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PREFACE

The progress of cellular immunology from descriptive phenomonology to the analysis of events in molecular and mechanistic terms is dependent in large part on the development of new technologies and new experimental systems. The five articles in this volume all deal to a greater or lesser extent with such developments and the new insights they have provided. They include (1) new methods for identifying major histocompatibility complex (MHC) determined leukocyte antigens and methods for the efficient detection and quantitation of immunoglobulins, (2) experimental activation of B cells and the use of lymphoid tumor cells to provide insights into normal cellular development and function, and (3) analysis of a hapten-induced immune response to reveal the intricacies of the regulatory systems controlling immune function.

In studying the development, functions, and regulatory interactions of the various kinds of lymphocytes, polyclonal activators of these cells have been invaluable. In the first chapter Waldmann and Broder review the use of polyclonal B cell activators in studying the regulation of human immunoglobulin synthesis, a field in which they have been prime movers. A thorough description of the various activators and their modes of action as well as the several techniques used to assay cellular activation is presented and critically evaluated. Since these agents differ in their actions, i.e., direct effect on B cells or action via stimulation of one or another regulatory cells, in total their use can provide the information necessary to define abnormalities in the function of both regulatory and immunoglobulin forming cells. From such studies have come clues suggesting that a variety of pathogenic mechanisms, some involving the B cells themselves and others affecting regulatory monocytes or T helper or T suppressor cells may be responsible for the disordered immunoglobulin synthesis in patients with immunodeficiency, autoimmunity, or malignancy.

Evidence of the dominant role played by the MHC in determining the immunologic potential and performance of an individual continues to accumulate. The MHC of man, the HLA system, controls two types of transplantation alloantigens (the HLA-A,B,C and the HLA-D/DR antigens) as well as some components of complement, immune response determinants, and susceptibility to a variety of diseases. The HLA-D/DR region is of particular interest since it appears to control early events of the immune response. In the second chapter Morling,

Jakobsen, Platz, Ryder, Svejgaard, and Thomsen review the biochemistry, genetics, and immunology of the HLA system and discuss the methods currently employed to define it. A new technique they have pioneered, i.e., primed lymphocyte typing (PLT) utilizing selected responder cells primed to homozygous typing cells, is presented and its advantages in defining D/DR and related antigens outlined. PLT has facilitated the typing of rare DR antigens, has permitted the detection of new DR antigens, and has led to the definition of a new set of DR-related antigens apparently coded for by genes located between the HLA-D/DR and GLO loci. Because of the versatility in application of PLT and recent technological refinements such as the development of monoclonal cell lines, this procedure should play a key role in the further unraveling of the D/DR region and its role in immunologic function.

Among the most useful reagents for the detection, quantitation, and isolation of immunoglobulins are the bacterial immunoglobulin receptor proteins particularly protein A of *Staphylococcus aureus*. In the third chapter Langone discusses this subject critically and in detail. The sources, methods of isolation, and the physicochemical properties of Staph A protein are described as well as its reactivity with the various Ig classes and subclasses from a variety of species. The biochemical characteristics of the interaction between Staph A and the Ig molecule are discussed thoroughly. Staph A also provides a versatile experimental tool for activation of humoral mediators of immunologic inflammation and stimulation of lymphocytes, both presumably via its interaction with the Fc portions of Ig heavy chains. Finally, the numerous analytical applications of Staph A protein to the measurement of a host of immunologic substances and events, i.e., Ig, antigen–antibody reactions, and immune complexes, to name a few, are described. This chapter provides important background and technical detail for anyone employing this valuable investigative tool.

The complexity of mechanisms regulating the immune response is being revealed primarily as a result of the exhaustive analyses of a few specific responses. In the fourth chapter Greene, Nelles, Sy, and Nisonoff present their findings concerning regulation of the response to the azobenzenearsonate hapten and compare them to related studies by others using different antigens. The suppressor control system emerging from these studies includes at least three separate sets of T suppressor cells interacting in large part via idiotype and antiidiotype events. The first cell (Ts_1) is activated by ligand and is idiotype positive, the second (Ts_2) is activated by Ts_1 or its factor and is antiidiotypic, the third (Ts_3) is activated by one or more factors from Ts_2 and is

idiotype positive. Although the interactions among the three sets of Ts cells have both H_2 and Igh restriction, the Ts_3 cell, once activated, appears to mediate its suppression in a relatively nonspecific mode. The possibility is raised of an extension of this suppressor system in which a parallel system initiated not by ligand but by antiidiotype might exist and cells of the two sets even interchange. Such a suppressor mechanism could operate both in humoral and in cellular immune responses.

In the last chapter Abbas reviews the use of lymphoid tumor cells in the study of normal lymphocyte biology. The diversity of the lymphoid system makes it particularly difficult to isolate and analyze uniform populations of lymphocytes. Lymphoid tumor cells offer a particularly good source of large numbers of near monoclonal lymphocytes, which in many instances appear to be reasonable counterparts of lymphoid cells in one or another stage of development or function. B cell tumors have provided examples of pre-B cells, nonsecreting B cells, and secreting B cells as well as subjects for the study of antigen presentation by B cells. T cell tumors have provided sources of T regulatory factors and targets for analysis of T cell interactions. Fusion-derived lines have produced a variety of effector factors and models for regulatory cellular interactions. The potentials and the pitfalls associated with this experimental approach to the study of lymphocyte differentiation and regulation are carefully evaluated in the light of the author's considerable first hand experience, and the subject is put into excellent perspective.

The editors wish to thank the authors of these excellent reviews for the considerable care, time, and effort they have devoted to their preparation. Finally, it is a pleasure to acknowledge the cooperation and assistance of the publishers, who have done much to ensure the quality of this series of volumes.

FRANK J. DIXON
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Polyclonal B-Cell Activators in the Study of the Regulation of Immunoglobulin Synthesis in the Human System

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I. Introduction

Many of the great advances in our understanding of the regulatory mechanisms that control human immunoglobulin synthesis have

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emerged from studies that have utilized polyclonal B-cell activators. These studies have been of value in defining the stages of differentiation of the cells of the B-cell series and in determining the role played by networks of immunoregulatory T cells and macrophages in the control of these maturational events. These studies have also brought to light new pathogenic mechanisms that underlie certain forms of primary immunodeficiency disease as well as autoimmune, malignant, and allergic disorders. Finally, they are providing the scientific basis for the development of new rational strategies for the treatment of these diseases.

The development of antibody-producing plasma cells from bone marrow stem-cell precursors involves a series of stages. The first identifiable stage involves a hierarchical rearrangement of the genes coding for the variable, diversity, and joining segments of the immunoglobulin heavy-chain genes (Sakano *et al.*, 1979; Early *et al.*, 1980). Following these initial rearrangements that affect the genes coding for heavy chains, there are rearrangements of the variable and joining segments of light-chain genes with kappa gene rearrangements preceding lambda ones (Hozumi and Tonegawa, 1976; Sakano *et al.*, 1979; Seidman *et al.*, 1979; Heiter *et al.*, 1980; Korsmeyer, 1981). After these rearrangements effective genes capable of inducing the synthesis of a particular heavy and a particular light chain are produced. As these changes progress at a DNA level, the stem cell matures into a pre-B cell with mu chains demonstrable in its cytoplasm and then into a B cell with surface markers characteristic of this cell (Cooper *et al.*, 1979). These markers include immunoglobulins acting as receptors for antigens. The union of appropriately presented antigen with surface immunoglobulin receptors triggers subsequent events, which include B-cell proliferation and terminal differentiation into antibody-synthesizing plasma cells. This process of B-cell maturation can also be activated in primates by the Epstein–Barr virus (Bird and Britton, 1979; Kirchner *et al.*, 1979; Rosen *et al.*, 1977) and by a series of plant and bacterial products termed polyclonal B-cell activators (Andersson *et al.*, 1972; Sultzer and Nilsson, 1972; Coutinho and Möller, 1973, 1975; Wu *et al.*, 1973; Waldmann *et al.*, 1974; Bona *et al.*, 1974; Dörries *et al.*, 1974; Möller, 1979). Many antigens and most, but not all, polyclonal activators require the presence of cooperating helper T cells and macrophages as well as the B cells to induce a maximal immunoglobulin response (Miller and Mitchell, 1968; Waldmann, 1977). More recently it has been recognized that certain monocytes and especially a network of T cells termed suppressor T cells act as negative regulators of B cells inhibiting the terminal matu-

ration of these B cells, thus inhibiting immunoglobulin synthesis (Gershon, 1974; Waldmann and Broder, 1977). It appears that the cells of the suppressor T-cell network emerge from the thymus as prosuppressor cells. These prosuppressor cells require an interaction with an inducer T cell as well as with an antigen or, alternatively, with a polyclonal activator to develop into final effectors of suppression (Waltenbaugh *et al.*, 1977; Feldman *et al.*, 1977; Tada *et al.*, 1978; Eardley *et al.*, 1978; Broder and Waldmann, 1978).

The majority of studies of immunoglobulin regulation *in vitro* in animal systems have examined the production of specific antibodies by immunocompetent cells stimulated by antigen *in vitro*. In contrast, the majority of studies in human systems have involved the use of polyclonal B-cell activators, since assays of antigen-induced specific antibody synthesis by the peripheral blood mononuclear cells *in vitro* in the absence of such activators have proved to be difficult in humans. Indeed, techniques that are antigen specific and that require no polyclonal activators, yet can be easily reproduced by many laboratories, are only just emerging. The studies with polyclonal activators have certain limitations, but they also have certain advantages over antigen-specific systems. In many cases they are not genetically restricted and thus permit the coculture of cells from unrelated individuals, a characteristic of great value in the analysis of monocyte function and of antigen nonspecific helper and suppressor T-cell activity from patients with disordered immunoglobulin synthesis. In addition, these antigen nonspecific approaches permit the definition of antigen nonspecific functions of clonal populations of regulatory cells, such as T-cell leukemias with retained helper or suppressor activity, without requiring that the particular antigen that is recognized by the antigen-specific T-cell receptor be identified. In addition, this approach has permitted the analysis of the activity of antigen-nonspecific helper and suppressor products produced and secreted by human mononuclear cells, cloned human T-cell lines, and human T-T-cell hybridomas.

In many of these studies of human cells pokeweed mitogen (PWM) has been utilized as the polyclonal B-cell activator. As noted below, the initiation of B-cell differentiation by PWM is a complex event requiring the presence of helper T cells and accessory cells. In addition, in certain circumstances PWM appears to activate prosuppressor T cells into effectors of suppression that inhibit the maturation of B cells. These characteristics are of value in many cases in understanding the cellular interactions involved in B-cell maturation, but in other cases these properties may make the interpretation of any disorders

observed with this system in health, and especially in disease, complex. For this reason many workers have studied other polyclonal activators of B cells, including those that differ from PWM in terms of their relative dependence on helper T cells and on accessory cells, and in terms of their propensity to activate prosuppressor cells into effectors of suppression (Bona *et al.*, 1974; Forsgren *et al.*, 1976; Bird and Britton, 1979; Möller, 1979; Montazeri *et al.*, 1980). In the present review, we will consider the different polyclonal B-cell activators that have been proposed in terms of these characteristics. We will emphasize our own experience in this area and will outline our views as to how the array of available polyclonal activators of human B cells can be used to develop rational approaches for categorizing disorders of human immunoglobulin synthesis as being due to intrinsic defects of B cells, as being due to disorders of helper T cells or accessory cells, or as being due to abnormalities of the suppressor cell network.

II. Techniques Used to Assess the Polyclonal Activation of B Cells into Immunoglobulin- and Antibody-Producing Cells

A variety of *in vitro* techniques have been developed to study polyclonal activation of human B cells. These techniques include procedures that measure B-cell proliferation and those that are directed toward quantitating immunoglobulin production by B cells as assessed by a variety of methods. In some methods the proportion of cells with immunoglobulin in their cytoplasm is determined. In other approaches immunoglobulin- or antibody-secreting cells are enumerated. Alternatively, the quantity of immunoglobulin or antibody synthesized and secreted into the culture media is determined. One of the earliest approaches to the analysis of B-cell activation was the study of B-cell proliferation assessed by demonstrating blastic transformation of these cells or by quantitating their thymidine uptake after exposure to polyclonal activators (Douglas *et al.*, 1967; Phillips and Roitt, 1973; Greaves *et al.*, 1974; Janossy *et al.*, 1976; Brochier *et al.*, Montazeri *et al.*, 1980). Many materials that activate B cells directly or indirectly also stimulate T-cell proliferation; therefore procedures must be used to limit the cellular proliferation in the cultures to the B cells. For helper T cell-independent polyclonal activators, the proliferative responses of rigorously T-cell-depleted mononuclear cell populations may be examined. When activators that require the presence of inducer T cells for B-cell activation are being studied, any T cells added to the system must be irradiated or be treated with an agent such as mitomycin C to prevent their proliferation. Moreover, one

must recognize that proliferation per se does not measure the terminal differentiation of B cells into plasma cells.

The demonstration of immunoglobulin in the cytoplasm of mononuclear cells cultured in the presence of polyclonal B-cell activators has also been used to define agents capable of activating B cells into immunoglobulin-synthesizing cells (Cooper *et al.*, 1971; Wu *et al.*, 1973). This has usually been studied using antibodies labeled with fluorochromes to determine the proportion of cultured cells with immunoglobulin in their cytoplasm at the end of the culture period. However, in other cases the uptake of radiolabeled precursors into cytoplasmic immunoglobulins (Geha *et al.*, 1974; Choi, 1977; Geha, 1979) or the quantitation of cellular immunoglobulin content by sensitive radioimmunoassays has been used in these analyses (Korsmeyer *et al.*, 1981). The approach using antibodies labeled with fluorochromes is relatively easy to establish but has certain limitations. It is a tedious procedure that is less sensitive than other approaches that measure immunoglobulin synthesis. It has a subjective component in the analysis of the proportion of positive cells and numerous potential causes of false positive results exist. It should be noted that the proportion of cells in a culture assayed as immunoglobulin-containing cells by this procedure usually far exceeds the proportion identified as immunoglobulin-secreting cells by the reverse hemolytic plaque technique discussed below. Despite these limitations this technique has been used successfully to obtain important information concerning B-cell activation and to define the nature of disorders in patients with abnormalities of immunoglobulin production (Wu *et al.*, 1973).

The Jerne hemolysis in gel plaque assay has been modified to determine the number of B cells that mature on stimulation into immunoglobulin-secreting cells by a so-called reverse hemolytic plaque assay or into specific antibody-secreting cells by direct plaque-forming-cell assays. The reverse hemolytic plaque assay may be used to determine the total number of cells in a culture producing and secreting immunoglobulin or, by modification, the number of cells secreting immunoglobulins of a particular isotype (Eby *et al.*, 1975; Gronowicz *et al.*, 1976; Friedman *et al.*, 1976; Ginsburg *et al.*, 1978; Kirchner *et al.*, 1979). Typically, in this procedure the lymphocytes obtained at the termination of the culture period are mixed with erythrocytes coated with protein A or with an appropriate antibody that is directed to human immunoglobulins. These cell mixtures are then incorporated into an agar layer. After a brief incubation period the plaques are generally developed by the sequential addition of antisera to human im-

munoglobulin of the isotype being examined and then complement. The reverse hemolytic plaque assay is a sensitive method that permits the determination of the proportion of cells producing immunoglobulin of a particular class rather than a particular antigenic specificity at a particular time point in the culture. In an alternative approach the antigen-specific antibody response of B cells polyclonally activated *in vitro* can be assessed with a direct plaque-assay system using sheep red cells or other erythrocyte targets (Fauci and Pratt, 1976a; Fauci and Ballieux, 1979; Fauci, 1979). This approach has the advantage of measuring the portion of a polyclonal immune response that is directed toward a specific antigen, but it yields relatively small numbers of plaques when compared to the reverse plaque assay and may not yield any plaques when the cells of some normal individuals are utilized.

Another approach used to analyze B-cell activation, the one that we have used most frequently in our own studies, is to quantitate the immunoglobulins or antibodies synthesized and secreted into the culture media by mononuclear cells stimulated by polyclonal activators (Waldmann *et al.*, 1974; Platts-Mills and Ishizaka, 1975; Stevens and Saxon, 1978). In this procedure the starting cell population is extensively washed, and the immunoglobulins synthesized and secreted into the medium by the mononuclear cells activated *in vitro* are then quantitated by sensitive double-antibody radioimmunoassay procedures (Waldmann *et al.*, 1974) or by ELISA procedures (Kelly *et al.*, 1979). Alternatively, specific antibody that is secreted by such cells can be measured by solid-phase radioimmunoassay or enzyme-linked immunoassay approaches (Stevens and Saxon, 1978). The limitations of these approaches are that they are more difficult to establish than are certain of the procedures discussed above and that they cannot be performed in the presence of human serum or other biological materials containing the human immunoglobulin molecules being studied. In addition, they cannot be used when the polyclonal B-cell activators being studied catabolize or bind the secreted human immunoglobulins. This latter problem comes into play when agents such as staphylococcal protein A or staphylococcal organisms of Cowan strain I, which produce protein A, are being studied, since these agents bind most IgG and certain IgM molecules.

The procedures involving assays of secreted immunoglobulins do, however, have certain significant advantages when compared to other approaches. They are not subjective, and they are very sensitive. They permit the quantitative analysis of the product of small numbers of immunoglobulin-secreting cells. This feature is quite important when

analyzing cocultures for suppression of immunoglobulin synthesis or when quantitating the production of a class of immunoglobulin such as IgE where small amounts of immunoglobulin are produced by cells in culture. Furthermore, this procedure can be used to determine the immunoglobulin or antibody secreted into the media throughout any time period of the culture (for example, from the onset to the termination of the culture period) and, therefore, is not subject to the problems of either plaque or cytoplasmic immunoglobulin analysis that determine immunoglobulin production at a specific time in the culture system. With these latter procedures, alternations in the time course of B cell activation in disease states may be incorrectly interpreted as alterations of the number of B cells that can be activated.

The various procedures for analysis of B-cell activation (that is, B-cell proliferation, immunoglobulin secretion at a cellular level, and the total immunoglobulin synthesized and secreted by the cells in culture) can be viewed as complementary techniques that measure different facets of B-cell activation. As noted below, each of the techniques can be modified so that it can be used to analyze the role of regulatory cells in controlling B-cell activation, including the definition of the requirement for monocytes and inducer T cells for a positive response and the determination of the role of inhibitory monocytes or of suppressor T cells as inhibitors of B-cell activation. Although a thorough analysis of the fine details of different techniques for B-cell activation is beyond the scope of this review, it must be emphasized that relatively modest variations in the technique used by different laboratories (for example, in the number of monocytes added to the system, in the nature of the serum used, in the shape of culture vessel employed, in the concentration of lymphocytes added to the system, or in the exposure of the T cells added to the system to ionizing irradiation) may produce marked variations in the results obtained. Certain of these variables will be discussed in the subsequent sections of this review when they are relevant to the issues being considered. An unrecognized variation in technique (such as using round as opposed to flat culture surfaces) can result in discordant results between workers in different laboratories.

III. Ability of Different Agents to Stimulate Human Peripheral Blood B Cells to Develop into Immunoglobulin-Producing and -Secreting Cells

Many agents have been studied to determine their capacity to function as polyclonal activators of normal human B cells. In our own studies of different potential B-cell activators, we compared the quantity

of IgG, IgA, and IgM synthesized and secreted into the medium by 2×10^6 unseparated mononuclear cells cultured for 7 or 12 days in flat-bottom vials with 1 ml of medium in the presence of various agents for each individual with the quantity of immunoglobulin secreted by an aliquot of the same population of cells cultured in medium containing fetal calf serum alone.

Peripheral blood mononuclear cells of normal individuals synthesized modest quantities of immunoglobulins when cultured in fetal calf serum in the absence of other polyclonal B-cell activators. In our studies of 17 normal individuals, the geometric means for synthesis of IgG, IgA, and IgM were 160, 226, and 214 ng per $\times 10^6$ lymphocytes, respectively, over a 7-day culture period. Immunoglobulin synthesis continued beyond day 7 despite the fact that cultures were neither fed nor rocked, so that by day 21 of culture the means for the total synthesis from the onset to the termination of the culture for IgG, IgA, and IgM were, respectively, 1073, 646, and 1622 ng per 2×10^6 lymphocytes in culture.

One of the most important and widely studied polyclonal activators used in evaluating the *in vitro* biosynthesis of human immunoglobulins is an extract of pokeweed (*Phytolacca americana*), a poisonous plant. It is possible to purify several proteins from the starting extract (Janossy *et al.*, 1976), which may individually or collectively exhibit very potent mitogenic activity for human lymphocytes. Most workers have used "pokeweed mitogen" (PWM) as a general designation for the starting extract.

In our studies immunoglobulin production by mononuclear cells was markedly stimulated when PWM was added at the initiation of the culture. In PWM-stimulated cultures, the geometric means for synthesis and secretion of IgG, IgA, and IgM were 1380, 1000, and 2344 ng per 2×10^6 mononuclear cells, respectively, for 7-day cultures and were 5888, 4786, and 7948 ng per 2×10^6 cells for 21-day cultures (Fig. 1). This represents a 4- to 11-fold increase in the mean immunoglobulin synthesis by lymphocytes by day 7 of culture for the different immunoglobulin classes in the presence of PWM as compared to the immunoglobulin synthesis by mononuclear cells cultured in medium containing fetal calf serum alone (Table I). An analysis of the time course of appearance of immunoglobulin molecules in the supernatants of cultures stimulated by PWM indicated that there was little synthesis and release of immunoglobulin by lymphocytes during the first 3 or 4 days of culture. However, after this time the concentration of immunoglobulin detectable in the culture media increased rapidly, indicating a maturation of B cells into the immunoglobulin-synthesizing and -secreting plasmacytoid cells.

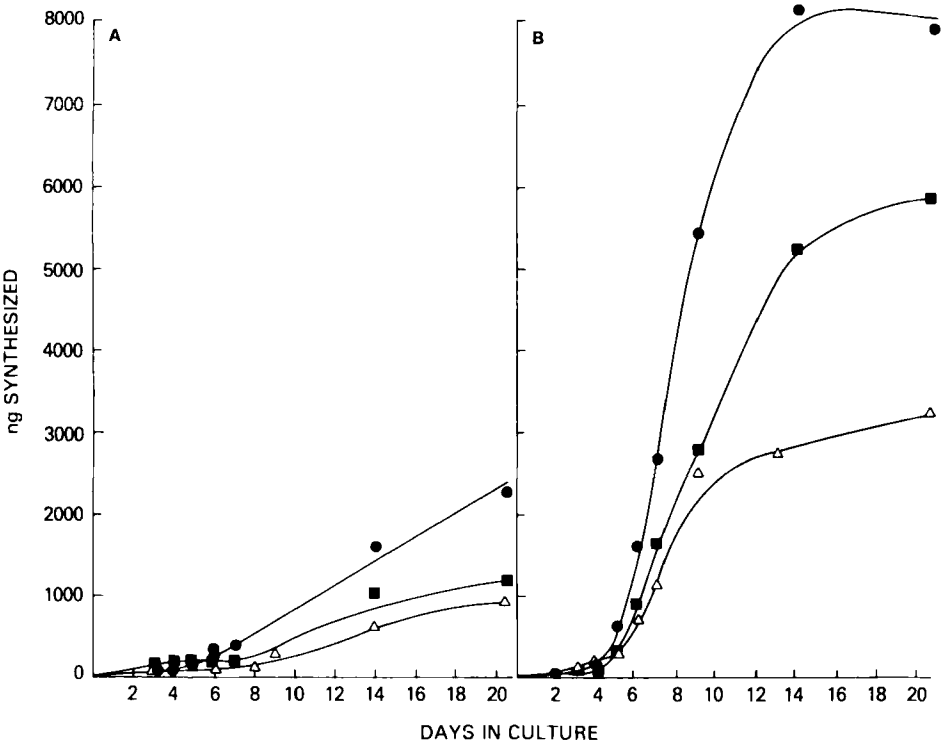

FIG. 1. Time course of synthesis of IgG (■—■), IgA (△—△), and IgM (●—●) by 2×10^6 peripheral blood lymphocytes in the presence of media containing fetal calf serum alone (panel A) or in the presence of pokeweed mitogen (panel B).

An implication of the time-course studies is that a culture period of at least 6 days should be used in human immunoglobulin biosynthesis experiments and that one can profitably extend the culture period beyond this day when one is measuring immunoglobulins synthesized from the onset to the termination of the culture by radioimmunoassays or enzyme immunoassays for immunoglobulins in the culture supernatants, since there is much greater synthesis and secretion of immunoglobulin by day 12 than there is by day 7 of culture. Such high levels of immunoglobulin in the culture supernatants are especially valuable when coculture studies to examine suppression are being performed. As noted below, the activation of B cells by PWM is a complex event requiring the presence of helper T cells and other accessory cells. In addition, in certain circumstances PWM appears to activate precursors of suppressor cells to become effectors of suppression that inhibit the maturation of B cells. These characteristics are of value in understanding the cellular interactions involved in B-

TABLE I
IMMUNOGLOBULIN SYNTHESIS BY PERIPHERAL BLOOD MONONUCLEAR CELLS STIMULATED BY POLYCLONAL ACTIVATORS

Activator[a]	IgG[b]	IgA	IgM
PWM	8.6 (1.2)*	4.4 (1.3)*	11.0 (1.3)*
Dextran sulfate			
1000 μg/ml	0.64 (1.5)	0.71 (1.4)	0.23 (1.5)
100 μg/ml	0.87 (1.6)	0.94 (1.4)	0.55 (1.5)
10 μg/ml	1.02 (2.2)	1.24 (2.3)	0.78 (2.0)
PVP			
500 μg/ml	0.92 (1.3)	0.73 (1.5)	0.91 (2.7)
Pneumococcal-polysaccharide III	0.67 (1.2)	0.72 (1.04)	0.79 (1.5)
Poly(A-U)			
300 μg/ml	1.27 (1.7)	0.98 (1.12)	1.05 (1.7)
PPD	0.58 (1.2)	0.70 (1.2)	0.64 (1.3)
Poly(I-C)			
300 μg/ml	1.31 (1.2)	0.89 (1.2)	2.29 (1.3)*
LPS			
1000 μg/ml	0.46 (1.3)	0.42 (1.3)	0.56 (1.5)
500 μg/ml	0.79 (1.2)	0.67 (1.2)	1.47 (1.5)
200 μg/ml	1.32 (1.2)	1.10 (1.04)	2.38 (1.2)*
50 μg/ml	1.34 (1.2)	1.32 (1.04)	2.32 (1.15)*
Staphylococcal organisms 10^6	—	2.07 (1.3)*	—
SLO, 100 μl of 1:3	3.52 (1.4)*	1.32 (1.2)	5.71 (1.3)*
SPL, 100 μl of 1:10	1.83 (1.3)	1.19 (1.1)	5.76 (1.3)*
EBV,	2.04 (1.3)*	1.97 (1.3)*	2.89 (1.2)*
NWSM			
10 μg/ml	2.66 (1.4)*	1.08 (1.4)	4.63 (1.4)*
50 μg/ml	8.50 (1.29)*	2.46 (1.5)*	11.9 (1.3)*
100 μg/ml	9.35 (1.3)*	4.65 (1.4)*	27.3 (1.1)*

[a] PWM, pokeweed mitogen; PVP, polyvinylpyrrolidone; poly(A-U), polyadenylic–polyuridylic acid; PPD, purified protein derivative; poly(I-C), polyinosinic–polycytidylic acid; LPS, lipopolysaccharide; SLO, Bacto-streptolysin O reagent; SPL, staphylococcal phage lysate; EBV, Epstein–Barr virus; NWSM, *Nocardia* water-soluble mitogen.

