suppressor lymphocytes (Small and Trainin, 1976; Umiel and Trainin, 1974). Using spontaneous lung carcinoma in mice as

the model system, Umiel and Trainin (1974) reported that co-transfer of tumor cells and splenocytes (or thymocytes) from tumor-bearing mice enhanced the incidence of tumor formation in recipients. Further studies indicated that thymus-derived cells were responsible for this tumor-promoting activity and that normal adult thymocytes were capable, although to a lesser degree, of similar activity. Adult thymectomy prior to tumor challenge resulted in decreased tumor incidence and growth rate. Subsequent studies with chemically induced tumors in mice showed that spleen and thymus cell populations sensitized to tumor antigens in vitro contained both cytotoxic and suppressor cells (Small and Trainin, 1976). Since cytotoxic cells could be generated, as well as suppressor cells, failure of an antitumor immune response could owe to a T cell-induced suppression of effector activity rather than a central failure of an immune response. Further evidence supporting the simultaneous development of cytotoxic and suppressor T lymphocytes was presented by Takei et al. in mastocytoma-bearing mice (1977). Suppressor cells were shown to be anti-Ig or carbonyl iron-insensitive, but were anti-theta sensitive. Additional studies demonstrating suppressor T cells have recently been reported in tumor-bearing mice and guinea pigs (Berczy and Sehon, 1979; Fujimoto et al., 1976; Schaaf-LaFontaine, 1978). For example, Fujimoto et al. (1976) reported that the T cell population probably homes to the recipient spleen. Aside from having a negative effect on the development of antitumor immunity, these cells reportedly can also repress nontumor-related cellular-related immune responses (Janeway, 1978).

5.2. Suppressor B Lymphocytes

B lymphocytes were initially implicated in suppression of antitumor immunity by Gorcyznski (1974) in Molony sarcoma-bearing mice. Anti-Ig bearing cells (presumably B cells) were responsible for reduced PHA responsiveness associated with progression of tumors. Those studies were further supported by Kilburn et al. (1974), who reported similar findings in Moloney sarcoma, polyoma, and chemically induced tumors. Eggars and Wunderlich (1975) furthered these studies by demonstrating that cytotoxicity toward allogeneic tumor targets was suppressed in methylcholanthrene-induced tumor-bearing mice. The suppressor cell was not anti-theta serum-sensitive, but was nylon wool-adherent, indicating it may have been a B lymphocyte. However, macrophages were not eliminated as a possible suppressor. The most solid evidence sup-

porting the existence of suppressive B cells was presented by Rudczynski and Mortensen (1978) in a mouse mammary tumor model. Nylon wool-adherent cells from these mice depressed the concanavalin A responsiveness of normal syngeneic lymphocytes. These cells were anti-IgG-sensitive and were unaffected by carbonyl iron or plastic adherence, indicating that they were not macrophages and most likely were B cells. The B lymphocyte's product, i.e., antibodies, may also have a role in the control of immunoresponsiveness in neoplasia. "Enhancing antibodies" are well-known for enhancing tumor or allograft survival (Hellström and Hellström, 1970; Takasugi and Hildeman, 1969), while tumor antigen–antibody complexes are also reported to suppress tumor-specific responses in animals and humans (Hellström et al., 1973; Sjögren et al., 1971; Tamerius et al., 1976). Although the mechanism by which these interactions prevent or diminish cellular immunity is not clearly defined, it is believed that such antibody complexes block interactions between tumor antigens on the tumor cell surface and cytotoxic lymphocytes.

5.3. Suppressor Macrophages

The role of macrophages as the "scavanger" of the body has lately been overshadowed by the demonstration of more significant activities with regard to antitumor as well as antibacterial immunity. As discussed earlier, these cells participate in tumor cell cytotoxicity and more recently have been demonstrated to contribute to the regulation of specific immune responsiveness. Kirchner et al. (1974) indicated that splenic macrophages from mice with tumors induced by Moloney sarcoma virus were capable of suppressing anti-sheep erythrocyte responses in vitro as well as depressing the PHA blastogenic response. Several studies with human cancer patients indicated that suppressor macrophages are found in individuals with depressed cellular immunity (Berlinger et al., 1976; Quan and Burtin, 1978; Zembala et al., 1977). When macrophages were removed from samples of peripheral blood of these patients, their T lymphocytes responded normally in vitro to PHA or con A, suggesting the presence of suppressor cells. Additionally, when these macrophages were mixed with normal peripheral blood lymphocytes, they depressed their ability to respond to mitogenic stimulation in vitro. The role of suppressor macrophages has been further substantiated in rats (Oehler et al., 1977) bearing Gross virus-induced lymphomas and in mice (Pope et al., 1976), MCA-induced sarcoma and spontaneous mammary adenocarcinomas

where suppression owed to cells lacking Ig or theta surface markers, but were plastic or nylon wool-adherent, as well as capable of phagocytizing carbonyl iron. Thus, suppressive macrophages *per se* appeared to be responsible for cellular immune depression in many tumor systems. These suppressor macrophages, however, did not appear to be exclusive to tumor systems, since normal spleens have been demonstrated to contain similar suppressive macrophages, although in lower numbers and with less activity.

The preceding discussion was not intended to imply that tumor-induced suppression of immunity arises exclusively from the expression of only one cell type or another. The interactions among the cells of the immune system are highly complex in terms of maintaining immunologic homeostasis, and there are many indications that multiple interactions occur during tumor-induced suppression (Broder et al., 1978; Ninnemann, 1978; Pope et al., 1978). In mice bearing murine plasmacytomas, the presence of suppressive macrophages has been demonstrated by several groups (Kolb et al., 1977; Katzmann, 1978). However, recent reports stressed that tumor-produced factors as well as macrophages and their products may be involved in depressed antibody responses to sheep erythrocytes. For example, Specter and Friedman (1976) demonstrated that plasmacytoma cells *per se* could depress in vitro antibody formation, as occurred with spleen cells from the tumor-bearing mice. Friedman et al. (1976) indicated further that substances in the serum or ascites fluids from plasmacytoma bearing mice also were immunosuppressive. Concurrently Zolla-Pazner and coworkers reported a soluble suppressive factor in plasmacytoma-bearing mice (Tanapatchaiyapong and Zolla, 1974). Subsequently this group, as well as several others, demonstrated that macrophages and tumor cells *per se* were immunosuppressive (Kolb et al., 1977; Katzmann, 1978; Zolla-Pazner et al., 1976). Thus macrophage- and tumor cell-produced soluble factors appeared responsible for immunosuppression in various degrees.

5.4. Tumor-Induced Suppression without Suppressor Cells

Three tumor systems investigated by the authors and co-workers have been demonstrated to result in suppression of cellular and/or humoral immune responses in the absence of any detectable host-generated suppressor cells. In all three systems the mechanism(s) of immune depression appears to be different as follows:

5.4.1. Virus-Induced Immunosuppression Friend leukemia virus (FLV) induces impairment of antibody formation to sheep erythrocytes as measured by depressed hemagglutinin titer and plaque forming cell (PFC) responses (Specter and Friedman, 1978), as well as suppressed antibacterial responses (Hirano et al., 1969). Virus-infected mice also expressed reduced cell-mediated immunity as measured by allograft survival prolongation, reduced lymphocyte blast transformation, and abrogation of migration inhibition (Specter and Friedman, 1978). Early events in antibody formation appeared most affected by FLV infection as assessed by cell transfer studies (Ceglowski and Friedman, 1969). Studies of in vitro antibody formation indicated that virus *per se* was responsible for reduction in the anti-erythrocyte PFC responses (Specter et al., 1976). Supernatant fluids of FLV infected spleen cell homogenates containing viruses were not suppressive if they were first neutralized with anti-FLV specific antisera, heated at 80°C for 10 min or passed through 300,000 molecular weight exclusion filters (Kateley et al., 1974). Passage of this virus suspension through an 0.45 diameter pore size filter did not remove immunosuppressive activity (Kateley et al., 1974). Additionally, supernatant fluids centrifuged at 100,000g to remove the virus were no longer suppressive of the PFC response (Specter, unpublished observations). Cellular debris from tumor cell homogenates could block suppression of intact splenocytes by cell-free virus preparations, indicating that availability of lymphoid cell receptors may be involved in virus activity that leads to suppression. By immunofluorescent antibody studies it was determined that the number of surface immunoglobulin (sIg) bearing cells in the spleen decreased as FLV-induced disease progressed (Bendinelli and Friedman, 1978). Studies with sIg class specific antisera indicated that IgG bearing cells were selectively affected by FLV infection (Specter, unpublished observations). These data may be interpreted to mean that the numbers of virus receptors on IgG-bearing cells were greater or that the effects might be on T lymphocytes, thus preventing the switch from IgM to IgG expression on spleen cell surfaces. Unlike suppression of PFC responses, depressed CMI in FLV infection could be associated with a soluble factor. Ceglowski and coworkers (unpublished observations) reported that 100,000g cell-free supernatants from FLV-infected spleen cell homogenates were capable of suppressing migration inhibitory factor production in vitro. Thus a soluble factor (cellular source unidentified) could be involved in CMI suppression, indicating multiple mechanisms of immunosuppression associated with FLV.

5.4.2. Immunosuppression Requiring Direct Cell Contact C57Bl/6 mice genetically resistant to FLV are susceptible to the FBL-3 lymphoma that was originally induced in this mouse strain. Although virus is not shed by this tumor, the virus can be induced to a mature form using X-irradiation and injection into susceptible newborn Balb/c mice (McCoy et al., 1967). This tumor strongly suppresses the PFC response to sheep RBCs in vitro and in vivo (Cimprich et al., 1978a,b). However, this tumor is only suppressive when intact, viable cells are in direct contact with responder splenocytes (Cimprich et al., 1978a). Homogenizing, heating, treating tumor cells with mitomycin C, or separation of responder lymphocytes from tumor cells by cell impermeable membranes all abrogated suppressive activity. The observations that co-cultivation of tumor and spleen cells separated by a molecular filter did not lead to suppressive activity strongly suggests that the decreased PFC response was not a result of nutrient depletion but of the lack of cell contact. Furthermore, in vitro addition of tumor cells as late as 72 h post-culture initiation resulted in suppression. Thus, unlike direct FLV suppression in susceptible mice, FBL-3 tumor cell-induced immunosuppression did not appear to affect early events in antibody formation.

5.4.3. Tumor-Produced Suppressive Factors Mastocytoma P815X2 is markedly immunosuppressive in DBA/2 mice. (Kamo et al., 1976). This tumor cell line is capable of suppressing anti-SRBC PFC responses, as well as delayed hypersensitivity, as measured by ear swelling using dinitrofluorobenzene sensitized mice (Kamo et al., 1976). Serum and ascites fluid from these mice yielded an immunosuppressive factor, although no suppressor cells were evident (Kamo and Friedman, 1978; Kamo et al., 1975b). Subsequent in vitro studies with tumor cell cultures yielded a similar suppressive factor(s). Initial studies with animal fluids and tissue culture supernatants suggested a factor having a molecular weight of approximately 25,000 daltons responsible for this activity. However, when tumor cells were cultured in the absence of serum, a peptide of approximately 2000–4000 mw was isolated (Kamo and Friedman, 1977). Additionally, when the ascites fluid was exposed to acid hydrolysis, it too yielded an active small molecular weight suppressive factor, thus explaining its association with larger substances in vivo. Apparently this substance complexed with serum proteins quite readily. Exposure to various enzymes indicated that both materials were peptides (Watanabe et al., 1978). This factor appeared to be very similar to the one described by Cooperband and

coworkers in human serum (1976). That factor has been named "Immunoregulatory Alphaglobulin" or IRA, and has a molecular weight of approximately 4000. IRA is present in normal serum of individuals (Cooperband et al., 1976), but tumor-bearing patients often show increased levels.

As stated earlier, plasmacytomas have been reported to produce a soluble factor that can depress humoral immunoresponsiveness. However, this factor does not appear to act directly on responding lymphocytes, but rather induces macrophages to also produce a soluble factor that has been named "plasmacytoma-induced macrophage substance" or "PIMS" that is the actual suppressive factor (Kennard and Zolla-Pazner, 1980). The mechanism of action of both of these factors remains to be delineated.

Specific factors associated with blockade of antitumor cytotoxic activity have been described by several investigators. Baldwin et al. (1972) demonstrated that sera from mice with chemically induced hepatomas blocked antitumor immunity. Subsequent studies have indicated that the blocking activity could be associated with antigen–antibody complexes (Morgan et al., 1978; Sjögren et al., 1971; Tamerius et al., 1976). Morgan et al. (1978) suggested that this complex-induced depression of cellular cytotoxicity could result from tolerance to tumor antigen *per se*. Certainly, one must conclude that the mechanisms of immunosuppression associated with the lack of tumor rejection are diverse and often complex. The vast spectrum of activities is indicative of the difficult task of inducing the immune system to recognize and reject tumors. These diverse immunosuppressive effects associated with tumors are, in part, an explanation to the many approaches developed to stimulate immunoresponsiveness as a possible therapeutic approach to tumor rejection.

6. Immunotherapy

Two major approaches to immunotherapy are generally emphasized: (a) specific, whereby tumor antigen is used to stimulate host immunity, and (b) nonspecific, which employs adjuvant substances capable of activating several compartments of the immune system. Specific immunotherapy is dependent on stimulating host immunity so that the tumor is more readily recognized by the host and therefore rejected. This has been performed in a variety of ways using autochthonous tumors and exposing host lymphocytes in vitro to the tumor and then injecting the cells back into the host

(Benjamini et al., 1976; Currie, 1972). Another approach has been to treat tumor cells with enzymes to alter cell surface components and/or to further expose potential antigens. Vaccines of *Vibrio cholerae* neuraminidase-treated tumor cells have been successfully used in animal models to induce tumor regression (Bekesi et al., 1976; Simmons et al., 1972). Although some of these experiments have been successful in animal models, there are very few successful reports of specific immunotherapy of this type in humans. Much of the failure of these trials may be attributed to the meager understanding of the immune response required for human tumor rejection in vivo and how the presence of tumors limits this immunity.

Nonspecific immunotherapy has achieved more widespread popularity in experimental models than clinical trials (Leventhal and Konior, 1977). The most popular material used has been Bacillus-Calmette-Guerin (BCG), a bovine tuberculosis strain. This material stimulates virtually all aspects of immune responsiveness and has been used with some success in melanoma (Morton et al., 1974), leukemia (Mathé, 1969) and epidermal tumors (Klein, 1973), yet overall statistics have indicated that these treatments are not as effective as expected (Carter, 1976; Leventhal and Konior, 1977). Other bacterial substances have been utilized with similar results, e.g., some positive effects in a few studies, but no generally successful treatments when the results were evaluated in toto.

It is anticipated that for successful immunotherapy to be achieved, a number of specific factors will have to be elucidated. The most obvious is a better understanding of host–tumor interactions. If the effects of tumors on immunoresponsiveness in humans are more clearly delineated, then appropriate manipulation of various relevant cell populations (e.g., removing suppressor cells or selectively stimulating cytotoxic effector cells) may lead to effective antitumor therapy. Another approach is combination therapy using mixtures of immunotherapy such as specific and nonspecific methods or combining immunotherapy with other modalities, e.g., chemotherapy or radiation. Whether these approaches become useful depends much upon empirical trials and perseverance.

7. Conclusions

The interplay of responses of various components of the immune system, tumors, and environmental factors creates a delicate balance. Whether the tumor is "successful" in becoming established

and progressing or the host defense responses are effective in preventing or rejecting the tumor appears to depend on how that balance is tipped. Determination of what causes the direction of imbalance is thought to be the key to understanding cell mediated immunity in tumor rejection. The cellular role of antitumor immunity has consistently been considered to be most important for tumor rejection. However, a shift has occurred in focusing attention on distinct cell classes that may be important in controlling tumors. It seems likely that the balance of the host–tumor relationship may shift in favor of the tumor under the influence of specific suppressor cells that may inhibit immunoresponsiveness. More recently, attention has also shifted to an understanding of the less well-defined cell classes involved in antitumor immunity, including natural killer cells and cells involved in ADCC. Whether these cells are more important or more active in tumor prevention or rejection remains to be determined. However, the presence of populations of cells capable of killing tumor cells in the absence of immunologic sensitization fits in well with the surveillance theory of tumor prevention. Future studies concerning cellular interactions involved in immune responsiveness and tumor rejection will most certainly be enlightening with regard to host defenses and cancer. Furthermore, experimental manipulations of various cell populations by immunological means will yield information which will undoubtedly lead to more effective antitumor therapies.

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Chapter 8

Transfer Factor and Other Factors in Leukocyte Dialyzates That Affect Cell-Mediated Immunity

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1. Introduction

While studying the transfer of tuberculin sensitivity by lymphoid cells and cell extracts, Lawrence made the original and highly important observation that dialyzable substances present in leukocytes have the ability to influence the expression of delayed hypersensitivity (Lawrence, 1955; Lawrence, 1963). In particular, Lawrence noted that a component of such dialyzates could appar-

ently "transfer" specific immunologic sensitivity from a highly sensitive donor to a nonsensitive recipient. This component of the leukocyte dialyzate was called "transfer factor." It is essential to understand that the component (s) responsible for this phenomenon of immunologic transfer represents only a minor fraction of the total dialyzate. Evidence has accumulated from several laboratories that indicate that a variety of other molecules present in the total leukocyte dialyzate are capable of affecting a number of parameters of cell-mediated immunity. The existence of specific transfer factor in such a heterogeneous milieu poses a problem in the evaluation of the active component(s) and in the interpretation of studies in which the crude dialyzate is employed, since the activity of individual components is likely to be affected by the action of other components in the mixture. Nevertheless, the administration of such dialyzates has repeatedly been shown to confer immunologic reactivity upon recipients who lack demonstrable sensitivity to a given antigen. A key issue in the interpretation of these experiments in which specific immunologic markers have apparently been transferred is to determine whether authentic transfer has been achieved, or alternately whether a weak and inapparent sensitivity to antigen has simply been amplified.