[b] Ratio of synthesis in cultures with activator to synthesis in cultures in fetal calf serum containing medium alone. The geometric mean of the ratio is shown in each case with the relative standard error of the geometric mean in parentheses.

* Significantly increased stimulation ($p < 0.05$) as compared to culture in medium alone.

cell maturation but make the interpretation of disorders observed in disease with this system complex. We and others have, therefore, attempted to find more T-cell-independent activators of human peripheral blood B cells. A series of agents that had been shown by others to be polyclonal activators of murine splenic B cells as well as new po-

tential B cell activators were studied in the *in vitro* biosynthesis system with human peripheral blood mononuclear cells. In these studies, the synthesis of the immunoglobulins by the peripheral blood mononuclear cells of an individual in the presence of an additive was related to the synthesis of immunoglobulins by the individual's cells cultured in medium containing fetal calf serum alone (Table I).

In our studies a number of agents that had been shown by others to be polyclonal activators of murine splenic B cells (Andersson *et al.*, 1972; Coutinho and Möller, 1973; Dörries *et al.*, 1974; Janossy and Greaves, 1975) were ineffective as stimulators of immunoglobulin synthesis when added to unseparated human peripheral blood mononuclear cells *in vitro*. Specifically, when dextran sulfate at 1000, 100, or 10 μg/ml, polyvinylpyrrolidone (PVP) at 500 μg/ml, polyadenylic–polyuridylic acid [poly(A-U)] at 3 and 300 μg/ml, purified protein derivative (PPD) at 100 μg/ml or pneumococcal polysaccharide type III at 150 μg/ml or antibodies to β_2-microglobulin were added to the cells in culture, there was no significant increase in the quantity of immunoglobulins synthesized. That is, for the 6–10 normal individuals studied the ratio of immunoglobulin synthesized in the presence of the additive as compared to that synthesized in the presence of fetal calf serum alone was not significantly greater than one.

Lipopolysaccharide (LPS), an excellent stimulator of polyclonal immunoglobulin synthesis and proliferation of B cells in the mouse spleen (Andersson *et al.*, 1972), did not stimulate IgG or IgA synthesis at any concentration utilized (50, 100, 500, 1000 μg/ml) when added to unseparated mononuclear cells. However, there was a modest (2.3-fold) but statistically significant ($p < 0.05$) stimulation of IgM synthesis at a concentration of 50 and 100 μg/ml but not at higher LPS concentrations. The pattern of immunoglobulin synthesis was similar with the agent polyinosinic–polycytidylic acid [poly(I-C)] used at 300 μg/ml, where there was a modest but statistically significant ($p < 0.05$) stimulation of the synthesis of IgM but not of IgG and IgA.

In previous studies Fauci and Pratt (1976b) saw no antibody response when human tonsillar cells were stimulated by dextran sulfate or type III pneumococcal polysaccharide. No stimulation of immunoglobulin synthesis was observed in one study (Smith *et al.*, 1971), whereas there was a modest response noted in others (Fauci and Pratt, 1976b; Ringdén *et al.*, 1979b; Rynnel-Dagöö *et al.*, 1979) when PPD was the stimulating agent. The addition of antibodies to β_2-microglobulin to peripheral blood cultures induced proliferation but not immunoglobulin synthesis (Ringdén and Möller, 1975). In most studies where it was tested on human peripheral blood or tonsillar cells,

there was little or no proliferative response following the addition of LPS and only marginal increases in antibody production by LPS-stimulated peripheral blood cells (Peavy *et al.*, 1970; Geha and Merler, 1974; Greaves *et al.*, 1974; Ringdén and Möller 1975; Fauci and Pratt, 1976a,b; Ringdén *et al.*, 1979a; Rynnel-Dagöö *et al.*, 1979). In some studies both polyclonal antibody secretion and DNA synthesis by LPS-stimulated human peripheral blood cells were observed when culture conditions were optimized (Kunori *et al.*, 1978; Miller *et al.*, 1978; Lawton, 1982; Levitt *et al.*, 1981).

A number of factors may be involved in the relative failure of many of these agents to act as polyclonal activators of human peripheral blood B lymphocytes in contrast to their effectiveness with splenic B cells of mice in addition to the potential species differences. One such factor that has been emphasized in the past is a possible difference in organ distribution of cells that can respond to these B-cell polyclonal activators since the studies discussed above involved human circulating blood cells as compared to studies on mouse splenocytes. Thus, although in many studies human peripheral blood cells could not be activated by LPS to synthesize immunoglobulins, splenic cells did respond (Ringdén and Möller, 1975; Gronowicz and Coutinho, 1976; Finkelman and Lipsky, 1979). Similarly, antibodies to β_2-microglobulin stimulated spleen cells to produce immunoglobulin but were not effective stimulators of immunoglobulin synthesis by peripheral blood lymphocytes (Ringdén and Möller, 1975). However, there are data to suggest that antibodies to β_2-microglobulin can trigger circulating human B cells to proliferate (Ostberg *et al.*, 1976). In addition, *Streptococcus pneumoniae* organisms were good stimulators of B cells in the adenoid but were much less effective when used as stimulators of peripheral blood cells (Ringdén *et al.*, 1979a).

The organ differences may reflect differences in the distribution of subsets of B cells considered in terms of their receptors for different activators or in terms of their stage of maturation. Alternatively, the organ distribution differences may reflect differences in the proportion of cells that can be activated to become suppressor cells by the activators used. It is clear that the action of circulating suppressor T cells or monocytes or their precursors may explain the failure of unseparated peripheral blood mononuclear cells to respond to certain agents (Rich and Chandler, 1976; Montazeri *et al.*, 1980; Lawton, 1982). The presence of unirradiated T cells in the unseparated peripheral blood populations that can be activated to become effectors of suppression may be a factor in the failure of such preparations to produce immunoglobulins in response to concanavalin A (Con A) (Dosch

et al., 1980). In addition the high concentration of monocytes may be an even more important factor when the responses of peripheral blood cells is considered. Lawton (1982) has shown that human peripheral blood cells will make a good IgM immunoglobulin biosynthesis response to LPS if these cells are depleted of their monocytes by passage through G-10 columns. Similarly, Montazeri and co-workers (1980) have shown effective responses by B cells to Con A and staphylococcal protein A if the peripheral blood cells have been depleted of monocytes. Whatever the reason for the low responses, these agents to date have been of relatively little value as B-cell activators in studies using unseparated peripheral blood lymphocytes in the analysis of the regulation of human B-cell immunoglobulin synthesis and in the study of these processes in disease states.

A series of other agents have been of greater value as B-cell activators (Table I). In our own studies Bacto-streptolysin O reagent (SLO; Difco Laboratories, Detroit, Michigan) used at a 1:30 to 1:60 dilution stimulated both IgM and IgG synthesis with stimulation ratios of 3.52 for IgG and 5.71 for IgM when compared to the synthesis by these cells cultured in media and fetal calf serum alone. In addition staphylococcal phage lysate (SPL) types I and III (Delmont Laboratories, Inc., Swarthmore, Pennsylvania), an agent that had been shown by Dean *et al.* (1975) to be both a T-cell and a B-cell mitogen, stimulated IgM synthesis (with a geometric mean stimulation ratio of 5.76) compared to cultures in media containing fetal calf serum alone but did not stimulate IgG or IgA production. Wheat germ agglutinin induced polyclonal synthesis of the three major immunoglobulin classes by unseparated peripheral blood mononuclear cells that was comparable to that induced by PWM (Greene *et al.*, 1981). Purified protein A (Forsgren *et al.*, 1976; Lipsky, 1980), protein A insolubilized on dextran beads (Ringdén and Rynnel-Dagöö, 1978; Ringdén *et al.*, 1979a), and formaldehyde and heat-treated *Staphylococcus aureus* Cowan strain I cells containing large quantities of protein A (Rodey *et al.*, 1972; Ringdén *et al.*, 1979a; Kim *et al.*, 1979) have been studied in terms of their capacity to function as polyclonal activators of B cells. Staphylococcal protein A (SPA) is a cell wall component of most strains of *Staphylococcus aureus* that binds most subclasses of IgG (Forsgren and Sjöquist, 1966) and some IgM proteins (Lind *et al.*, 1975). These agents could not be used to study IgG and IgM synthesis in our own studies because the immunoglobulin binding characteristics of protein A interfered with radioimmunoassay for secreted IgG and IgM. When evaluated for their capacity to augment IgA biosynthesis, *S. aureus* Cowan strain I organisms gave a stimulation ratio of

2.07 ($p < 0.05$) when 10^6 organisms were added to 2×10^6 unseparated peripheral blood mononuclear cells. The purified protein A from *S. aureus* did not lead to significant stimulation of IgA synthesis in these studies.

In studies of others, conflicting results have been obtained using soluble SPA as a potential B-cell mitogen or polyclonal activator of B cells from peripheral blood. Soluble protein A has been variously said to be a poor mitogen (or even an immunosuppressant) (Williams and Kornwall, 1972), to be a good T-cell mitogen (Schuurman *et al.*, 1980), to be a mitogen of both B and T cells (Sakane and Green, 1978; Ringdén and Rynnel-Dagöö, 1978), and to be an agent that stimulates DNA and immunoglobulin synthesis by purified B cells, directly (Ringdén and Rynnel-Dag öö, 1978) or indirectly with the requirement of a helper T cell (Lipsky, 1980; Romagnani *et al.*, 1978). In most studies, soluble SPA appears to stimulate splenic lymphocytes to synthesize immunoglobulins, but it has been a very poor stimulator of peripheral blood B cells when unseparated mononuclear cell preparations were examined (Ringdén *et al.*, 1979a; Rynnel-Dagöö *et al.*, 1979). In contrast to these results, Lipsky (1980) demonstrated that SPA stimulated IgM synthesis of peripheral blood lymphocytes as well as did PWM, whereas the IgG synthesis was less than that observed with PWM. The effectiveness of the response in the latter studies may be explained by the fact that a monocyte depletion step was included in these studies. Support for this explanation comes from the observations of Montazeri and co-workers (1980), who noted that there was essentially no antibody production by unseparated peripheral blood mononuclear cells in response to SPA, whereas there was an excellent response in macrophage-depleted peripheral blood mononuclear cell preparations. Furthermore, as discussed more fully below, protein A appears to be an activator of B cells that is suppressed in this action not only by monocytes in peripheral blood (Montazeri *et al.*, 1980), but also in many cases by induced suppressor T cells as well (Lipsky, 1980). The best responses to SPA are obtained with B cells cultured in the presence of both irradiated or mitomycin C-treated T cells and modest numbers of monocytes.

The pattern of stimulation with Cowan strain I organisms is quite different from that with protein A, especially if the organisms were effectively treated with formalin to prevent leakage of SPA into the culture. These organisms were shown to be effective stimulators of B-cell, but not T-cell, proliferation and were shown to activate the B cells of peripheral blood into cells synthesizing antibodies of various specificities (Forsgren *et al.*, 1976; Romagnani *et al.*, 1978; Sirianni *et*

al., 1979; Schuurman *et al.*, 1980; Kasahara *et al.*, 1980; Pryjma *et al.*, 1980). In contrast to purified protein A, Cowan strain I organisms are effective stimulators in T-cell-depleted cultures. The assumption that Cowan strain I organisms act as B-cell activators solely by displaying protein A in an insoluble form has been challenged since the Wood strain 46 of *S. aureus* that does not produce this protein was also shown to stimulate peripheral blood lymphocytes and splenic cells to produce antibodies (Ringdén *et al.*, 1977, 1979a).

The Epstein–Barr virus (line B95-8 supernatant) has been shown to induce the synthesis of all three major classes of polyclonal immunoglobulins in human peripheral blood as assessed by reverse-hemolytic-plaque formation (with a peak noted on day 8), or as assessed by radioimmunoassay of immunoglobulin secreted into culture supernatants (Rosen *et al.*, 1977; Kirchner *et al.*, 1979; Bird and Britton, 1979). To serve as a polyclonal activator of B cells, live virus must penetrate the cells, perhaps at a site closely associated with the complement receptor. Treatment of the virus by agents, such as ultraviolet light, that inactivate the virus also inhibits its capacity to stimulate B cells (Bird and Britton, 1979). In some studies there was a proportional dominance of IgM secretion (Bird and Britton, 1979), whereas in others this IgM predominance was not observed (Kirchner *et al.*, 1979). Although strain B95-8 of the Epstein–Barr virus (EBV) has been used in most studies, other strains also are effective, but in some cases have different T-cell requirements for B-cell activation (Tosato *et al.*, 1980a). Normal unseparated cord blood lymphocytes produced considerable quantities of IgM when stimulated by EBV, but not when stimulated by PWM (Rosen *et al.*, 1977; Bird and Britton, 1979; Tosato *et al.*, 1980b).

In our own studies the addition of EBV (the culture supernatant of cell line B95-8) to unseparated normal peripheral blood mononuclear cell cultures gave immunoglobulin biosynthesis stimulation ratios of 2.04 for IgG, 1.97 for IgA, and 2.89 for IgM as compared to cultures in fetal calf serum alone. Although these stimulation values were significantly elevated ($p < 0.05$) for each class of immunoglobulin studied in the presence of EBV, the stimulation was not of the same magnitude as that observed when PWM was added to unseparated cells. These observations of polyclonal stimulation by EBV are in accord with those of Kirchner *et al.* (1979) and Tosato and co-workers (1980b).

A most promising polyclonal activator of B cells is the water-soluble mitogen extracted from *Nocardia opaca* (NWSM). NWSM has been shown to be an excellent stimulator of the proliferation of B cells and of polyclonal immunoglobulin synthesis by mouse, rabbit, guinea pig,

and monkey spleen cells (Bona *et al.*, 1974). In addition it has been shown to stimulate the proliferation of human lymphocytes obtained from the spleen, tonsils, cord blood, and peripheral blood of normal individuals (Brochier *et al.*, 1976; Lethibichthuy, 1978; Bona *et al.*, 1979). In these studies, NWSM stimulated the proliferation of enriched B cells, but not T cells, from the peripheral blood. It has been shown that polyclonal B-cell activators do not necessarily stimulate all B cells and that the target of one B-cell activator may not be the same as that of another B-cell activator (Gronowicz and Coutinho, 1975; Shek *et al.*, 1976; Bona *et al.*, 1979; Ringdén *et al.*, 1979a; Ault and Towle, 1981). For example, using the BUDR and light cell-suicide technique, it has been shown that the B-cell population stimulated by NWSM to prolifeate was not identical with the one stimulated by PWM (Bona *et al.*, 1978, 1979).

In additional studies, NWSM was shown to stimulate unseparated peripheral blood cells to secrete antibodies to a variety of antigens as assessed by plaque techniques. In our own studies, lymphocytes cultured for 12 days with 100 μg of NWSM (obtained from Dr. Constantine Bona) had geometric means for the synthesis and secretion of IgG, IgA, and IgM of 1623, 1226, and 3016 ng per 2×10^6 lymphocytes in culture, respectively. The mean immunoglobulin stimulation ratios in the presence of this agent as compared to cultures in media and fetal calf serum alone were 9.4 for IgG, 4.7 for IgA, and 27.3 for IgM—values quite comparable to those obtained for PWM.

IV. T-Cell Requirement for Immunoglobulin Synthesis by B Cells

A. Difference in T-Helper-Cell Requirement for Different Polyclonal Activators

The different polyclonal activators of immunoglobulin synthesis differ with respect to their T-cell requirement for B-cell activation. It is critically important to consider the rigor of the T-cell depletion obtained with the various methods used in different reports in interpreting conclusions concerning the T-cell requirement for different activators, since many B-cell activators are absolutely T-cell dependent but require only very small numbers of such T cells for their action. In our own studies, populations of B cells and monocytes freed of T cells were prepared by a two-step procedure that takes advantage of the observation that normal human T cells pass through anti-$F(ab')_2$ immunoabsorbent columns and form spontaneous rosettes with sheep erythrocytes, whereas normal B cells are selectively retained by these

immunoabsorbent columns and do not form sheep cell rosettes (Chess *et al.*, 1974; Broder *et al.*, 1976). In the first step, mononuclear cells were passed over an anti-F(ab')$_2$ column. After passage of the T cells through the column the B cells and macrophages were eluted by the addition of soluble immunoglobulin and agitation of the column. The few contaminating T cells in the B-cell preparation were then removed from these preparations by an overnight sheep red blood cell rosetting procedure. These preparations, containing B cells and macrophages, did not synthesize detectable immunoglobulin when cultured in media with fetal calf serum or when stimulated by PWM. The results of studies on the T-cell requirements of the different lectins in the present series are shown in Table II. It had been shown previously that immunoglobulin synthesis by human peripheral blood lymphocyte stimulated with PWM requires helper T cells (Keightley *et al.*, 1976; Janossy *et al.*, 1977; Siegal and Siegal, 1977; Gmelig-Meyling *et al.*, 1977; Hirano *et al.*, 1977; Moretta *et al.*, 1977; Lipsky *et al.*, 1978; Ballieux *et al.*, 1979). In our own studies (Table II), rigorously T-cell-depleted B cells stimulated with PWM had geometric means for synthesis of 52 ng for IgG, 42 ng for IgA and 92 ng for IgM per 10^6 B cells in culture for 12 days. The addition of 0.5×10^6 purified T cells to 0.5×10^6 normal autologous B cells in these PWM-stim-

TABLE II
REQUIREMENT FOR HELPER T CELLS FOR B-CELL IMMUNOGLOBULIN SYNTHESIS STIMULATED BY DIFFERENT POLYCLONAL ACTIVATORS

	T-depleted B cells (1×10^6) in 12-day culture[b]			B cell (0.5×10^6) + autologous T cells (0.5×10^6)		
Activator[a]	IgG	IgA	IgM	IgG	IgA	IgM
No lectin (9)[c]	21 (1.4)	40 (2.2)	39 (2.9)	—	—	—
PWM (11)	52 (1.4)	42 (1.2)	92 (1.2)	1622 (1.6)	2534 (1.6)	4180 (1.4)
SLO (3)	19 (1.2)	29 (1.2)	67 (1.8)	2322 (1.3)	2597 (2.3)	7612 (1.6)
SPL (6)	20 (1.2)	39 (1.2)	43 (2.2)	1334 (1.4)	1855 (2.0)	7247 (1.8)
NWSM (11)	118 (1.5)	110 (1.6)	465 (1.5)	716 (1.6)	629 (1.6)	3879 (1.5)
EBV (6)	424 (1.7)	745 (2.5)	2093 (2.0)	251 (1.5)	516 (2.0)	2736 (1.5)
PWM-induced (7) helper factor	374 (1.4)	640 (1.9)	1063 (1.5)	—	—	—

[a] PWM, pokeweed mitogen; SLO, Bacto-streptolysin 0 reagent; SPL, staphylococcal phage lysate; NWSM, *Nocardia* water-soluble mitogen; EBV, Epstein-Barr virus.

[b] Geometric mean of nanograms synthesized is shown in each case with the relative standard error of the geometric mean in parentheses.

[c] Number of normals studied is shown in parentheses.

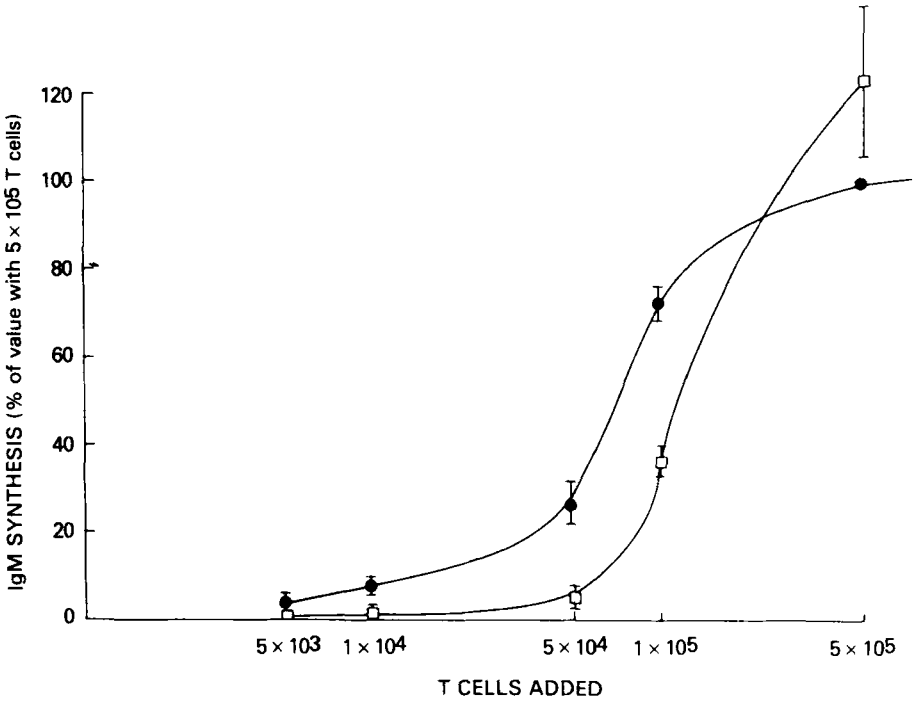

FIG. 2. Pokeweed mitogen-stimulated IgM synthesis by 5×10^5 B cells from five normal individuals cultured with varying numbers of autologous unirradiated (●—●) or irradiated (2000 R) T cells (□—□). All values are normalized to the synthesis value obtained with 5×10^5 B cells and 5×10^5 unirradiated T cells.

ulated cultures restored the capacity of these B cells to produce large quantities of all three major classes of immunoglobulins with geometric means for synthesis of 1622 ng for IgG, 2534 ng for IgA, and 4180 ng for IgM. Helper activity could also be seen when smaller numbers of normal T cells were added (Fig. 2). For example, the means for the PWM-stimulated synthesis of IgG, IgA, and IgM by 0.5×10^6 normal B cells cultured with only 5×10^4 autologous T cells were 75%, 34%, and 32%, respectively, of the values observed when B cells were cultured with 5×10^5 autologous T cells. The addition of as few as 10^4 T cells to 0.5×10^6 B cells produced an augmentation of immunoglobulin synthesis. Streptolysin O (SLO)-stimulated B cells synthesized very little immunoglobulin, and there was 100-fold increment of immunoglobulin synthesis, similar to that seen with PWM, observed when T cells were added to the B cells (Table II), supporting the view that SLO is a T-cell-dependent activator of B cells. In previous studies, Dean and co-workers (1975) reported that staphylo-

coccal phage lysate (SPL) was both a T- and a B-cell mitogen. In those studies—in contrast to the observations with Con A, phytohemagglutinin (PHA) or PWM—SPL stimulated B-cell mitogenesis in the absence of added T cells. However, in the present study we noted that B cells rigorously depleted of T cells by the two-step procedure did not synthesize immunoglobulin molecules when stimulated by SPL. There was a marked augmentation of immunoglobulin synthesis for all immunoglobulin classes ranging from 47- to 170-fold (Table II) when T cells were added to the B-cell cultures stimulated by SPL indicating that this activator is also a helper T-cell-dependent stimulator of B cells. In studies of soluble staphylococcal protein A by Lipsky (1980) and of wheat germ agglutinin by Greene *et al.* (1981), a similar requirement for T cells in order to obtain immunoglobulin synthesis and secretion by B cells was observed. Thus PWM, SLO, SPL, soluble staphylococcal protein A, and wheat germ agglutinin may be viewed as polyclonal activators of B cells that have an absolute requirement for helper T cells. *Nocardia* water-soluble mitogen (NWSM) has previously been shown to be an excellent stimulator of the proliferation of B cells and of polyclonal immunoglobulin synthesis by mouse and rabbit splenic cells (Bona *et al.*, 1974). In addition, it has been shown to stimulate the proliferation of purified B cells (but not T cells) of human peripheral blood (Brochier *et al.*, 1976; Lethibichthuy *et al.*, 1978; Bona *et al.*, 1979). In our own studies NWSM stimulated purified B cells to synthesize immunoglobulin with geometric means for synthesis of 118 ng for IgG, 110 ng for IgA, and 465 ng for IgM. It should be emphasized, however, that the quantity of immunolobulin synthesized was increased by a factor of five when T cells were added to the cultures (Table II). Therefore, for the system used in our own studies, NWSM would be viewed as only a relatively T-cell-independent stimulator of immunoglobulin synthesis.

As noted above, *Staphylococcus aureus* organisms of Cowan strain I stimulate B cells to differentiate into immunoglobulin and antibody-synthesizing and -secreting cells. This stimulation by *S. aureus* organisms appears to be at least partially T-cell indpendent, although in some cases the procedures used for T-cell depletion were limited to a single sheep red blood cell rosetting procedure. In addition, Kim and co-workers (1979) reported that the plaque-forming cell response of human B cells induced by *S. aureus* was augmented after the addition of T cells. The Epstein–Barr virus (EBV) appears to be a helper T-cell-independent B-cell activator. In the studies of Kirchner and coworkers (1979) and Bird and Britton (1979) the synthesis of immunoglobulin and antibodies by T-cell-depleted B-cell preparations was as

great as or greater than that of unseparated cell populations. In our own studies (Table II) EBV was shown to be an effective stimulator of rigorously T-cell-depleted B-cell preparations. In addition, the synthesis of immunoglobulins by the B cells cultured with EBV was not augmented when autologous T cells were added to the cultures. It should not be concluded that the response of B cells to EBV remains unaffected by the presence of T cells in all cases since, as discussed further below, in many circumstances suppressor T cells that inhibit B-cell activation are induced in the EBV system. A final series of agents that are effective in inducing immunoglobulin synthesis by purified B cells includes soluble products of stimulated T cells, such as the helper factor(s) secreted after exposure of T cells to such agents as PWM (Janossy and Greaves, 1975; Insel and Merler, 1977; Stevens *et al.*, 1979) and allogeneic or autologous cells or antigen (Geha *et al.*, 1977; Chiorazzi *et al.*, 1979a,b; Friedman *et al.*, 1980; Reinherz *et al.*, 1980b). For example, in our studies, B cells rigorously depleted of T cells produced immunoglobulin when cultured in the presence of a helper factor secreted by PWM-stimulated T cells, with geometric means for synthesis of 374 ng for IgG, 640 ng for IgA, and 1063 ng for IgM per million B cells in culture for 12 days. As discussed below, the availability of agents that are relatively or completely T helper cell independent, such as NWSM, *S. aureus* organisms, EBV, and T cell helper factors are of considerable value in analyzing potential helper T cell defects in disease and in defining the target of suppressor influences (especially in determining whether their action is on B cells and macrophages, on the one hand, or on helper T cells on the other hand).

B. Radiosensitivity of T-Helper-Cell Function: Evidence for at Least Two Populations of Helper T Cells Involved in Polyclonal Activation of B Cells

Immunoglobulin synthesis stimulated by PWM is quite radiosensitive. This radiosensitivity appears to be due in large part to the sensitivity of B cells to irradiation. There was an over 95% inhibition of synthesis of each class of immunoglobulin when 0.5×10^6 B cells were X-irradiated with 500, 1000, or 2000 R (Fig. 3A) and cocultured with an equal number of unirradiated autologous T cells and PWM. By contrast, there was little inhibition of the immunoglobulin synthesis when the cocultured T cells used to provide help were irradiated with up to 2000 R (Fig. 3B). The relative radioresistance of helper T cell function when the T:B cell ratio in cultures is one to one or greater has been emphasized by Siegal and Siegal (1977), Keightley and co-workers (1976), Saxon and co-workers (1977), and ourselves (Broder *et al.*, 1976), and has been used extensively to distinguish de-

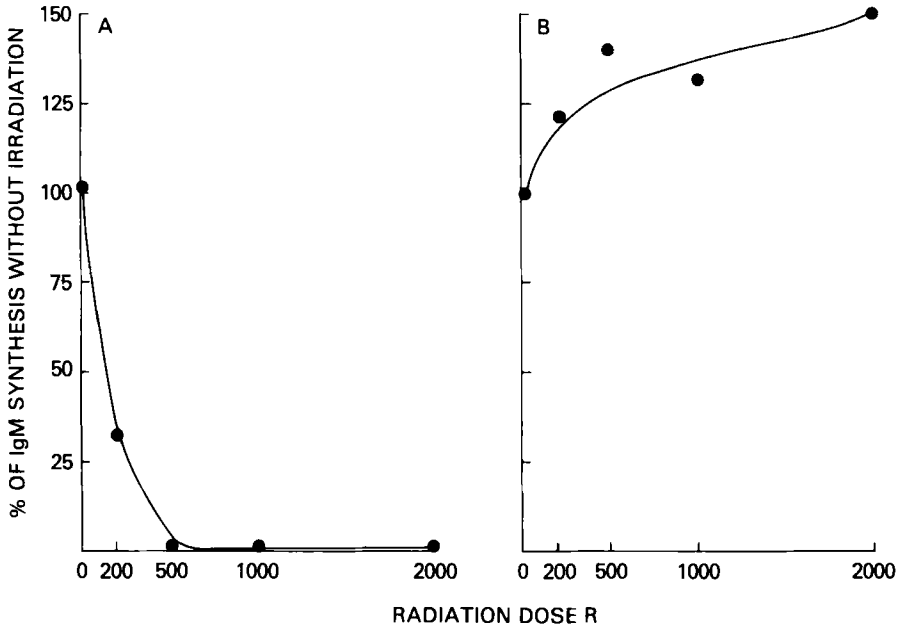

FIG. 3. Effect of varying doses of irradiation on B cells (panel A) or on T cells (panel B) prior to coculture in pokeweed-mitogen-stimulated IgM synthesis. In all cases, 5×10^5 T cells were cultured with 5×10^5 autologous B cells.

ficiencies of helper function from excessive suppressor T-cell activity since these latter functions are more radiosensitive in most cases. It should be noted, however, that when the T cells used to provide help were serially diluted, evidence for some degree of radiosensitivity was observed (Fig. 2). By using serial dilutions of T-cell populations it was shown that when 1×10^5 or fewer T cells were cocultured with 5×10^5 B cells, larger numbers of irradiated T cells than unirradiated T cells were required for the same immunoglobulin synthesis by a constant number of B cells. This apparent radiosensitivity of T-cell help when examined with higher dilutions of T cells was also observed by Thomas and co-workers (1980), who studied helper function of human T-cell subsets in PWM-induced stimulation of B-cell differentiation and by Lipsky (1980), who analyzed T-cell helper function in a staphylococcal protein A-stimulated *in vitro* system. Furthermore Nagaoki *et al.* (1981) showed that T helper function is relatively radiosensitive when NWSM, a stimulator that does not activate pro-suppressor cells, is examined. The apparent radiosensitivity of T-cell help when examined at moderate to low T:B cell ratios may reflect a requirement for proliferation of T cells to exert optimal help at such ratios, may reflect a heterogeneity of different independent T helper

cell populations in terms of their inherent radiosensitivity, or may reflect a requirement for the interaction of two T cells, including one that is radiosensitive, for the generation of optimal helper cell activity.

There is a body of evidence emerging that at least two types of helper T cells exist and that these helper T cells differ in terms of their radiosensitivity. For example, there is evidence in murine systems for the existence of at least two helper subsets of lymphocytes within those cells bearing the maturation antigen Lyt-1 (Marrack and Kappler, 1975; Janeway *et al.*, 1977; Tada *et al.*, 1978; Keller *et al.*, 1980; Takatsu *et al.*, 1980; Agarossi *et al.*, 1981). Both Tada *et al.* (1978) and Keller *et al.* (1980) have distinquished Ia(positive), antigen-nonspecific helper T cells and Ia(negative), antigen-specific helper T cells. These cells differed in terms of their frequency, nylon wool column adherence, dose responsiveness to antigens, and mechanisms of action as well. More recently, Reinherz and co-workers (1981) have demonstrated subpopulations of inducer T cells within the helper T-cell subset (helper cells were defined by the monoclonal antibody termed OKT4) that differ in terms of their expression of Ia-like antigens. These subsets of helper T cells were noted in a system requiring tetanus toxoid antigenic stimulation. Only the antigen-induced Ia-positive subset of $OKT4^+$ T cells alone could produce a nonspecific polyclonal helper factor (LMF) whereas both $OKT4^+$ Ia-positive and $OKT4^+$ Ia-negative inducer cells could induce significant amounts of immunoglobulin secretion by B cells when restimulated by tetanus toxoid. However, the presence of both OKT4-positive, Ia-positive, and $OKT4^+$ Ia-negative inducer T cells was required to generate maximal immunoglobulin production by the B cells in this antigen-driven system. In other studies of the $OKT4^+$ helper T cell population involved in the regulation of B-cell differentiation, Thomas *et al.* (1980) demonstrated that helper activity within the OKT4-positive population was radiosensitive and that only at high T:B ratios could this radiosensitivity be overcome. In addition, the $OKT4^+$ population required for induction of suppressor cells from the OKT8-positive population was also shown to be radiosensitive.

We have used a monoclonal antibody (anti-Tac) to separate two populations of T cells activated by preculture with PWM that differ in terms of their radiosensitivity when analyzed for helper activity in the PWM-induced *in vitro* immunoglobulin biosynthesis system (Uchiyama *et al.*, 1981b). The anti-Tac monoclonal antibody used in these studies reacts with a 120,000-dalton[1] antigen on human periph-

[1] Determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.

eral blood T cells that have been activated by lectins such as Con A, PWM, and PHA, by exposure to antigens, or by culture with allogeneic cells (Uchiyama *et al.*, 1981a). The Tac antigen is not demonstrable on normal T cells prior to activation, or on macrophages or B cells. T cells precultured with PWM and fractionated into Tac-positive and Tac-negative cell populations by a staphylococcal protein A-coated burro red blood cell rosette method were studied for helper and suppressor T-cell activity in the PWM-induced immunoglobulin production system. After PWM activation, Tac-positive T cells possessed suppressor cell activity that was radiosensitive as well as helper T cell activity that was resistant to 2000 R irradiation. On the other hand, the Tac-negative cells manifested only helper-cell activity. However, the helper activity of the Tac-negative cell populations was abrogated or markedly diminished following 2000 R irradiation (Uchiyama *et al.*, 1981b). Considering the data from all the studies, it appears that at least two populations of inducer T cells for B-cell activation exist and that these differ in terms of their surface characteristics (e.g., reactivity with anti-Ia and Tac antisera) and in terms of their radiosensitivity.

V. Differences in Polyclonal B-Cell Activators in Terms of Their Capacity to Activate Prosuppressor T Cells into Suppressor Effector Cells

A distinct group of interacting T cells constitutes a suppressor cell network that inhibits antigen or polyclonally induced B cell maturation and immunoglobulin synthesis. Cells of this suppressor system appear to emerge from the thymus and have an interim existence as inactive precursors, which may be referred to as prosuppressor cells. Such prosuppressor cells can be activated into fully functional regulatory cells, the suppressor-effector cells, by a variety of means. In many cases such prosuppressor T cells must interact with a different set of inducer (or activating) T cells before differentiating into the effector cells (Feldman *et al.*, 1977; Tada *et al.*, 1977; Broder *et al.*, 1978, 1981; Eardley *et al.*, 1978). A number of agents that we have been considering as polyclonal B-cell activators also activate prosuppressor T cells to become functional suppressor effectors. This appears to be the case for PWM (Broder *et al.*, 1976, 1978; Gomez de la Concha *et al.*, 1977; Saxon *et al.*, 1977; Moretta *et al.*, 1977; Uchiyama *et al.*, 1981b), Con A (Dutton, 1972; Rich and Pierce, 1974, Haynes and Fauci, 1977), PHA (Winger *et al.*, 1977), staphylococcal protein A (Lipsky, 1980), and under certain circumstances EBV (Blaese and Tasato, 1981; Bird and Britton, 1979), but not NWSM (Bona *et al.*, 1979).

One of the observations reported by others (Gomez de la Concha *et al.*, 1977) and ourselves (Broder *et al.*, 1976) that suggested that PWM

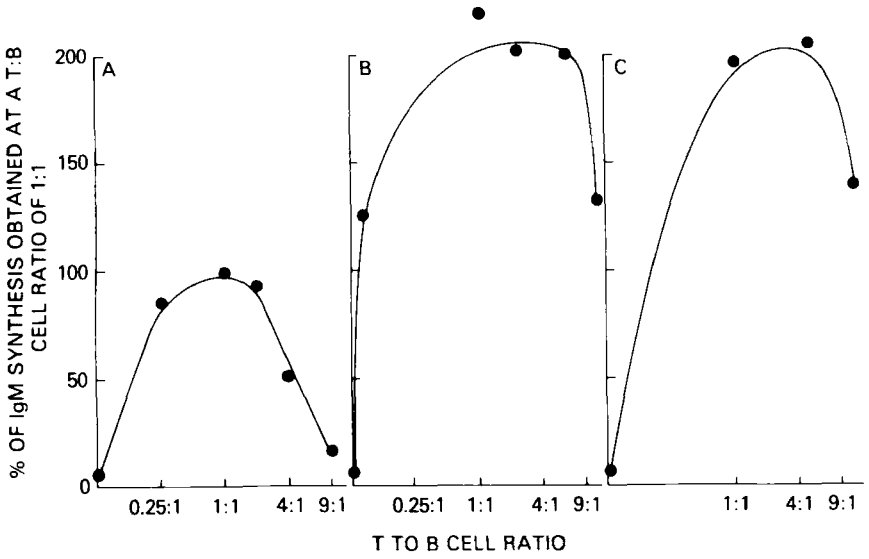

FIG. 4. (A) Effect of varying ratios of autologous T:B cells in culture on immunoglobulin synthesis per B cell in culture. (B) Effect of varying ratios of irradiated (2000 R) T:B cells on immunoglobulin synthesis. (C) Effect of varying ratios of T:B cells in culture on immunoglobulin synthesis in the presence of 10^{-5} *M* hydrocortisone sodium succinate. Cultures are in the presence of pokeweed mitogen. All values for the four individuals studied are normalized to the value (indicated as 100% value) obtained for that individual at a ratio of one unirradiated T cell to one B cell.

could induce the activation of prosuppressor cells into effectors of suppression was the reduction of immunoglobulin synthesis noted in PWM-stimulated cultures when the ratio of T:B cells was increased. As discussed above, when T cells were added to B cells in equal numbers, marked augmentation of the PWM-induced immunoglobulin synthesis per B cell in culture was observed. However, in the present study when the ratio of T cells added to B cells at a constant number of total cells exceeded 4:1, there was a progressive suppression of the synthesis of all immunoglobulin classes until at a ratio of 9:1 the mean synthesis of immunoglobulin per B cell in culture was only 22% of the peak level (Fig. 4A). It should be emphasized that this suppression was observed with only certain lots of PWM and fetal calf serum. The suppression, when observed, was present in these coculture experiments with allogeneic T cells as well as with autologous T cells. A comparable pattern of reduced immunoglobulin synthesis at high T:B cell ratios was observed when progressively increasing numbers of normal T cells (i.e., from 4×10^5 up to 1.6×10^6) were added to a constant number of B cells (2×10^5 cells). We have made a number of ob-

servations that support the view that the inhibition of immunoglobulin synthesis in PWM-stimulated cultures at high T:B cell ratios reflects the activation of prosuppressor cells within the T cell population into effectors of suppression. Irradiation of the T cells with 2000 R prior to coculture with the B cells and PWM, prevented the inhibition of immunoglobulin synthesis at high T:B ratios (Fig. 4B). The synthesis of IgM per B cells in culture at a ratio of nine unirradiated T cells to one B cell was only 22% of that observed at a one T cell to one B cell ratio (Fig. 4A). However, the synthesis was increased to 132% of this value if the T cells used at T:B cell ratios of 9:1 had been irradiated prior to coculture. In addition, when 10^{-5} *M* hydrocortisone was added to these cultures, the immunoglobulin synthesized at a T:B cell ratio of 9:1 was greater than the synthesis of T and B cells cultured in the absence of hydrocortisone at a ratio of 1:1 (Fig. 4C compared to 4A). As noted below, there was no inhibition of immunoglobulin synthesis at high T:B cell ratios when NWSM was the sole activator of immunoglobulin synthesis. However, the NWSM-stimulated immunoglobulin synthesis was reduced by 98% when PWM was added to these NWSM-stimulated cells cultured at T:B cell ratios of 9:1. Finally, when Sézary T leukemic cells, which frequently function as helper cells, were added to B cells, no suppression of immunoglobulin synthesis was observed at high T:B cell ratios.

These observations taken together are consistent with the view that normal peripheral blood T cells include a distinct subpopulation of prosuppressor cells that can be activated by PWM to function as suppressors of immunoglobulin synthesis. In addition, prosuppressor cells appear to be more sensitive to X-irradiation in terms of their activation to become suppressor effector cells and in terms of their interactions with B cells than are helper T cells. The relative radiosensitivity of suppressor T cells, or at least the generation of suppressor T cells from prosuppressor cells in the PWM system, was anticipated and is supported by a number of previous studies. Siegal and Siegal (1977) have shown that immunoglobulin synthesis by B cells and cocultured T cells is significantly augmented when the T cells are irradiated with 1000–3000 R when T:B cell ratio in the culture is 1:1 or greater. Although corticosteroids have multiple functions that affect immunoglobulin synthesis by human peripheral blood lymphocytes, the view that they inhibit the activation of suppressor T cells is supported by the observation of Saxon and co-workers (1978), who demonstrated that corticosteroids administered *in vivo* inhibited the generation of activated suppressor T cells that was usually observed at high T:B cell ratios.

A number of studies have been performed to define the subpopula-

tions of T lymphocytes that can be activated by PWM to become suppressors of immunoglobulin production. Moretta and co-workers (1977, 1978) demonstrated that T cells with receptors for IgG (Tγ cells) suppressed the differentiation and proliferation of B cells in the pokeweed system when the Tγ cells had prior interactions with immune complexes. The suppressor activity of the Tγ cells was radiosensitive. It should be noted, however, that T cells with receptors for IgM (Tμ cells) that were viewed as helper cells for PWM-induced differentiation of B lymphocytes *in vitro* also showed the pattern of inhibition of immunoglobulin synthesis at high T:B cell ratios suggesting that such cells could be activated by PWM to become suppressor cells (Pichler and Broder, 1981). This view is in accord with the observation that Tμ cells can be activated by preculture with Con A to become active suppressors in this system (Hayward *et al.*, 1978; Haynes and Fauci, 1978).

Functionally distinct subsets of human T cells have also been isolated, using the monoclonal antibodies, OKT4 and OKT8, by Reinherz, Schlossman, Chess, Kung, Goldstein, and their co-workers (Reinherz *et al.*, 1979a, 1980a; Thomas *et al.*, 1980). The OKT8$^+$ populations was shown to contain radiosensitive cells that were effective in suppressing B-cell differentiation, whereas the helper activity was exclusively contained within the OKT4$^+$ population. In certain studies (Thomas *et al.*, 1980) an OKT4$^+$ inducer population had to collaborate with an OKT8$^+$ population in order to generate suppressor effectors. It should be emphasized that Thomas and co-workers reported that at high T:B cell ratios unirradiated OKT4$^+$ cells inhibited the antibody production by B cells in a pattern quite similar to that observed with unfractionated T cells. This suggests that OKT4$^+$ cells contained precursors of suppressor T cells as well as inducer T cells. When the OKT4$^+$ cells were irradiated, they did not show this pattern of inhibiting B-cell immunoglobulin synthesis at high T:B cell ratios. Of considerable interest, Thomas and co-workers showed that two radiosensitive cells, one within the OKT4$^+$ population and the other within the OKT8$^+$ populations collaborate to generate suppressor effectors.

Agents classically viewed as polyclonal T lymphocyte activators, such as Con A and PHA, have been shown to be more effective activators of suppressor precursor cells than is PWM (Dutton, 1972; Rich and Pierce, 1974; Haynes and Fauci, 1977). The incubation of T lymphocytes with Con A, PHA, and to a lesser extent PWM for 24–96 hours has led to the activation of suppressor cells that inhibit the immunoglobulin synthesis of peripheral blood mononuclear cells stimulated by PWM (Haynes and Fauci, 1977; Uchiyama *et al.*, 1981b). In most studies the optimal mitogenic doses of Con A and PHA did not

stimulate polyclonal B cell immunoglobulin synthesis by unseparated peripheral blood mononuclear cells. As noted below, the regulatory role of circulating suppressor monocytes is one factor in the failure of these activators to be effective stimulators of immunoglobulin production by such unseparated mononuclear cell populations. However, Bird and Britton (1979) have demonstrated that these agents were effective in the functional activation of adult human lymphocytes into immunoglobulin-secreting cells when used at approximately one-tenth the optimal mitogenic dose. Furthermore Dosch and co-workers (1980) showed that the hemolytic plaque-forming cell response to PHA was markedly augmented when irradiated T cells rather than unirradiated T cells were added in culture with purified B cells and that a plaque-forming cell response to Con A was observed when irradiated T cells, but not when unirradiated T cells, were used as the source of helper T-cell activity.

Another of the T helper cell-dependent polyclonal activators of human B cells, staphylococcal protein A (SPA) was shown by Lipsky (1980) to have a greater capacity to trigger suppressor cell function than does PWM. In these studies, Lipsky showed that the failure to produce immunoglobulins when stimulated with high concentrations (>5 μg/ml) of SPA as compared to low concentrations (<1 μg/ml) and the failure to respond at high T:B cell ratios could be ascribed to the action of suppressor T cells activated by SPA. In addition, the hyporesponsiveness to stimulation by some normal individuals was shown to be due to the action of suppressor T cells activated by this agent. The generation of suppressor T cell effectors on exposure to SPA, like that induced by PWM, Con A, or PHA, could be abolished by inhibiting T-cell DNA synthesis. In general, mitogen-triggered suppressor T cells are not specific in their action for the activating mitogen. For example, SPA-triggered suppressor T cells are able to suppress the PWM-induced generation of immunoglobulin-secreting cells. Thus, in summary, each of the T helper cell-dependent polyclonal activators of B cells appears to be capable of activating prosuppressor T cells into effectors of suppression in addition to their capacity to function as inducer cells for B-cell maturation. The net effect depends on the balance between the antagonistic regulatory T cell functions activated by a particular agent. Suppression tends to be favored over help when high doses of the polyclonal activators are utilized and when the cells are cultured at a high T:B cell ratio. This generation of T suppressor effector cells appears to require cellular proliferation and is thus more sensitive to irradiation or to the action of mitomycin C than is helper T-cell activity.

The Epstein–Barr virus (strain B 95-8) has been shown to be an in-

ducer of polyclonal immunoglobulin synthesis that does not require the cooperative interaction of helper T cells or monocytes. Although EBV activation of B cells is independent of helper T cells, it should not be concluded that the B-cell response to EBV remains unaffected by the presence of T cells. It is clear that the EBV-induced B-cell activation is quite responsive to suppressor T-cell signals. For example, Thorley-Lawson and co-workers (1977) showed that the establishment of permanently growing B-cell lines from adults was significantly delayed when unseparated mononuclear cells were used rather than T-cell-depleted B cells. Furthermore, Blaese and Tosato (1982) have demonstrated a number of suppressor T-cell systems in normal humans that regulate EBV activation of B cells. In these studies normal adults who were immune to EBV (i.e., EBV seropositive), but who had not experienced their infection with virus recently, demonstrated suppression that appeared late during the culture period. When T cells from such subjects were mixed with autologous B cells stimulated with EBV, there was no observable effect on B-cell immunoglobulin synthesis as assessed by the reverse hemolytic-plaque assay for the first 7–10 days of culture. However, by days 10–14 of culture a striking late-appearing suppression emerged, so that by day 14 the response of EBV-stimulated B cells cultured with T cells averaged only 10% of that of B cells cultured in the absence of T cells. This late-acting T suppressor cell activity was abolished by irradiation of the T cells or by the addition of hydrocortisone to the cultures. Furthermore, the addition of cyclosporin A to the cultures abolished this suppressor T-cell activity. In contrast to the observations with seropositive individuals, when T cells from seronegative individuals were added to autologous B cells stimulated with EBV, no effect in either a positive or negative direction was observed during the 14 days of coculture.

One of the critical differences between the EBV and PWM systems of B-cell activation is on the effects observed when allogeneic cells are mixed in cocultures. For most of the effective polyclonal activators, including PWM, the combinations of allogeneic cells did not result in net augmentation or suppression of the mean B-cell immunoglobulin synthetic response to the polyclonal activator (Waldmann *et al.*, 1974; Saxon *et al.*, 1977). However, when similar allogeneic cell-mixing experiments were performed using EBV as the B-cell stimulant, a very different effect was observed (Blaese and Tosato, 1982). Strong suppression of the expected reverse plaque response to EBV was observed when allogeneic cells that were not matched at the HLA-A or B locus were cocultured. By contrast the cocultures of cells

of subjects matched at HLA-A and -B locus antigens but mismatched at the HLA-DR locus did not demonstrate suppression. Thus, in contrast to the other polyclonal activators where allogeneic cocultures had been of considerable value, this allogeneic suppressor response limits the use of EBV in coculture studies of immunoregulatory function to individuals who are identical at the HLA-A and -B loci.

Nocardia water-soluble mitogen contrasts with the polyclonal activators discussed above in terms of its capacity to activate prosuppressor T cells. When NWSM was the polyclonal stimulant used in the culture, we observed no suppression of immunoglobulin synthesis at high T:B cell ratios. In these cultures immunoglobulin synthesis per B cell progressively increased as more T cells were added. With NWSM as the activator, the IgM synthesis per B cell in culture at T:B cell ratios of 9:1 was 3.66-fold greater than that observed when the T:B cell ratio was 1:1. As we have already discussed, it is clear that this lack of suppression at high T:B cell ratios did not reflect the inability of B cells stimulated by NWSM to respond to suppressor signals, since the addition of PWM to the NWSM stimulated cultures at a T:B cell ratio of 9:1 led to a 98% inhibition of the immunoglobulin synthesis observed with NWSM alone. Thus, NWSM appears to be a most promising activator of B cells that is relatively T helper cell independent and does not have the propensity to activate prosuppressor T cells into effectors of suppression. This latter characteristic is of value in further defining the suppressor cell (i.e., prosuppressor, suppressor inducer, suppressor effector) that is disordered when an abnormality in the suppressor T-cell network is demonstrated in coculture studies of patient and normal cells stimulated with PWM.

VI. Regulatory Role of Circulating Monocytes in B-Cell Activation by Polyclonal Stimulators

A. Monocyte Requirement for B-Cell Activation by Different Polyclonal B-Cell Stimulators

Cells of the monocyte and macrophage lineage play critical roles both as accessory cells required for the inducation of many types of immune responses and as negative regulators that suppress immune reactions (Oehler *et al.*, 1978). A monocyte requirement for an optimal immune response has been suggested for the *in vitro* antibody responses of mice to a number of antigens and for the proliferation of murine lymphocytes to mitogens and antigens (Mosier, 1967; Shortman and Palmer, 1971). Similarly, human lymphocytes have been

shown to require monocytes for an optimal proliferative response to various antigens (Oppenheim *et al.*, 1968; Alter and Bach, 1970). On the basis of early studies using monocyte depletion, it was concluded that there is no monocyte requirement for PWM-induced immunoglobulin and antibody synthesis (Saxon *et al.*, 1977). However, more recently it has been shown that there is a monocyte dependence for the proliferation and differentiation of human B cells that is induced by some of the polyclonal B-cell activators, including PWM. Blaese *et al.* (1977) were able to abrogate PWM-induced B-cell differentiation by producing an extreme monocyte depletion. In these studies the peripheral blood mononuclear cells were depleted of monocytes by a combination of carbonyl iron phagocytosis and buoyant density centrifugation followed by passage over a column of siliconized glass beads. Knapp and Baumgartner (1978) obtained similar results by passing peripheral blood mononuclear cells through nylon wool columns to remove monocytes. In both of these studies, mononuclear cells, including B cells in addition to monocytes, were removed by the depletion procedures utilized, and studies to demonstrate that immune responses could be reconsitituted by supplementing the depleted cells with fresh monocytes were not performed.