There has been considerable interest in the use of crude leukocyte dialyzates in clinical situations in which cell-mediated immunity is absent or has been compromised. Examples of such applications include the primary immunodeficiencies, cancer, and certain infectious diseases. However, conclusions regarding the clinical efficacy of leukocyte dialyzates are clouded by inconsistencies in overall results. Nevertheless, it is clear that in some instances skin-test reactivities were transferred from normal recipients sensitive to a given antigen to patients lacking such sensitivity and clinical improvement occurred, but the factors leading to successful use of the material were not understood. In view of the heterogeneous nature of crude leukocyte dialyzates, it would seem likely that the effects of the active substance were being influenced by other substances in the mixture, and that the overall effectiveness of leukocyte dialyzates in these instances might be expected to vary from one person to another. Isolation and purification of the active components would clearly be of value in understanding the "specific" transfer phenomenon originally reported by Lawrence, as well as the activities of other molecules in the leukocyte dialyzates that affect the expression of cell-mediated immunity. Moreover, it is highly likely that these other substances do affect the activity of the

immunologically "specific" components when these are administered in vivo.

Several components of dialyzable leukocyte extracts can be assayed by in vitro techniques. At the present time, in the absence of purification of the components involved, it has not been possible to determine the relationship between molecules active in vitro assays and those affecting the in vivo expression of delayed hypersensitivity, in either a specific or nonspecific fashion.

2. The Transfer Phenomenon

Although the importance of cell-mediated reactions in the immune system is now widely recognized, the demonstration that delayed hypersensitivity could be transferred by cells was the result of the pioneering investigations of Chase (1965). Contact sensitivity, the tuberculin reaction, and graft rejection are now recognized as prototypes of cell-mediated immunity. Additionally, cell-mediated immunity represents a principal immune defense mechanism against a variety of microorganisms, viruses, fungi, protozoa, and parasites.

The first transfer of cell-mediated immunity was demonstrated in the guinea pig (Landsteiner and Chase, 1942). Transfer of cutaneous sensitivity to picryl chloride was achieved in the guinea pig by injecting peritoneal exudate cells from a guinea pig that had been sensitized to picryl chloride into a nonsensitized guinea pig and challenging the recipient guinea pig with picryl chloride. The recipient guinea pigs acquired skin sensitivity by virtue of such cell transfers. The same result could not be produced by transfer of serum. The acquired skin sensitivity was of limited but significant duration, and was referred to as the transfer of drug allergy. Subsequently, it is also shown that the tuberculin reaction could be transferred by peritoneal exudate cells, as well as spleen and lymph-node cells (Chase, 1945). Moreover, the intensity of tuberculin sensitivity in the recipient depended on the degree of reactivity of the donor and the number of donor cells employed.

Following these classic studies, Lawrence demonstrated that human peripheral leukocytes could transfer delayed hypersensitivity to tuberculin from sensitive donors to nonsensitive recipients (Lawrence, 1949). Such transfers were reproducible, and sensitivity persisted in the recipients for up to 2 years. Similar transfers could be achieved using diphtheria toxoid and other microbial, fungal, and viral antigens (Lawrence, 1974). Moreover, whole cells

were not an absolute requirement for such transfers; extracts of peripheral leukocytes were as effective (Lawrence, 1955). Moreover, extracts prepared from donors who were sensitive to more than one antigen could transfer multiple sensitivities to the recipient. It was also noted that such conversions occurred in the absence of the production of circulating antibody (Uhr et al., 1956).

A further advance was made by Lawrence's demonstration that only a fraction of the whole-cell extract was required. When such extracts were dialyzed, the resulting dialyzates were very effective in mediating such transfers (Lawrence, 1963).

A principal difficulty in the interpretation of the phenomenon of transfer of immunologic reactivity by extracts or dialyzates of white blood cells is the question of prior sensitivity of the recipient. Intradermal challenge with antigens as a test for delayed hypersensitivity has two major disadvantages: (a) it is relatively nonsensitive; therefore, it is possible to display no reaction to an antigen while being weakly immune to it (Dvorak et al., 1974); and (b) sensitivity to certain skin-test antigens can be stimulated by repeated antigenic challenge. This is particularly true in individuals who are weakly sensitive to the antigen but do not display skin reactivity upon initial challenge (Rapaport et al., 1960).

Owing to these considerations, it would appear to be very difficult to clearly demonstrate the transfer of "specific" reactivity to an antigen. It is equally possible that the action of leukocyte dialyzates is simply to amplify an existing weak sensitivity in a given recipient. This possibility is especially evident if one is using microbial antigens or other antigens that are widely distributed in the environment, and to which the probability of exposure is high.

One way to attempt to resolve this dilemma involves the use of individuals residing in areas endemic for the antigens. In one report, donors were selected from an area endemic for *Coccidioides immitis*; leukocyte dialyzates were prepared from such donors and administered to individuals who were lifelong residents of a geographic area free of the organism (Rapaport et al., 1960). The donors were determined to be permanent residents of endemic areas having moderate sensitivity to *Coccidioides immitis*. The recipients had not been in an area endemic for coccidioidomycosis and gave no reaction to undiluted coccidioidin, whereas the donors reacted to a 1/100 dilution. Leukocyte dialyzates were injected intradermally and subcutaneously into the skin overlying the shoulder; the challenge was performed at a site distant from that of injection. Thirteen of 16 recipients converted to a coccidioidin-positive skin test. As a control, leukocyte dialyzates were prepared from a nega-

tive donor residing in a nonendemic area. Of the recipients tested with this preparation one converted to a positive skin test for coccidioidin, and a number of other recipients of this "negative" preparation displayed an erythematous response to this antigen. No biopsies of these lesions were performed, so that it is not possible to determine whether these recipients displayed a histopathologic picture consistent with that of a delayed reaction to this antigen. It would seem particularly informative to biopsy erythematous reactions, which can represent a weak but significant delayed hypersensitivity response (Sokal, 1975).

An alternative approach is to use an antigen that is seen infrequently in nature. Ideally, synthetic antigens would be the most desirable type of "neoantigen" since prior exposure to such antigens would be highly unlikely. Constraints on human investigations make this approach difficult, if not impossible. Hence, there has been considerable interest in the use of keyhole limpet hemocyanin (KLH) as an antigen, since exposure to this antigen would seem to be limited. The use of KLH as a marker for immunologic conversion, using "transfer factor" or dialyzable leukocyte extracts, was introduced originally by Zuckerman et al. (1974). However, it is important to note that 5–10% of the population appears to react to this antigen upon intradermal challenge wthout known prior exposure. This may be the result of consumption of seafood or processed foods made from materials derived from marine sources. It would seem, therefore, that KLH may not be a true neoantigen and that one cannot use it to distinguish between specific "transfer" and amplification of prior sensitivity. Burger and coworkers have prepared leukocyte dialyzates from donors immunized with KLH and used these materials in recipients who have not been intentionally immunized with KLH. Leukocyte dialyzates prepared from the same donors prior to immunization with KLH serve as an appropriate control (Burger et al., 1977). These workers have reported that a fraction of the leukocyte dialyzate obtained from immunized donors is capable of transferring KLH sensitivity to randomly selected, nonimmunized recipients. These results remain to be confirmed and do not eliminate the possibility that the recipients used in these studies may have had unrecognized sensitivity to KLH.

It is of value to note that recent reports indicate that considerable caution must be applied in the interpretation of specific "transfers" mediated by leukocyte dialyzates. Neidhart et al. (1978) have directed attention to a high degree of false-positive skin-test conversions in such studies and, in particular, have noted that injection of saline may lead to positive reactions to ward KLH in recipi-

ents who have previously not been intentionally immunized with KLH but who presumably had acquired sensitivity to this antigen in the past.

3. Clinical Use of Leukocyte Dialyzates or Transfer-Factor Preparations

The reported ability of leukocyte dialyzates to transfer sensitivity from one individual to another led to the obvious suggestion that such materials might be useful in correcting defects in cell-mediated immunity and/or immunodeficiencies occurring in patients as a result of their disease process. Leukocyte dialyzates have several properties that make it particularly useful in these applications: (a) leukocyte dialyzates are natural products, and (b) they are cell-free preparations devoid of histocompatibility antigens and therefore, they cannot lead to graft-host reactions (Polmar, 1973) as can occur with whole cells. Moreover, the active molecules appeared to be small and presumably would be relatively simple to isolate and synthesize (Lawrence, 1963). Accordingly, leukocyte dialyzates have been employed as an immunotherapeutic maneuver in a variety of microbial and fungal infections, conditions displaying immunodeficiency (as in the Wiskott–Aldrich syndrome), and in the treatment of a variety of malignancies (Arala-Chaves et al., 1974; Fudenberg et al., 1974; Good et al., 1962; Lawrence, 1970). The most dramatic instances of the therapeutic effectiveness of leukocyte dialyzates are in patients suffering from chronic infections, such as chronic candidiasis (Lawrence, 1972; Schulkind et al., 1972; Rocklin et al., 1970; Valdimarsson, 1972), in which some patients appear to be transfer-factor dependent and in which the clinical improvement after receiving leukocyte dialyzates is dramatic.

In the application of leukocyte dialyzates to other immunodeficiency states, the results have been less consistent and may reflect the complexity of the disease process as well as the complexity of the leukocyte dialyzate preparations. It is assumed that the active components of leukocyte dialyzates act predominantly on T cells, and leukocyte dialyzates would be expected to be most helpful in deficiencies involving the T cell arm of the immune system rather than in those conditions in which deficiency is principally or exclusively in the B cell arm. However, it is important to note that leukocyte dialyzates appear to act effectively only in those cases in which the deficiency in T cell function is not absolute. These dialyzates have not been effective in athymic conditions.

The rationale for the use of leukocyte dialyzates in cancer is based on the assumption that such dialyzates can stimulate a patient's cell-mediated immune responsiveness against his own tumor. Variable success has been achieved in this regard (Oettgen et al., 1971; LoBuglio et al., 1973; Fudenberg et al., 1974; Spitler et al., 1976). Since the dialyzates are complex mixtures, it is possible that activity of stimulatory components may be masked by suppressive substances in the preparation, or that suppressor cells may be activated by other components in the mixture, and that such suppressor-cell activation may prevent the expression of immune responsiveness against the tumor. In general, the administration of leukocyte dialyzates to patients has not been associated with adverse effects, although instances of malignant or pseudomalignant lymphoproliferation have occurred following treatment with transfer factor (Graziano and Dwyer, 1978). These rare instances occurred in patients with underlying diseases that had the potential to develop into frank malignancies. However, bona fide adverse reactions have occurred in patients with leprosy who were treated with leukocyte dialyzates. In these cases, lepromatous lesions became painful and "activated" following administration of these materials. Although this presumably reflects immunologic activity in these lesions, the clinical effects are of reasonable severity and, therefore, limit enthusiasm for the use of leukocyte dialyzates as immunotherapy in leprosy.

4. Biochemical Characterization

It is clear that in order to advance the study of leukocyte dialyzates and to resolve some of the difficulties inherent in the interpretation of the clinical effectiveness of such material, purification of the active components is essential. Isolation, of course, implies that suitable assays are available to test for the components in question. In general, the only suitable assays involve in vivo administration of the materials to human volunteers and patients. Since these materials must be tested in human recipients, there are necessary constraints on the fractionation procedures that can be employed. In particular, chromatography using polyacrylamide matrices or techniques involving the introduction of ampholines should be used with great caution. Despite these constraints, highly useful information has been obtained over the past few years regarding the biochemical composition of leukocyte dialyzates. The most significant observation obtained to date is that more than one biologically

active molecule capable of affecting cell-mediated skin reactions is present in the dialyzates (Gottlieb et al., 1977; Tomar et al., 1977; Burger et al., 1977; Krohn et al., 1976).

One approach to the separation of leukocyte dialyzates is gel filtration on Sephadex G-10. The Sephadex matrix is a polydextran compound, and leukocyte components separated on these columns can be used in human recipients without unusual or special precautions. Sephadex is available in different forms, having distinct capabilities of resolving molecules of different molecular weights. Both Sephadex G-10 and G-25 can be employed to resolve compounds of less than 2000 molecular weight. It is helpful to remember, in using these materials, that elution should be carried out using a volatile buffer such as ammonium bicarbonate so that the fractions recovered from the column can be concentrated without leaving a high-salt residue. This is important, since the final material, because of its low molecular weight, cannot be dialyzed to remove salts. All materials recovered for use in human recipients should be filtered through a 0.45-μm membrane filter, tested for contamination with endotoxin by a procedure such as the limulus assay (Fine, 1973), and cultured to confirm sterility before use in human recipients. Column effluents are routinely characterized by absorption at 260 and 280 nm to detect molecules containing nucleic acids and amino acids, respectively. Increased sensitivity in measuring components bearing primary amines, including most amino acids and polypeptides, can be achieved using the fluorescamine assay (Udenfriend et al., 1972).

Since the chemical nature of active molecules is unknown, routine absorbance profiles only provide a reference. Peaks often represent a number of different molecules of similar size and require further separations to isolate active components.

4.1. Dermal Transfer Activity

Fractionation of leukocyte dialyzates has been undertaken by several laboratories for the purpose of isolating the component(s) responsible for the "transfer" of dermal sensitivity. Components of leukocyte dialyzates from appropriately sensitized donors have been administered both systemically and locally in combination with antigens to which the donor or recipient were sensitive. Several reports have appeared that describe the identification of components in the dialyzates exhibiting transfer activity. Unfortunately,

the elution characteristics of such materials seem to vary with different laboratories.

Neidhart et al. (1973) reported separation of leukocyte dialyzates on G-25 Sephadex and recovered transfer activity in a peak that eluted in a volume five-fourths that of the total volume (V_t) of the column. Using local transfer of tuberculin sensitivity to assay for transfer-factor activity, it was found that reactivity to this fraction plus partially purified tuberculin protein (PPD) was greater than reactivity to the whole dialyzate plus PPD.

Burger et al. (1977) have separated leukocyte dialyzates on Sephadex G-25, but reported different elution charateristics from that of Neidhart. The material responsible for "specific" transfer activity eluted before the total volume of the column. Transfer activity was recovered before this peak to two antigens PPD and KLH. Burger et al. and other groups have pursued further purifications of the active fractions using isoelectric focusing and high-pressure reverse-phase chromatography. Indeed, Burger et al. have proposed a tentative model for a "transfer factor" specific for KLH based on enzymatic sensitivities of a subfraction of the dialyzate prepared by high pressure liquid chromatography (Burger et al., 1979). Although a chemical structure can not be rigorously proved by enzymatic studies, the data from Burger's group are consistent with the properties one would expect an antigen-specific transfer factor to have.

More recently, Borkowsky and Lawrence (1980) have identified substances in the dialyzate that are able to complex with antigen bound to PVC plates. The material which associated with antigen is recovered by treatment with 8*M* urea and is able to affect a leukocyte migration inhibition (LMI) assay in an antigen-specific fashion. These authors have claimed that this assay measures "transfer factor" activity, though it is well to note that the material recovered in this way, which is active in the LMI system, has not yet been shown to have "transfer factor" activity in vivo.

The work of investigators who have attempted to isolate the "specific" transfer components has led to the identification of several other components in the dialyzate that affect delayed hypersensitivity, in addition to the component mediating "specific" transfer (Vandenbark et al., 1977, Krohn et al., 1976; Gottlieb et al., 1977). These activities may influence the activity of "specific" transfer factor, and indicate that other substances in leukocyte dialyzates may be important mediators or regulators of cellular immunity.

4.2. Other In Vivo Activities of Fractionated Dialyzed Leukocyte Extract

In addition to the component mediating "specific" dermal transfer, other components display important effects on the expression of delayed hypersensitivity. Our laboratory has recently noted that a component present in leukocyte dialysates is capable of inducing an intradermal reaction in the *absence* of antigen, which grossly and morphologically resembles delayed hypersensitivity. The intradermal reaction to this compound appears as soon as 3 h lowing injection, and the reaction is maximal at 16–18 h. Another component of the dialyzate distinct from this antigen-independent mediator or inducer of delayed-type responses, is capable of amplifying a reaction of intradermal delayed hypersensitivity to antigens to which the recipient has been previously sensitized (Gottlieb et al., 1980). From these studies, it is clear that latent sensitivities can be expressed following systemic injection of leukocyte dialyzates. Moreover, it is of interest that both the "amplifier" and the antigen-independent mediator are capable of displaying these reactivities when given back to individuals who donated the leukocytes.

It is likely that these other substances contribute to the overall "transfer-factor" phenomenon by amplifying and/or otherwise modulating the functional expression of the "specific" dermal transfer component. It would seem highly important to determine how these components operate individually and collectively in vivo in mediating or otherwise influencing the expression of delayed hypersensitivity.