In more recent studies by Rosenberg and Lipsky (1979), Montazeri and co-workers (1980), and Gmelig-Meyling and Waldmann (1981) it was shown that the capacity of PWM-stimulated human peripheral blood mononuclear B cells to mature into immunoglobulin- and antibody-secreting cells was markedly diminished by monocyte depletion and that immunoglobulin synthesis could be restored upon addition of appropriate numbers of purified monocytes. These studies support the requirement for monocytes for PWM-induced differentiation of B cells. A number of differences in the culture conditions would appear to underlie the differences between the observations in the more recent studies suggesting monocyte requirement and the earlier studies where this requirement was not demonstrable. These differences in the culture conditions involve differences in the extent of monocyte depletion, differences in the number of cells cultured, and differences in the geometry of the culture vessels utilized. Many of the studies demonstrating a monocyte requirement for obtaining responses by PWM-stimulated B cells used a two-step procedure to produce extreme monocyte depletion whereas in the earlier studies there was a 1–4% monocyte contamination of the cells cultured with PWM. Another feature emphasized by Rosenberg and Lipsky (1979) is that the demonstration of monocyte dependence was simplified by using culture conditions that limited cell-to-cell interaction. That is, monocyte

dependence was most obvious when low numbers of cells were cultured (i.e., 50,000 cells per microtiter well) and when flat-bottomed wells or vials as opposed to U-bottomed wells or round-bottomed test tubes were used.

When other polyclonal B cell activators were studied, it was shown that monocytes were required for the response to certain B-cell activators but not for others. In the studies of Gmelig-Meyling and Waldmann (1981) it was shown that the immunoglobulin synthetic responses to SPL and SLO were markedly depressed after monocyte depletion of peripheral blood mononuclear cells and that the responses were reconstituted when purified monocytes were added to the cultures. In contrast to the findings with these activators, NWSM did not require monocytes in order to stimulate immunoglobulin production. A similar monocyte independence was observed by Bona and co-workers (1979) when NWSM-induced B-cell proliferation was examined. In addition, the response to EBV appears to be independent of monocytes. These data are interesting since NWSM and EBV are activators that are relatively helper T-cell independent and also appear to be independent of monocytes, whereas the polyclonal B-cell activators (including PWM, SPL, and SLO) that are monocyte dependent are also helper T cell dependent as well.

It is still not clear what factors are involved in the monocyte dependence of the B-cell stimulation by polyclonal activators. The diminished responsiveness of monocyte-depleted peripheral blood mononuclear cells could not be ascribed to cell death, since removal of monocytes had no adverse effect on the viability of lymphocytes in general and of B lymphocytes in particular. The total living cell yields and the number of B cells were similar in cultures with and without monocytes (Gmelig-Myeling and Waldmann, 1981). Macrophage accessory cell function might involve transmission of information via direct cell-to-cell contact during a process similar to antigenic presentation, for instance by macrophage presentation of the mitogens to the helper T lymphocytes or B lymphocytes. Alternatively, the macrophages may function by the production of a series of soluble factors (e.g., interleukin I) that modify lymphocyte responses (Wood and Cameron, 1975; Wood, 1979; Aarden *et al.*, 1979; Oppenheim *et al.*, 1980).

The action of the monocytes and their secreted factors may theoretically be directly on the B cells or alternatively on the regulatory T-cell system. Evidence for a direct action on B cells includes the demonstration by Hoffman and co-workers (1979) in mice that appropriately activated macrophages produce a soluble factor that converts antigen-

stimulated B cells to antibody-secreting cells without requiring T cells. In addition, Dimitriu and Fauci (1978) have identified a factor released from human monocytes stimulated by supernatants of mixed lymphocyte cultures that augments the PWM-driven generation of anti-sheep red cell antibody secreting cells in human peripheral blood lymphocyte cultures. This monokine was specifically absorbed by a B-cell-enriched subpopulation, but not a T-cell-enriched population, supporting the view that the B cell was the target for that monocyte-derived factor. Finally, it has been easiest to demonstrate monocyte factor-augmented immunoglobulin production in T-cell-depleted B-cell systems in studies performed with cells from animals or from man (Wood, 1979). Alternatively, there is also considerable evidence favoring a different target for the action of certain monocyte factors, specifically the helper T cell. In the human system, Rosenberg and Lipsky (1980) have demonstrated a macrophage factor that augmented immunoglobulin production by macrophage-depleted mononuclear cells stimulated with PWM. However, they found that the macrophage factor was unable to enhance PWM-induced generation of immunoglobulin-secreting cells in T-cell-depleted cultures. Furthermore, the enhancing effect of the monocyte supernatant was restored when small numbers of T cells were added back to the T-cell-depleted cultures. These studies, as well as other similar studies in experimental animals, indicate that macrophage factors may act by stimulating T-cell help or by acting in concert with T-cell signals to stimulate final B-cell differentiation. The demonstration that monocytes are required for the optimal immunoglobulin biosynthetic response of B cells to polyclonal stimulants that are helper T cell dependent, but not those that are helper T cell independent, would be in accord with this conclusion.

B. Suppression by Monocytes of the B-Cell Proliferation and of Immunoglobulin Synthesis Induced by Polyclonal B-Cell Activators

Monocytes and macrophages play an important suppressive role in the regulation of the immune response in addition to their previously recognized roles as effector cells in their own right and as accessory cells enhancing immune responses (Parkhouse and Dutton, 1966; Yoshinaga *et al.*, 1972, Baird and Kaplan, 1977; Laughter and Twomey, 1977; Unsgaard and Lamvik, 1977; Wing and Remington, 1977; Bona *et al.*, 1979; Hem, 1979; Rinehart *et al.*, 1979; Youdim, 1979; Wilson and Remington, 1979; Oehler *et al.*, 1977). Such suppressor macrophages have been found in normal humans and in animals bearing

virus- or carcinogen-induced tumors as well as those stimulated *in vivo* with certain bacterial products (e.g., *Corynebacterium parvum*, bacillus Calmette-Guérin) (Oehler *et al.*, 1978). Many T-cell functions, especially those involving T-cell proliferation, such as the induction of cytotoxic T cells or the proliferative response to mitogens or antigens, can be suppressed by highly activated macrophages or even by resting macrophages in sufficient numbers. More recently it has been demonstrated that monocytes from normal individuals may inhibit the proliferation of human B cells and their differentiation into immunoglobulin-secreting cells (Knapp and Baumgartner, 1978; Montazeri *et al.*, 1980; Gmelig-Meyling and Waldmann, 1981). In addition, in certain diseases including multiple myeloma and common variable immunodeficiency, monocyte suppressor cells may play a role in the pathogenesis of the immunoglobulin deficiency (Broder *et al.*, 1975; Siegal *et al.*, 1976; Ashman *et al.*, 1980).

The suppressive capacity of large numbers of monocytes on B lymphocyte activation has been demonstrated in studies where augmented polyclonal activator stimulated immunoglobulin synthesis by peripheral blood mononuclear cells was observed following monocyte depletion. In these studies the readdition of large numbers of monocytes led to suppression of B-cell proliferation and immunoglobulin synthesis. Knapp and Baumgartner (1978), Montazeri and coworkers (1980), and Gmelig-Meyling and Waldmann (1981) observed a 3- to 8-fold augmentation of PWM-stimulated immunoglobulin synthesis by normal peripheral blood mononuclear cells when the percentage of monocytes was reduced from normal levels to approximately 5% of the cultured mononuclear cells. The augmentation of the immunoglobulin synthesis on monocyte depletion was not limited to PWM but was observed with all polyclonal B cell activators examined including SLO, SPL, NWSM, and EBV. In addition, Montazeri and co-workers have shown that certain mitogens, namely staphylococcal protein A and Con A, that were not previously recognized as inducers of differentiation of B cells in human peripheral blood cells could induce significant B-cell maturation when added to monocyte-depleted cultures. Partial monocyte depletion led to a 30- to 100-fold increase in the generation of anti-SRBC forming cells with purified SPA addition, a 10- to 100-fold augmentation when Con A was the activator, and a 7-fold increase when PHA was the stimulant as compared to stimulated cultures that were not monocyte depleted. Similarly, Levitt *et al.* (1981) and Lawton (1982) have described substantial immunoglobulin synthesis by lipopolysaccharide (LPS)-stimulated peripheral blood B cells if the cells were partially monocyte depleted, but not if unse-

parated peripheral for the production of an anti-SRBC response by peripheral blood mononuclear cells stimulated by SRBC in studies by Luzzati and co-workers (1976) and by such cells cultured in association with allogeneic T cells in studies by Montazeri *et al.* (1980).

Each of the procedures used in the experiments considered above led to the depletion of other cells as well as monocytes. Thus, it was important to exclude the possibility that another kind of suppressor cell was removed along with the monocytes (e.g., sticky suppressor T cells), and the possibility that depletion of adherent cells led to the enrichment of B-cell subsets with a high proliferative capacity. The role of the monocytes as the suppressor cells was confirmed by the observation that the addition of purified monocytes to the monocyte-depleted or unseparated cells led to a suppression of immunoglobulin production (Knapp and Baumgartner, 1978; Rosenberg and Lipsky, 1980; Gmelig-Meyling and Waldmann, 1981). This suppression was observed with each of the polyclonal activators examined including the T-cell-dependent activators PWM, SPL, and SLO as well as the relatively T helper cell-independent activators NWSM and EBV. (Gmelig-Meyling and Waldmann, 1981).

Macrophages were effective as suppressors when prepared by a variety of methods including purification by adherence to plastic and glass surfaces, adherence to G-10 columns, preparation by Percoll gradients, and enrichment by T-cell depletion. Rigorous T-cell or B-cell depletion of the macrophage preparations did not abrogate their capacity to suppress. Although the number of macrophages required for suppression depended in part on their state of activation and the system used, it was noted that immunoglobulin production by cells cultured at 2×10^6 mononuclear cells per milliliter in flat-bottomed vials was almost completely abrogated when sufficient numbers of autologous monocytes were added to monocyte-depleted lymphocytes to bring these cultures to 35% monocytes (Gmelig-Meyling and Waldmann, 1981). The monocytes had to be added near the initiation of the cultures to produce suppression, suggesting that the suppressing monocytes act by inhibiting early events in B-cell proliferation and differentiation.

The suppression by monocytes of peripheral blood B-cell activation is not limited to inhibition of differentiation into immunoglobulin- and antibody-synthesizing cells, but has been shown to affect B-cell proliferation as well. In this regard the addition of excessive numbers of monocytes has been shown to inhibit LPS-induced B-cell proliferative responses (as well as other kinds of mitogen-induced proliferative responses) in mice and rats (Yoshinaga *et al.*, 1972; Kirchner *et al.*,

1974, 1976) and to suppress the [^{3}H]thymidine incorporation of cultures of PWM-stimulated human B cells and irradiated T cells (Montazeri *et al.*, 1980) as well as cultures of peripheral blood mononuclear cells stimulated by anti-IgM antibodies (Bona *et al.*, 1979; Montazeri *et al.*, 1980) or cultures stimulated by NWSM systems, where the [^{3}H]TdR incorporation is mainly accounted for by B cells (Bona *et al.*, 1979; Chiorazzi *et al.*, 1980). In a number of cases, suppression of B-cell proliferation was observed with the addition of numbers of monocytes that were not sufficient to affect T-cell proliferation.

Various treatments of the monocyte have been evaluated to determine their effects on the monocyte-induced suppression. Viable monocytes were shown to be required. However, the monocyte suppression was resistant to 8000 R irradiation and to treatment with anti-Ia type serum and complement (Gmelig-Meyling and Waldmann, 1981). In animal model systems of monocyte suppression, the suppressive effect was sensitive to protein synthesis inhibitors and inhibitors of glycolysis, but not to inhibitors of DNA synthesis. It is apparent from animal studies that highly activated macrophages are more effective than minimally activated macrophages as inhibitors of immune responses (and as inhibitors of replicating cell lines of diverse origins) although high numbers of minimally activated macrophages could suppress lymphocyte proliferation (Keller, 1974, 1975; Diener and Lee, 1977; Oehler *et al.*, 1978). Similarly in the human immunoglobulin synthesis system it was observed that macrophages prepared by a number of methods, including those that did not require macrophage adherence and thus led to the minimal possible monocyte activation (including the use of monocyte-enriched cells produced by depletion of cells rosetting with SRBC as the only separation technique), were effective as suppressor cells. In addition, suppression was observed with all polyclonal B-cell activators examined. Most polyclonal B-cell activators could theoretically act as monocyte activators as well. However, EBV does not directly bind to monocytes (Jondal *et al.*, 1976) and thus would presumably not do so. These observations support the view that high numbers of monocytes that have not been specially activated inhibit the proliferation and differentiation of human B cells.

The mode of action and the cellular target of monocyte suppressors has been examined in the *in vitro* immunoglobulin synthesis system. It has been shown in several systems that the monocytes do not "suppress" by cytotoxic reactions, nor do they act by simply binding the polyclonal activators or by binding or catabolizing the secreted immunoglobulins. In some cases, the apparent regulatory action of macro-

phages on B cells may be via T-cell-derived factors (Rich and Pierce, 1973; Tadakuma *et al.*, 1976; Ptak, 1978; Larsson and Blomgren, 1979). For example, Con A-stimulated T cells have been shown to secrete a soluble immune response suppressor (SIRS) that does not inhibit B cells directly but has its suppressor signals transmitted via macrophages (Pierce *et al.*, 1975). However, neither suppressor T cells nor their factors appear to be required for all monocyte suppressor effects. As noted above, excess monocytes suppress the B-cell proliferation induced by anti-IgM antisera and by the relatively T-cell-independent polyclonal activators NWSM and EBV. In addition, Gmelig-Meyling and Waldmann (1981) have observed that excessive numbers of monocytes inhibit immunoglobulin synthesis by rigorously T-depleted B cells that were stimulated by T helper cell-independent activators, and thus monocytes appear to be able to produce a suppressive effect directly on B cells. Monocytes have been shown to secrete a number of products that inhibit lymphocyte proliferation. One of these groups of molecules, the prostaglandins, has been implicated in the suppressive effects of monocytes on T-cell proliferation systems (Goodwin *et al.*, 1977). However, the addition of the prostaglandin synthetase inhibitor indomethacin to systems where excessive numbers of monocytes suppressed immunoglobulin synthesis either had no effect or led to only a partial reversal of the suppression, suggesting that additional factors are involved (Gmelig-Meyling and Waldmann, 1981).

Although the exact mechanism for the monocyte-dependent suppression of different immunological responses remains to be defined, the potential suppression by monocytes must be considered in interpreting *in vitro* immunoglobulin biosynthesis systems, especially when studying human peripheral blood mononuclear cells with their high monocyte contents. The presence of monocyte suppressors may clearly be one of the factors that is involved in the failure of human peripheral blood mononuclear B cells to respond to activators that are effective stimulators of antibody production by mouse splenocytes (e.g., LPS). Similarly, the differing proportions of monocytes in different lymphoid organs may be one of the factors involved in their differential capacities to produce immunoglobulin when stimulated with polyclonal activators. Finally, as discussed below, the capacity of large numbers of monocytes from normal individuals to inhibit immunoglobulin synthesis by polyclonally activated B cells must be considered in interpreting the potential pathogenic role of monocytes in the suppression of the immunoglobulin synthesis observed in different disease states.

VII. Application of Polyclonal Activators of B Cells to the Analysis of the Pathogenic Defects That Lead to Disordered Immunoglobulin Synthesis

The development of a panel of polyclonal activators of B cells that differ in terms of their requirement for helper T cells and in terms of their propensity to activate prosuppressor T cells into effectors of suppression has been of value in defining the pathogenic defects that lead to abnormalities of immunoglobulin levels. With these techniques one can often differentiate disorders of immunoglobulin synthesis in disease that are due to helper-T-cell disorders from those due to abnormalities of the suppressor cell network, and in turn from those due to intrinsic defects of B cells. In the area of disorders of the suppressor cell network, abnormalities of non-T cells as well as T cells have been identified. When the T-cell system is involved, one can determine if the abnormality involves prosuppressor cells, suppressor inducer cells, or the final effectors of suppression. In addition, such studies have been of value in identifying the target of suppressor T-cell action. Overall, these approaches have been of considerable value in providing clues to the nature of the pathogenic defects in an array of primary immunodeficiency states, in the immunodeficiency associated with malignancy and in autoimmune and allergic disorders. In addition, they have lead to the demonstration that monoclonal expansions of leukemic T cells may retain immunoregulatory helper or suppressor function. Studies with these agents are thus providing insights into the nature of the interacting cells controlling immune responses and into the pathogenesis of different disease states.

A. Use of Polyclonal Activators of B Cells for the Analysis of Disordered Helper-T-Cell Activity

Although the majority of patients with reduced immunoglobulin levels are presumed to have a defect of the B cell–plasma cell system (Geha *et al.*, 1974; Cooper *et al.*, 1975; Siegal *et al.*, 1978; Gomez de la Concha *et al.*, 1977), others have been shown to be due to excessive suppressor T cell activity in the PWM-induced *in vitro* biosynthesis system (Waldmann *et al.*, 1974, 1976a,b, 1980a,b; Siegal *et al.*, 1976, 1978) and in yet other cases to have diminished T helper cell function (Seeger *et al.*, 1976; Broder *et al.*, 1976). In general, assays of helper T cell activity have taken advantage of the T helper cell requirement in the PWM stimulated *in vitro* immunoglobulin biosynthesis system. The ability of a patient's T cells to augment PWM-stimulated immunoglobulin synthesis when added to rigorously T-cell-depleted normal B cells has been used as a test for the patient's helper T cell capac-

ity. Other T-cell-dependent activators, such as SLO, SPL, SPA, WGA described in this review can also be used in similar coculture studies that involve patient's T cells and normal B cells to assess antigen nonspecific-T cell helper activity.

The basic assumption underlying this approach is that allogeneic T cells can help B cells to differentiate into immunoglobulin-producing cells. This assertion appears to be fulfilled for these activators, since it has been shown in the PWM system that allogeneic normal T cells (and their soluble products) provide help to normal B cells to essentially the same degree as do autologous T cells (Yata *et al.*, 1978; Saxon *et al.*, 1977; Hirano *et al.*, 1977; Stevens *et al.*, 1979). This lack of genetic restriction may reflect the fact, noted above, that T cells stimulated by PWM and certain other polyclonal activators secrete molecules that will induce B cells to differentiate into immunoglobulin-secreting cells. It should be noted that Yata and co-workers (1978) have demonstrated that T cells from an apparently normal subject whose sister had died of severe combined immunodeficiency disease, could not collaborate with HLA-incompatible allogeneic B cells *in vitro* although her T cells collaborated with autologous B cells as well as with B cells from her histoidentical brother. They suggested that the mechanism that in normal cases overcomes HLA antigen incompatibility, perhaps the secretion of genetically unrestricted helper factors, was defective in this case and that an additional mechanism of T-helper-cell action in PWM-induced B-cell maturation that requires HLA compatibility exists. As discussed above, allogeneic T cells cannot be added to cultures containing EBV since suppressor cells are induced in such cocultures (Blaese and Tosato, 1981). In addition, histocompatibility between T cells, macrophages, and B cells is required for antigen-induced specific antibody production in certain *in vitro* human culture systems (Misiti and Waldmann, 1981).

The presence of excessive numbers of precursors of suppressor T cells or of activated suppressor effector T cells among the patient's T cell population to be evaluated can obscure helper T cell activity in these coculture studies. One approach directed toward the detection of helper T cell activity in the T cells of patients with excessive prosuppressor cell activity is to irradiate the patient's cells to be evaluated for helper-cell activity prior to the coculture with normal B cells, thus taking advantage of the relative radiosensitivity of prosuppressor T cells and the relative radioresistance of helper T cells. However, activated suppressor T cells resistant to 2000 R irradiation may occur in some patients with common variable immunodeficiency or with immunodeficiency following bone marrow transplantation (Waldmann

et al., 1981; Korsmeyer *et al.*, 1982). In addition, as noted in Fig. 2, the activity of helper cells is not fully resistant to exposure to 2000 R. When radioresistant suppressor T cells were present, it has been necessary to isolate T-cell subpopulations such as Tμ cells or cells that react with monoclonal antisera defining T helper populations (e.g., OKT4$^+$ cells or LEU-3$^+$ cells) from the patient under study in order to assay for helper activity. By means of these approaches (use of irradiation of T cells or the use of isolated T-cell subpopulations), we have demonstrated that helper and excessive prosuppressor T-cell activity can coexist in human peripheral blood T-cell populations.

The demonstration of absent or markedly reduced helper cell activity in the patient's T cells when cocultured with normal B cells and a T-cell-dependent polyclonal activator is a necessary requirement for defining a patient as having a helper T cell defect as the primary factor in the pathogenesis of extreme immunoglobulin deficiency. However, it is certainly not a sufficient one. A second requirement is that the patient's B cells should be normal. That is, they should produce immunoglobulins normally when cultured in the presence of polyclonal activators that are relatively T cell helper cell independent (e.g., NWSM, *Staphylococcus aureus* Cowan strain I organisms, EBV, and the helper factor secreted by normal T cells after their exposure to such agents as PWM). In addition, the B cells from the patient should produce immunoglobulin on stimulation with T-cell-dependent activators when they are cocultured with normal T cells. Although the T cells of many patients with primary immunodeficiency diseases or with immunodeficiency following bone marrow transplantation showed diminished helper cell activity when cocultured with normal B cells, only a minority fulfilled the second requirement for the definition of the helper cell defect as the sole factor leading to the abnormality of immunoglobulin synthesis. In our own experience, patients fulfilling both of the criteria include certain patients with severe combined immunodeficiency with B cells, patients with diminished T-cell activity, thymic abnormality, and reduced immunoglobulin levels (i.e., the Nezeloff's syndrome), a single patient over 60 years old studied with common variable immunodeficiency, a very small proportion of patients with ataxia-telangiectasia and reduced IgA levels, and a minority of patients with hypogammaglobulinemia following bone marrow transplantation (Broder *et al.*, 1976; Korsmeyer *et al.*, 1982; Waldmann *et al.*, 1981). Others have also demonstrated defective helper T cell activity in similar groups of patients (Seeger *et al.*, 1976; Siegal *et al.*, 1978) as well as in patients with the immunoglobulin deficiency associated with chronic lymphocytic leukemia

(Fu *et al.*, 1978). Diminution of helper-T-cell activity in association with either disordered B-cell function or excessive suppressor-T-cell activity or both has been observed more widely.

The *in vitro* biosynthesis system with PWM has also been used to determine whether neoplastic T cells have helper activity when they are cocultured with B cells from normal individuals or from the patient (Broder *et al.*, 1976; Berger *et al.*, 1979). The fact that only very small numbers of normal T cells are required for a B-cell response to PWM (Fig. 2) must be taken into consideration in these analyses. When this was not taken into account, as is true in some reports, the apparent helper-cell activity that was observed may not have been due to the action of the neoplastic T cells, but may have been due to helper activity provided by small numbers of nonneoplastic normal T cells that contaminated the neoplastic cells. For example, if 0.5×10^6 normal indicator B cells were cocultured with equal numbers of a patient's peripheral blood T cells to be assayed for helper activity and the neoplastic cells were contaminated with but 2% of normal nonneoplastic T cells (i.e., 10^4 normal T cells), significant immunoglobulin synthesis by the B cells would be expected and could be due to the helper activity of these 10^4 contaminating nonneoplastic T cells alone (Fig. 2). Because of this possibility of contaminating normal T cells, only very high count T-cell leukemias can be easily evaluated for helper function with the techniques presently available. In addition, serial dilutions of the neoplastic T cells and a normal T cell population should be performed in parallel.

Helper activity that is comparable to that provided by the normal T cells should be demonstrable with the neoplastic T cells cultured at low neoplastic T-cell to normal B-cell ratios before the neoplastic T cells can be confidently classed as helper T cells. This may unavoidably require that certain neoplastic populations with weak helper activity be classified as lacking helper activity. In addition it is important to show that the neoplastic T cells are capable of helping autologous as well as allogeneic B cells in the mitogen-induced system. Using such criteria, we have defined helper activity in 6 of 14 patients with the Sézary T cell leukemia and in one of 20 patients with T-cell acute lymphocytic leukemia (Broder *et al.*, 1976, 1980). Others have subsequently shown helper T cell activity in related neoplastic-cell populations (Berger *et al.*, 1979). When examined with monoclonal antibodies, Sézary T cells have been shown to bear the antigens recognized by anti-OKT3 and OKT4 antibodies that define mature inducer T cells but not those recognized by antibodies OKT5 and OKT8, which define suppressor cells (Kung *et al.*, 1981). Thus these

cells have been shown by functional and phenotypic analysis to be mature T cells dedicated to helper interactions with B cells.

B. Use of Polyclonal Activators of B Cells for the Analysis of Disordered Suppressor-T-Cell Activity

1. Excessive Suppressor-T-Cell Activity

In general, evidence for excessive suppressor-T-cell activity in patients with immunoglobulin deficiency states has been obtained utilizing coculture techniques. In this approach circulating mononuclear cells or T cells from a patient undergoing study are cocultured with circulating mononuclear cells from normal individuals or with normal B cells plus irradiated normal T cells in the presence of PWM or other polyclonal activators. The synthesis of immunoglobulins by the two subjects in coculture is related to the sum of the contribution expected from these subjects determined from the immunoglobulin synthesis by the cell populations of these individuals when cultured alone. We consider a depression of immunoglobulin production in the coculture system to less than 50% of the expected value to represent a moderate, and to less than 20% as a profound, suppressor effect. An abnormal number or an abnormal state of activation of suppressor T lymphocytes has been observed in this way using the PWM system in a subset of patients with common variable immunodeficiency (Waldmann *et al.*, 1974, 1980a,b, 1981; Siegal *et al.*, 1976; Witemeyer *et al.*, 1976; Herrod and Buckley, 1979), in patients with hypogammaglobulinemia associated with a thymoma (Waldmann *et al.*, 1975; Siegal *et al.*, 1976), in patients with hypogammaglobulinemia following bone marrow transplantation (Reinherz *et al.*, 1979b; Lum *et al.*, 1981; Korsmeyer *et al.*, 1982), in patients during EBV-induced infectious mononucleosis (Tosato *et al.*, 1979; Haynes *et al.*, 1979; Johnsen *et al.*, 1979), and in some patients with acute T-cell lymphocytic leukemia (Broder *et al.*, 1978, 1981) or with the form of subacute adult T-cell leukemia of Japan (Uchiyama *et al.*, 1978). IgA class-specific suppressor cells have also been demonstrated in a subset of patients with selective IgA deficiency (Waldmann *et al.*, 1976a; Atwater and Tomasi, 1978; Schwartz, 1980).

A number of critical questions have been raised concerning this *in vitro* demonstration of disordered suppressor-T-cell activity that are common to all of these groups of patients. These issues will be considered with special reference to common variable hypogammaglobulinemia. Common variable immunodeficiency is a heteroge-

neous group of disorders characterized by hypogammaglobulinemia and an increased incidence of infections but variable patterns of clinical manifestations. Clearly the patients are also heterogeneous in terms of the basic pathogenic mechanisms that lead to the defect in immunoglobulin synthesis. The majority of patients appear to have intrinsic defects in their B cell–plasma cell system, but other subsets have abnormalities of helper T cells or of the system of suppressor cells. In 1974, using the coculture of peripheral blood lymphocytes from patients with common variable immunodeficiency and normal lymphocytes in a PWM-stimulated *in vitro* biosynthesis system, we suggested that, in a subset of patients, common variable immunodeficiency is caused or perpetuated by an abnormality of suppressor T cells that act to inhibit B-cell maturation and antibody production. In the intervening years we have expanded our series to include 60 patients with this syndrome. In this series, the PWM-stimulated synthesis of immunoglobulins by normal cells was suppressed by over 50% when cocultured with equal numbers of peripheral blood mononuclear cells of 44% of the patients with common variable immunodeficiency and by 80–100% when incubated with the cells of 18% of the patients (Waldmann *et al.*, 1980b, 1981). The observation of suppression in such cocultures between peripheral blood lymphocytes from patients with common variable hypogammaglobulinemia and cells from normal individuals has been confirmed by a number of laboratories (Siegal *et al.*, 1976; Broom *et al.*, 1976; Herrod and Buckley, 1979; Morito *et al.*, 1980; Ashman *et al.*, 1980). However, a considerable number of issues have been raised concerning these observations. The first issue relates to the nature of the suppressing cell in the circulation of the patients. In our initial studies, as well as in most studies by others, T cells obtained from the peripheral blood of the patients were effective as suppressors. In some cases the non-sheep cell rosetting cells (i.e., B cells and especially monocytes) also manifested suppressor cell activity usually, but not always, in association with excessive suppressor T cell activity (Siegal *et al.*, 1976; Ashman *et al.*, 1980; Waldmann *et al.*, 1981).

Another question that has been raised concerning the coculture system relates to whether a mixed leukocyte reaction between the patient's cells and the allogeneic normal cells contributes to the suppression observed, especially in light of the fact that the cells of some patients will suppress the synthesis of the cells of some normals but not of others. A mixed leukocyte reaction does not appear to be an adequate explanation for the observed suppression since

no net suppression was observed when the cells of unrelated normals were cocultured (Waldmann *et al.*, 1974) and since the cells of a patient with hypogammaglobulinemia were shown to suppress immunoglobulin synthesis of a normal HLA-MLC matched twin (Siegal *et al.*, 1976). In our own experience with 10 mixtures of allogeneic lymphocytes from normal individuals in PWM-stimulated cultures, the mean observed synthesis of IgM, IgG, and IgA ranged from 95 to 106% of the expected values. In addition, as discussed below in a number of cases, mononuclear cells from a suppressor agammaglobulinemic patient could be induced to secrete IgM normally if they were depleted of T cells. In each of these cases the addition of autologous T cells to the culture system at a T:B cell ratio of 1:1 led to suppression of this immunoglobulin synthesis. Even considering these observations, suppression observed in allogeneic mixtures should be confirmed, whenever possible, using techniques that do not involve allogeneic cells.

Another issue that was raised by Gomez de la Concha and co-workers (1977) concerning the coculture assay system with normal and hypogammaglobulinemic lymphocytes followed their observations discussed above that suppression was observed when normal T and B cells were cocultured at high T:B cell ratios in certain lots of fetal calf serum (FCS) and PWM. They suggested that such high T-cell to effective B-cell ratios might be produced when the mononuclear cells of patients with hypogammaglobulinemia are cocultured with normal lymphocytes. Although this may be a problem with cells of patients that suppress modestly in coculture, this problem can be obviated by using lots of PWM and FCS in flat-bottommed vials where suppression at high T:B cell ratios is not observed. However, in light of these concerns, suppression should be demonstrated at T:B cell ratios that are physiological (e.g., 4:1 or lower) before one concludes that an abnormality of suppressor cells is demonstrable. Using this approach we have shown that an abnormal T-cell to effective B-cell ratio is not an adequate explaination for the profound suppression we observed with the five patients we studied most extensively (Waldmann *et al.*, 1981). We have observed one patient whose circulating T cells dramatically suppressed the PWM-stimulated immunoglobulin synthesis of the indicator cells (normal B cells and irradiated normal T cells) at a patient T-cell to normal B-cell ratio of 1:10. In addition, all the patients we have studied whose unseparated cells profoundly suppressed normal mononuclear cell immunoglobulin synthesis suppressed at patient T-cell to normal B-cell ratios of 1:1 when separated populations were analyzed. This experience has been confirmed by

others and a high T-cell to effective B-cell ratio does not appear to be an adequate explanation for the profoundly increased suppressor cell activity observed in a subset of patients with common variable immunodeficiency.

An additional issue that can be addressed with the use of a variety of polyclonal activators that differ in their propensity for activating prosuppressor cells to suppressor effectors relates to the nature of the cell abnormality of the suppressor cell network that may exist in these patients. With some patients with common variable immunodeficiency, we have seen suppression when PWM is the polyclonal activator used in the coculture studies but not when another B-cell activator, NWSM, was used (Bona *et al.*, 1979; Waldmann *et al.*, 1980a,b, 1981). In these cases when both PWM and NWSM were added to the system simultaneously, suppression was observed. Since PWM has the propensity to activate prosuppressor cells whereas NWSM does not, we view the patients with this pattern of suppression in coculture as having increased numbers of circulating prosuppressor cells that require PWM for their activation to become suppressor effectors. We have also identified a group of patients where suppression in coculture was observed when any polyclonal activator of B cells was utilized including PWM, NWSM, SLO, and SPL. This observation has led us to the view that these patients have abnormally increased numbers of circulating activated suppressor-effector T cells. A similar analysis of leukemic T cells with retained suppressor activity has shown that some of these leukemias represent expansions of prosuppressor T cells (Broder *et al.*, 1978, 1981), whereas others represent expansions of more mature cells that are suppressor-effectors. In the former case the leukemic cells require an interaction with an unirradiated normal T cell or a T-cell inducing factor before showing marked suppression in the PWM-driven immunoglobulin biosynthesis system (Broder *et al.*, 1981).

An additional issue concerns the target of suppressor T-cell activity. In most animal systems, carrier-specific suppressor T cells appear to act on helper T cells (Gershon, 1974; Herzenberg *et al.*, 1975). However, this may be not be the case for many of the antigen-nonspecific suppressors observed in the human systems. It should be noted that, when suppressor T cells from a patient are mixed with normal unseparated cells, the target of the suppression (i.e., B cells, macrophages, or helper T cells) cannot be defined. However, advances in the culture systems involving the use of both T helper cell-dependent and independent polyclonal activators have permitted a resolution of the issue of the target of antigen-nonspecific suppressor cells in certain cases.

In some cases suppressor cells appear to inhibit polyclonally activated B cell responses indirectly by inactivating the requisite helper T cell. For example, Moretta and co-workers (1979) have reported that the suppressor function of activated Tγ cells for PWM-induced B-cell differentiation involves the production of a soluble factor that inhibits the helper effect of the Tμ cells. This Tγ-produced suppressor factor is incapable of inhibiting the B-cell immunoglobulin synthesis if the regulatory helper activity is not provided by intact T cells, but is instead provided by a soluble inducer factor secreted by Tμ cells precultured separately. We have observed a potentially similar pattern of suppression of immunoglobulin synthesis induced by T-helper-dependent polyclonal activators, but not by T-helper-independent activators when the cells of a patient with the Japanese suppressor T-cell leukemia were examined. It is important in the study of patients who show this pattern to include cultures to which both the T-independent polyclonal activators and PWM are added. Such cultures are required to distinguish patients who have an excessive number of circulating prosuppressor T cells that require PWM for their activation on the one hand, from those who have circulating activated suppressor T cells with helper T cells as their targets on the other. With the former group of patients suppression should occur when PWM is added to the cultures in addition to the T helper cell-independent activator, whereas with the latter group of patients no such suppression should be observed. In contrast to the patients discussed above, in certain cases the target of the suppressor T cell action appears to be a non-T cell (i.e., the B cell or macrophage). In such a case that we have studied (Waldmann *et al.*, 1980b), T cells from a patient with common variable immunodeficiency inhibited the immunoglobulin synthesis by purified normal B cells and macrophages stimulated by a T-cell-helper factor. In such cases the helper T cell cannot be the target of the suppression, since these cells have been eliminated from the system. Similarly, the target of the autologous suppressor observed late in EBV-stimulated culture by Blaese and Tosato (1981) must be a non-helper T cell and is presumably a B cell, since EBV is a helper T-cell-independent activator.

A final and most critical question relating to the demonstration of suppressor T lymphocytes in patients with common variable hypogammaglobulinemia is whether the development of such suppressors is a primary pathogenic mechanism causing the hypogammaglobulinemia in any of these patients or whether all the patients have a primary defect in their B cells with the secondary development of circulating suppressor T cells. This issue is especially pertinent, since

excessive suppressor T-cell activity has been demonstrated by a number of groups including our own in patients with X-linked agammaglobulinemia and in patients with thymoma and hypogammaglobulinemia (Siegal *et al.*, 1976; Herrod and Buckley, 1979; Waldmann *et al.*, 1975, 1976b). In addition, Blaese and co-workers (1974) have demonstrated activated suppressor T cells in the circulation of chickens that were made hypogammaglobulinemic by bursectomy and irradiation at hatching. However, it is possible that suppressor T cells may play a role in the pathogenesis of the hypogammaglobulinemia of patients with thymoma and hypogammaglobulinemia and even, as suggested by the work of Dosch and co-workers (1977), of those with X-linked infantile hypogammaglobulinemia. In addition, in the bursectomized bird model of agammaglobulinemia syngeneic normal birds became agammaglobulinemic following the adoptive transfer of T cells from the bursectomized birds (Blaese *et al.*, 1974). This latter observation indicates that suppressor T cells are capable of shutting down the process of immunoglobulin production *in vivo* and supports the possibility that the suppressor cell phenomenon may have real biological significance as an aberrant control mechanism for immunoglobulin production in pathologic states.

In order to address the significance of the suppressor T cells in the patients with profound suppression in coculture further, we separated the T cells from the B cells and macrophages of these patients using rosetting procedures and evaluated the capacity of the B cells to produce immunoglobulins following PWM stimulation. In most, but not all, of these cases with profoundly increased suppressor activity, the patient's B cells depleted of T cells made immunoglobulin molecules when stimulated with the T helper cell-independent activator EBV and when cocultured with normal T cells to provide helper function with PWM as well. In addition, the B cells of the patients studied synthesized immunoglobulins in the presence of PWM and autologous Tμ cells, but not with unseparated autologous T cells. In each case examined, when the patient's unseparated T cells were added to the patient's B cells and normal or autologous helper T cells in the presence of PWM at a ratio of one patient T cell to one patient B cell, the immunoglobulin synthesis was suppressed, with a mean suppression of 88% (Waldmann *et al.*, 1980a,b, 1981). Thus, this subset of patients appears to have B cells that can produce IgM *in vitro* normally if they were freed of their suppressor T cells. These studies do not prove that this subset of patients have suppressor T cells that are the sole cause of their immunoglobulin deficiency. However, such patients do fulfill the minimal criteria for such biologically meaningful

suppressors; that is, that the patient's B cells can produce immunoglobulin molecules when cultured with autologous helper T cells and furthermore this immunoglobulin synthesis is suppressed when the patient's unseparated T cells are introduced to this culture system at a physiological T cell to B cell ratio.

2. *Deficient Suppressor-T-Cell Activity*

Several different systems involving the use of polyclonal activators have been adapted to detect deficiencies of suppressor-T-cell function and have been applied to the study of regulatory T cells in autoimmune disorders. The most widely employed systems involve the preincubation of T cells to be analyzed for suppressor capacity with a polyclonal activator such as Con A that normally induces prosuppressor cells to become activated effectors of suppression (Dutton, 1972; Rich and Pierce, 1974; Shou *et al.*, 1976; Haynes and Fauci, 1977; Schwartz *et al.*, 1977; Bresnihan and Jasin, 1977). After the preincubation step, the cells to be assayed for suppressor effector function are washed to remove the activator and are then cocultured with an indicator population. When the action on the B-cell system is to be analyzed, the ability of preincubated cells to suppress PWM-driven immunoglobulin or antibody production is usually assessed, although several other kinds of *in vitro* immune reactions can be inhibited as well. Such Con A-activated suppressor T cells must be added relatively early in the course of PWM-stimulated lymphocyte cultures for maximal effect.

A number of variations have been utilized in the preculture system to activate the suppressor cells. In some systems, PWM (Uchiyama *et al.*, 1981b) rather than Con A has been used as the activator. Indeed, under certain conditions it is possible to activate a population of suppressor cells by preincubation in media supplemented with FCS or AB human serum without the addition of a mitogen such as Con A (Schwartz *et al.*, 1977; Lipsky *et al.*, 1978). However, the culture conditions and the duration of the preincubation period can influence the kind of suppressor activity that appears to emerge. For example, Lipsky *et al.* (1978) were able to induce suppressor T cells capable of inhibiting PWM-driven immunoglobulin production by cocultured autologous or allogeneic lymphocytes when a 7-day preincubation period was used to generate suppressor cells. On the other hand, Schwartz *et al.* (1977), using a 48-hour preincubation period, observed that suppressor cells generated in the absence of Con A showed the most pronounced inhibitory activity in coculture with autologous lymphocytes, whereas suppressor cells generated in the presence of

Con A were active against both autologous and allogeneic lymphocyte populations.

A second approach to detect a deficiency of suppressor T cell activity does not involve a preincubation period, but rather the addition of the mitogen, to activate suppressor cells, directly to the indicator culture system (Miller and Schwartz, 1979). Several laboratories have used the capacity of Con A to inhibit immunoglobulin production when added directly to PWM-stimulated lymphocyte populations as an index of suppressor cell potential. The interpretation of such an approach is more complex than that with the preculture technique, since the mitogen could act to inhibit B-cell function directly as well as indirectly by activating suppressor T cells. Another approach takes advantage of the fact that normal suppressor T-cell function may be suppressed in PWM-induced immunoglobulin systems when they are cultured at high T:B cell ratios (James *et al.*, 1980). Failure to see suppression at such ratios in the PWM system has been interpreted as indicating diminished suppressor-T-cell capacity. A final approach takes advantage of the observation that suppressor cells that appear late in EBV-stimulated mononuclear cell systems are derived from seropositive individuals (Blaese and Tosato, 1981). The failure to observe such suppressor-cell activation is interpreted as a defect in this form of suppressor cell system.

Utilizing the Con A pulsing system, most laboratories have observed diminished suppressor-cell capacity in patients with systemic lupus erythematosus (Messner *et al.*, 1973; Abdou *et al.*, 1976; Bresnihan and Jasin, 1977; Fauci *et al.*, 1978; Sakane *et al.*, 1978; Morimoto, 1978; Miller and Schwartz, 1979; Clough *et al.*, 1980) and in patients with chronic active hepatitis (Hodgson *et al.*, 1978). A failure of suppressor T-cell function has been demonstrated in patients with primary biliary cirrhosis (James *et al.*, 1980) as well as in a subset of patients with common variable immunodeficiency and autoimmune manifestations (Nelson *et al.*, 1976) using the PWM-induced immunoglobulin biosynthesis system at high T:B cell ratios. In addition, patients with rheumatoid arthritis have been shown to be deficient in the EBV-induced late-appearing suppressor cell (Tosato *et al.*, 1981). Although these studies suggest that suppressor T cell dysfunction may occur in association with a variety of disease states usually considered to be autoimmune, it cannot be concluded that the disorders of suppressor T cells are necessarily the primary underlying defect in these disorders. In this regard, in many animal-model systems of autoimmunity, excessive activation of B cells has been viewed as the most dramatic and universal disorder (Dixon *et al.*, 1980). In humans high

numbers of circulating plaque-forming cells assessed without *in vitro* culture appear to characterize these disorders, supporting a role for excessive activation of B cells in these diseases (Ginsburg *et al.*, 1979; Blaese *et al.*, 1980). It has been difficult, however, to use polyclonal B-cell activators to demonstrate hyperactive B-cell function in patients with autoimmune disorders (Bobrove and Miller, 1977; Nies and Louie, 1978; Ginsberg *et al.*, 1979). Indeed, the majority of such patients have low PWM-stimulated immunoglobulin and antibody synthesis. This latter observation may reflect an activation of B cells in response to specific antigens *in vivo* with a diminution of those precursor cells that can be further activated with polyclonal stimulators *in vitro*.

C. Use of Polyclonal B-Cell Activators to Define Disorders of Regulatory Monocytes

Monocytes have a critical bimodal effect on the response of human B cells to polyclonal activators and, through a combination of enhancing and suppressing effects considered above, play a role in regulating the immune response. Abnormalities of the number or state of activation of monocytes may potentially contribute to disorders of immunity in disease states including disorders of B-cell differentiation and of immunoglobulin synthesis. Depressed B-cell proliferation into immunoglobulin-synthesizing cells was observed with polyclonal B-cell activators when there was a profound depletion of monocytes or when culture conditions limiting cell contact were used. In light of these observations, deficiencies of functional monocytes in the culture must be considered in interpreting abnormal *in vitro* immunoglobulin biosynthesis in disease in three general circumstances: (*a*) in the presence of high-count lymphocytic leukemias where the absolute number of monocytes may be normal, but where these cells are diluted by large numbers of leukemic cells; (*b*) marked depletion of the absolute numbers of circulating monocytes; and (*c*) disorders of monocyte function that lead to abnormalities in their ability to enhance B-cell activation.

We have noted a basic pattern of immunoglobulin synthesis in response to polyclonal activators in each of the circumstances with monocyte deficiency. The mononuclear cells of patients with this characteristic pattern synthesized immunoglobulin when cultured in media containing fetal calf serum alone or in cultures stimulated by NWSM. In these cultures the synthesis of IgA tended to be normal or high whereas that of IgM was quite reduced, so that the ratio of IgA to IgM synthesis was high. Furthermore, the addition of PWM to cultures of

these mononuclear cells either had no stimulating effect or even suppressed immunoglobulin synthesis. It should be noted that this pattern of immunoglobulin production, including the responses to PWM, is not specific for disorders associated with diminished monocyte function but is also observed in association with a number of other conditions especially those considered to be autoimmune (e.g., systemic lupus erythematosus, rheumatoid arthritis) (Ginsburg *et al.*, 1979).

The major factors involved in the diminished immunoglobulin response to polyclonal activators by peripheral blood mononuclear cells of patients with the lymphocytic leukemias reflect abnormalities intrinsic to the B cells, increased suppressor cell activity of the leukemic cells themselves, diminished helper T-cell activity or diminished proportions of normal B cells in the associated nonleukemic populations. However, diminished numbers of monocytes added to the culture system must be considered as well, since the proportion of monocytes in the mononuclear cell population may be markedly reduced to a fraction of 1% owing to dilution by large numbers of leukemic B or T lymphocytes in these patients with high-count leukemias. Patients with the low-count Sézary T-cell leukemia showed the pattern of high IgA to IgM synthesis and suppression by PWM discussed above (Broder *et al.*, 1977). This may reflect the helper activity of these leukemic cells or could conceivably be related to diminished numbers of monocytes added to the system.

Patients with aplastic anemia may have extreme monocytopenia (i.e., 0–1% of mononuclear cells) and normal numbers of circulating lymphocytes. These patients have provided an opportunity to study monocyte requirements for immune responses that do not require depletion procedures that might affect cells other than monocytes. The patients studied with aplastic anemia have had normal T-cell proliferative response to nonspecific mitogens, but markedly deficient responses to antigens (Twomey *et al.*, 1973). When the lymphocytes of such patients were cultured with macrophages obtained from HLA-MLC matched siblings, reconstitution to normal *in vitro* responsiveness to antigens could be demonstrated (Twomey *et al.*, 1973). Patients with aplastic anemia as well as a few with common variable immunodeficiency were also shown to have a markedly diminished capacity to produce a monocyte-derived B-cell differentiation factor (Finelt and Hoffman, 1979). The patients with aplastic anemia that we studied showed the pattern of *in vitro* immunoglobulin synthesis response to NWSM and PWM discussed above. A similar pattern was observed with the cells of patients with the Wiskott–Aldrich syn-

drome, who may have defective macrophage function in this assay (S. Broder, R. M. Blaese, and T. A. Waldmann, unpublished results) as well as in assays of monocyte chemotactic responses (Altman *et al.*, 1974) and of monocyte-mediated ADCC (Poplack *et al.*, 1976).

As noted above, normal monocytes can have strongly suppressive effects on immunoglobulin and antibody production by human B lymphocytes stimulated by polyclonal activators if they are added in sufficient numbers to the cultures. This observation has been used by some to exclude the possibility that monocyte suppression could play a role in the pathogenesis of diminished immunoglobulin synthesis in disease states. This does not appear to be a valid conclusion, for an exaggeration of the normal regulatory mechanism either due to excessive numbers of monocytes or especially an increase in their state of activation might play a role in the pathogenesis of immunodeficiency. Great care to include appropriate controls for such studies is required to show that any suppression that is observed in the disease state occurs with monocyte proportions that do not suppress when normal monocytes are used. Furthermore, it must be shown that augmentation of mitogen-induced immunoglobulin synthesis following monocyte depletion is greater when studying the peripheral blood mononuclear cells of the patients than when the cells of normal individuals are examined.

Suppression of T-cell proliferation due to excessive adherent suppressor cells (probably monocytes acting either directly or indirectly through an interaction with other regulatory cells) has been proposed for certain patients with anergy associated with widespread fungal infections (Stobo, 1977) tuberculosis (Ellner, 1978), solid tumors (Berlinger *et al.*, 1976; Yu *et al.*, 1977; Jerrells *et al.*, 1978), Hodgkin's disease (Twomey *et al.*, 1975; Goodwin *et al.*, 1977b), and in some cases of common variable immunodeficiency (Goodwin *et al.*, 1978). It has been proposed by Goodwin and co-workers (1977b, 1978) that the anergy associated with monocyte suppression in some patients with common variable immunodeficiency and in those with Hodgkin's disease is due to the excessive production by monocytes of prostaglandin E2 which suppresses T cell function.

Inhibition of polyclonal B cell activation *in vitro* by suppressor monocytes has been reported in patients with multiple myeloma (Broder *et al.*, 1975; Broder and Waldmann, 1979), some patients with common variable immunodeficiency (Siegal *et al.*, 1976; Ashman *et al.*, 1980), and in patients with sarcoidosis (Katz and Fauci, 1978). Patients with sarcoidosis were reported by Katz and Fauci (1978) to have a deficiency of SRBC plaque-forming cell capacity following poly-

clonal activation with PWM. In these studies removal of monocytes from the mononuclear cell suspensions from patients with sarcoidosis lead to a marked augmentation of the plaque-forming cell response. No such augmentation was observed following monocyte depletion of normal cells, suggesting that a suppressor monocyte was present in the circulation of the patients. As noted above, patients with common variable immunodeficiency represent a complex group of diseases with different pathogeneic mechanisms. It has been shown in many studies that the cells of some patients with this disorder suppress immunoglobulin synthesis of PWM-stimulated normal cells cocultured with them. In our own experience, all patients who showed profound suppression in coculture had T-cell suppressors (Waldmann *et al.*, 1976b, 1981). The non-T cells did not suppress in two of the five cases examined most extensively, gave modest suppression in one case (23% suppression) and moderate suppression in two cases (43 and 58% suppression).

In studies by others, nonrosetting cells (presumably monocytes) from some patients were shown to act as suppressors (Siegal *et al.*, 1976; Ashman *et al.*, 1980). In most cases these monocyte suppressors were associated with T-cell suppressor cells, whereas in rare cases they were the sole suppressor cell demonstrable. The suppression by non-T cells might reflect the activation of monocyte suppressors by activated suppressor T cells. Alternatively, the apparent suppression by the non-T cells may reflect an increased proportion of monocytes in the peripheral blood mononuclear cell preparations from the patients if they were lymphocytopenic, since, as noted earlier, suppression by sufficient numbers of monocytes of even normal individuals can lead to suppression in coculture. Finally, monocyte suppressors could play a role in the pathogenesis of the hypogammalobulinemia. In one case the removal of adherent cells lead to an increase in the number of immunoglobulin-producing cells in a patient with common variable immunodeficiency (Siegal *et al.*, 1976). However, even in this case, the adherent monocytes did not suppress immunoglobulin synthesis when added to normal cells. It is clear, however, that the possibility of adherent cell suppressors should be considered in any study of the immunoglobulin synthetic capacity of patients with common variable immunodeficiency, since special cell separation techniques, other than T-cell depletion alone, may be required to differentiate between abnormal suppressor monocyte activity and an intrinsic B cell defect as the primary problem in those patients whose non-T cells produce no immunoglobulin response when stimulated by polyclonal B-cell activators. This is especially true in light of a preliminary report that

the mononuclear cells of essentially all patients with common variable immunodeficiency could be induced to become antibody-secreting cells if they are passed through G-10 columns before exposure to antigen and activators (Pollack *et al.*, 1981).