4.3. In Vitro Activities

A number of other components of leukocyte dialyzates have been shown to have a variety of effects on in vitro parameters of cell-mediated immunity. Although some of these effects are clearly defined, the relationship of any one of these activities to mediators of dermal transfer remains to be determined (Ascher et al., 1974; Burger et al., 1976a; Andron and Ascher, 1978). Leukocyte dialyzates have been shown to contain materials that augment antigen-triggered proliferation of human lymphocytes in vitro (Ascher et al., 1974). In contrast, specific transfer of new immunologic sensitivities to naive lymphocytes has not been consistently demonstrated in vitro. Indeed, the magnitude of augmentation observed in vitro has been shown to be determined by the state of sensitivity of the donor (Ascher et al., 1974; Burger et al., 1977). Other studies have

shown that leukocyte dialyzates contain other components that suppress or modify the uptake of ^{3}H-thymidine by mitogen- and antigen-stimulated cells (Saito et al., 1977; Burger et al., 1976b). In general, it appears that stimulation of lymphocyte function by leukocyte dialyzates, as monitored by the uptake of ^{3}H-thymidine, is a result of components in the mixture that act nonspecifically (Hamblin et al., 1976a, b; Ascher and Andron, 1976).

There have also been reports describing the effects of dialyzable leukocyte extracts on the formation of E rosettes. A component in leukocyte dialyzates appears to increase the number or the arrangement of sheep-cell receptors on T cells (Holzman and Lawrence, 1977). Some studies report that the number of active rosette cells is correlated with immune competence (Wybran et al., 1973; Horowitz et al., 1975).

Chemotactic activities are also present in the bulk leukocyte dialyzate and can be partially purified by gel filtration. The relationship between the chemotactic activities and components mediating skin sensitivity also remains to be determined (Gallin and Kirkpatrick, 1974).

5. Experimental Considerations

Since the effects of leukocyte dialyzates are complex, it is helpful to summarize some considerations that are important in planning these kinds of studies.

5.1. Donor

Donors are chosen on the basis of their specific skin-test sensitivities to given antigens. Specific selection is dependent on the antigenic markers that the investigator wishes to transfer. It was noted by Lawrence, and confirmed by the general experience of those working in the field, that in order to achieve a successful transfer, the donor must display a high degree of sensitivity to antigen. For systemic transfers, dialyzates prepared from a minimum of 5×10^8 leukocytes are required. Local dermal reactions *can be achieved* with 5×10^7 to 1×10^8 cells.

5.2. Recipient

For demonstration of specific transfer by leukocyte dialyzates obtained from highly sensitive donors, recipients are generally tested

in advance to demonstrate their sensitivity or lack of sensitivity to a given antigen. Lack of skin-test reactivity to an antigen(s) is not absolute evidence of lack of sensitivity of the recipient to that antigen. This is a particularly critical point to be aware of in the interpretation of these studies, since the suggestion that leukocyte dialyzates act specifically is absolutely dependent on the demonstration that the recipients are truly nonsensitive to that antigen. Since it is difficult, if not impossible, to rigorously prove that a recipient is truly immunologically naive, the contention that leukocyte dialyzates act specifically must be tempered by this reservation. Moreover, prior skin testing with certain antigens (tetanus toxoid, streptokinase, and PPD) may result in stimulation of weak pre-existing immunity, so that repeated challenge with these antigens at a later date might yield positive reactions based on such stimulation.

In selection of patients for therapy with leukocyte dialyzates, it is generally assumed that the active components act on T lymphocytes. This, in fact, may not be completely true, and detailed studies on the effects of these several components on T cells, B cells, and macrophages are needed. It is clear, however, that patients who are completely deficient in T cells, or who are athymic at birth, are not likely to be good candidates for therapy with leukocyte dialyzates unless they are first reconstituted with thymus tissue and/or thymic cells. Evidence of some T cell functions, such as production of migration-inhibition factor (MIF) or responsiveness of peripheral blood cells to phytohemag glutinin (PHA), is a favorable indication of potential responsiveness to leukocyte dialyzate components.

The cellular and molecular basis of most immunodeficient states, particularly those involving cell-mediated immunity, are not well understood. Additional information concerning the type of defect, its location in particular subpopulations of T cells, etc., as well as a clear understanding of how the leukocyte dialyzate components interact with distinct cell subpopulations, is needed before leukocyte dialyzates or components contained therein can be used in a rational manner for immunotherapeutic purposes.

5.3. Preparation of Leukocyte Material

Blood is obtained from the donor before skin testing. Postponement of skin testing eliminates the possibility of influencing the immune status of the donor. After the blood is donated, the donors are then screened to determine their sensitivities by testing against a battery of microbial and fungal antigens to which the public is normally exposed. Blood may be kept separate for preparation of

leukocyte dialyzates from particular individuals, or pooled if a common antigenic marker is to be studied.

Dialyzable leukocyte extracts are prepared according to the method of Lawrence with minor modifications. Anticoagulated blood is collected to a volume of approximately 450 mL. Leukocytes are separated from the RBC by dextran sedimentation. The recovery of leukocytes from this amount of blood is consistently $(1–2) \times 10^9$.

Since the active components of leukocyte dialyzates have not been isolated, it is difficult to quantitate the amount of a given component in the dialyzate. Conventionally, clinicians employing leukocyte dialyzates refer to units of dialyzate, a unit being that amount of dialyzate recovered from one unit (450 mL) of whole blood.

This amount of whole blood generally contains $(1–2) \times 10^9$ leukocytes. Another convenient way to refer to the amount of a given component present in a leukocyte dialyzate is to refer to the number of cells needed to produce a given number of doses of a component. Since the chemical nature of active components are not known, it is reasonable to refer to the activity of a given component in terms of the number of units or cells required for preparation.

From the leukocytes, components having a molecular weight of less than 12,000 are initially obtained. The leukocytes are disrupted by freeze-thawing in the presence of DNAase and $MgSO_4$. The lysate is then dialyzed through tubing having a cutoff of 12,000. The dialyzate is recovered and lyophilized. It is important that dialysis be performed against a medium that will not affect ionic strength upon lyophilization. Thus, a volatile buffer or water is preferred. Other methods of obtaining materials having molecular weights of less than 12,000 have been introduced to reduce the length of processing time (Burger et al., 1974). These include the use of ultrafiltration through membranes of defined size. The material obtained by dialysis or ultrafiltration is a heterogeneous composition of molecules and activities of less than 12,000 molecular weight. Commonly referred to as the "crude" material (or "crude transfer factor), it is the preparation that has most often been used in the past for clinical and research studies. It is also the starting material for further separations.

Two general approaches are used to study the effects of leukocyte dialyzates. In the classic type of study described by Lawrence, leukocyte dialyzate prepared from one unit (450 mL) of whole blood, and representing 5×10^8 cells, is injected subcutaneously in

the upper deltoid area of the recipient's arm. Skin testing for appropriate marker antigens is then carried out within 24 h or as late as 2 weeks following administration of the dialyzate at sites distant from the site of inoculation. This type of procedure is referred to as "systemic" administration.

Alternatively, smaller amounts of dialyzate can be employed locally. Leukocyte dialyzate obtained from as few as 5×10^7 cells can be combined with antigen (selected on the basis of donor sensitivity and recipient nonsensitivity) and injected intradermally at convenient sites on the forearm or the back of a recipient. Local transfers do not generally lead to systemic conversion and permit a large number of tests to be performed in the same individual.

6. Implications for Immunotherapy

It is clear that leukocyte dialyzates contain a number of molecules having potent effects on the expression of cell-mediated immunity, both in vivo and in vitro. Although a great deal of attention has been focused over the past 25 years on the identification and isolation of components of these dialyzates that mediate "specific" transfer of immunologic reactivity from sensitive donors to nonsensitive recipients, there is concern among some investigators in the field that such "transfers" may in reality be instances of amplification of weak and inapparent sensitivities in the recipient. The identification of other components in the mixture that augment, suppress, or otherwise modify the expression of delayed hypersensitivity in vivo and in vitro provides a basis for possible use of these materials in clinical situations wherein amplification or suppression of cell-mediated immunity is desirable. Indeed, if specific transfer factors do exist, their action in vivo is almost surely influenced by these other components that act nonspecifically. From the point of view of developing new types of immunotherapeutic agents, it would in principle be simpler to manufacture and test a single agent that nonspecifically amplified reactivity to a number of antigens than to synthesize a number of individual molecules, each having a unique specificity.

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Chapter 9

Hybridization of Lymphocytes

Techniques and Applications

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1. Introduction

One of the major problems in immunology has been to raise homogeneous antibodies of high concentration that are specific for a given antigen.

When most antigens are injected into an animal the resulting antisera are complex mixtures of antibodies, reflecting the isotypic (class) allotypic, and idiotypic heterogeneity characteristic of immunoglobulins (Kabat, 1976). Even when highly purified immunogens are used for immunization the extensive variation in potential antigenic sites presented by most immunogens results in a heterogeneous collection of antibodies.

With complex mixtures of antigens, such as entire living cells, bacteria, or viruses, immunization occasionally gives an antiserum

that can be made operationally specific by intensive absorptions with a variety of selected tissues or cell types, but this procedure, besides being very time-consuming, is far from fool-proof and is frequently unsuccessful.

Thus it was a revolutionary technical advance when Köhler and Milstein (1975) first described their cell-fusion protocol for the production of permanent cell lines producing monoclonal antibodies to a predetermined antigen (Köhler and Milstein, 1975). In principle, their technique took advantage of previous somatic cell hybridization procedures in order to immortalize one antibody-secreting cell from the spleen of a mouse. Since a given antibody-secreting cell makes antibodies of entirely one specificity, it follows that once such a cell line has been established all of the progeny will make exactly the same antibodies. The big trick was to fuse the antibody-secreting cell with a tumor cell line, so that the resulting hybrid not only continued to synthesize antibody, but also displayed the neoplastic character of the tumor cell, namely, the dual features of tissue culture adaptation, and growth as a solid tumor in appropriate strains of mice. To generalize then, spleen cells from recently immunized mice serve as a source of antibody-secreting cells and are hybridized with a tissue-culture-adapted plasmacytoma cell line. The latter has especially been established as an 8-azoguanine-resistant line according to the original procedure of Littlefield (1964); as such, it lacks certain enzymes and will not grow in Littlefield's selective medium, which contains hypoxanthine, aminopterin, and thymidine (HAT medium). The spleen-cell partner of the fusion, however, dies normally in tissue-culture medium, but does contain the enzymes necessary for continued growth of the plasmacytoma in the HAT medium. Upon fusion, therefore, complementation occurs so the resulting hybridoma retains the immortal character of the plasmacytoma, and is able to grow in the HAT medium since the spleen-cell genome contributes the necessary enzymes.

In practice, of course, the first stage of a hybridization protocol realizes a heterogeneous mixture of hybrids, which simply reflects the heterogeneity of antibody-secreting cells in the donor spleen at the time of fusion. For this reason, single hybrid cells are selected and grown up, a procedure that is known as cloning and that ensures production of monoclonal antibody by the clonal progeny.

Monoclonal antibodies of any desired specificity can therefore be prepared given spleen cells from appropriately immunized mice. In the past few years there has been a marked increase in the use of this technique, and monoclonal antibodies to soluble anti-

gens, viral antigens, and cell-surface antigens have been produced. Furthermore, T cell hybrids have been obtained by fusing immunized T cells with T cell lymphomas. Although results have been less spectacularly successful with T cell hybrids, nonetheless one may confidently predict that future work along these lines will provide useful information on the biochemistry and function of T cell subpopulations.

2. B Cell Hybrids

2.1. Cell Lines

The first cell line to be successfully used by Köhler and Milstein (1975) was originally derived by Potter, who injected mineral oil into the peritoneum of a Balb/c mouse (see Potter, 1972, for review). It secreted an IgG_1 mouse myeloma protein with K light chains and was called MOPC 21; upon adaption to tissue culture by Horibata and Harris (1970) it was renamed P3X63. The next step was to prepare an 8-azoguanine-resistant mutant suitable for fusion (P3-X63-Ag8 or, for short, X63) (Köhler and Milstein, 1975) that would nonetheless continue to synthesize and secrete the IgG_1 myeloma protein. The 8-azoguanine-resistant cell line, being defective in hypoxanthine-guanine phosphoribosyl-transferase (HGPRT), will not grow in the selective HAT (hypoxanthine, aminopterin, and thymidine) medium of Littlefield (1964). The reason for this is that the folic acid analogue aminopterin blocks the biosynthesis of purines and pyrimidines. Under these conditions, the myeloma cells, being deficient in the enzyme HGPRT, are unable to substitute hypoxanthine and thymidine for nucleic acid synthesis until fusion with a lymphoid cell provides the relevant enzymes. A similar variant (MPC11-X45-6TG or, for short, X45) was derived by Scharff's group from a mouse plasmacytoma secreting IgG_{2b} with K light chains (Margulies et al., 1976). Both cell lines have been successfully used for fusion with mouse spleen cells in many laboratories, and in a direct comparison X63 and X45 gave similar yields of antibody-producing hybrids (Yelton et al., 1978).

Unfortunately, upon fusion of these cell lines with antibody-producing mouse spleen cells, an element of heterogeneity is introduced by the formation of hybrid molecules as a result of random mixing of the two heavy and light chains of both parental cells (Köhler and Schulman, 1978; Köhler et al., 1978). This problem can be reduced by using a variant of the cell line that has been selected for lack of heavy chain production. For example, the cell line

P3-NS1-1 Ag4-1 (for short, NS-1) was derived in Milstein's laboratory from X63. It no longer produces γ1 heavy chains but continues to produce light chains (Köhler et al., 1976). The latter do compete with the light chains of the spleen-cell half of the parent, and so once again some molecular heterogeneity is introduced by the fusion. The NS-1 line fuses well but this is not necessarily the case with all similarly derived variants. To illustrate this point, we cite experiments from Scharff's laboratory in which the fusing properties of subclones of an X45 line were demonstrated to vary widely (Yelton et al., 1978). In fact, a very major problem in the technology of hybridization is that a cell line chosen for its ability to hybridize well may suddenly or slowly become quite unsuitable. The exact molecular events responsible for this change have not been defined, but the practical lesson is very clear: always maintain a stock of the original cell line in the frozen state in the event of such changes occurring. Relevant to this point is that infection of the cell line by mycoplasma may drastically change its properties, and thus continuous monitoring for mycoplasma is essential (see Kenny, 1973; Barile, 1973).

Recently two cell lines that synthesize neither heavy nor light chains have been derived from X63 cells. In one case a subclone of X63 was fused with a Balb/c mouse spleen cell to yield an Ig-negative cell line (Sp2/0 Ag 14) (Shulman et al., 1978). In the other case, a variant of X63 was selected for deletion of both heavy- and light-chain synthesis (P3-X63-Ag8.653) (Kearney et al., 1979). Subject to obvious considerations, such as their efficiency in fusing with spleen cells, totally Ig-negative, HAT-sensitive cell lines of this type would appear to be the most suitable choice for the preparation of monoclonal antibodies by the hybridoma approach. Mouse myeloma cell lines can be used both for intraspecific and interspecific hybridization. For example, X63 has been fused to syngeneic and allogeneic mouse spleen cells (Köhler and Milstein, 1976; Köhler et al., 1977a) and to rat spleen cells (Galfrè et al., 1977) with similar high fusion rates. However, hybrids derived from fusion of mouse myeloma cells with rabbit, human, and frog lymphocytes occur at very low frequency and do not exhibit stable immunoglobulin secretion (Buttin et al., 1978; Hengartner et al., 1978a; Kennett et al., 1978; Köhler and Shulman, 1978). This can be accounted for by the preferential loss of non-mouse chromosomes. Interspecific hybridization also has the disadvantage that the resultant hybridomas cannot be grown easily in vivo, and then only in special strains of animals such as nude mice. Because of these restrictions, it is more convenient to fuse parental cells, which are phylogenetically

closely related. Thus the derivation of hybridomas secreting human antibodies will almost certainly have to await the production of an appropriately drug-marked human plasma cell line.

An 8-azoguanine-resistant mutant of the rat myeloma (Y3-Ag 1.2.3) has been recently selected and successfully fused with rat spleen cells (Galfrè et al., 1979). Since the amount of serum or ascitic fluid that can be obtained from tumor-bearing rats is about 20 times higher than the amount obtainable from mice, there is a very clear-cut advantage to using the rat system.

Finally, as noted above, we cannot emphasize too strongly that mutants, revertants, or spontaneous variants that may have lost the ability to fuse and to generate antibody-forming clones can arise at a high frequency (Coffino and Scharff, 1971; Margulies et al., 1976; Adetugbo et al., 1977; Yelton et al., 1978). It is advisable that the line to be used be tested for fusion in a simple system in which antibody-producing frequency is already known , e.g., anti-sheep red blood cells. It is inadvisable to keep the myeloma cells in culture for long periods of time, since under these conditions cultures may become overgrown by nonsuitable variants. A cell line that is satisfactory for fusing with immune lymphoid cells ideally should be frozen down, and working stock should be maintained for only a limited period of time in continuous culture (e.g., 3 months). When that time has elapsed, it is best to go to freezer stocks and start again from samples laid down at the time the line was received and tested.

2.2. General Hybridization Protocol

Spleen cells from immunized mice are mixed with appropriately selected HAT-sensitive myeloma cells, and fusion is prompted by the addition of Sendai virus or polyethylene glycol. Hybrids are selected for by growing the cells in HAT medium, and supernatants from cultures exhibiting growth are assayed for the presence of antibody. Cells present in positive cultures are submitted to a cloning procedure, and the resulting clones are propagated in vivo or in vitro, depending on the circumstances. The most important point to grasp is that a clone of cells secreting a desired antibody can be easily lost in the early stages prior to cloning as a result of overgrowth by other clones (clonal competition), or as a result of early chromosome loss. Consequently, the rule to observe is to clone as early as possible. This necessitates the development of rapid and sensitive procedures for the detection of antibody in culture supernatants.