Patients with multiple myeloma were the first individuals to have suppressor monocytes suggested as a pathogenic factor in the impaired synthesis of polyclonal (non-paraprotein) immunoglobulins observed (Broder *et al.*, 1975; Waldmann *et al.*, 1976b; Broder and Waldmann, 1979). Such patients have reduced circulating levels of polyclonal immunoglobulins in the serum and demonstrate impaired antibody formation following antigenic stimulation. We have used the *in vitro* model of immunoglobulin production to study the humoral immunodeficiency of such patients. The peripheral blood mononuclear cells from patients with multiple myeloma synthesized reduced quantities of immunoglobulin on PWM stimulation with means for synthesis of IgG, IgM, and IgA of only 9–25% of normal. A number of factors including the action of monocyte suppressors, nonmonocyte suppressor cells, as well as intrinsic B-cell disorders play a role in the immunodeficiency observed. When the peripheral mononuclear cells of these patients were cocultured with normal cells they suppressed the immunoglobulin synthesis of the normal cells by 65–100% in four of the seven patients studied. The suppressor cells were shown to be radioresistant and were removed by the carbonyl iron technique. These studies as well as those in the mouse models of myeloma (Kolb *et al.*, 1977; Krakauer *et al.*, 1977; Katzmann, 1978) support the view that excessive numbers of activated monocyte suppressors contribute to the polyclonal immunodeficiency associated with multiple myeloma.

D. Use of Polyclonal Activators of B Cells for the Analysis of Defects Intrinsic to the B Cell–Plasma Cell System

In many disease states, disorders of immunoglobulin production reflect disorders intrinsic to the cells of the B cell–plasma cell series. A minimal requirement for the identification of a B-cell defect is the demonstration that the patient's peripheral blood mononuclear cells do not produce normal quantities of immunoglobulin when stimulated by polyclonal activators *in vitro*. This is not a sufficient condition for categorizing the defect as being intrinsic to the B cell, for a comparable result would be observed when there are deficiencies of required helper T cells or monocytes or when there is excessive activity of suppressor monocytes or of the suppressor T-cell network. Thus,

to determine that there is an intrinsic B cell defect one must fulfill a second requirement, that is, the demonstration that there is failure of immunoglobulin synthesis by rigorously T cell- and monocyte-depleted B cells of the patient when stimulated by such relatively T-cell- and monocyte-independent activators as EBV, NWSM, or *Staphylococcus aureus* Cowan strain I organisms. In addition, it should be shown that the patient's purified B cells cultured in the presence of requisite numbers of normal T helper cells and monocytes cannot produce immunoglobulin molecules when stimulated by such T helper cell-dependent, polyclonal activators as PWM, WGA, SLO, or SPL. Many of the primary and secondary immunoglobulin deficiency states have demonstrable B-cell defects that fulfill these two criteria.

In our own experience we have defined at least some patients with the following disease categories as having intrinsic B-cell defects: X-linked agammaglobulinemia, common variable immunodeficiency, selective IgA deficiency, IgA deficiency associated with ataxia–telangiectasia, hyper-IgM syndrome with reduced IgG and IgA, and in patients after bone marrow transplantation. Others have used similar approaches and have demonstrated B-cell disorders in association with a wide variety of primary and secondary immunoglobulin deficiency diseases. Frequently the B-cell defects in these diseases are associated with abnormalities of regulatory T-cell circuits as well.

VIII. Summary

A series of agents have been demonstrated to function as polyclonal activators of human B cells. Such agents are effective in activating B cells to proliferate and to differentiate *in vitro* into immunoglobulin-synthesizing and -secreting cells. Such B-cell differentiation is induced when unseparated human peripheral blood mononuclear cells are stimulated with pokeweed mitogen, streptolysin O, staphylococcal phage lysate, wheat germ agglutinin, staphylococcal protein A, staphylococcal organisms producing large quantities of protein A, the Epstein–Barr virus, or *Nocardia* water-soluble mitogen. The PWM, SLO, SPL, WGA, and SPA do not stimulate immunoglobulin synthesis by rigorously T-cell-depleted B cells, whereas such B-cell populations are stimulated to produce immunoglobulin when staphylococcal organisms, EBV, or NWSM are added to the system. Thus PWM, SLO, SPL, WGA, and SPA are clearly T-cell dependent whereas NWSM, EBV, and staphylococcal organisms are relatively helper T cell-independent activators of immunoglobulin synthesis by B cells. A series of distinct subsets of T helper cells that differ in terms of radiosensiti-

vity, surface antigens on activation, and binding to nylon columns appear to act synergistically with the B cells in facilitating the response to T-cell-dependent activators. Each of the T-cell-dependent activators of B cells appears to be dependent on monocytes as well. Another important difference among the polyclonal activators is in their propensity to activate precursors of suppressor T cells into effectors of suppression. For example, there is an activation of prosuppressor T cells in cultures stimulated by PWM or SPA that have a high T:B cell ratio or that have a high concentration of the activator. In contrast, NWSM does not lead to the activation of suppressor cells under these conditions. In many cases when activators such as SPA, Con A, and PHA with a very high propensity for activating precursors of suppressor cells are examined, irradiated T cells must be used in place of unirradiated T cells in order to see B-cell activation. The high numbers of monocytes that are present in normal peripheral blood inhibit B-cell stimulation with many of the activators studied. Indeed, when certain activators are used monocyte depletion is required before significant immunoglobulin synthesis can be observed.

The development of techniques to study polyclonal activation of B cells into immunoglobulin and antibody-producing cells along with the availability of polyclonal activators that differ in terms of their requirements for helper T cells and monocytes and in terms of their propensity to activate prosuppressor cells into effectors of suppression has been of great value in defining the regulatory cellular interactions that control B-cell maturation. Furthermore, such studies have provided clues suggesting new pathogenic mechanisms for the disordered immunoglobulin synthesis in patients with immunodeficiency, autoimmunity, and malignancy. Abnormalities of each of the major types of cells of the immune system have been shown to play a pathogenic role in the disordered immunoglobulin synthesis observed in different disease states including disorders intrinsic to the B cell–plasma cell series itself, disorders of regulatory monocytes, defects in helper T cell function as well as abnormalities of the network of suppressor T cells that control B-cell maturation.

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ADVANCES IN IMMUNOLOGY, VOL. 32

Typing for Human Alloantigens with the Primed Lymphocyte Typing Technique

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I. The Major Histocompatibility Complex in Man: HLA

A. INTRODUCTION

All vertebrates studied have been found to possess one major histocompatibility complex (MHC) in addition to many minor histocompatibility systems. In man, the MHC is called the human leukocyte A (HLA) system, whereas, for example, it is H-2 in mice, AgB in rats, and B in chickens. The MHCs control strong transplantation antigens, play a fundamental role in the cooperation of cells in thymus-dependent immune responses, contain immune response (*Ir*) genes and some complement coding genes, and control susceptibility and resistance to a diversity of diseases. For example, the HLA system contains at least four segregant series (HLA-A, B, C, D/DR) of HLA antigens, and they alone constitute the most polymorphic genetic system known in man at the present time.

The HLA-A, B, C, and DR antigens can be recognized with serological techniques. The HLA-D antigens are identified by the so-called mixed lymphocyte culture (MLC) reaction (MLR) in which lymphocytes from two different individuals are mixed and cultured *in vitro*. This reaction is based on the facts that lymphocytes carry both HLA-D antigens and receptors for foreign HLA-D antigens not possessed by the lymphocyte donor, and that lymphocytes with such receptors under appropriate conditions will respond *in vitro* by proliferation, which can be measured by the uptake of certain substances. By preventing the stimulator lymphocytes from one of the two donors from dividing, e.g., by X-ray treatment, only the response of the other

donor's responder lymphocytes will be allowed to occur. This is the one-way MLR, which is used among others in HLA-D typing by means of homozygous typing cells (HTCs). For example, *HLA-Dw1/1* homozygous stimulator cells will only be recognized as foreign and induce proliferation of the responder cells when these lack the HLA-Dwl antigen. In contrast, the HLA-Dwl-positive responder cells will not respond to the *HLA-Dw1/1* HTCs. Accordingly, the assignment of a given HLA-D specificity is based on a nonresponsiveness of the cells to be typed toward the HTCs. Clearly, this is not an entirely satisfactory approach, because nonresponsiveness may be seen in other cases. Furthermore, it may be difficult to obtain HTCs for rare specificities. It was therefore an important step forward when the so-called primed lymphocyte typing (PLT) technique was developed.

The PLT test is based on the fact that primed memory cells are generated *in vitro* after 1–3 weeks in MLC if the MLR is positive. When new stimulator cells are added to such primed cells, an accelerated proliferative response may take place (so-called secondary MLR) if the new (secondary) stimulator cells share certain antigens with the stimulator cells used in the primary MLC (Fig. 1). If the memory cells have a sufficient degree of specificity, the secondary MLR can be used for typing: PLT.

In addition to memory cells with proliferative capacity, cytotoxic cells may develop during a primary MLR, and they may be used in cell-mediated lympholysis (CML) assays.

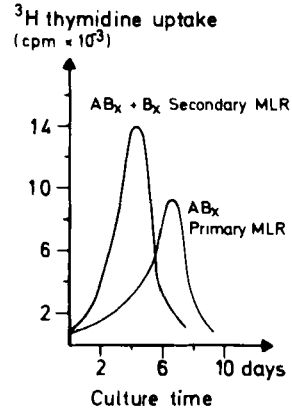

FIG. 1. Primary MLR (AB_x) of A responder lymphocytes stimulated with irradiated B lymphocytes (B_x), and accelerated secondary MLR (AB_x + B_x) of *in vitro* primed AB_x cells restimulated with B_x. The primed AB_x cells were obtained after 10 days in primary MLR (AB_x).

TABLE I
ABBREVIATIONS IN PRIMARY AND SECONDARY MIXED LYMPHOCYTE REACTION (MLR)

Symbols	Interpretation
A	Unirradiated responder lymphocytes
A_x, B_x	Irradiated stimulator lymphocytes
AB_x	A cells stimulated with B_x in primary MLR
AA_x	A cell stimulated with autologous cells
$AB_x + B_x$	A cells sensitized to B_x in primary MLR are restimulated with the original priming cells (B_x)
$AB_x + A_x$	Restimulation with autologous cells
$AB_x + C_x$	Restimulation with cells allogeneic to both the primary responder and stimulator

This review will mainly summarize the works dealing with the identification of human lymphocyte activating determinants (LADs) recognized by secondary MLR with special reference to the genetics of the PLT in man. As HLA-region gene products are the major stimulants in secondary MLR, the properties of the HLA factors will be summarized as well as the cell-mediated lympholysis in man. Typing for LADs with the PLT is a field in a very active phase of research, and the new information that will emerge may change our views on the PLT. However, we find that the technical state of the PLT has now reached a point where it may be taken seriously as a cellular typing system superior to the conventional HLA-D typing with HTCs.

B. COMPONENTS OF THE HLA SYSTEM AND THEIR INHERITANCE

There are three major classes of HLA characters: alloantigens, thymus-dependent immune responses, and some components of the complement cascade (Fig. 2).

The HLA antigens are of two kinds: the HLA-A,B,C antigens and the D and DR antigens.

The three classical segregant series of HLA antigens, A (LA or FIRST), B (FOUR or SECOND), and C (AJ or THIRD) series are found on all nucleated cells and on blood platelets. They are readily detected by serological techniques and have been called the SD (serologically detectable) or class 1 antigens.

The HLA-D antigens are the determinants that are principally responsible for the stimulation in the MLR. The HLA-D antigens are defined by MLC technique with homozygous typing cells (HTCs) as

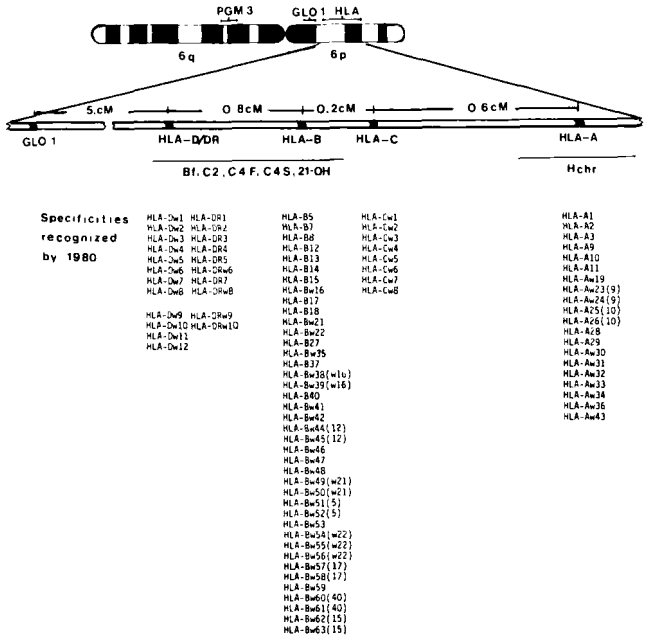

FIG. 2. Linkage relationship of HLA genes on chromosome No. 6 and HLA antigen specificities recognized by 1980. A number in parentheses after an antigen shows that this antigen represents a "split" of the antigen indicated in the parenthesis, e.g., Aw23 and Aw24 are both "splits" of A9. (From Ryder *et al.*, 1981.)

stimulating cells or reference cells. These antigens constitute a segregant series. These determinants have previously been called MLC antigens, LD (lymphocyte defined) determinants, or LADs (lymphocyte-activating determinants).

The HLA-DR antigens also constitute a segregant series of antigens, which are closely related—and perhaps partly identical—to the HLA-D antigens. The HLA-DR antigens are serologically defined but can be recognized only with special serological methods. These antigens are differentiation antigens because they are present on, for example, macrophages and B lymphocytes, but not on T lymphocytes in the blood. These antigens are called class 2 antigens and correspond to the Ia (immune-region associated) antigens in mice.

There is increasing evidence that the HLA system contains *Ir* (*Immune response*) genes, which control the specific immune responsiveness toward certain antigens (Levine *et al.*, 1972; Blumenthal *et al.*, 1974; Bergholtz and Thorsby, 1977; Hansen *et al.*, 1978). Such *Ir* genes have been found in the MHCs of all vertebrates investigated, including

monkeys (for review, see, e.g., Benacerraf and Germain, 1978; Snell, 1978).

The complement components controlled by genes in the HLA region are the second, C2 (Fu *et al.*, 1974), and the fourth, C4 (Rittner *et al.*, 1975; Teisberg *et al.*, 1977; O'Neill *et al.*, 1978), components of the classical pathway, and properdin factor B (or Bf) (Allen, 1974) of the alternative pathway.

The red blood cell antigens Bg^a, Bg^b, and Bg^c are strongly associated with—and perhaps identical to—the HLA antigens B7, B17, and A28, respectively (Morton *et al.*, 1969, 1971).

The Chido (Ch) and Rodgers (Rg) antigens on red blood cells are most probably two different types of complement C4 (O'Neill *et al.*, 1978).

The enzyme 21-hydroxylase, the lack of which results in congenital adrenal hyperplasia, is functionally coded by a gene located close to the HLA-B locus (Dupont *et al.*, 1977, 1980). Idiopathic hemochromatosis is an autosomal recessive disorder closely linked to HLA (Simon *et al.*, 1977; Kravitz *et al.*, 1979), but the precise location and action of the hemochromatosis gene(s) is unknown.

HLA genes inherited from one parent constitute an HLA haplotype (Ceppellini *et al.*, 1967) (haplos = single), and thus every individual has two HLA haplotypes—one maternal and one paternal. Each haplotype carries one gene for each HLA locus (e.g., one *HLA-A*, one *HLA-B*, one *HLA-C*, and one *HLA-DR* gene). The inheritance of HLA factors is exemplified in Table II. Usually, the HLA haplotypes are handed on unaltered to the offspring, but occasionally, genetic recombination (crossing-over) takes place (Table III and Fig. 3) between the loci containing genes coding for known HLA factors (Kissmeyer-Nielsen *et al.*, 1969), and such crossing-over has allowed determination of the order of HLA loci on the short arm of chromosome No. 6 (Fig. 2). The best estimate of the recombination frequency between HLA-A and B is 0.87 centimorgan (Belvedere *et al.*, 1975). The A-C and the C-B distances are approximately 0.7 and 0.2 centimorgan, respectively (Nielsen *et al.*, 1975). The recombination frequency between B and D is about 0.5 centimorgan (Keuning *et al.*, 1975; Thorsby *et al.*, 1975).

The genes coding for the HLA factors are syntenic (i.e., found on the same chromosome) with phosphoglucomutase-3 (PGM_3) (Lamm *et al.*, 1971), and glyoxylase (GLO) (Weitkamp and Guttormsen, 1976). There is increasing evidence that some determinants that may be recognized with the PLT technique are coded by genes situated between HLA-D/DR and GLO (Mawas *et al.*, 1978, 1980; Shaw *et al.*, 1980a,b). However, it seems most reasonable to consider these PLT factors to be part of the HLA complex.

TABLE II

INHERITANCE OF HLA-A AND -B ANTIGENS WITHIN A FAMILY[a,b]

	Antigens										
	A series				B series					Genotype (haplotype/haplotype)	
	HLA-A1	A2	A3	A9	B5	B7	B8	B12	Phenotype	In full	Abbreviated
Father	+	−	+	−	−	+	+	−	HLA-A1, 3; B7, 8	*HLA-A1, B8/A3, B7*	*a/b*
Mother	−	+	−	+	+	−	−	+	HLA-A2, 9; B5, 12	*HLA-A2, B5/A9, B12*	*c/d*
1st child	+	+	−	−	+	−	+	−	HLA-A1, 2; B5, 8	*HLA-A1, B8/A2, B5*	*a/c*
2nd child	+	−	−	+	−	−	+	+	HLA-A1, 9; B8, 12	*HLA-A1, B8/A9, B12*	*a/d*
3rd child	−	+	+	−	+	+	−	−	HLA-A2, 3; B5, 7	*HLA-A3, B7/A2, B5*	*b/c*
4th child	−	+	+	−	+	+	−	−	HLA-A2, 3; B5, 7	*HLA-A3, B7/A2, B5*	*b/c*

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

[b] Plus and minus signs indicate presence and absence, respectively, of the antigen in question. Note that in the children the paternal haplotype is usually given before the maternal. In the phenotypes, A series antigens are given before B series antigens.

TABLE III

CROSSING-OVER WITHIN THE HLA SYSTEM[a,b]

	HLA antigens													
	A series			B series				C series		Phenotype			Genotype	
Relative	A1	A2	A3	B7	B8	B22	B27	C1	C2	A	B	C	A,C,B/A,C,B	
Father	+	+	−	+	−	−	+	−	+	1,2	7, 27	2	*1, −, 7/2, 2, 27*	*a/b*
Mother	+	−	+	−	+	+	−	+	−	1, 3	8, 22	1	*1, −, 8/3, 1, 22*	*c/d*
1st child	−	+	+	−	−	+	+	+	+	2. 3	22, 27	1, 2	*2, 2, 27/3, 1, 22*	*b/d*
2nd child	+	−	+	+	−	+	−	+	−	1, 3	7, 22	1	*1, −, 7/3, 1, 22*	*a/d*
3rd child	+	+	−	−	+	−	+	−	+	1, 2	8, 27	2	*2, 2, 27/1, −, 8*	*b/c*
5th child	+	−	+	−	−	+	+	+	+	1, 3	22, 27	1, 2	*1, 2, 27/3, 1, 22*	*a:b/d*
6th child	+	−	−	+	+	−	−	−	−	1	7, 8	—	*1, −, 7/1, −, 8*	*a/c*

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

[b] Plus and minus signs indicate that an antigen is present and absent, respectively. Only the antigens present in the family have been shown. Letters A, B, and C have been omitted from the phenotypes and genotypes for simplicity. Small letters *a*, *b*, *c*, and *d* indicate the parental HLA haplotypes. The fifth child is a paternal A:C recombinant.

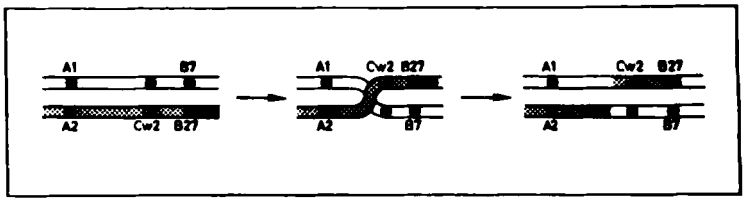

FIG. 3. Graphical illustration of the crossing-over as it took place during the meiosis in the father in Table III. The recombinant *HLA-A1,Cw2,B27* haplotype was inherited by the fifth child. (From Svejgaard *et al.*, 1979.)

HLA genes at nearby loci are not randomly distributed in the haplotypes, but some combinations of HLA genes tend to occur more frequently together than is to be expected by chance (Table IV). This phenomenon is called *linkage disequilibrium* or *gametic association.* It is crucial for the understanding of the genetics of the HLA system, and it explains many of the problems in the unraveling of this system both in the past and at the present time. Linkage disequilibrium may be illustrated as follows: The frequencies of the *HLA-B8* and *HLA-DR3* genes in the Danish population are 0.1250 and 0.1389, respectively. If there were linkage equilibrium between *B8* and *DR3*, the frequency of the haplotypes carrying both these genes would be $0.1250 \times 0.1389 = 0.0174$. The actual frequency of the *HLA-B8,DR3* haplotype is 0.1007, i.e., almost six times higher than that expected. Obviously, this phenomenon is reflected at the phenotype level: if an individual carries the HLA-B8 antigen, there is a much higher probability that he also possesses the DR3 antigen than if he is B8-negative. Every HLA factor discovered so far shows linkage disequilibrium with at least one other HLA factor controlled by a gene at a nearby HLA locus (Table V). There may also exist so-called "super-haplotypes" containing more than two HLA-genes in linkage disequilib-

TABLE IV
LINKAGE DISEQUILIBRIUM

Haplotype			Frequency (%)	
A	B	DR	Observed	Expected[a]
A1	*B8*		9.8	2.1
A3	*B7*		5.4	2.1
	B8	*DR3*	10.1	1.7
	B7	*DR2*	7.2	2.0

[a] The expected haplotypes were calculated under the assumption of no association.

TABLE V
HLA-B,DR AND -B,D HAPLOTYPE FREQUENCIES AND DELTA VALUES IN DANES[a]

Haplotype	Frequency × 10^3	Delta (Δ) × 10^3 [b]	Haplotype	Frequency × 10^3	Delta (Δ) × 10^3 [b]
DR1, Bw35	32	23***	*Dw1, Bw35*	26	18
DR2, B7	72	52***	*Dw2, B7*	75	58
DR3, B8	101	83***	*Dw3,B8*	122	98
DR4,B15	59	38***	*Dw4,B15*	48	35
DR4,B40	29	11*			
DR4,Bw44	32	10*			
DR5,B18	9.2	6.5**	*Dw5,B18*	9.2	7.3
—			*Dw6,B40*	25	19
DR7,B13	16	13***	*Dw7,B13*	14	12
DR7,B14	5.2	3.4*			
DR7,B17	32	27***	*Dw7,B17*	26	22
DR7,B21	6.4	4.9*	*Dw7,B21*	9.7	8.1
DR7,Bw44	21	7.0*	*Dw7,Bw44*	29	17
—			*Dw8,B27*	8.0	6.3
DRw8,Bw39	3.0	2.2*			
DRw10,B37	1.4	1.4**			

[a] Data from Jakobsen *et al.* (1981) and Thomsen *et al.* (1982).

[b] *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; all B,D-associations, $p < 0.01$. $\Delta = h - p_1 \times p_2$, where h = haplotype frequency, p_1 and p_2 are the *B* gene and *D/DR* gene frequencies, respectively.

rium, e.g., *A23,Cw3,B44* (Nielsen *et al.*, 1975). The deficiency of the complement factor C2 is a recessive trait coded by a C2° gene closely associated with D/DR2 and B18 and to some extent A25, and the haplotype *A25,B18,D/DR2,C2°* may be considered a super-haplotype (Fu *et al.*, 1974, 1975). The blood group and complement systems Chido/Rodgers/C4 (cf. above) are also closely associated with HLA-B antigens, e.g., Chido-negative (C4S-) is associated with B44 (Dupont *et al.*, 1978).

HLA is considered to be a genetic system (Mi and Morton, 1966) because (*a*) the HLA factors are coded by closely linked genes; (*b*) the factors are nonrandomly associated (i.e., show linkage disequilibrium) in the population; and (*c*) the functions of most HLA factors seem to be related in various ways. In this review, we will use the term HLA for all factors controlled by genes within or at a close distance from the *HLA-A,B,C, D/DR* genes. Today, at least 70 different HLA factors have been identified. Many millions of different HLA phenotypes may be created, and the most common phenotypes occur with frequencies of less than 1% in the Caucasian population.

C. Biochemistry of the HLA Antigens

The HLA-A, B, C antigens are composed of two chains that are non-covalently bound: a heavy chain (MW ≃44,000) and a light chain (MW ≃11,500). The light chain is identical to β_2-microglobulin (β_2-M), which is coded for by genes at chromosome No. 15 (Goodfellow *et al.*, 1975). The heavy chain carries the alloantigen specificities and is coded for by genes at chromosome No. 6 (Lamm *et al.*, 1974; van Someren *et al.*, 1974; Francke and Pellegrino, 1977). The HLA-A, B, and C antigens are very much alike, and the genes coding for the heavy chains of A, B, and C series antigens may have arisen by gene duplication (Bodmer, 1972). The antigens from each of the three series were shown to exist as separate molecules on the cell surface membrane (Bernoco *et al.*, 1972; Kourilsky *et al.*, 1972; Solheim *et al.*, 1973). The heavy chain is a glycosylated polypeptide, and the complete amino acid sequence of, e.g., HLA-B7 has been established (Orr *et al.*, 1979b) in intact antigens from solubilizations of membranes with non-ionic detergents. The external part of the papain-solubilized HLA-B7 molecules consist of 271 residues. The carboxy-terminal part of the molecule (3500 daltons) is anchored inside the cell by a cluster of basic, positively charged residues (arginine and lysine). The "internal part" also includes a high proportion of hydroxyl amino acids (serines) that may become phosphorylated. Phosphorylation and dephosphorylation of high-energy intermediates may prove to be one of the functions of HLA molecules in transmitting signals from the external to the internal part of the cells. The intramembranous part of HLA-A, B consists of about 25 amino acids, all of which are hydrophobic.

The remainder of the molecule has been subdivided into three main parts: α_1 (residues 1 to 90), α_2 (residues 91 to 180), and α_3 (residues 181 to 271). One disulfide loop is found in α_2 and one in α_3. The region of the disulfide loop in the α_3 part shows some homology to both β_2-M and to the constant domain of immunoglobulin (Ig) (Orr *et al.*, 1979a). The amino acid sequence of α_3 is highly conserved among different HLA-A, B antigens. The α_1 and the α_2 parts show some homology, and they both contain clusters of differences, which most probably reflect the alloantigen specificities. The α_3 part of the heavy chain and β_2-M may interact in a manner similar to the two constant domains of Ig.

At the time of writing, less is known about the HLA-DR antigens. HLA-DR antigens are composed of two polypeptide chains—a heavy (α) chain (MW ≃34,000) and a light (β) chain (MW ≃29,000), which

are both different from the heavy A, B, C chains and β_2-M (Barnstable *et al.*, 1978). Both chains are glycosylated and bound tightly to each other by noncovalent bindings, and they are both transmembranous proteins (Kaufmann and Strominger, 1979). The HLA-DR specificities seem to be determined by the light chain (Kaufmann *et al.*, 1980; Shackelford and Strominger, 1980); the gene for this chain must then be in the HLA complex.

Amino acid sequencing has been performed only for smaller parts of the N termini of the α and β chains. These studies, however, have shown that both the α and the β chain of DR show sequence homology to the α and the β chain of I-E/C in the mouse (Strominger, 1980).

In the mouse, at least two genetic subregions exist that code for Ia molecules (which have a structure very similar to that of DR antigens): (*a*) the *I-A* subregion that codes for A_α, A_β, and E_β; and (*b*) the *I-E/C* subregion coding for E_α. The A_α and A_β chains are noncovalently linked together, and so are the E_α and E_β chains. There is extensive serological polymorphism among the alleles coded by genes in the *I-A* region and some polymorphism at the *I-E/C* region. If the A_α/A_β and the E_α/E_β chains were inherited as classical codominant traits, there would be a maximum of four different Ia molecules in heterozygous (F_1 hybrids) mice (two A chains and two E chains). However, there is some evidence that the paternal E_α chain may also combine with the maternal E_β chain, and vice versa (Fig. 4). Thus, a heterozygous animal may express four different E molecules (for review, see Uhr *et al.*, 1979). It has become clear that the E_α locus often contains a "null" allele, which does not give rise to any E_α chains (see Klein *et al.*, 1981).

In man, there is accumulating—but still circumstantial—evidence based on biochemical and serological investigations that the "DR antigen" preparations used for these investigations contain several sets of molecules that may be the product of separate genetic loci (Tosi *et al.*, 1978; Kimura *et al.*, 1979; Charron and McDevitt, 1979; Tanigaki *et al.*, 1980; Shackelford and Strominger, 1980; Strominger *et al.*, 1981). Because of the strong homology between all MHCs, it is likely that the HLA region also controls two sets of Ia-like antigens (one corresponding to I-A and one to I-E antigens) and that all four genes (two α and two β genes) are within HLA.

D. Biology of the HLA System

The HLA system is the strongest histocompatibility system in man. Skin grafts between HLA-identical siblings survive much longer than grafts between siblings nonidentical for HLA (Amos *et al.*, 1969; Cep-

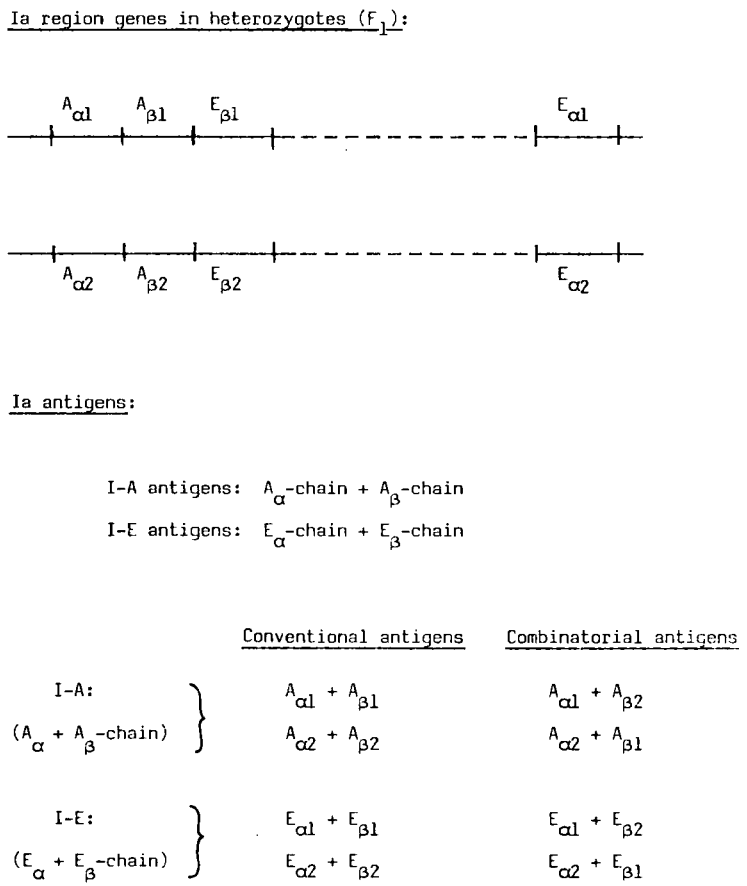

FIG. 4. Example of combinatorial (or "hybrid") antigens in mice. The I-A antigens $A_{\alpha 1}$ + $A_{\beta 2}$ and $A_{\alpha 2}$ + $B_{\beta 1}$, and the I-E antigens $E_{\alpha 1}$ + $E_{\beta 2}$ and $E_{\alpha 2}$ + $E_{\beta 1}$ have been coded for by genes in transposition (e.g., $A_{\alpha 1}$ and $A_{\beta 2}$).

pellini *et al.*, 1969). Kidney grafts in siblings identical for HLA, in general, have a very good graft survival compared to HLA-nonidentical sibpair combination (van Rood, 1967). For these reasons among others, HLA is considered to be the major histocompatibility complex (MHC) in man.

MHC systems in vertebrates have been known since 1948 (Snell, 1948). In the mouse, viral leukemia is associated with certain MHC (H-2) factors (Lilly *et al.*, 1964), and this knowledge led to the studies of associations between HLA and disease. The first possible association was found between Hodgkin's disease and HLA (Amiel, 1967), but the first definite associations (with psoriasis and celiac disease)

were not discovered until 1972 (Russell *et al.*, 1972; Falchuk *et al.*, 1972). The strongest association observed until now is between HLA-B27 and ankylosing spondylitis (Brewerton *et al.*, 1973; Schlosstein *et al.*, 1973), but in most cases the associations have been demonstrated to be strongest with HLA-D and DR antigens (Svejgaard *et al.*, 1980). The associations between HLA and diseases indicate that HLA factors have other functions than those related to transplantation. At the present time, the possible *Ir* genes (cf. below) are the best candidates for the explanation of the associations.

From a biological point of view, the most fascinating function of MHCs from various species concerns various immune responses. The so-called Ir (immune response) determinants have been studied most extensively in animals—mice in particular (e.g., McDevitt and Benacerraf, 1969; Shearer, 1974; Doherty and Zinkernagel, 1975; Rosenthal, 1978; Klein *et al.*, 1981), but there is little doubt that HLA controls similar determinants. In the following, extrapolation from the results of animal studies to the situation in man will be done.

The immune responses controlled by HLA are the so-called thymus-dependent responses that are responsible for cell-mediated immunity and IgG antibody formation toward a variety of foreign antigens. The key to understanding the role of HLA in these responses is the extensive cooperation that successively takes place between various cells (Fig. 5). For example, macrophages take up foreign antigen and present it to so-called T-helper lymphocytes, which when thus activated liberate lymphokines and help B lymphocytes to develop into plasma cells producing large amounts of IgG toward the foreign antigen in question. Activated T-helper lymphocytes can also help other T lymphocytes to develop into cytotoxic T cells capable of lysing cells carrying the foreign antigen (e.g., a hapten or a virus) on their surfaces. It now appears that the HLA-D/DR molecules are involved in the presentation by macrophages of foreign antigen to the T-helper lymphocytes, which apparently respond only to foreign antigens when presented on cells carrying self HLA-D/DR molecules (Bergholtz and Thorsby, 1977; Hansen *et al.*, 1978). Moreover, it is believed that certain HLA-D/DR antigens present certain foreign antigens more effectively than others, which would make individuals possessing these HLA-D/DR antigens "high responders" to the foreign antigens in question. The HLA-ABC antigens appear to be involved in the lysis by cytotoxic T lymphocytes of haptenated or virus-infected cells (Shearer, 1974; Zinkernagel and Doherty, 1974; Dickmeiss *et al.*, 1977; Goulmy *et al.*, 1977; McMichael *et al.*, 1977): in order for these T lymphocytes to lyse their target cells, they must recognize both for-

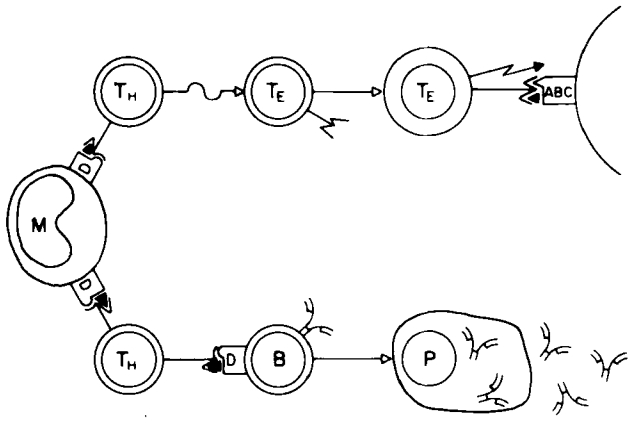

FIG. 5. Simplified theoretical model of thymus-dependent immunity: M, Macrophage; T_H, T-helper lymphocyte; T_E, T-effector lymphocyte; B, B lymphocyte; and P, plasma cell. The foreign antigen, symbolized by the black triangle, is taken up by the macrophage and presented together with HLA-D antigen to T-helper cells, which, when thus activated, can trigger precursor cells of T-effector cells to become active killer cells. These cells react both with foreign antigen and with HLA-ABC antigen on the target cell. The same or another set of T-helper cells helps B lymphocytes to transform into IgG antibody-producing plasma cells. This figure shows only one of the several possible models for the cellular cooperation (combined presentation and a single T-cell receptor), but it could be replaced by any of the five other models illustrated in Fig. 6. (From Svejgaard *et al.*, 1979.)

eign antigens and HLA-ABC molecules on the target cells. Again, certain ABC antigens seem to generate more effective lysis of target cells carrying a given foreign antigen than do other ABC antigens (Dickmeiss *et al.*, 1977; Goulmy *et al.*, 1977; McMichael *et al.*, 1977). According to these concepts, the HLA-D/DR and ABC antigens are two different classes of immune response (Ir) determinants that are involved in different steps of the thymus-dependent immune response. It is also possible that HLA controls immune suppressor (Is) determinants, which can decrease the immune response to certain foreign antigens. In mice, such Is determinants seem to exist within the *Ia* region (Benacerraf and Germain, 1978).

The mechanism by which the interaction between the T cell, on the one hand, and foreign antigen and MHC antigen takes place is not known. At the time of writing, there are still six different possibilities (Fig. 6).

Thus, it appears that the antigens recognized by MLR, anti-DR antibodies, and PLT cells are involved in the earliest phase of the specific immune response: the presentation by macrophages of foreign anti-

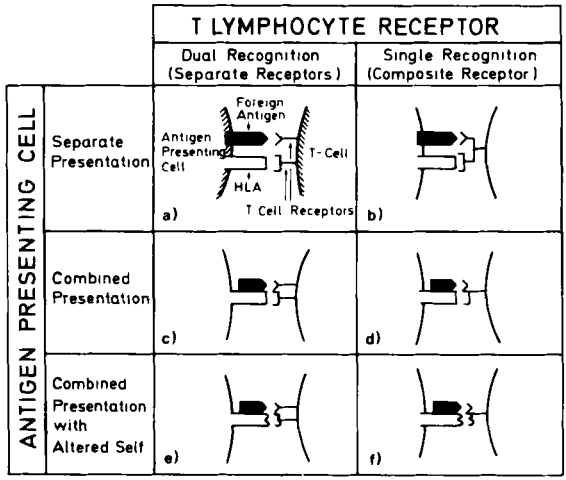

FIG. 6. Models for molecular interactions in thymus-dependent immunity. There may be one (b, d, and f) or two (a, c, and e) receptors on the T lymphocyte, and, on the antigen-presenting cell, the foreign antigen may be separate (a and b) or combined (c, d, e, and f) with an HLA molecule; in the latter case, an alteration of the HLA molecule may take place (e and f). When the T lymphocyte is a T-helper cell, the antigen-presenting cell is a macrophage and the HLA molecule is probably HLA-D/DR antigen. If it is a T-effector lymphocyte, the antigen-presenting cell may, for example, be a virus-infected cell and the HLA molecule is an HLA-A, B, or C antigen. (From Svejgaard *et al.* 1979).

gen to the T-helper lymphocytes. One of the crucial questions presently is whether a physical interaction takes place between foreign antigen and the D/DR antigens (combined presentation in Fig. 6), but there is evidence from studies of guinea pigs that this may indeed be the case (Rosenthal, 1978; Werdelin, personal communication). If this is so, there may be a certain specificity already at the level of this interaction, which may possibly lead to a change of the D/DR antigens making them look like "altered self" antigens (Fig. 6), perhaps resembling certain allo-D/DR antigens. According to this concept, alloreactivity (including the response to MHC transplantation antigens) merely reflects the basic role of MHC in the immune response.

The complement components Bf, C2 and C4, which are controlled by genes in the HLA system, are crucial for the defense against some microbial organisms and possibly for the elimination of immune complexes.

All the above-mentioned functions of HLA are related to immunological functions and/or the defense against foreign organisms. It seems logical that such related functions are governed by closely

linked genes. However, the HLA complex also contains genes that control factors considered to be nonimmunological, e.g., the 21-hydroxylase enzyme (Dupont *et al.*, 1977) and factors responsible for the development of idiopathic hemochromatosis (Simon *et al.*, 1977; Kravitz *et al.*, 1979). It may be anticipated that other factors—immunological as well as nonimmunological—coded by genes in the HLA region will be found. In the mouse, the H-2 system seems to influence the testosterone concentration in the blood, the weight of testes and thymus, and the amount of cyclic AMP in liver cells (for review, see, e.g., Ivanyi, 1978).

II. HLA-A, B, C, and DR Serology

A. HLA-A, B, C Antigens

The first leukocyte antigen (MAC = HLA-A2 + A28) was described by Dausset (1958), who showed that a group of sera from multitransfused patients agglutinated leukocytes from approximately 60% of the French population, but not leukocytes from the serum donors. The definition of serologically detectable leukocyte antigens was greatly facilitated when Terasaki and McClelland (1964) introduced the more sensitive and reproducible microlymphocytotoxicity test. In 1965, both Dausset *et al.* and van Rood *et al.* suggested that the majority of the leukocyte antigens belonged to one genetic system, which was later named the HLA system [the first (*A*) *H*uman *L*eukocyte antigen system (WHO, 1968)]. Kissmeyer-Nielsen *et al.* (1968) first suggested that the HLA antigens defined at that time belonged to only two segregant series (LA and FOUR, now A and B). Later, Sandberg *et al.* (1970) suggested the existence of a third segregant series of HLA antigens (AJ, now C).

The genetic loci coding for the HLA antigens have been mapped on the short arm of chromosome No. 6 (Lamm *et al.*, 1974; van Someren *et al.*, 1974; Francke and Pellegrino, 1977), and the order of the loci is B-C-A (Löw *et al.*, 1974) when reading from the centromere (Fig. 2).

HLA-ABC typing is done by means of specific HLA antisera that are mixed with peripheral blood lymphocytes. If the HLA antibodies react with HLA antigens on the cell surface, the addition of complement (usually rabbit serum) will lead to membrane damage, which can be visualized under the microscope because lymphocytes with a destroyed or damaged membrane take up certain dyes, such as trypan blue and eosin. Until now, almost all HLA antibodies used for typing has been obtained as sera from humans who have been sensitized

by pregnancy, transfusion, transplantation, and/or planned immunization. Probably all such sera contain mixtures of various populamunization. Probably all such sera contain mixtures of various populations of HLA antibody molecules. At the time of writing, enormous efforts are made to produce monoclonal antibodies *in vitro,* which may revolutionize the field of HLA typing. However, it can be anticipated that most monoclonal antibodies will show varying degrees of cross-reactivity (cf. Section II,C), just as is the case for most HLA antibodies in serum. The validity of typing reagents, whether monoclonal or serum antibodies, has to be established by testing known panels of HLA typed cells. Apart from cross-reactivity, two other serologic phenomena may cause problems in HLA-ABC typing: (*a*) two (or more) different HLA antibodies present in the same serum may act synergistically, i.e., react more strongly with cells carrying both of the corresponding HLA antigens (Svejgaard, 1969a; Ahrons and Thorsby, 1970); and (*b*) homozygous cells carry more of the corresponding HLA antigen (gene dose effect) and react more strongly with the equivalent antibody than do heterozygous cells (Svejgaard, 1969b; Ahrons and Thorsby 1970).

At the time of writing, 20 A antigens, 40 B antigens, and 8 C antigens (Fig. 2) have been named by the HLA nomenclature committee ("Histocompatibility Testing, 1980").

B. HLA-DR Antigens

In 1973, van Leeuwen *et al.* demonstrated the existence of leukocyte antigens that were closely associated with the cellularly defined MLR determinants that were later named the HLA-D antigens (cf. Section III,D). The determinants are present on monocytes and B lymphocytes but not on T lymphocytes (Winchester *et al.*, 1975). They can be demonstrated with MLR inhibition, immunofluorescent, and lymphocytotoxicity techniques (van Leeuwen *et al.*, 1973; van Rood *et al.*, 1975; Winchester *et al.*, 1975). In order to obtain readable results, van Rood *et al.* (1975) removed the T lymphocytes by rosetting with sheep red blood cells (SRBC) before performing the cytotoxicity test.

A modification of this method is now widely adopted, and the B-lymphocyte alloantigens are now called HLA-DR antigens [HLA-*D* Related (WHO 1978)]. At the time of writing, 10 HLA-DR antigens have been defined (Table VI), and the sum of gene frequencies of known antigens is approximately 0.9 in Caucasians ("Histocompatibility Testing, 1980").

HLA-DR typing is considerably more difficult and time-consuming

TABLE VI
FREQUENCIES OF HLA-D AND -DR ANTIGENS AND GENES IN DANES[a]

HLA-D (N = 389)			HLA-DR (N = 704)		
	Frequency			Frequency	
	Antigen (%)	Gene		Antigen[b] (%)	Gene
Dw1	20.8	0.111	DR1	19.3	0.102
Dw2	27.0	0.146	DR2	28.3	0.153
Dw3	29.0	0.160	DR3	25.9	0.139
Dw4	25.0	0.134	DR4	34.7	0.192
Dw5	11.6	0.060	DR5	11.7*	0.060
Dw6	13.9	0.072	DRw6	17.9**	0.094
Dw7	17.7	0.093	DR7	20.9	0.111
Dw8	7.2	0.037	DRw8	8.7	0.044
Blank		0.187	DRw9	1.7***	0.009
		1.000	DRw10	0.71	0.004
			Blank		0.092
					1.000

[a] Data from Jakobsen *et al.* (1981) and Thomsen *et al.* (1982).
[b] Number of individuals investigated. * N = 545; ** N = 368; *** N = 58.

than ABC typing, partly because of the necessary B-lymphocyte enrichment and partly because good DR antisera still are rare: many sera containing DR antibodies also contain ABC antibodies, which have to be removed by absorption with blood platelets (which lack DR antigens). Often, pooled platelets from many donors are used for these absorptions, the results of which are not always reproducible. Although the quality of available DR antisera has increased steadily, some DR antigens (e.g., DRw6) are still difficult to define.

Cross-reactivity, synergism, and gene dose effects are also seen in DR serology (Bodmer *et al.*, 1977; Madsen *et al.*, 1981).

In the mouse, there are at least two segregant series of Ia antigens (cf. Section I,C), and several claims have been made for the existence of segregant series of HLA-linked B-lymphocyte alloantigens in man in addition to the DR antigens (van Rood *et al.*, 1977; Park *et al.*, 1978; Duquesnoy *et al.*, 1979; Tosi *et al.*, 1978; Fuller *et al.*, 1978). For example, the high-frequency antigens belonging to the so-called MB and MT series may either be supertypic determinants of the DR antigens (i.e., constitute cross-reacting antigens) or be controlled by one or more separate loci. Perhaps the most convincing evidence of a separate series is the recombination between DR4 and LB13 described

by van Leeuwen *et al.* (1980). However, it would be satisfying to see more recombinants involving other DR and MB-MT antigens before the latter are accepted as constituting a separate series. Nevertheless, even if the evidence of more than one HLA locus for B-cell alloantigens is still mainly circumstantial, it is, in fact, very likely that the HLA system contains more than one locus for DR-like antigens. Thus, there is most probably at least one α and one β gene in HLA, and probably there are two of each (cf. Section I,C). However, the degree of polymorphism may be limited for some of these loci.

C. Serological Cross-Reactions

The existence of cross-reactive HLA antibodies was first suggested by Ceppellini *et al.* (1967) and later unequivocally demonstrated by Dausset *et al.* (1968) and Svejgaard and Kissmeyer-Nielsen (1968). Cross-reactivity is an important phenomenon in serology as well as in secondary MLR. It plays a crucial role in the selection of specific primed lymphocyte typing (PLT)-cell combinations for typing purposes. (cf. Sectons X,B and XII,C). Cross-reactions in PLT, however, are not as well recognized as they are in HLA serology, and, therefore, we shall briefly describe the basic phenomenon as exemplified by HLA serology.

The cross-reaction between HLA-A2 and A28 was discovered by the investigation of a serum containing both anti-A2 and anti-A28 reactivity. The serum was obtained from an individual who had most probably been immunized only with A2 (Svejgaard and Kissmeyer-Nielsen, 1968; Svejgaard, 1969a). The serum reacted with all A2-positive and some A2-negative cells. The antibody activity was completely absorbed by all positively reacting cells whether or not the cells were A2-positive. The observations were made at a time when the specificity A28 was not yet recognized. The results lead to the conclusion that the serum contained "cross-reactive" antibodies against A2 and a "new" antigen (later called A28).

It is of importance to note that (*a*) cross-reacting antibodies may be found after immunization with only one of the cross-reacting antigens; and (*b*) cross-reactions are usually restricted to antigens belonging to the same segregant series, but may also be found between antigens belonging to different series (Legrand and Dausset, 1975; Kostyu *et al.*, 1980). It should also be stressed that, owing to the frequent occurrence of cross-reacting HLA antibodies, a serum (or reagent) is only "monospecific" until the opposite has been proved, i.e., by finding a more "narrowly" reacting antibody. Thus "monospecificity" is operationally defined.

Cross-reactivity between, e.g., A2 and A28 is now easily understood considering the extreme homology in the amino acid sequence between the A2 and the A28 molecules (Strominger, 1980), which supports the concept of Kabat (1956) that cross-reactivity is due to two antigeneic specificities having a structure in common.

III. Mixed Lymphocyte Culture Reaction (MLR)

A. Introduction

The basis for the accelerated proliferative response in secondary MLR is that the events in the primary MLR and the phenomena observed in primary and secondary MLR are very similar to each other. Therefore, we shall briefly mention some of the basic observations in the primary MLR (for review, see, e.g., Dupont *et al.*, 1976).

In 1963, Bain *et al.* and Hirschhorn *et al.* reported that lymphoblasts and mitosis occurred when lymphocytes from two different human individuals were mixed *in vitro*. The lymphocyte transformation phenomenon had first been demonstrated *in vitro* by Nowell (1960), who reported that lymphoblasts appeared when phytohemagglutinin (PHA) was added to cultures of leukocytes. Bain *et al.* (1964) reported that blast transformation could not be induced by plasma, erythrocytes, or platelets. Furthermore, they demonstrated that lymphoblast transformation did not occur between monozygotic twins, whereas most other MLR combinations, including, some dizygotic twin pairs as well as unrelated individuals, caused lymphoblast transformation. These findings started the unraveling of the genetic background of the MLR (see below). Bain *et al.* (1964) had also suggested that the MLR was an immunological reaction that might be of value for allografting in man. The same point was made by Bach and Hirschhorn (1964), who suggested that the MLC response might be an *in vitro* parameter for the degree of histocompatibility between two individuals.

B. Technical Innovations in MLR

The MLC technique was greatly facilitated when Bain *et al.* (1964) introduced the uptake of radioactive labeled [^{14}C]thymidine as a measure of the blast transformation. Thymidine is incorporated into the DNA during mitosis.

Uptake of radioactive labeled thymidine has become the method of choice for the quantitation of the strength of lymphocyte proliferation in MLR. The spontaneous uptake of [^{3}H]thymidine or [^{14}C]thymidine in unstimulated lymphocyte cultures is low. The uptake of radioactive

labeled thymidine in stimulated cultures can be determined usually between day 3 and day 7. Quantitative comparisons of thymidine uptake should be done by pulse labeling and detection during the exponential phase of the proliferation (Sørensen *et al.*, 1969). The exponential phase of MLC proliferation in conventional culture systems is usually between day 4 and day 6 (Bondevik *et al.*, 1974; duBois *et al.*, 1974; Jørgensen and Lamm, 1974), but the culture conditions may affect the time course.

Another innovation of great importance was the introduction of the one-way MLC. In a normal MLC, the lymphocytes from each of the two individuals may act as both responders and stimulators (two-way MLC). When one of the lymphocyte populations is treated with either radioactive irradiation (Kasakura and Löwenstein, 1965) or mitomycin C (Bach and Voynow, 1966), these lymphocyte cannot undergo normal mitosis and are unable to incorporate radioactive labeled thymidine when stimulated by mitogens. Therefore, the total uptake of radioactive labeled thymidine in a one-way MLC is a measure of the proliferation of the nontreated (responder) lymphocytes when stimulated with the irradiated or mitomycin C-treated (stimulator) lymphocytes.

Hartzman *et al.* (1971) developed the microculture systems and the semiautomatic multisample harvesting system (Hartzman *et al.*, 1972), which have saved much time and many resources of lymphocytes and other reagents.