Once a clone has been selected and preferably recloned, it should be frozen down. It may be grown up for production of antibody, either in vitro or in vivo, depending on the participants in the original fusion. Syngeneic mouse–mouse (e.g., Balb/c spleen cells × NS-1) or rat–rat hybridomas can be grown in mice or rats of the same inbred strain. Allogeneic mouse–mouse hybrids can often be maintained in the corresponding F1 hybrid. For example, the anti-Thy 1-2 hybrid made by the fusion of immune AKR mouse spleen cells with the HAT-sensitive Balb/c lines NS-1 grows well in sublethally irradiated Balb/c × AKF mice (Lake et al., 1979).

Xenogeneic combinations are usually kept in vitro, and the antibody must then be recovered from culture supernatants. A recently described tissue culture medium (Iscove and Melchers, 1978) is apparently very good for the growth and secretion of hybridoma cells (Andersson and Melchers, 1978). Obviously it is easier to prepare monoclonal antibody from tumors maintained in vivo rather than in vitro. A useful point is that mice previously injected with pristane are more permissive to the growth of tumor cells than normal mice (Potter et al., 1972).

2.3. Optimizing Fusion Parameters

The optimization of conditions leading to routine successful production of hybridomas specific for a given antiserum are particularly important, especially in the case of poor immunogens or antigens available only in low amounts, such as most cell-membrane antigens.

Many parameters may have to be varied before the optimal conditions for immunization and hybridization are defined. We shall examine some of them below.

2.3.1. Physiological State of the Parental Cells

We have already indicated that myeloma cells vary in their fusion frequency and, once having fused, in their capacity to support continued antibody production by the hybrid. Because of the instability of myeloma cell lines and the possibility of introducing such instability in the hybrids, it is not advisable to fuse cells that have been continuously maintained in culture for very long periods of time.

A further variable is introduced by the possibility that the fusion potential of cells may vary at different stages of the cell cycle. For example, Hansen and Stadler (1977) have shown that spontaneous mitotic cells of a mouse lymphoid cell line are preferentially fused by polyethylene glycol. In their experiments, however, cells

arrested in mitosis by colcemid block did not fuse in the presence of polyethylene glycol. On the other hand, polyethylene-glycol-induced mouse leukemia cells fused most efficiently when in the G1 stage of the cell cycle (Vaughan et al., 1976). Finally, myeloma cells in the midlogarithmic phase of growth fuse better than cells in the stationary phase (Andersson and Melchers, 1978; Kennett et al., 1978; Levy et al., 1978; Oi et al., 1978), and therefore should be fused at this phase of growth.

A further important precaution to ensure that the HAT-sensitive myeloma cell line is free from mycoplasma, since this type of infection may alter the physiological state of the cell so that it is no longer suitable for fusion. Mycoplasma, as is well known, cause chromosome aberrations and interfere with nucleic acid synthesis in the host cell (Russell, 1966; Stanbridge et al., 1969).

The differential fusion frequency of myeloma and leukemic cells at different phases of the cell cycle must also raise the possibility of similar properties in the antibody-secreting half of the hybridoma. In addition, the origin and composition of the lymphoid cell population may vary: adult cells, unfractionated or fractionated from spleen, lymph nodes, or peripheral blood have all been used, as well as neonatal spleen cells or cells grown in vitro from monoclonal spleen fragments [see *Curr. Top. Microbiol. Immunol.* 81, (1978)]. However, it must be emphasized that hybrid formation is the result of a selective fusion of the myeloma with antigen-stimulated B lymphocytes. Fusion of 8-azoguanine-resistant myeloma cells with other types of differentiated cells such as T cells has failed to result in Ig production by the hybrids (Goldsby et al., 1977; Hämmerling, 1977) or in new differentiated properties (reviewed by Davidson, 1974; Gordon, 1975).

Studies with fractionated subpopulations of human lymphocytes showed that fractions containing antigen-stimulated cells fused, whereas fractions containing precursor B cells did not (Schwaber, 1975). Similar results have been obtained with fractionated mouse B cells: only a pool of large, dividing cells secreting Ig gave rise to successful fusions. In contrast, small lymphocytes did not fuse (Andersson and Melchers, 1978).

An elegant confirmation of these findings comes from recent studies by Clark and Lake (1979), who conclusively demonstrated a positive correlation between the number of antibody-secreting cells in a spleen and the number of hybridomas making the same antibody. Taking spleens from mice immunized against the Thy-1 antigen of murine T cells, they measured the number of cells secreting antibody by an appropriately designed plaque assay. The

same suspensions were fused, and the resultant number of cultures positive for anti-Thy-1 was recorded. Their strikingly simple observation was that the frequency of hybrids was related to the frequency of plaque-forming cells or, quite simply, the highest yield of positive hybridoma cultures was obtained when spleen cells were fused at the peak of the plaque-forming cell (PFC) response. Consequently, any procedure that enriches for PFC, be it immunization or cell separation, will increase the chances of preparing hybridomas.

The fact that B cells stimulated in vitro with mitogens (Andersson and Melchers, 1978) or with antigens (Hengartner et al., 1978a) gave rise to large numbers of Ig-producing hybridomas again suggests that activated B cells preferentially fuse. However, not all types of activated B cells necessarily fuse equally well. The reason for saying this is that there is some evidence to suggest that a recently activated B cell hybridizes more efficiently than a mature plasma cell (Hämmerling et al., 1978; Köhler and Shulman, 1978). Indeed recent studies indicate that the dividing pre-B cell will fuse with myeloma cells essentially as well as the dividing and activated mature B cell (Burrows et al., 1979). This point should be carefully borne in mind if the experimenter is considering enriching for antibody-secreting cells prior to hybridization.

2.3.2. Immunization Strategy We have already discussed the observation that the yield of antibody-secreting hybridomas is directly correlated with the percentage of cells producing the desired antibody. Thus, the preferred strategy is to first determine the immunization protocol for producing maximum numbers of PFC and to fuse at the peak of the PFC response. An appropriate immunization schedule should consider a number of variables: the most immunogenic dose, the choice of an adjuvant, the route and number of immunizations, and the intervals between priming and boosting. (See W. M. Hunter's article in Weir, 1978).

The immunization protocol varies, of course, in relation to the immunogenicity of the antigen used and to the form, soluble or particulate, in which it is given. For example, with the aim of obtaining antibodies against cell-surface antigens it is possible to immunize with whole cells, purified membranes, or isolated antigens. The cells and membranes may be a heterogeneous mixture, such as spleen or peripheral blood, or a homogeneous population, such as a tumor cell line. Quite clearly, the more purified the antigen is, the greater the possibility of raising the corresponding antibody-

secreting hybridoma. Of course, a completely pure immunogen is not required since the great advantage of hybridoma technology is that it allows the investigator to select the specificity he desires from a heterogeneous response.

Although it is difficult to generalize about immunization protocols it is worth mentioning that in some experiments primed and once-boosted mouse spleen cells were more effective than hyperimmune spleen cells in generating antibody-producing hybrids (Oi et al., 1978). Also there is some evidence to suggest that IgG- and IgM-secreting PFC are equally effective in fusing (Hengartner et al., 1978a). Thus it is possible to arrange immunization protocols that are optimal for a chosen antibody class, and then expect to see this reflected in the classes of antibody secreted by the resultant hybridomas.

Finally, one possible way to increase the frequency of PFC to a given antigen is to include a step of immunization in vitro, as was successfully done by Hengartner et al., (1978a), using sheep red blood cells as the antigen. In this situation one might reasonably expect the antigen-stimulated lymphocytes to survive better in vitro than the unstimulated cells, thereby leading to an enrichment of PFC in the culture.

2.3.3. Hybridization Technology Although, as indicated above, the yield of hybridomas producing specific antibodies depends very much on the input of PFC, nonetheless the success of the fusion also depends upon continued growth following the fusion event. This in turn depends upon a large number of variables as well as strict attention to technical details. Some aspects of this phase of the fusion are discussed below.

The basis of effective production of hybridomas is a good sterile tissue culture technique in conjunction with carefully selected reagents. The culture medium is fairly standard for mammalian cells, usually consisting of the commercially available RPMI 1640 or Dulbecco's modified Eagle's medium, fortified with fetal calf serum (10–15%), glutamine, and nonessential amino acids.

The serum is a critical reagent and should be carefully checked. Normal procedure is to arrange for samples of several fetal calf serum lots to be supplied from a company in conjunction with a promise to reserve stocks until testing has been done. The simplest test is simply to look for cell growth over a series of cell dilutions. The serum that supports growth at the lowest initial seeding density is the best. Ideally, hybridoma cells should be used for this test, but if they are not available the myeloma half of the pa-

rental cells will do. Also, it is recommended to include in the test a survey of fetal calf serum with known efficacy in hybridization.

In general, commercially available sterile plastic tissue culture pipettes, flasks, dishes, etc., should be used. If, however, "in-house" washed glassware is to be used, then the washing procedure should be carefully supervised and controlled so that cells are not killed by, for example, inadequate removal of detergent from glassware.

For facilitation of the actual fusion event, most investigators use polyethylene glycol (PEG). The use of Sendai virus (Harris and Watkins, 1975) is limited by difficulties in its preparation and batch-to-batch variations in efficiency (Pontecorvo, 1975; Davidson and Gerald, 1977). Also, PEG is 100–300-fold more effective than virus in promoting fusion (Gefter et al., 1977), particularly of human lymphoid cells (Pontecorvo, 1975; Davidson and Gerald, 1976; Davidson et al., 1976; Steplewsky et al., 1976; Vaughan et al., 1976; O'Malley and Davidson, 1977).

The molecular weight as well as the concentration of PEG have been shown to affect the efficiency of hybridization, and the optimal combination that produces no obvious toxic effects seems to be PEG 1000 at 50% (Davidson et al., 1976). However, preparation of widely different molecular weights (600, 1500, 4000, 6000) have been used by different groups with apparently similar success. Evidence has been presented that the addition of dimethyl sulfoxide to the PEG increases the production of viable, multinucleate cells (Norwood et al., 1976; Hales, 1977). Other parameters such as time of exposure, temperature, pH, and cell densities have been reported as crucial in affecting the yield of viable hybrids (Davidson et al., 1976; Steplewsky et al., 1976; Vaughan et al., 1976; Gefter et al., 1977). Because PEG toxicity has been found to be different for different cell lines (Gefter et al., 1977) and PEG lots vary in degree of purity, the optimum conditions for each batch of the PEG of choice should be estimated in relation to the cell line to be used.

Given that parameters for hybridization have been optimized, and that a good fetal calf serum has been selected, is there anything else that can be done to ensure the survival of hybridoma cells, particularly in those first few critical days? The answer is probably yes, by the addition of unrelated bystander or feeder cells, which serve, so it is thought, to supply undefined essential nutrients to "condition" the medium. Previously, and in other systems, it has been shown that the addition of "feeder" cells have an enhancing effect on the growth of cells of different nature plated at low population

density (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; Coffino et al., 1972; Lernhardt et al., 1978; Andersson et al., 1977).

This enhancing effect is probably a result of the liberation of a nonspecies-specific, growth supporting factor into the medium (Anderson et al., 1977). In hybridoma technology, a variety of different cell types have been used as feeders. Thus syngeneic, allogeneic, or xenogeneic cells, such as fibroblasts, thymocytes, macrophages, peritoneal exudate cells, peripheral lymphocytes, or total spleen cells, have been used [see *Curr. Top. Immunol. Microbiol.* **81** (1978)] and shown to considerably increase the fusion and cloning efficiency. For the same purpose, medium from fibroblast monolayer or thymocyte culture can be used. The addition of medium harvested from growing suspension cultures of the mouse myeloma parent has been used without reproducible success (Coffino et al., 1972; Zurawsky et al., 1978).

Hybridoma recovery depends on the number of lymphoid cells used. In a good experiment, the expected fusion frequency is in the range of one hybridoma per 10^5–10^6 normal spleen cells. The relative number of parental cells present in the hybridization step is not highly critical. Most investigators use a spleen/myeloma ratio of 10/1, but lower ratios have also been used with similar or slightly better results (Buttin et al., 1978; Oi et al., 1978; Clark and Lake, 1979).

Any comparative studies should consider many variables and that the fusion frequency varies in relation to the strain, species, and cell combination used (Vaughan et al., 1976).

2.3.4. A Cell Fusion Protocol

There is no standardized protocol, the method of choice also depending on the kind of cells to be fused. Detailed particulars of various authors are most conveniently located in *Current Topics in Microbiology and Immunology*, **81** (1978).

The protocol currently in use in our laboratory is outlined below. We do not claim any originality for the procedure. On the contrary, it derives from several published methods, and is included merely to serve as an example and to give the reader a place to start organizing his own working protocol.

Reagents

Media. RPMI 1640 is obtained from Gibco and used (a) without additions or (b) with fetal calf serum (FCS) at 15% (v/v) or 20% (v/v), penicillin (100 I.U./mL), streptomycin (100 μg/mL), 2m*M* L-glutamine, and 5×10^{-5} *M* β-mercaptoethanol.

The selective HAT and HT media are prepared in the RPMI 1640–20% FCS medium by addition of 50X concentrated stock mixtures prepared in sterile double-distilled water. Final concentrations required in the media are 4×10^{-4} *M* hypoxanthine (H), 4×10^{-7} *M* aminopterin (A), and 1.6×10^{-5} *M* thymidine (T). The concentrated stocks are kept frozen.

Polyethylene glycol 1000. This is autoclaved and mixed with an equal weight of RPMI 1640 without additions. The pH of the solution is adjusted to 7.4 with sodium hydroxide if necessary.

Spleen cell suspension. Appropriately immunized mice are killed and their spleens aseptically removed and dissociated in RPMI 1640–15% FCS by teasing with mouse-tooth forceps. Aggregates are removed by passage of the suspension through a metal sieve. The cell suspension is washed twice with RPMI 1640–15% FCS, once with RPMI 1640 without additions, and finally adjusted to contain 10^8 cells/mL in RPMI 1640 without additions. All of these operations are conducted in the cold.

Myeloma cell line. The cells are maintained in RPMI 1640–15% FCS and harvested by centrifugation in the mid-logarithmic phase of growth [about $(3–5) \times 10^5$ cells/mL], washed twice with RPMI 1640 without additions, and suspended in the same medium at 10^7 cells/mL. Here, the washings are done in the medium at room temperature.

Hybridization. Spleen cells (10^8) and myeloma cells (10^7) are mixed in a 50 mL conical, plastic centrifuge tube, centrifuged, and all of the supernatant is carefully removed. The cells pellet is loosened by tapping the tube gently, and 1 mL of 50% PEG 1000 is added drop by drop over a period of 1 min. During the addition the cells and PEG are mixed by agitation and by stirring with the end of the pipette used for dispensing the PEG. After 1 min more, during which time the cells are gently agitated, 1 mL of RPMI without additions is added, again dropwise and with mixing over a period of 1 min. The mixture is then diluted every minute by sequential additions of 2, 4, 8, and 16 mL of RPMI 1640 without additions. The entire operation is conducted at a temperature of 37°C, using prewarmed reagents and a water bath. The cells are centrifuged and resuspended in RPMI 1640–20% FCS–HAT (at 3×10^6 per mL), and 0.1 mL aliquots are dispensed into the wells of a microtiter plate containing 96 flat-bottomed wells. Addition of about 10^4 Balb/c mouse peritoneal exudate cells into each well improves the fusion frequency. These cells may also be added later during the first week of incubation. Cultures are incubated at 37°C in a humidified incubator gassed with 7% CO_2 in air. After 3–4 days a further 0.1 mL of

RPMI 1640–20% FCS–HAT medium is added to each well. This process, termed "feeding," is repeated every 3–4 days over the first 2 weeks. At these later times, 0.1 mL of culture fluid is first removed prior to adddition of the fresh medium. The feeding is continued for 1–2 weeks with RPMI–20% FCS–HT medium, and then with RPMI–20% FCS. The HAT and HT media are always made up on the day of use. Hybrids appear in the cultures from the first week on.

When the growing hybrids cover one-third to one-half of the bottom of the well, supernatants are collected for screening. Positive cultures at this stage should be expanded by transfer to the larger 24 × 2 mL Costar tissue culture plates. At the same time, we remove cells for cloning by limiting dilution on peritoneal feeder cells in the microtiter plates. In our experience the transfer of positive cultures from microtiter plates to Costar wells is the most capricious stage, one at which seemingly fast growing cultures may die off or, conversely, sluggishly growing cultures may survive. For this reason we usually add 10^4–10^5 mouse peritoneal exudate cells to each Costar well if the cells are not growing vigorously. When the cells have grown up in the Costars, they are then transferred to large plastic tissue culture flasks. Cells are then harvested and frozen away in FCS/dimethyl sulfoxide (9/1). It is advisable to check that a given culture remains positive for secreted antibody at each transfer stage, since, as mentioned above, clonal overgrowth and/or chromosome loss frequently causes loss of desired hybrids.

2.4. Screening

As soon as evident growth of hybridoma cells is observed in the cultures, screening for the desired antibody specificity should be carried out. The reason for screening as early as possible is that each culture will generally contain several different hybridoma clones, and thus clonal competition and overgrowth can conceivably lead to loss of a desired hybridoma. Since the original spleen cell suspension will contain antibody-secreting cells, it is important to ensure that such cells, if they remain unfused, do not confuse the screening procedure by giving rise to positives. Normally, this does not occur because antibody-secreting lymphoid cells die rapidly under the conditions employed and, moreover, any antibody they secrete in the first few days of culture tends to be diluted out by the replacing of the medium, i.e., feeding. An absolutely sure way of avoiding the problem is to subculture hybridoma cells prior to screening, but this does take time, and therefore the possibility of loss through clonal competition arises. Our experience tells us that

the advantages of screening primary cultures outweigh any risk of false positives resulting from the presence of antibody secreted by unfused splenic cells.

In principle, the screening procedure can be done with either the hybridoma cells or the cultured supernatants. In either case, the test is for secreted antibody. Obviously, the method of choice with cells is the formation of hemolytic plaques, using as targets red cells on which the relevant antigen has been coated. This is not always feasible, and in any case not all hybridoma antibodies necessarily fix complement in such a way as to cause the efficient cell lysis required for the formation of hemolytic plaques (Howard et al., 1978).

Because of these problems, few investigators screen at the level of the cells, preferring instead to look for antibody in the culture supernatants. A recent approach, however, in which screening and cloning are conducted simultaneously, deserves to be mentioned. L. A. Herzenberg and his colleagues at Stanford University have combined the relevant antigen to microscopically small fluorescent plastic beads and then added these to hybridoma cultures. The derivatized beads stick to hybrid cells secreting the complementary antibody and are singly selected for growing up with the aid of the fluorescent-activated cell sorter (Parks et al., 1979). This is clearly a powerful tool and is only limited by whether or not the appropriate fluorescent bead can be prepared.

Screening for antibody in supernatants can be done in a variety of ways, all based on existing techniques such as radioimmunoassay, agglutination, hemolysis, rosette formation, or the ELISA test (see Weir, 1978).

Soluble antigens may be coupled to red cells, and then used in agglutination or rosette-forming or complement-mediated tests. Perhaps the simplest of these is to add such antigen-coated red cells directly to hybridoma cultures, and then look directly for agglutination, as was done by Claflin and Williams (1978) when screening for anti-phosphoryl-choline antibodies. Alternatively, soluble antigens may be fixed to plastic as a prelude to screening either by solid-phase radioimmunoassay (Klinman and Taylor, 1969) or ELISA assay (Voller et al., 1976). Both of these assays depend on revealing the hybridoma antibody bound to the plastic surface with an antibody directed at the antibody secreted by the hybridoma cell. For mouse–mouse hybrids an anti-mouse Ig antibody would be used, whereas an anti-rat Ig antibody is necessary when the antibody-secreting part of the hybrid is a rat lymphoid cell. In the latter case the anti-rat reagent should be first absorbed

with insolubilized mouse Ig to remove cross-reacting specificities for mouse Ig. Such antibodies could clearly give rise to false positives if the other half of the hybrid synthesizes mouse Ig.

The indicator antibody may be marked in a variety of ways, depending on the assay. It may be coupled to iodine for radioimmunoassays, to alkaline phosphatase for the ELISA test, or to red cells for agglutination assays. All of these procedures are standard methods and may be found in textbooks (e.g., Weir, 1978). For routine sensitive and qualitative assay of soluble antigens that stick to plastic, the ELISA assay is certainly the quickest. Here, the antibody is coupled to alkaline phosphatase, and its presence, once combined with the target antigen, is revealed by hydrolysis of a chromophoric substrate.

A small variation to the procedures is introduced if the antigen is particulate (e.g., cells), when perhaps the use of ^{125}I-labeled antibody as used by Williams and his colleagues (Williams et al., 1977) is the quickest, particularly if the results are revealed by autoradiography rather than by counting (Parkhouse and Guarnotta, 1978). For those who prefer not to use radioisotopes, a useful alternative is to couple a fluorescence dye (fluorescein or rhodamine) to the antibody probe. In this case the readout is visual inspection of the particles or cells, and if the target antigen is indeed located on cells, fluorescence has the added advantage of indicating the proportion of cells that possess the relevant antigen. Taking the above mentioned into account, the radiobinding assay is still the most convenient method to measure the actual number of antigenic sites on a particle or cell. Using indicator antibody–red-cell complexes in conjunction with particulate antigens to form rosettes as a test for hybridoma antibodies is, in our view, tricky and time-consuming.

Any discussion of methods used to reveal antibody bound to cells would be incomplete without mentioning the obvious application here of complement-mediated lysis. However, it should be emphasized that not all antibody classes fix complement and, as stated above, even those that do do not necessarily efficiently cause cell lysis (Howard et al., 1978). As Howard and his colleagues (1978) have elegantly indicated, efficient complement-mediated lysis often does not occur with monoclonal antibodies. In fact, it is frequently found that lysis demands the combination of two monoclonal antibodies of differing specificities, yet which are directed toward the same cell-surface antigen.

Finally, suitable choice of the antibody probe does allow the experimenter to select the class (i.e., heavy-chain isotype) of antibody. For example, use of appropriately prepared indicator anti-

bodies specific to the μ heavy chain will only reveal IgM antibodies. In this respect, mention should be made of protein A as a tool for screening. Protein A is a component of the cell wall of certain strains of the bacterium *Staphylococcus aureus*, and has the property of binding to some, but not necessarily all, of the IgG classes of mammalian antibodies (Goudswaard et al., 1978). Like antibody, it can of course be coupled with ^{125}I, fluorescein, or red blood cells and thus be used for screening. Obviously protein A will only reveal those particular IgG antibody classes. Nonetheless, it must be emphasized that antibodies binding to protein A are exceedingly easy to purify—simply through the use of a column of protein A–sepharose—and this could be a very important factor. For example, consider the case of a rat–mouse hybridoma restricted to passage in vitro, and yet secreting a useful antibody with reactivity for protein A. In this case it would be possible to easily purify the antibody from the culture fluid, in spite of the presence of relatively vast amounts of fetal calf serum proteins.

2.5. Selection and Maintenance of Positive Clones

The concentration at which the cells are plated to obtain a successful fusion is usually so high that several different hybrids will often grow in the same well. If, as seems the case, different cell lines may proliferate at different rates (clonal competition), overgrowth of faster dividing clones will occur. Heterogeneity of the cell population can also result from genetic instability *within* one clone (see Section 2.6). For all of these reasons, once a positive culture has been identified it should be cloned as soon as possible, so that the desired antibody is not lost.

Different cloning methods can be used; which include cloning by limiting dilution, either in soft agar or in liquid cultures, by micromanipulation (Paul, 1975), or by using the fluorescence-activated cell sorter (Parks et al., 1979). Details on cloning by limiting dilution methods can be found in *Current Topics in Microbiology and Immunology* 81 (1978). Since some lines may have low cloning efficiency, cloning by different methods may be necessary. In many cases, the use of feeder cells increases the cloning efficiency to 100%. Cloning by the use of the fluorescence-activated cell sorter has the advantage of selecting rare antibody-producing clones without the laborious screening and repeated subculturing. This technique could become the method of choice in the future. We should stress that more than one cloning is advisable to ensure the isolation of individual antibody-producing clones.

Once cloning has been done, the selected hybridomas can be expanded and propagated in vitro or in vivo as indicated in Section 2.2 above.

2.6. Loss of Antibody Production

The chromosome number of hybrids relatively soon after fusion is often close to the sum of the chromosome number of the parental cells. However, if the hybrids are grown for some time in vitro chromosome loss occurs, and there is the possibility of disappearance of the specific antibody. With intraspecific hybrids, the chromosome loss is usually small, but in interspecific hybrids there can be extensive and preferential loss of chromosomes of one parent. This phenomenon, also referred to as chromosome segregation, is well known in somatic cell genetics and is used to map the chromosome location of genes. Thus human and rodent hybrid cells, in which human chromosomes are preferentially lost (Weiss and Green, 1967), should allow assignment of the genes responsible for human immunoglobulin heavy and light chains, as has been done for the mouse (Hengartner et al., 1978b). For establishing stable hybridomas secreting immunoglobulins, therefore, intraspecific hybridization is preferred.

The phenomenon of chromosome loss depends not only on species used, but also on the type of parental cell used (Ringertz and Ege, 1977). In addition, chromosome segregation and loss may be more rapid during early stages after fusion. At later times, the chromosome number may be more stable. In fact the rate at which the process of chromosome loss (and loss of its production) occurs tends to decrease with time after hybridization (Ruddle et al., 1970; Schall and Rechsteiner, 1978). Furthermore, with closely related species the rate is usually slow, whereas with cells from different classes chromosome segregation is very rapid.

As already stressed in other sections of this chapter, in order to obtain stable clones it is important to clone at as early a stage as possible. Moreover, since loss of activity is likely to occur at any time during the life history of a hybrid, frequent quality controls for antibody production and recloning of the productive clones are advisable in order to maintain positive lines.

Finally, it should be said that loss of antibody production by hybridomas does not invariably result from chromosome loss. Elimination or mutation of structural or regulatory genes in the cluster coding for immunoglobulins could conceivably also cause the same phenotypic change.

3. T Cell Hybrids

The fusion of functional T cells with appropriately derived T-lymphoma cell lines will be a major step toward solving many of the most fundamental and unsolved problems in cellular immunology; for example, the properties of different subsets of T cells, the nature of the T cell receptor for antigen and its relationship to other surface molecules, the physicochemical properties of T cell-derived factors, and the control mechanisms exerted by these factors in the regulation of immune responses.

However, at the present time T cell fusion presents some difficulties. Enriched, highly purified populations of T cells that express a particular marker or a specialized function ideally should be used, but these are difficult to obtain. A further problem is that the screening stage in T cell hybridization involves laborious functional tests. Attempts to produce T cell hybrids have been reported, but in many cases the hybrids have been unstable and/or without any of the functions characteristic of T cells [Goldsby et al., 1977; Hämmerling, 1977; Kontiainen et al., 1978; see also *Curr. Top. Microbiol. Immunol.* 81 (1978)].

In general there has been failure to obtain hybrids with cytotoxic activity (Goldsby et al., 1977; Köhler et al., 1977b; Hämmerling, 1977; Di Pauli, and Di Pauli, 1978), whereas the greatest success has been in establishing hybrid cell lines secreting T cell suppressor factors. The relative failure to obtain functional T cell hybrids could simply reflect the fact that the HAT-sensitive cell lines used (EL4 and BW 5147) are inappropriate. It seems likely that for successful fusion to occur both of the parental cell types should be closely matched in terms of surface markers and state of differentiation. Thus helper, suppressor, and killer T cells may very well have to be fused with different and appropriately chosen HAT-sensitive T cell partners. If this is so, then the use of different cloned T cell lines (Gillis and Smith, 1977), and a range of other T cell tumor lines, will be a crucial factor in the successful production of a representative selection of T cell hybridomas.

The major successful aspect of T cell hybridization has been, so far, the preparation of T hybridomas producing suppressor factors. Antigen-specific and nonspecific factors have been obtained with suppressor activity on antibody production (Kishimoto et al., 1978; Kontiainen et al., 1978; Taniguchi and Miller, 1978; Watanabe et al., 1978; Taniguchi et al., 1979; Taussig and Holliman, 1979; Taussig et al., 1979) or on delayed-type hypersensitivity responses (Hewitt and Liew, 1979). It is to be hoped that physicochemical and

biological analysis of these presumed monoclonal factors will provide important information for the understanding of the mechanism of suppressor T cell action. However, a note of caution should be given, for among the factors so described there certainly are some curious differences; for example, the wide variation in molecular weight. Whether these differences reflect a truly significant heterogeneity of suppressor factors or simply technical problems will, no doubt, soon be resolved by experimentation. Nonetheless, we can, to be sure, anticipate with considerable pleasure an imminent clarification of the complexity of T cell biology through the application of hybridoma technology.

4. Applications and Future Perspectives

Hybridoma technology, because of the enormous potential of its applications, has deservedly attracted interest from fields as diverse as neurology, virology, clinical medicine, and industry. The major advantage of the method, we should restate, is that it allows the investigator to select the antibody he wants from a heterogeneous response. It is, therefore, not surprising that those working in complex problems of cell interaction have seized upon the method as the key to unlock the door of their Pandora's box. We shall briefly summarize the more obvious uses of hybridomas, which fall into two broad categories: analytical and clinical. Both of these, of course, have industrial interests.

As analytical tools, monoclonal antibodies are, par excellence, the reagents of choice. They can be used not only to detect, but also to quantitate or purify the corresponding antigen. Given cell-surface atigens, and in conjunction with the fluorescent-activated cell sorter, the corresponding monoclonal antibody can be used in order to select out for study that particular cell type.

The power of the hybridoma tool is most evident, in fact, in unraveling the complexity of those molecules that constitute and characterize the diverse cell types comprising a multicellular organism. Its exploitation will result in a wave of new information and new concepts. In principle, there is no reason to doubt (1) that monoclonal antibodies to all cell-surface molecules can be feasibly produced and (2) that this can be done without the old and tedious practice of absorbing a given anti-tissue antibody with every other type of tissue that the investigator can lay his hands on and without the necessity of purifying the antigens. Applications of such antibodies are enormous. At present one major thrust is in the defini-

tion of differentiation antigens characteristic of different cell types, and in the definition of tumor antigens. For example, is a tumor antigen a new or modified surface molecule, or is it simply an existing molecule displayed by a restricted cell type, perhaps only at a certain stage of its cell cycle? The answer to this old question will surely be answered soon. Similarly, given antibody probes directed toward appropriate surface molecules, we can expect major advances in our understanding of cell–cell interactions in a variety of fundamental situations, for example, the nervous and immune systems. In conjunction wth these types of advances we can also confidently predict a very rapid increase in our knowledge of the chemistry of membrane components, simply because the monoclonal antibodies will provide the tool for their isolation.

Finally, monoclonal antibodies will provide the answer in situations where molecules are very difficult to purify, present in low amounts, or generally not very immunogenic, e.g., hormones, factors, or interferons.

The clinical potential of hybridomas is vast and as yet largely untapped. We shall consider two aspects: diagnostic and therapeutic.

On the diagnostic side there are some very obvious examples: reagents for immune response and radioimmunoassay of hormones, bacteria, viruses, parasite antigens, serum proteins, etc.; reagents for identifying HLA types, rare blood groups, and various differentiated cell types, in health and disease. Already the ball has started to roll. For example, a mouse hybridoma has been prepared that recognizes human cortical thymocytes, but not normal peripheral lymphocytes (McMichael et al., 1979). Interestingly, reactive cells were found in the periphery in some cases of acute lymphatic leukemia (Bradstock et al., 1979). The antibody is therefore of clear diagnostic value, and, together with similar reagents yet to come, will be useful in detection and management of human leukemias.

There is little advantage in listing the total literature covering the various types of hybridomas described to date, particularly since by the time this work appears there will be many, many more. We shall, instead, simply mention some selected cases that may be of general and introductory interest. Hybridomas with specificity to glycoproteins coded for by the major histocompatability locus of mouse (Hämmerling et al., 1978; Oi et al., 1978) and man (Barnstable et al., 1978) obviously represent the first of many similar reagents we can expect to see. The same can be said of hybridoma antibodies to teratocarcinoma cells (Knowles et al., 1978; Stern et al., 1978) and various differentiated cell types in, for example, the nerv-

ous system (Walsh and Eisenbarth, 1979) and the immune system (Williams et al., 1977; Levy et al., 1978; Springer et al., 1978; Trowbridge, 1978; Trucco, 1978).

Analysis of the antigenic variants that constantly arise in populations of viruses, bacteria, and parasitic protozoa will be considerably facilitated by development of the relevent hybridoma antibodies. Already there are examples of this for influenza virus (Gerhard and Webster, 1978; Koprowsky et al., 1978) and malaria (Howard et al., 1979).

An interesting application of monoclonal antibodies in the field of virus epidemiology has recently been described (Webster et al., 1979). Using a panel of monoclonal antibodies, these workers identified five antigenic sites on the surface of influenza virus. Mutations at these sites are responsible for the phenomenon of antigenic shift in the virus, and thus the incredible specificity of the hybridoma antibody provides a precise tool for the study of this process. Furthermore, the fine-structure antigenic analysis made possible by the development of anti-influenza-virus monoclonals allowed a remarkable piece of detective work, namely that the virus A/USSR is identical to the virus prevalent in 1947 and 1948.

Therapeutic use of monoclonal antibodies is currently limited by the species barrier, the major monoclonals being of rat or mouse origin. It is reasonable to suppose, however, that it will not be long before an appropriate 8-azoguanine-resistant human cell line will be developed and successfully fused with human antibody-secreting cells, perhaps activated in vitro. Until that time, monoclonal antibodies directed toward toxins, drugs, poisons, or autoantibodies could conceivably be used in an extracorporeal manner. The antibody could be coupled to a insoluble support and then used as a filtering device for the blood. One example of an antibody that could be used this way is the anti-tetanus-hybridoma antibody of Zurawsky et al. (1978).

In cases where virus infection is lethal, such as rabies, the murine monoclonal antibodies would have a value, in spite of the species barrier and its associated problems. From this point of view it should be noted that murine monoclonal antirabies antibodies do give protection to mice when infected intracerebrally with a lethal dose of the virus (Wiktor and Koprowski, 1978).

Finally, given that the species problem can be obviated, there are two very clear immediate therapeutic roles for monoclonal antibody: (1) removal of cancer cells by coupling a lethal substance to the appropriate hybridoma antibody and (2) manipulation of the immune response. Monoclonal antibodies directed toward a defined antibody idiotype would allow control of the corresponding

antibody response in a specific way, whereas an anti-T cell subset antibody could allow gross modulation of the entire immune response. In either case there are precedents for up or down modulation. Thus a monoclonal anti-idiotype antibody could in principle be used as a vaccine (up modulation) or, perhaps in autoimmune conditions, as a suppressor (down modulation). In the latter case the action would presumably be via activation of the relevant suppressor T cell.

Finally, a whole new realm of modulation of both T- and B-lymphocyte responses can be readily conceived if and when the potential of T cell hybrids is realized.

Notes Added in Proof

Since the writing of this review there has been a considerable growth of activity in areas of hybridomas technology of direct relevance to clinical medicine. Two HAT sensitive human cell lines have been obtained and successfully hybridized with human cells. The U-266AR$_1$ myeloma cell line (now designated SKO-007) has been selected from a human myeloma by Olsson and Kaplan (*Proc. Natl. Acad. Sci. USA* 77, 5429, 1980) and fused with spleen cells from patients with Hodgkin's disease. The resulting hybridomas produce antibody against the chemical hapten 2,4-dinitrochlorobenzene used to sensitize the patients. Another line, GM 1500 6TG-Al 1, derived from a human B cell line by Croce, Linnenbach, Hall, Steplewski, and Koprowski (*Nature* 288, 488, 1980), has been fused with peripheral lymphocytes, and hybridomas secreting antibodies to measles virus have been derived.

Another major achievement has been the production of a large number of monoclonal antihuman reagents. Among the many now commercially available, perhaps the best known are the OK series of Schlossman and coworkers (information available from Ortho Pharmaceutical Corporation). This panel of monoclonal antibodies identifies subpopulations of human white blood cells and has already proved of great value in human research. We may confidently predict further expansion in the production and use of monoclonal antibodies in human and veterinary medicine.

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Chapter 10

Immunologic Tests for Diagnosis and Monitoring of Defects in Cell-Mediated Immunity

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1. Introduction

The recent explosion of knowledge about the functions of the human immune system—in its cellular and molecular aspects—has permitted more frequent diagnosis and more precise definition

of clinical immunodeficiencies. The definition of particular defects by immunological and biochemical means has in addition led to the development of rational therapeutic approaches, such as replacement therapy and immunomodulation (Fudenberg and Wybran, 1980). Advances in these areas have been applied to the diagnosis and treatment of defects in cell-mediated immunity (CMI) in human patients.

CMI is mediated by thymus-derived (T) lymphocytes. During the past decade the availability of syngeneic mouse strains and highly specific antisera has led to precise enumeration and characterization of murine T cell subsets (Cantor and Boyse, 1975, 1977a). Characterization of the murine major histocompatibility complex (MHC) and its products on the lymphocyte membrane (Klein, 1978) has shed further light on the regulation and control of the immune response: helper, suppressor, killer, and inducer T cells have been defined antigenically, and other T cell subpopulations will undoubtedly be delineated in the future. Although for obvious reasons it has been difficult to perform similar studies in humans, certain lymphocyte markers and functional tests have been developed and have been used to analyze human T cell subpopulations. More recently, the development of monoclonal hybridoma antisera, produced by the fusion of murine plasma cells (antibody-secreting) with murine myeloma cells (nonsecreting) has led to the production of exquisitely specific (monoclonal) reagents with reactivity against human T cell subsets.

Since the description of the duality of the immune system, immune deficiency diseases have been divided into defects of either the T cell system (or its subsets) or the B cell system. Such a classification may still be valid, although in most instances there are actually overlapping syndromes. Furthermore, deficiencies of either cellular or humoral immunity or both may in many instances reflect abnormal regulation of the immune response. Undoubtedly, definitions of clinical immune deficiencies in terms of immunoregulation will in the future be added to, or possibly replace, the present classification system.

In the following sections, in vivo and in vitro aspects of CMI are discussed, including the in vivo dynamics of granuloma formation, classical delayed-type hypersensitivity (DTH), and cutaneous basophil hypersensitivity (CBH). Laboratory tests for the evaluation of CMI in man, in particular lymphocyte morphology and function, as well as regulation, and the types of clinical immune deficiencies that result from detectable abnormalities of CMI are described.

2. Anatomy of the Immune System

Lymphoid cells are organized into the various lymphoid tissues throughout the body, varying in complexity from loose collections of lymphocytes, as in the tracheal submucosa, to the intricate anatomical organization of the lymph node and spleen. Most lymphoid tissues, with the exception of the thymus, consist of a three-dimensional branching latticework of reticular cells and the reticular fibers they produce, with the lymphoid cells interspersed among them. This structure is well suited to facilitate interactions between cells and with antigen as the blood and lymph filter through it.

The lymphoid system can be divided functionally into three compartments: (a) the stem cells, which are the progenitors of all classes of lymphocytes; (b) the central lymphoid organs, where immature lymphocytes develop into either T or B cells; and (c) the peripheral lymphoid system, containing lymphocyte populations processed by the central lymphoid organs and generating the immune response after antigen challenge. In birds the central lymphoid organs are the thymus (T) and the bursa of Fabricius (B); in humans also, T cells are derived from the thymus, but there is no human equivalent of the bursa and B cells are considered to be derived from the bone marrow or an anatomically vague "bursal equivalent." The human peripheral lymphoid system consists of spleen, lymph nodes, gut-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), and a less well-defined collection of lymphocytes in the submucosa or lamina propria of many organs.

The thymus is the first lymphoid organ to appear in birds and in most mammals. At about the ninth week of human gestation (Kay et al., 1962), lymphoid cells appear in the epithelial thymic tissue and undergo rapid proliferation, producing a predominantly lymphocellular cortex with scattered epithelial cells and a predominantly epithelial medulla with interspersed lymphocytes. The cortex is subdivided by connective tissue extending from the fibrous capsule inward toward the medulla. The medulla contains a population of mature lymphocytes as well as the Hassall's corpuscles, small islands of partially hyalinized epithelial cells whose function is not known (Fig. 1). In humans, the thymus is structurally complete by 20 weeks of gestation. It reaches maximum size at birth and thereafter undergoes a gradual age-related involution (Boyd, 1932).

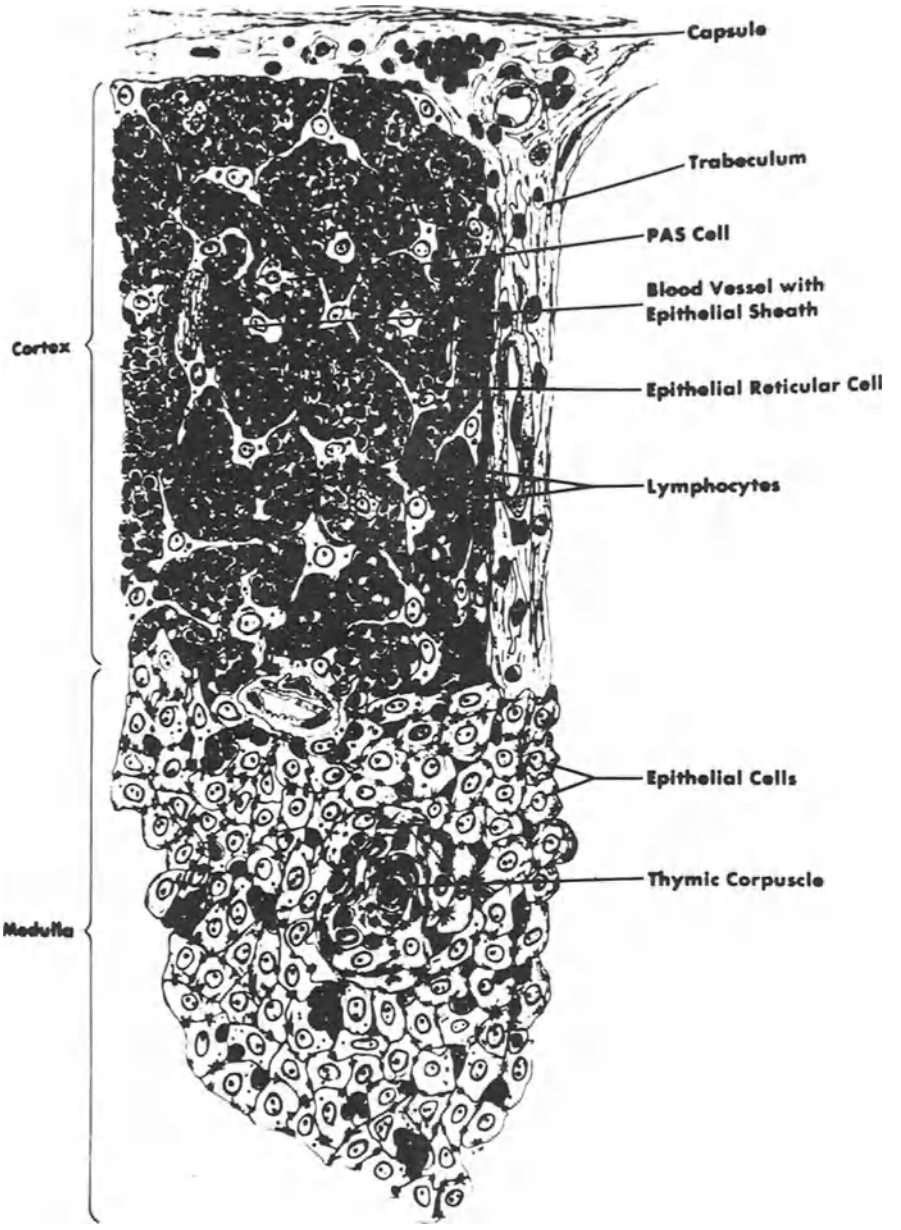

Fig. 1. Thymus, portion of lobule. The cortex is heavily infiltrated with lymphocytes. As a result, the epithelial cells become stellate and remain attached to one another by desmosomes. The medulla is closer to a pure epithelium, although it too is commonly infiltrated by lymphocytes. A large thymic corpuscle consisting of concentrically arranged epithelial

The lymph node can be thought of as an anatomic unit consisting of a heterogeneous lymphocyte population supported by a reticular framework and surrounded by a capsule. Lymph nodes begin to form in the human embryo as early as 11 weeks of gestation and are generally complete at birth (Papiernik, 1978). The structure of a typical lymph node is illustrated in Fig. 2.

The spleen originates during the fifth week of gestation, but is not completely organized and mature until after birth. The organ can be divided into the white pulp, islands containing aggregates of lymphoid cells, and the surrounding red pulp, consisting primarily of broad vascular sinuses filled with blood (Fig. 3). The immune functions of the spleen occur primarily in the white pulp, where a reticular superstructure supports a cylinder of lymphocytes, the periarteriolar lymphocyte sheath (PALS), which is a thymus-dependent area (Weiss, 1964) analogous to the paracortical areas of the lymph node. Lymphoid follicles lie in and around this area; as in the lymph node, these are B cell-dependent regions. A loose collection of lymphoid cells surrounds the sheath and follicles. This area, the marginal zone, contains many branching reticular cells and appears to be the main site of antigen capture (Nossal and Ada, 1971).

Lymphoid tissue is also present along the entire alimentary tract (GALT) and along the entire respiratory tract (BALT). These tissues consist mainly of small lymphocytes, singly and in clusters or follicles, along with some more highly organized structures, such as the Peyer's patches in the ileum, the densely packed lymphoid nodules of the appendix, the tonsils, and typical lymph nodes that drain the parenchyma of the lung. In addition, the bone marrow, though not a pure lymphoid organ, does provide stem or precursor cells of lymphocytes and monocytes (macrophages). Lymphocytes consist of 5–20% bone marrow cells and 2–3% monocytes. Lymphopoiesis is active in the marrow throughout life. Data in mice indicate that the bone marrow contains two populations of lymphocytes, a large population that is produced endogenously and then migrates out, and a smaller recirculating pool of long-lived cells (Miller and Osmond, 1975).

We are concerned here with disorders of CMI, the functional arm of the immune system that is mediated by T cells, and the

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cells is figured. The corpuscle and trabecula are rich in connective tissue fibers (mainly collagen) and contain blood vessels and variable numbers of plasma cells, granulocytes, and lymphocytes. [Reprinted with permission from Weiss, L. (1972), *The Cells and Tissues of the Immune System: Structure, Function, and Interactions*, Prentice-Hall, New Jersey. p. 81.]

Fig. 2. Topography of the lymph node. (A) T and B areas. C, cortex; Pc, paracortex; MS, medullary sinuses; MC, medullary cords; GC, germinal center with "cap" of dense dendritic reticular cells; PF, primary follicle; H, hilum; a, artery; v, vein; pcv, postcapillary venul; el, efferent lymph; al., afferent lymph; IS, intermediary sinus; PS, peripheral sinus. (B) Speculative

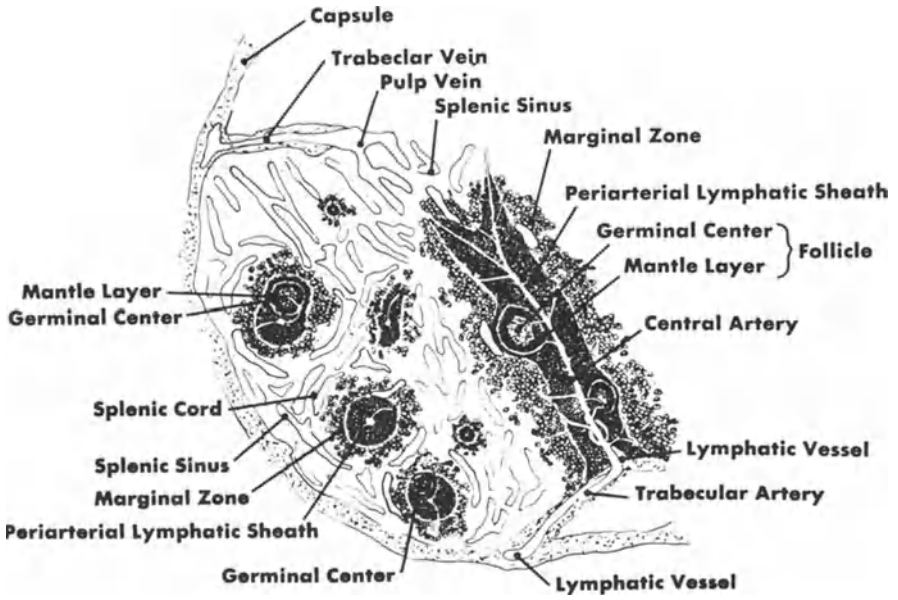

Fig. 3. Schematic view of spleen. [Reprinted with permission from Weiss, L., and M. Tavassoli (1970), *Sem. Hematol* 7, 372. (Grune and Stratton, New York).

assays by which such orders are delineated. Although it is not known what relationship in vitro testing and analysis have to the in vivo differentiation process, studies conducted for the most part in mice have permitted a tentative delineation of the functional and antigenic maturation of T cells (Fig. 4). More recently, Schlossman and coworkers have characterized the stages of human intrathymic differentiation by the use of monoclonal antibodies (see Section 4.2, below).

In mice and chickens, studies of chromosomal markers have shown that yolk sac cells, and later fetal liver cells, populate the thymus (Moore and Owen, 1967; Stutman and Good, 1971). Dramatic evidence that fetal liver cells can function similarly in humans has been provided by reports of successful immunologic reconstitution of several children with combined immunodeficiency by transplantation of fetal liver cells (Keightly et al., 1975; Horowitz and Hong, 1977). Despite its gradual involution with age, the thymus ap-

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traffic route. O = B cells, O = T cells (larger cells, lymphoblasts), ? = little or no direct evidence for this route but represents the most likely. [Reprinted with permission from Greaves, M. F., J. J. T. Owen, and M. C. Raff (1974), *T and B Lymphocytes*, American-Elsevier, New York, p. 34.]

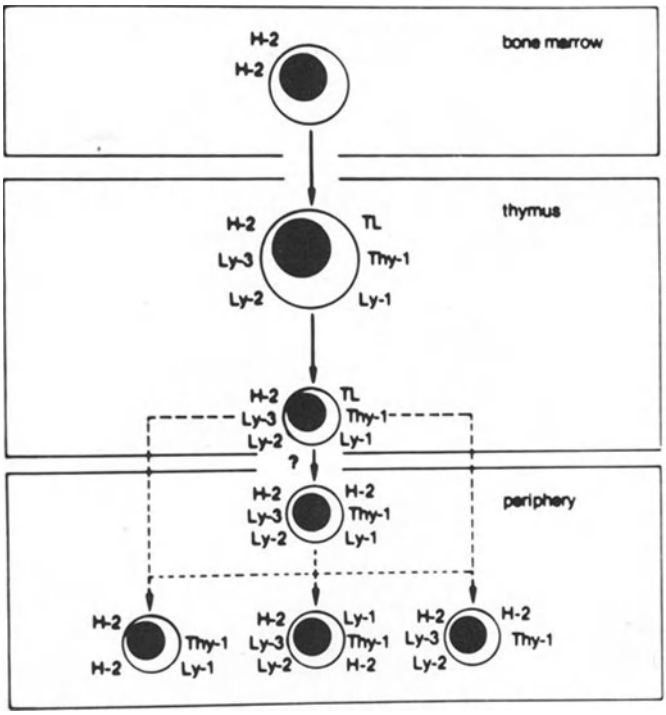

Fig. 4. Differentiation of mouse T cells with acquisition of differentiation antigens. Dashed lines indicate possible alternative routes of differentiation. TL, Ly-1, Ly-2,3, Ly-1,2,3, and Thy-1 are T cell differentiation antigens found on the cell surface. H-2 antigens are found on cell surfaces in most tissues of the mouse. Boxes indicate distinct anatomical compartments. [Reprinted with permission from Hood, L. E., I. L. Weissman, and W. B. Wood (1979), *Immunology*, Benjamin/Cummings, Menlo Park, CA., p. 13.]

parently continues to function in the processing of T cells; however, in adults the source of the lymphoid precursor cells is the bone marrow, and the processing is less efficient (Airokawa and Makinodan, 1975). Adult thymectomy in humans produces no immediate alteration of immune function, but in mice a detectable deficiency becomes apparent 5–7 months later (Metcalf, 1965), along with changes in the histology of lymphoid tissues suggestive of loss of T cell function (Metcalf, 1960). In addition, the development of peripheral T cell populations in the lymph nodes is intimately related to the presence and maturation of thymic cells. In humans, this relationship is reflected, for example, in the paracortical lymphocyte depletion characteristic of the congenital thymic aplasia syndrome of DiGeorge.

The acquisition of immune functions usually attributed to T lymphocytes (see below) occurs throughout pre- and postnatal development (Davies, 1969; Stutman, 1977). In vitro responsiveness to phytohemagglutinin (PHA) and concanavalin A (con A) is seen at 2–3 weeks of gestation and increase with fetal age. Mixed lymphocyte culture (MLC) reactivity to allogeneic cells and graft-vs-host (GVH) reactivity are present in neonatal thymocytes, as is T helper cell function. After birth, there is a gradual increase in T cell immune competence in both lymph nodes and spleen. The thymus appears to influence peripheral T cell maturation via thymic hormones, as suggested by the identification of thymosin, a small polypeptide rich in glutamic and aspartic acid, in a variety of animals and in man (Aiuti and Wigzell, 1980). This influence includes the appearance of T surface antigens, enhanced response to mitogens, and immunoglobulin production in athymic mice. In humans, the level of thymic hormone(s) is relatively high during the first 30 years of life and then gradually decreases with age (Bach, 1973). Thymic hormone infusion has been reported to be associated with some restoration of immune function in a patient with thymic hypoplasia (Wara et al., 1975).

3. In Vivo Manifestations of Cell-Mediated Immunity

Subcutaneous administration of certain antigens induces characteristic histopathological lesions. These are classified into three major types: granuloma formation, delayed-type hypersensitivity (DTH), and cutaneous basophil hypersensitivity (CBH). Failure to appropriately manifest these reactions is suggestive of defective CMI in vivo.

3.1. Granuloma Formation

Granuloma formation can be observed in nonsensitized humans following the administration of mycobacterial antigens. A great number of other antigens also induce granuloma formation, which is an indication of CMI in the nonsensitized host.

First described by Koch (1891), granuloma formation is now recognized as a highly specific hypersensitivity reaction to certain types of antigens. Its essential feature, which distinguishes granulomatous inflammation from other types of chronic inflammatory reaction, is the appearance of cells of the reticuloen-

dothelial system (RES), including mononuclear phagocytes, histiocytes, epithelioid cells, and giant cells (Epstein, 1967). The organized epithelioid cell granuloma can be distinguished pathologically from colloidal foreign body reactions, and can be produced experimentally in humans by the use of chemically defined antigens (Shelley and Hurley, 1957; Epstein et al., 1962, 1963). The presence of epithelioid cells and giant cells and their organization into tubercles are the hallmarks of granuloma formation.

Zirconium salts (Epstein et al., 1962, 1963) and beryllium (Sneddon, 1955; Norris and Peard, 1963) have been shown to induce granuloma formation in sensitized human subjects, and positive responses to skin testing with these antigens but not others are considered diagnostic for zirconium hypersensitivity and berylliosis, respectively. In sarcoidosis, the reliability of the Kveim test is established, giving less than 2% false positives, and purified Kveim antigen has been shown to produce granuloma formation in sarcoidosis patients regardless of environmental variables (Siltzbach, 1964). Granulomatous hypersensitivity is also characteristic of the tuberculoid variety of leprosy, and apparently is associated with protection from the more severe lepromatous type (Epstein, 1967). Lepromin antigen generally produces a delayed granuloma in patients with tuberculoid leprosy, but also in many normal individuals, and histologically the reaction is not a true organized epithelioid cell granuloma (Rees, 1964; Epstein, 1967). Tuberculosis is the classic granulomatous disease, and granulomatous hypersensitivity appears to be involved in the complex immune response; however, the exact nature of the granulomagenic antigen(s) has not been determined.

3.2. Delayed-Type Hypersensitivity

In 1891 Koch demonstrated that injection of nonviable tubercle bacilli into sensitized guinea pigs resulted in mononuclear cellular infiltration at the site of injection that reached its peak after 24–48 h. The occurrence of a similar reaction was later observed in humans, and skin testing became the standard method for evaluating DTH reactions. Indeed, skin testing is still the most widely used clinical test for the in vivo evaluation of CMI to mycobacterial antigens.

The DTH lesion is characterized morphologically by erythema and induration of varying degree. Activation of the coagulation cascade with fibrin deposition is responsible for the induration (Edwards and Rickles, 1978), which does not occur with cellular infiltration alone, as seen in CBH. The test is performed by intra-

cutaneous administration of small amounts of antigens, such as purified protein derivative from tuberculin (PPD). The evaluation is made by measuring the diameter of induration (and not of the erythema) after 48 and 72 h. Some quantitation of CMI is possible by using different dilutions of the antigens, but repeated testing may itself induce CMI to the antigen (Ramsey et al., 1977). A minimal diameter of induration of 10 mm indicates a positive reaction. In the absence of a reaction, either the patient is not sensitized or the failure is suggestive of defective CMI in vivo. Therefore, testing in such a situation should include substances that induce comparable reactions without prior sensitization.

Defective in vivo manifestation of CMI as assessed by intracutaneous testing may be transient in a number of situations. This observation was first reported by von Pirquet (1908), who observed the absence of tuberculin reactivity in patients with measles. A similar suppression of this CMI manifestation in vivo can be observed during other infections or vaccinations (Brody et al., 1964), or in patients with protein malnutrition (Douglas and Schopfer, 1976), or after treatment with steroids or irradiation.

3.3. Cutaneous Basophil Hypersensitivity

Whereas tuberculin-type DTH reactions are the prototype of CMI after immunization with adjuvants containing mycobacteria, there are also DTH reactions in humans to intradermal injections of proteins without concurrent exposure to mycobacteria. These reactions are less intense, less prolonged, and mainly erythematous. They were orginally known as Jones-Mote reactions (Mote and Jones, 1936). By specialized histological techniques it has been demonstrated that basophils are the most prominent infiltrating cells (Dvorak, 1976). Hence these manifestation of CMI are now called cutaneous basophil hypersensitivity (CBH); however, it should be pointed out that tuberculin-type DTH may also show large numbers of basophils (Askenase and Atwood, 1976).

Although CBH reactions have been studied in a variety of species, the clinical significance of CBH in the evaluation of human T cell immune deficiencies has not been studied in detail. CBH is the normal cellular response to a variety of antigens administered without supplementation with mycobacterium-containing adjuvants (Dvorak, 1976). Cell transfer experiments in guinea pigs have demonstrated that the cells mediating this reactivity are probably T cells; however, antibodies seem to be necessary as well (Askenase, 1976; Askenase et al., 1976). Thus, T cells might participate in the complex regulation of basophil numbers in the tissue.

3.4. General Considerations

The dynamics of cellular and humoral events leading to mononuclear cellular infiltration and activation of the coagulation system in DTH after antigenic challenge are not fully understood. The interaction of immune complexes with the complement system and activation of coagulation via the Hagemann factor have been described (Ratnoff, 1969), but it is not understood why such activation occurs only in tuberculin-type DTH and is absent in CBH. Furthermore, there may be particular immunological reactions within the skin. The existence of a particular skin-associated lymphoid tissue system (SALT) has recently been suggested (Streilein, 1978). In addition to this possible specialized traffic of lymphocytes passing through the skin, there are cellular components within the epidermis that may contribute to the peculiarities of the skin as a specialized organ for antigen presentation and processing. Langerhans cells have been known to be a component of the epidermis for more than a century (Langerhans, 1868). Recently, it has been demonstrated that Fc and C3b receptors are present on the surface of these cells (Stingl et al., 1977), as well as Ia antigens (Frelinger et al., 1979). In addition, their bone marrow origin has been demonstrated in the murine model (Katz et al., 1979). In other experimental systems their role in antigen recognition and processing has been studied (Silberberg et al., 1976), and the results suggest that they may be monocyte-derived. The role of the SALT and of Langerhans cells in systemic immunological mechanisms remains to be elucidated and it may be that investigation with specialized techniques will provide better definitions of DTH and CBH and their relationship to CMI in the evaluation of patients.

4. In Vitro Tests of Cell-Mediated Immunity

4.1. Membrane Receptors on T Cells

Lymphocytes can be divided into subpopulations on the basis of membrane surface antigens or markers. Typically, B lymphocytes possess membrane immunoglobulin (Warner, 1974; Ross, 1979), and antigens (Winchester et al., 1975), receptors for complement (Ross et al., 1978), and receptors for the Fc portion of immunoglobulins (Dickler, 1974). Peripheral blood T cells uniformly lack Ia antigens and complement receptors. The simplest and most widely used assay for T cells is rosette formation with sheep erythrocytes (E rosettes) (Bloom and David, 1976). About 60–80% of

the lymphocytes in the peripheral circulation have receptors for sheep erythrocytes and are classified as T cells.

The "active" E rosette assay (Wybran and Fudenberg, 1971) has also been used extensively as a sensitive indicator of CMI in man (Wybran and Fudenberg, 1973; Wybran et al., 1973; Fudenberg et al., 1980). The T cells that form active rosettes are part of the E rosette-forming population and have been reported to correlate more closely with the clinical status of patients with immune deficiencies than the "total" E rosette assay or in vivo assays of delayed-type hypersensitivity (Horowitz et al., 1975). Increased active rosette formation by lymphocytes exposed to antigen has been correlated with skin reactivity in vivo (Felsburg and Edelman, 1977). This assay has also been used to assess the effects of immunotherapeutic agents, such as thymosin (Wybran et al., 1975), levamisole (Ramot et al., 1977), and dialyzable leukocyte extracts (transfer factor) (Nekam et al., 1977). A number of studies have shown that these "active T cells" have unique physical characteristics, including differences in sialic acid content of their membrane (Yu, 1975); and the inhibition of active rosette formation after exposure to fragments of sheep erythrocytes suggests the presence of a unique receptor on their surface (Taniguchi et al., 1976). Recent studies suggest that the subset of active T cells is heterogeneous and contains cells that function in MLC recognition as well as cytotoxic killing of allogeneic lymphocytes (Robbins et al., 1981).

A rosette-formation assay is also used for the detection of two subpopulations of T cells with receptors for the Fc portions of IgG and IgM molecules, respectively, designated T-gamma and T-mu (Moretta et al., 1975). After initial isolation of the T cell population by E rosette formation and dissociation by neuraminidase treatment, the T-gamma and T-mu subpopulations are quantitated by rosette formation with ox (or chicken) erythrocytes coated with rabbit IgG or IgM antibodies. The T-gamma cells can be identified in freshly isolated blood, whereas the IgM Fc receptors are saturated with plasma IgM and thus T-mu cells can be detected only after overnight incubation in medium. It was originally thought that T-mu cells were helper and T-gamma suppressor cells, but further investigations have shown that both subpopulations are antigenically and functionally heterogeneous (Reinherz et al., 1980a). Imbalances in these two subsets have been reported in agammaglobulinemia of common variable immunodeficiency, selective IgA deficiency, severe combined immunodeficiency following bone marrow transplantation, ataxia telangiectasia, and some lymphoproliferative disorders (reviewed by Gupta and Good, 1980).

Imbalances of circulating T-gamma and T-mu subsets have also been correlated with alterations in T cell subpopulations detected by monoclonal antibodies (see below). T-mu cells can be stimulated in vitro to mature into specific cytotoxic effector cells and to perform helper and suppressor functions for B cell maturation following PWM stimulation; the gamma receptor is expressed on T cells activated in vivo, which are refractory to further in vitro stimulation (Pichler and Broder, 1981).

Another rosette-formation assay detects a subpopulation of "interactive" T cells by their ability to bind to cells of a B lymphoid line (Goust et al., 1978). The Raji cell line is currently used for this assay (Goust et al., 1980). The T cell lineage of these interactive rosette-forming cells is shown by the fact that the highest percentage of rosettes is observed with fresh thymocytes and with E rosette-forming cells from peripheral blood, whereas non-E rosette-forming cells do not bind Raji cells (Goust et al., 1980). Interactive T cells are reduced in patients with Guillain-Barre syndrome or active multiple sclerosis (Goust et al., 1980); the finding of an inverse correlation with increased levels of T-gamma cells in the latter group suggests the possibility that interactive T cells may have helper functions (J. M. Goust and H. H. Fudenberg, in preparation).

4.2. Antigenic Surface Markers on T Cells

Cell surface antigens specific for both B cells and T cells have recently been characterized by the use of several different types of antisera. Heterologous antisera have been used to show that cells of the B lineage, from very immature B cells to plasma cells, express a marker that is analogous to the Ia marker of murine lymphocytes (Hoffman et al., 1977). Almost all T cells are Ia negative, but a subpopulation may express this marker after exposure to antigenic stimuli (Evans et al., 1978; Fu et al., 1978). Heterologous antisera that react exclusively with T cells have also been produced. For example, two heterologous antisera have recently been developed to antigens found specifically on T cells, designated TH_1 and TH_2; these are roughly equivalent to the Ly antigens in mice. TH_2 is expressed on both cytotoxic and suppressor T cells, and TH_1 is expressed on helper T cells (Evans et al., 1977; Reinherz and Schlossman, 1979). However, such heterologous antisera are now regarded by many investigators as obsolete, since monoclonal antibodies against these determinants (see below) are completely free of cross-reacting specificities and can theoretically be produced in unlimited amounts.

More recently, it has been shown that sera from some patients with certain autoimmune disorders react selectively with some normal T cells and not with other subpopulations. To date, three such autoantisera have been described (Strelkauskas and Robbins, 1981): (a) Autoantibodies in sera from patients with juvenile rheumatoid arthritis (JRA) react with a subset of T cells that respond well to con A but weakly to PHA, do not have strong cytotoxic activity, and do respond to soluble antigens (the JRA^+ subset); (b) autoantibodies present in the sera of a small fraction of patients with systemic lupus erythematosus (SLE) react with a subset of T cells that have the ability to enhance (help) immunoglobulin production by B cells (the SLE^+ subset); and (c) autoantibodies in the serum of one patient with agammaglobulinemia (AGG) have been shown to react with a subset of T cells which, like the SLE^+ subset, have helper functions, but which also have the ability to recognize and kill allogeneic lymphocytes (the AGG^+ subset). The reactivity of such autoantisera can be analyzed by immunofluorescence or by rosette formation with antibody-coated sheep cells (Strelkauskas et al., 1975); the latter technique can be used for isolation of the various subsets for functional studies.

Finally, a series of monoclonal antibodies to T cell-membrane markers have been developed in the past few years and used for the delineation of functional subsets of T cells (Reinherz and Schlossman, 1980). The reactivity of these antibodies with human thymocytes at different stages of maturation is shown in Table 1. Anti-T1 is a monoclonal antibody reactive with 100% of peripheral T cells, but only 10% of thymocytes. The $T1^+$ thymocytes are the only thymocytes capable of MLC reactivity. Anti-T3 has essentially identical reactivity. Anti-T4 reacts with 75% of thymocytes and 60% of peripheral T cells; it appears to identify a helper or inducer subset of peripheral blood T cells, which also are the only peripheral T cells that show a proliferative response to soluble antigens. This $T4^+$ subset is roughly equivalent to the TH_1^+ (TH_2^-) subset defined by heteroantisera. Anti-T5 and anti-T8 react with about 80% of thymocytes and 20–30% of peripheral T cells; anti-T5 appears to identify a subset with both suppressor and cytotoxic capacity, similar to the TH_2^+ subset. Anti-T6, anti-T9, and anti-T10 react almost exclusively with thymocytes and not with peripheral T cells. The earliest thymocytes appear to bear T9 and T10 markers, or T10 alone; the T10 antigen is apparently lost when the cells leave the thymus for the peripheral compartment. It has been suggested that the $T5^+$ subset in man is analogous to the murine Lv2,3 subset, which mediates both cytotoxic and suppressor functions, and that the

Table 1
Stages of Human Thymocytes as Defined by Reactivity with Monoclonal Antibodies[a]

Cell stage	Cell type	Antibody reactivity
I. Early thymocyte	Thy1	$T10^+$
	Thy2	$T10^+$, $T9^+$
	(Thy3)[b]	
II. Common thymocyte	Thy4	$T10^+$, $T6^+$, $T4^+$, $T5^+$, $T8^+$
	(Thy5)[b]	
	(Thy6)[b]	
III. Mature thymocyte	Thy7	$T10^+$, $T1^+$, $T3^+$, $T4^+$
	Thy8	$T10^+$, $T1^+$, $T3^+$, $T5^+$, $T8^+$

[a]Adapted from Reinherz, E. L., and S. F. Schlossman (1980), *Cell* 19, 821.
[b]Hypothetical transition cell between stages.

human $T4^+$ subset is analogous to the murine Ly1 subset, which has helper functions (Cantor and Boyse, 1977b). The $T4^+$ and $T5^+$ subsets make up about 80–90% of the peripheral blood T cells; the remainder appear to be equivalent to the JRA^+ subset defined by autoantisera, which probably has feedback regulatory function (Strelkauskas et al., 1978). Monoclonal antibodies with specificities similar to those of the anti-T reagents are now available commercially from several manufacturers (anti-OKT, anti-Leu).

These anti-T cell antisera have already been applied clinically to human immunodiagnosis in, for example, acute T cell leukemias (Reinherz et al., 1979, 1980b). In immune deficiency diseases, the monoclonal anti-T reagents have been used successfully in the differential diagnosis of patients with acquired agammaglobulinemia owing to (a) lack of B cells, (b) deficiency of helper T cells, or (c) excess of activated suppressor T cells (Reinherz and Schlossman, 1980). In addition it is possible that antisera to specific functional subsets of T cells, if derived from human–human hybridomas (Croce et al., 1980), could prove useful as immunotherapeutic

agents for selective removal of regulatory subsets in patients with autoimmune or immunodeficiency diseases due to immunoregulatory defects.

4.3. Functional Assays

4.3.1. Lymphocyte DNA Synthesis by ^{3}H-Thymidine Uptake The most widely used assay for lymphocyte function in vitro has been the measurement of "blast transformation" after stimulation with mitogen or antigen. Originally, the transformation of stimulated lymphocytes in culture was determined morphologically by the appearance of blast cells, but the development of quantitative assays of incorporation of radioisotope-labeled precursors has made these assays more reliable and easier to standardize (Douglas, 1971). In general, the thymidine incorporation assay is performed with leukocytes isolated by unit gravity sedimentation over dextran or Ficoll-Hypaque, followed by removal of neutrophils by adherence to plastic. Optimal DNA synthesis response to mitogens is usually seen within 2–4 days of incubation, whereas the response to antigenic stimulation requires somewhat longer, about 4–7 days. In most laboratories, microassay techniques (Oppenheim and Schechter, 1980) are used. Results are expressed either as counts per minute (absolute counts) or as a stimulation index comparing the counts in stimulated and unstimulated cultures.

Lymphocyte DNA synthesis assays have been used both for the evaluation of cell-mediated immune function in immunodeficient patients and for monitoring the effects of therapy (e.g., Kazimiera et al., 1973; Lawrence, 1974; Rotter and Trainin, 1975). However, in recent years a number of investigators have found that assays for mediator production (especially LIF; see below) are in general more sensitive indicators of CMI status and apparently provide a better correlation with in vivo skin test results (Felsburg et al., 1976; Wilson et al., 1979; Fudenberg et al., 1980) for evaluation of antigen-specific responses.

4.3.2. Lymphocyte Protein Synthesis by ^{3}H-Leucine Incorporation This technique is generally similar to the assay for lymphocyte DNA synthesis, but requires a shorter incubation time. For the most part it is considered to be less sensitive than DNA synthesis assays (Oppenheim and Schechter, 1980).

4.3.3. Assays for Suppressor Cell Function Spontaneously occurring suppressor cell activity in unseparated leukocytes

is usually assayed by changes in B cell responses to pokeweed mitogen (PWM) or other mitogens, as measured by enumeration of plaque-forming cells (Fauci and Pratt, 1976; Gronowicz et al., 1976; Ginsburg et al., 1978), enumeration of cells containing intracytoplasmic immunoglobulin (Cooper et al., 1971), or quantitation of immunoglobulin secreted into the culture supernatant (Munoz et al., 1980). Co-culture of separated lymphocyte subpopulations with normal allogeneic cells is used to determine the spontaneous suppressor activity of the test cells (Haynes and Fauci, 1978).

It has been shown that exposure to con A or to sodium periodate results in the induction of suppressor cell activity for B cell function in unseparated leukocyte cultures (Schwartz et al., 1977; Haynes and Fauci, 1977; Galanaud et al., 1980; Goust et al., 1981). The above assays can be applied for measurement of suppressor cell activity after a suitable induction period.

4.3.4. Macrophage Migration Inhibitory Factor (MIF)

When sensitized lymphocytes are activated by specific antigen in vitro, they produce MIF, a soluble mediator of CMI that inhibits macrophage migration (David et al., 1964). Failure to produce MIF in response to antigenic stimulation in vitro indicates lack of sensitization of the donor to that antigen. Assays for MIF production generally measure the migration of indicator cells, usually guinea pig peritoneal macrophages, in capillary tubes. Two variations are in wide use: in the direct or one-step method, lymphocytes from the donor to be evaluated are mixed with the indicator cells in capillary tubes and specific antigen is added, and MIF production is assessed by measuring the inhibition of migration of the indicator cells after 24 h compared with that in controls in the absence of antigen; in the indirect or two-step method, donor lymphocytes are first incubated with antigen for 1–2 days, and the cell-free culture supernatant is then added to the indicator cells in capillary tubes.

Although the MIF assay has been used to detect cell-mediated immune defects in a variety of clincial situations (e.g., Rocklin, 1974), and has been shown to correlate with clinical response in immunodeficient patients following immunotherapy (Fudenberg et al., 1974), it has several disadvantages. Because it is difficult to obtain sufficient quantities of human monocytes as indicator cells, guinea pig cells are used, and this raises questions about the interpretation of some results; more importantly, MIF can be made by either T or B cells in humans (Rocklin et al., 1974), as well as by

nonlymphoid cells (Tubergen et al., 1972; Papageorgiou et al., 1972), and the significance of this mediator for in vivo CMI is not clear.

4.3.5. Leukocyte Migration Inhibitory Factor (LIF) Another mediator of CMI secreted by activated lymphocytes is LIF, which selectively inhibits the random migration of polymorphonuclear neutrophils (PMN). Unlike MIF, LIF is not secreted by nonlymphoid cells nor by B cells in the absence of monocytes or T cells (Bigazzi, 1979; Rasanen et al., 1979; Bergstrand, 1979). LIF production by stimulated lymphocytes is measured by leukocyte migration inhibition (LMI) assay. The first human LMI technique, described by Bendixen and Soborg (1969), employed capillary tubes of peripheral blood lymphocytes placed in chambers containing medium and antigen. This capillary tube method was later replaced by a direct method in which leukocytes were mixed with antigen in wells cut into semi-solid agarose in a Petri dish (Clausen, 1971). As with the MIF assay, an indirect or two-step method using supernatants from stimulated leukocytes has also been developed. More recently, an agarose microdroplet technique has been introduced (McCoy et al., 1977), which allows better quantitation.

The LMI assay is generally regarded as the most sensitive parameter available for measurement of antigen-specific CMI. The results have been shown to correlate with in vivo skin test results (Felsburg et al., 1976; Wilson et al., 1979), and the greater sensitivity of the LMI assay is suggested by the fact that in subjects repeatedly exposed to small amounts of antigen for skin testing, LMI results may indicate lymphocyte responsiveness to the antigen even while skin tests remain negative (Ramsey et al., 1977). LIF production in response to antigen or mitogen may be impaired or absent in primary immunodeficiencies involving defects of CMI, and defective LIF production in response to specific antigen has been demonstrated in several cases of "antigen-selective" immune deficiencies associated with recurrent severe infections with a single organism (Metcalf et al., 1981).

The LIF assay has also been used to test the effects of immunotherapeutic agents on patients' cell in vitro, as a guide to in vivo administration. For example, dialyzable leukocyte extracts (transfer factor) have been shown to be capable of inducing *de novo* CMI in previously unresponsive lymphocytes when added with antigen in the LMI assay (Wilson and Fudenberg, 1979; Borkowsky and Lawrence, 1979), and quantitation of the responsiveness has been used for the construction of dose–response curves for the defini-

tion of "potency units" of this biological agent (Wilson and Fudenberg, 1981).

5. Clinical Immunodeficiencies

Increased susceptibility to infectious agents including mycobacteria, fungi, parasites, and viruses is clinically the prominent feature of T cell deficiencies. There is considerable variation in the severity and extent of the diseases, ranging from overwhelming infections with ordinarily benign agents such as cytomegalovirus in severe deficiencies of CMI to localized fungal infections in more subtle forms of T cell disorders. Graft-vs-host (GVH) reactions of varying degree after transfusion of allogeneic cells may be the first manifestation of defective CMI. In addition to infection and GVH, there is generally a markedly increased prevalence of lymphoproliferative malignancies in such patients (Fudenberg, 1968, 1971; Fudenberg et al., 1981). Autoimmunity may be another manifestation of defective CMI and may in addition directly contribute to T cell failure (e.g., by formation of lymphocytotoxic antibodies). The clinical phenomenology of defective T cell immunity is, therefore, very heterogeneous and complex.

In the past decade attempts have been made to replace the descriptive clinical classification of defective T cell immunity by a more functional classification. Such a classification is based on the better understanding of the maturation and differentiation pathway of T cells experimentally obtained in the murine model, and subsequently in humans (see above). Such an approach has been made possible by the definition of surface markers differentially displayed by T cells during maturation. In the following discussion clinical immune deficiencies will be classified into three major categories, namely, clinical manifestation of immunodeficiency related to (1) failure of prethymic T cell development, (2) intrathymic failure of maturation, and (3) postthymic T cell failure, or defects of regulation.

5.1. Prethymic Failure of T Cell Differentiation

This heterogeneous group of diseases includes all forms of severe combined immunodeficiency (SCID) syndromes. There may be agenesis of hematopoietic stem cells including lymphoid and myeloid cell lines at one end of the extreme and (theoretically) selective T cell agenesis at the other end.

5.1.1. Reticular Dysgenesis This most severe form of SCID was first described by DeVaal and Seynhaeve (1959). The disorder is characterized by a developmental defect of hematopoietic stem cells with agenesis of T and B lymphocytes as well as granulocytes in the bone marrow. Erythropoiesis and thrombopoiesis usually are normal. Death due to failure of specific (CMI and humoral immunity) and nonspecific (phagocytosis) mechanisms of host defense usually occurs within the first days of life. In some cases suppressor T cells appear to suppress the maturation of stem cell precursors.

5.1.2. Developmental Defect of T and B Cells This form of SCID has been named the "Swiss Type" since it was first described by the Swiss authors Glanzmann and Riniker (1950). The absence of lymphoid stem cells characterizes this syndrome, whereas the development of other hematopoietic stem cells usually is not affected. As in reticular dysgenesis, there is virtual absence of T and B cells, with a dysplastic thymus without Hassall's corpuscles and, of course, depletion of lymphoid elements in the peripheral lymphoid organs. Agammaglobulinemia may also be seen. An autosomal recessive and an X-linked form of inheritance have been described.

In about 20% of the cases, this form of SCID is associated with adenosine deaminase (ADA) deficiency, which is inherited as an autosomal recessive defect (Giblett et al., 1972). ADA catalyzes the conversion of adenine to inosine. Children with ADA deficiency lack both the low-molecular-weight and various high-molecular-weight forms of the enzyme, yet have normal levels of the "conversion protein." There is experimental evidence that the failure of lymphoid cell development results from toxic effects caused by the intracellular accumulation of adenosine or adenosine nucleotides (Coison et al., 1979). A small but unknown percentage of SCID is associated with purine nucleophosphorylase (PNP) deficiency (Giblett et al., 1975; Coison et al., 1979), another enzyme of the purine metabolism that catalyzes the conversion of inosine to hypoxanthine. A similar toxic effect of purine metabolites is suggested. B cell function is less severely affected than T cell function in the enzyme deficiency syndromes in SCID.

5.1.3. Developmental Defect of T Cells Nezelof et al. (1964) first described a 14-month-old child with a lifelong history of infections clinically resembling SCID, with the exception that the child had normal levels of serum immunoglobulins. Subsequently

other patients with "Nezelof syndrome" have been reported (e.g., Buckley et al., 1967; Rubenstein et al., 1971). These patients show considerable heterogeneity, and many also demonstrate deficiency of humoral immunity (impairment of antibody response, deficiency of selective Ig classes); there is usually some IgM, but levels of IgG and IgA may be low. There is experimental evidence that the B cell system may be normal, but that there is a lack of T cell help in inducing normal antibody responses by B cells (Seeger et al., 1976). The basic defect is not known. Failure of the development of progenitors of T cells and failure of thymic factors to induce T cell maturation have been discussed as theoretical possibilities. Successful grafting of bone marrow in some situations (Seligmann et al., 1974) and reconstitution of normal T and B cell function following thymic transplants (Hong et al., 1976) suggest that both theoretical possibilities may indeed occur.

Clinically, children with this form of immunodeficiency may present symptoms later in childhood; recurrent upper respiratory tract infection, otitis media, and tonsillitis may be major features. In some patients an atypical response to live virus vaccines, such as smallpox, is observed as the first symptom. Fungal infections occur in all cases. The clinical course may be indistinguishable from other forms of SCID.

5.2. Failure of Intrathymic Maturation or of Thymic Development

Normal T cell development depends on normal thymic function. Hence, any disorder affecting thymic function will be expressed as some impairment of T cell function. It can again be anticipated that the clinical phenomenology of these disorders is extremely complex. Such deficiencies may be manifested as complete failure of T cell function (such as in some forms of Nezelof syndrome) or as subtle impairment of immunoregulation. There are some classical forms of immunodeficiency within this group, such as the DiGeorge syndrome and ataxia telangiectasia. As mentioned above, some forms of the Nezelof syndrome may also belong to this group. In addition, some forms of CVID with hypogammaglobulinemia owing to excessive suppressor activity of T cells may be classified here.

5.2.1. The DiGeorge Syndrome

This disorder, first described by DiGeorge (1965) as congenital hypoparathyroidism with tetany and associated infection, results from abnormal embryogen-

esis of the third and fourth pharyngeal pouches during the first three months of gestation. This faulty development leads to abnormalities of the ears and face (hypertelorism, antimongoloid eye slant, peculiar form of the mouth), congenital heart disease, and hypoplasia of the parathyroids and thymus.

The thymic abnormality, which is the important pathogenetic factor for the defective CMI, is variable ranging from hypoplastic thymus to thymic agenesis (Lischner and DiGeorge, 1969). There is usually a normal number of peripheral blood lymphocytes, but enumeration of T cells shows marked reduction. A depletion of the thymus-dependent areas in the peripheral lymphoid organs is characteristic, and the extent of in vivo and in vitro defects of T cell immunity is heterogeneous; humoral immunity seems to be normal, although some patients fail to mount a normal antibody response to selected antigens. The results of therapeutic trials with cultured thymic epithelium support the concept that the major pathogenetic defect is the absence of intrathymic T cell maturation (Wara et al., 1975).

Clinically, the earliest symptom is usually hypocalcemic tetany after birth. Heart disease, such as Fallot's tetralogy, may be another non-immunological complication. Recurrent infection primarily of the upper respiratory tract, diarrhea, and failure to thrive characterize the clinical evolution of this disorder. The children are at high risk of sudden death.

5.2.2. Ataxia Telangiectasia

Ataxia telangiectasia is characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, and recurrent upper respiratory tract infections. There is a high incidence of neoplastic diseases. In the first description of the syndrome by Syllaba and Henner (1926), its multisystem character was recognized. Their patients, two males and one female, suffered in addition to the symptoms mentioned above from athetosis, muscular weakness, goiter, genital hypoplasia, and pigmentation of the skin. Boder and Sedgwick (1957) characterized the disease entity, now designated ataxia telangiectasia. Since that time the multisystem character of this disorder has repeatedly been confirmed, with symptoms including ovarian dysgenesis, testicular atrophy, other endocrine disorders such as pituitary failure, and abnormalities of carbohydrate metabolism consisting of glucose intolerance and insulin resistance. Signs of autoimmunity (Ammann and Hong, 1971), including autoantibodies to insulin receptors (Bar et al., 1978), may be present and contribute to the endocrine disorder. Chronic sinopulmonary infection in ataxia telangiectasia may

ultimately lead to structural alteration of lung architecture with respitory insufficiency. Malignancy appears in about 10% of the patients, primarily lymphoproliferative malignancies and brain tumors.

The immunological deficiency involves both T and B cell immunity, varying from patient to patient and also within the same patient. Low or deficient levels of serum IgA and IgE are detected in up to 80% of the patients studied. IgG is usually within normal limits. Monomeric IgM is present in up to 80% and may be responsible, for technical reasons, for the reported elevation of serum IgM levels (Stobo and Tomasi, 1967). T cell immunity is consistently impaired, but the extent of impairment is variable. A consistent feature is the absence or hypoplasia of the thymus, usually without Hassall's corpuscles. Lymphoid depletion of the central and peripheral lymphoid organs is typical.

The underlying pathogenetic mechanism in not known. The thymic abnormality is unlikely to account for the multisystem character of the disease. Recently, defective DNA repair mechanisms have been observed in two patients (Kraemer, 1977). This observation may explain the high frequency of spontaneous chromosomal breaks, the sensitivity to irradiation, and the development of cancer, as well as CMI deficiency in ataxia telangiectasia, and the hypothesis of faulty tissue differentiation suggested by Auerbach (1960) may be related to impaired DNA repair mechanisms.

5.3. Defects of Regulation

In a number of patients with common variable hypogammaglobulinemia or with isolated Ig deficiencies, increased suppressor T cell activity has been demonstrated (Waldmann et al., 1978). Comparable suppression of Ig production has been observed in patients with hypogammaglobulinemia and thymoma, as well as in some children with the X-linked form of agammaglobulinemia (Waldmann et al., 1974, 1978; Siegal et al., 1976; Dosch et al., 1978). Evidence for such a mechanism was obtained from co-culture experiments and in part also from the observation by Soothill et al. (1968) that corticosteroid treatment was followed by normalization of Ig levels in vivo.

Finally, a group of patients from a single family have been described whose clinical manifestations include fatal infectious mononucleosis, aproliferative disorders, and lymphoproliferative disease (Purtilo et al., 1979). This "new" syndrome, designated X-linked recessive lymphoproliferative syndrome (XLRLS) or Duncan syn-

drome, appears to be related to altered lymphocyte populations and, possibly, a defect in natural killer cells (Sullivan et al., 1980).

6. Conclusion

The above classification of cellular immune defects in terms of T cell maturation has been made possible by relatively recent advances in our understanding of the cellular components of the human immune system, due largely to the development and application of the in vitro methods described earlier in this chapter. As further technical advances are made, it is likely that this classification will again be superseded. At present it appears that many of these clinical disorders will ultimately be attributed to aberrations in immunoregulatory mechanisms. When such mechanisms are understood in more detail, further refinements in methods for evaluation of patients with defective CMI should result. More importantly, approaches to therapy of such patients should become both more specific and more effective.

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