C. Genetics of the MLR

The twin studies of Bain *et al.* (1964) suggested that the MLR was under genetic control, and the studies of Bain and Löwenstein (1964) and Bach and Hirschhorn (1964) confirmed this assumption. Bach and Voynow (1966) showed that one-fourth of sib-pairs were mutually negative in MLR, indicating that the determinants responsible for the MLC stimulation were coded by genes at either one locus or at closely linked loci. Furthermore, the determinants must be inherited as codominant traits.

Bach and Amos (1967) and Amos and Bach (1968) showed that the MLR was negative between sibs who shared serologically detectable HLA antigens (Table VII), and these findings suggested that the MLR was controlled by genes controlling the SD HLA antigens or by genes closely linked to the SD-coding genes. In the mouse, similar observations had been made by Dutton (1966), who found that inbred mice identical for the serological detectable H-2 antigens were mutually negative in MLR, while the MLR was positive when the mice differed for H-2 antigens.

TABLE VII
MIXED LYMPHOCYTE CULTURE (MLC) TEST IN A FAMILY[a,b]

Responder		Stimulator						
Relationship	HLA genotype	F_x *a/b*	M_x *c/d*	1_x *a/c*	2_x *a/c*	3_x *b/c*	4_x *b/d*	U_x *x/y*
Father	*a/b*	0	++	+	+	+	+	++
Mother	*c/d*	++	0	+	+	+	+	++
1st child	*a/c*	+	+	0	0	+	++	++
2nd child	*a/c*	+	+	0	0	+	++	++
3rd child	*b/c*	+	+	+	+	0	+	++
4th child	*b/d*	+	+	++	++	+	0	++
Unrelated	*x/y*	++	++	++	++	++	++	0

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

[b] The test is a "chessboard" between untreated responder and X-irradiated (X) stimulator lymphocytes from all family members and an unrelated control (U). Letters *a*, *b*, *c*, *d*, *x*, and *y* indicate different HLA haplotypes determined by ABC typing. Zero indicates absence, and one and two plus signs indicate various degrees of stimulation in the MLC tests.

A gene dose effect in the MLR was demonstrated in family studies. Albertini and Bach (1968) showed that relatives differing for two HLA haplotypes elicited a stronger MLR than did relatives with one HLA haplotype difference.

The MLR between unrelated individuals identical for serologically detectable HLA antigens was in general positive (Kissmeyer-Nielsen *et al.*, 1970; Sørensen and Nielsen, 1970; van Rood and Eijsvoogel, 1970), indicating that the known SD antigens could not be responsible for the MLR.

Plate *et al.* (1970) studied families with genetic recombinations in the HLA region and found that the MLR followed the HLA-B antigens when recombinations took place between HLA-A and -B. This observation was confirmed by Dupont *et al.* (1971). In another family, Yunis and Amos (1971) found that the MLR was positive between two children who had inherited the same HLA-A and B antigens from their parents, and these observations suggested that a maternal recombination had occurred between HLA-B and a locus (or closely linked loci) carrying genes controlling the MLR.

The observations led to the hypothesis of the existence of a separate locus for the genetic control of the MLR (now HLA-D). The existence of this locus has now been amply confirmed; it is located between *HLA-B* and the centromere (Fig. 2).

D. HLA-D Typing with Homozygous Typing Cells

In 1973, it became possible to type for MLR determinants by the homozygous typing cells (HTC) technique. Bradley *et al.* (1972) had demonstrated that lymphocytes from MLR homozygous pigs did not stimulate lymphocytes from other pigs sharing one or two MLR haplotypes with the stimulator. The concept of using HTCs as reference cells for MLC-typing purposes was formulated by Mempel *et al.* (1973a). If an HTC is used as the stimulator toward a panel of responders, the responses can be divided into high and low responses. The low responses are taken to indicate that the responder lymphocytes possess the same MLR determinant(s) as the stimulating HTC.

The method has several drawbacks but was immediately taken up by Jørgensen *et al.* (1973) and van den Tweel *et al.* (1973), who used HTCs from inbred individuals (first-cousin offspring) as reference cells, and by Dupont *et al.* (1973c), who, like Mempel *et al.* (1973b), used HTCs from outbred individuals.

Typing for the MLR determinants is done in large experiments preferentially including several HTCs of different specificities and several different responders. During the Sixth International Histocompatibility Workshop (1975) it became possible to define eight different specificities of MLR antigens. These eight antigens seemed to belong to one series of antigens, which functionally were coded by one locus in the HLA region (Joint Report, 1975), and the MLR determinants were named HLA-D antigens (WHO, 1975; "Histocompatibility Testing, 1975").

After the Eighth International Histocompatibility Workshop (1979/1980) twelve HLA-D antigens were defined ("Histocompatibility Testing, 1980"), and the sum of gene frequencies of detectable HLA-D antigens is approximately 0.60 in Caucasians.

E. The Interpretation of MLR Data for HLA-D Typing

From a theoretical point of view, the assignment of an HLA-D specificity is made when the cells to be typed are nonresponsive toward the appropriate HTC(s). However, in the majority of cases—including family combinations—the HTC induces a weak stimulation of the responder cells, even when the responder cells carry the HLA-D antigen of the HTC. The realization of this fact has led to the concept of the typing response (Jørgensen *et al.*, 1974; Joint Report, 1975), which is a pragmatically defined weak MLC response.

It has been suggested that the weak but definitely positive typing response may be caused by blastogenic factors released from the

HTCs (Janis *et al.*, 1970; Jørgensen *et al.*, 1974), which may induce proliferation of the responder cells. Another plausible explanation of the typing response is the possible heterogeneity of the HLA-D antigens. There has even been formulated a concept of "multiple MLC determinants on the same HLA-haplotype" (Dupont *et al.*, 1973b). This concept predicts that the final stimulatory (or antigenic) capacity in the MLR is governed by several closely linked genes in the HLA region.

It would appear from the model of the *D/DR* region in Fig. 9 that HLA-D typing with HTCs is typing for common combinations of different *D/DR* region products rather than for single determinants.

On the other hand, typing responses may be seen when responder cells are specifically tolerant to HTCs with which they do not share any HLA-D antigens (Thomsen *et al.*, 1976, 1978; Kristensen and Jørgensen, 1978). These observations illustrate one of the problems of HLA-D typing: We draw conclusions based on observations of the *lack* of reactivity.

The primary aim of using the typing response is to make the antigen assignment reproducible and biologically meaningful, judged by the segregation of the HLA-D antigens in families as well as by the predictive value of the MLR between unrelated individuals who are assigned to possess the same HLA-D antigen.

The most commonly used methods for evaluating the results of HLA-D typing are the stabilized relative response (SRR) (Ryder *et al.*, 1975), which is also called the double normalized value (DNV), and the cluster analysis of Piazza and Galfré (1975). These two methods include normalizations of both the responder cell function and the stimulator cell function. Both the SRR and the cluster analysis compensate for the greater part of the variability in the MLR, but still leave us with the typing response.

F. Predictive Value of HLA-D Typing

If the HLA-D antigens were completely identified, and if no other factors influenced the MLR, all unrelated individuals sharing HLA-D antigens should be mutually MLR-negative. Jørgensen *et al.* (1975) have demonstrated that this is not the fact, and this point has been confirmed by others (e.g., Grosse-Wilde *et al.*, 1975; Thorsby *et al.*, 1975). Only 50–80% of HLA-D identical unrelated individuals have weak MLR (corresponding to the typing response toward HTCs), and less than one-third of the pairs have "negative" MLR.

The reason for the relative poor predictive value of HLA-D typing in the MLR is probably caused by (*a*) the problems of the assignment

of a specificity based on a "typing response" (cf. above); (*b*) heterogeneity of the HLA-D antigens; and (*c*) the influence of MLC reactivity caused by non-HLA-D antigens.

G. Weak MLR Loci

It has been suggested that genetic factors other than HLA-D antigens may cause weak but definitely positive response in MLR. Family studies of children with recombination between *HLA-A* and *-B* or between *HLA-B* and *-D* loci have indicated that weak MLR may be induced between HLA-D (and B/D) identical sibs who are HLA-A disparent owing to genetic recombination (Eijsvoogel *et al.*, 1972; Mempel *et al.*, 1973a; Thorsby *et al.*, 1973; Dupont *et al.*, 1974). Netzel *et al.* (1975) and Bijnen *et al.* (1977) have quantitated the MLR that take place between (*a*) HLA-different sibs; (*b*) HLA-identical sibs; and (*c*) sibs identical for HLA-B/D but different for HLA-A owing to recombination. The MLR of the HLA-A disparate sibs was significantly higher than the completely HLA-identical sibs, supporting the concepts of an *HLA*-A-linked MLR locus. The response between HLA-A disparate sibs were, however, only 10% of the response of two haplotype-different sibs.

Formal typing for these postulated HLA-A-associated weak MLR determinants has not been peformed. Weak MLR has also been observed in a pair of HLA-A, B, C, D, DR identical sibs, in one of whom a recombination had taken place between *HLA-D/DR* and *GLO*, indicating that determinants coded by gene(s) between *HLA-D/DR* and *GLO* may govern the MLR (Mawas *et al.*, 1978).

It should be kept in mind that, in other species, genetic factors not linked to MHC giving rise to MLR have been identified, e.g., the Mls system in mice (Festenstein *et al.*, 1971). The Mls system has a limited polymorphism. An equivalent system may be found in man.

IV. Cell-Mediated Cytotoxicity (CMC)

Although many features of the cytotoxic response in secondary cultures of primed lymphocytes are very similar to the secondary proliferative response, we shall only summarize the basic phenomena in the CMC, as this item has been reviewed elsewhere (e.g., Kristensen *et al.*, 1982).

A. *In Vivo* Sensitization

Cytotoxicity may be mediated by lymphocytes themselves without the involvement of antibodies or complement factors. This phenome-

non was first described by Govaerts (1960), who investigated the destruction *in vitro* of cultured kidney cells by lymphocytes from dogs that had been transplanted with allogeneic kidney grafts. Similar phenomena were observed in humans, and the major targets for the CMC after allogeneic *in vivo* sensitization were suggested to be the HLA antigens (Lundgren, 1969); Lundgren *et al.*, 1970). *In vivo*, cytotoxic T lymphocytes (CTL) may be found in the peripheral blood few days after an allogeneic stimulation, e.g., skin grafting. The CTLs reach a maximal activity about day 10, and the activity decreases during the following 2–4 weeks (Biesecker, 1973; Cerottini and Brunner, 1974). The result of *in vivo* sensitization is called *direct* CMC.

The direct CMC is a valuable tool for the *in vitro* investigation of the actual capacity of CMC *in vivo* of an individual. The presence of sensitized CTLs *in vivo* may have clinical relevance in organ transplantation in man. There is strong evidence that even hyperacute rejection of allotransplants, e.g., kidney grafts, may be caused not only by donor-specific circulating cytotoxic antibodies (Kissmeyer-Nielsen *et al.*, 1966), but also by the presence of CTLs specifically reacting with donor histocompatility antigens (Kristensen *et al.*, 1976).

B. *In Vitro* Sensitization

In vitro, cytotoxic T lymphocytes may be generated in MLR between unsensitized individuals as first demonstrated in the mouse (Häyry and Defendi, 1970; Hodes and Svedmyr, 1970) and in humans (Solliday and Bach, 1970; Hardy *et al.*, 1970). In these studies, it was shown that immunospecific cytotoxic effector T lymphocytes are produced in the MLR between allogeneic individuals, and that the CTLs can specifically destroy cells syngeneic with the stimulator cells. This is called *indirect* CMC. Lymphocytes may be used as a target for CMC, i.e., indirect cell-mediated lympholysis (CML). In most investigations, PHA-stimulated day 3 lymphoblasts have been used because they are more sensitive to CMC than are nonactivated lymphocytes (Lightbody *et al.*, 1971). Despite the increased sensitivity to lysis, this procedure has certain drawbacks, since alloantigens recognized in CMC may not be expressed to the same degree in PHA-stimulated lymphoblasts as they are on lymphocytes or other cells, and conversely PHA blasts may express determinants not present on resting cells. The function of HLA-D/DR as important target determinants in CMC was not recognized for years owing to the use of PHA-stimulated lymphoblasts (Albrechtsen *et al.*, 1979; Feighery and Stastny, 1979; Johnsen, 1980a).

C. Genetic Control of the Inductive Phase in Indirect CML

The strong cytotoxic response in indirect CML generally necessitates a positive MLR or alloantigen activation due to incompatibility for HLA-D antigens (Eijsvoogel *et al.*, 1973a–c). However, CTLs are not necessarily produced in MLR between HLA-D disparent individuals—or in cultures with positive MLR (Eijsvoogel *et al.*, 1973a–c; Bach *et al.*, 1973), and conversely, CML activity may be demonstrated in combinations where no MLR were demonstrated in the primary cultures between seemingly unsensitized individuals (Mawas *et al.*, 1973).

Considering the complexity of the genetic control of the MLR, it is not surprising that HLA-D/DR may not account for the induction of all CML activity, and the sensitivity of the MLC and CML techniques may not be equal. Furthermore, *in vivo* sensitization cannot be excluded when unexpected "indirect" CML activity is observed.

D. Genetic Control of the Target Determinants in Indirect CML

The target determinants in indirect CML are under genetic control, and the targets of major importance in man are HLA-coded (Miggiano *et al.*, 1972) as had been suggested by Lightbody *et al.* (1971). The major targets on PHA-stimulated lymphoblasts are HLA-A, B, C antigens and/or determinants coded by genes closely linked to genes coding for HLA-A, B, C (Eijsvoogel *et al.*, 1973a–c; Bach *et al.*, 1973; Kristensen *et al.*, 1975). In general, the CTLs generated during a primary MLC do not show precisely the same specificity as that displayed by presently known HLA-A, B, C, DR antibodies (Grunnet *et al.*, 1975; Johnsen, 1980b). However, more recently CTL clones obtained from an individual sensitized by repeated, planned immunizations have shown exactly the same specificity as certain monospecific HLA antibodies (Kristensen *et al.*, 1981c). Moreover, it has been demonstrated that at least some of the cross-reactivities characteristic for ABC antibodies also can be detected by the indirect CML (Kristensen *et al.*, 1981b). These observations strongly indicate that the ABC antigens can be targets for CTLs. HLA-D/DR antigens have also been shown to function as targets for CMC on Epstein–Barr virus (EBV)-transformed B-cell lines (Albrechtsen *et al.*, 1979), monocytes (Feighery and Stastny, 1979) and pokeweed mitogen (PWM)-stimulated B-lymphoblast-enriched cell suspensions (Johnsen, 1980a).

Determinants different from the known HLA-A, B, C, D/DR antigens may also act as targets in indirect CML, as suggested by (*a*) the

positive CML between unrelated individuals matched for known HLA-A,B antigens (Schapira and Jeannet, 1974); (*b*) lysis of target cells sharing no HLA antigens with the primary stimulator (Kristensen *et al.*, 1974); and (*c*) the demonstration in family studies of CML not directed toward HLA antigens (Mawas *et al.*, 1974).

There is good evidence that at least two separate CML loci exist in the HLA region: one locus between *HLA-A* and *-D/DR* but different from *HLA-B* (Kristensen and Mossin, 1982; Kristensen *et al.*, 1981a), and another locus between *HLA-B* and *GLO* but different from *HLA-D/DR* (Johnsen *et al.*, 1982).

However, the genetics of the indirect CML may be complicated by phenomena such as gene complementation as indicated by the studies of Schendel *et al.* (1978b). In these studies, CTLs were generated that could lyse only family members with certain haplotype constellations, while the cells from parents of these individuals were not lysed.

The indirect CML technique is a powerful tool for the cellular definition of histocompatibility antigens (for review, see, e.g., Kristensen *et al.*, 1982).

V. Proliferative Response of Primed Lymphocytes (Secondary MLR)

Responder lymphocytes cultured with allogeneic stimulator lymphocytes may become "immunized"—or "primed"—in MLC *in vitro*. When such primed lymphocytes are cocultured with further stimulator cells in a "new," secondary MLC, the primed lymphocytes will respond with a proliferative response. This secondary MLR reaches the maximum strength faster than does a normal (primary) MLR—giving rise to the accelerated proliferative response of primed lymphocytes (Fig. 1).

This phenomenon was first described in the mouse. Andersson and Häyry (1973) showed that specific priming of mouse thymus-dependent lymphocytes to allogeneic cells *in vitro* led to an accelerated proliferative response of the primed cells when restimulated in secondary MLC with the primary stimulator. These authors also demonstrated that the secondary MLR was governed by the MHC in the mouse (H-2): an accelerated proliferative response was only provoked by restimulating with lymphocytes sharing H-2 cross-reactive determinants with the original, primary stimulating lymphocytes.

In man, the accelerated proliferative response of lymphocytes primed *in vitro* toward allogeneic cells was first shown by Fradelizi and Dausset (1975), Svedmyr (1975), and Zier and Bach (1975). In the first experiments (Fradelizi and Dausset, 1975; Svedmyr, 1975), there

was no evidence that lymphocytes from third-party individuals could restimulate in secondary MLR, while Zier and Bach (1975) showed that third-party cells could restimulate primed cells. It was soon demonstrated that the secondary MLR is under genetic influence and that HLA—the HLA-D region inparticular—controls the majority of the reactions observed in the secondary MLR (Mawas *et al.*, 1975; Sheehy *et al.*, 1975). Sheehy and co-workers (1975) suggested that alloantigen-primed cells might be used for typing for lymphocyte defined (LD) determinants: primed "LD" typing, or primed lymphocyte typing (PLT).

The following sections will deal with the genetics of the secondary MLR with the main emphasis on the use of primed lymphocytes for typing for human alloantigens. In this section, we shall briefly summarize some of the basic phenomena in the secondary MLR.

A. Kinetics of Secondary MLR

In primary MLC, the maximum blastogenic transformation is reached on about day 6–7 (Sørensen *et al.*, 1969). The number and fraction of blast cells gradually decreases, and after 2–3 weeks only small lymphocytes are found. When additional stimulator cells are added to the cultures, the lymphocytes are transformed into blasts, with a maximum transformation response 2–3 days later (Andersson and Häyry, 1973; Häyry and Andersson, 1974; Fradelizi and Dausset, 1975; Svedmyr, 1975). If third-party cells—sharing no MHC antigens with the primary stimulator cells—are added to the primed cell cultures, the kinetics of the blastogenic response is comparable to that of a primary response (peak at day 6–7). The amount of incorporated radioactive thymidine is proportional to the degree of blastogenic transformation, and Fig. 1 illustrates the responses in primary and secondary MLR.

The responding cells in secondary MLR are, as in primary MLR, T lymphocytes (Andersson and Häyry, 1973; Fradelizi and Dausset, 1975). The cells responsible for the accelerated proliferative response in secondary MLR are the progeny of the lymphocytes that have undergone blast transformation in the primary MLR: day 7 lymphoblasts isolated at the peak of the response by velocity sedimentation show an accelerated proliferative response when restimulated with the original stimulators; in contrast, small lymphocytes from day 7 do not respond to the primary stimulator but may respond with a primary response to third-party cells (Andersson and Häyry, 1973; McDonald *et al.*, 1974; Fradelizi *et al.*, 1977).

The specific accelerated proliferative response of primed cells is

due mainly to an increased fraction of antigen-reactive cells; it is thus a quantitative phenomenon as well as a qualitative one. In secondary MLR in the mouse, up to 90% of the primed cells may respond to the original antigen as compared to only 3–5% in primary MLR quantitated by hydroxyurea block (Häyry and Andersson, 1974). In humans, the number of responder cells necessary to demonstrate significant proliferation is 5–20 times lower in secondary MLR than it is in primary MLR (Corley, 1977a; Singal, 1980). Experiments with serial dilutions of primed cells have demonstrated that the specificity of the primed cell cultures is strongly correlated to the fractions of antigen reactive cells toward different allogeneic cells (Corley, 1977b; Wank *et al.*, 1977).

B. Role of Lymphocyte Subpopulations and Other Cell Populations in Secondary MLR

The responding cells in secondary MLR are T lymphocytes as judged by the reaction with sheep red blood cells (SRBC; E-rosetting) in humans (Fradelizi and Dausset, 1975; Wollman *et al.*, 1980) and anti-θ plus complement in the mouse (Andersson and Häyry, 1973). The responding T lymphoblasts also express autologous HLA-DR antigens (DeWolf *et al.*, 1978; Evans *et al.*, 1978; Suciu-Foca *et al.*, 1978) as well as MLR activating determinants (Suciu-Foca *et al.*, 1978). In man, the alloantigen-activated T blasts respond to T-cell mitogens like PHA, Con A, PWM, and leuco-agglutinin A (DeWolf *et al.*, 1979b; Malissen *et al.*, 1979; Charmot *et al.*, 1980).

The major stimulating cells in secondary MLR are B lymphocytes—including EBV-transformed B-cell lines—and monocytes (Reinsmoen *et al.*, 1977b; Baron *et al.*, 1979; Wollman *et al.*, 1980); but other cells, e.g., allogeneically activated T cells (Wollman *et al.*, 1980) and epithelial cells (Moen *et al.*, 1980), may also stimulate in secondary MLR.

Evidence has accumulated that, in primary MLR, the HLA-D/DR antigens of the stimulating cells may be transferred to the responding cells (Nagy *et al.*, 1976), and there is some evidence that alloantigen-activated lymphocytes—probably carrying stimulator antigens—may stimulate autologous lymphocytes in primary and secondary MLR (Gänsbacher *et al.*, 1980).

The major stimulating antigens are either the D/DR antigens or antigens closely associated with D/DR antigens. Blocking of antigens on stimulator cells with anti-DR antisera directed toward the stimulator cells inhibits the stimulatory capacity of the stimulator cells. This is apparently true even for heterozygous stimulator cells, where the primed cells seem to react with antigens controlled by one HLA hap-

lotype and the anti-DR antisera react with antigen coded by the other HLA haplotype (Hirschberg *et al.*, 1977; Suciu-Foca *et al.*, 1978; DeWolf *et al.*, 1978, 1979c).

Cell populations with inhibitory effect on MLR may arise in secondary MLR (Sasportes *et al.*, 1978a; Sheehy *et al.*, 1979). The phenomenon may be induced by T-suppressor cells that suppress the response of the T-responder cells directly or by the secretion of a soluble substance that can mediate the phenomenon ((Sasportes *et al.*, 1978a, 1979, 1980). There is also some circumstantial evidence that the inhibitory effect might be mediated by cytotoxic cells (Sheehy *et al.*, 1979).

VI. Genetics of Secondary MLR

In the mouse, the major reaction in secondary MLR is genetically controlled by the mouse MHC: H-2 (Andersson and Häyry, 1973). The antigens recognized include (*a*) highly polymorphic determinants coded by the *I-A* region (Fathman *et al.*, 1977; Peck and Wigzell, 1978); (*b*) determinants with limited polymorphism coded by the *I-E/C* region (Peck and Wigzell, 1978; Peck, 1980); (*c*) highly polymorphic determinants coded by genes in the *K* and *D* regions (Peck and Wigzell, 1978, Peck *et al.*, 1978); and (*d*) non-MHC coded determinants associated with the Mls system (Peck *et al.*, 1977a).

The recognition of these alloantigens may be extremely specific (Fathman and Nabholz, 1977): some clones of parental lymphocytes primed toward F_1 stimulator cells react in secondary MLR only toward F_1 cells, but not against cells from the other parental strain. Thus, these primed cells recognize combinatorial or "hybrid" antigens (cf. Fig. 4). There is also some evidence that primed cells may specifically recognize unique homozygous MLR determinants (Fathman and Hengartner, 1978; Hengartner and Fathman, 1980).

The recognition of Mls determinants in secondary MLR seems to depend on the sharing of *H-2* gene products between the primary and secondary stimulator cells, thus lending support to the involvement of "self" MHC determinants in the presentation of non-MHC antigens (Peck *et al.*, 1977a,b).

In man, genes in the *HLA-D* region control the major reactions in secondary MLR (Mawas *et al.*, 1975; Sheehy *et al.*, 1975). These genes include the genes coding for the series of HLA-D and -DR antigens, but the *HLA-D* region most probably codes for other alloantigens, and there may even be multiple genes in the *HLA-D* region coding for determinants recognized in primary and secondary MLR as suggested

by Dupont *et al.* (1973b) and Bach *et al.* (1975). The secondary MLR seems to be controlled also by HLA genes telomeric to *HLA-B* and by other HLA genes situated between *HLA-D/DR* and *GLO* (and possibly belonging to the *HLA-D* region). In addition, other determinants, e.g., the blood group antigen Lewis, may be recognized in secondary MLR (see below).

Most of these determinants are thought to be inherited in a simple mendelian way as dominant or codominant traits. A gene dose effect has been suggested by Bach *et al.* (1976b) and Hirschberg *et al.* (1976), but the results have not been confirmed by others (e.g., Bradley *et al.*, 1977b; Jaramillo *et al.*, 1977; Pawelec *et al.*, 1977). Differences in the techniques may account for the discrepant results, and the problem is not solved. The discovery of combinatorial antigens recognized in mouse secondary MLR (see above) suggests that similar phenomena may exist in human MLR.

The genetics of secondary MLR in man is, thus, rather complex, and we shall in this section discuss some observations that are the basis for our present knowledge of the genetics of the human secondary MLR.

A. Role of the HLA-D Region

Primed cells may be generated between family members disparate for one HLA haplotype (Fig. 7). Such primed cultures are called haplotype-primed lymphocytes. These primed lymphocytes in general show a secondary MLR against the specific restimulator cells (a/c) as well as to lymphocytes from all family members carrying the HLA haplotype, *c*, of the stimulator (Mawas *et al.*, 1975; Sheehy *et al.*, 1975). If a gametic recombination (crossover) takes place between the *B* and the *D* locus in an individual, haplotype-primed cells can in most cases be generated only if the responder and the stimulator differ for the part of the chromosome carrying the *D* allele (Fig. 2), but not if they share the *D* allele and differ for the *A*, *B* alleles (Mawas *et al.*, 1975). Similarly, haplotype-primed cells react only with cells from *B/D* recombinant family members if the secondary stimulator share the *D* region with the primary stimulator (Mawas *et al.*, 1975; Bradley *et al.*, 1976).

These early studies of a few families including individuals in which *HLA-B/D* recombinations had taken place showed that genes in the *HLA-D* region control the major reactions both in the inductive phase (primary MLR) and in the secondary MLR.

The determinants responsible for primary and secondary MLR are either identical or very closely associated to each other, since a positive primary MLR is a prerequisite for a secondary MLR (Mawas *et*

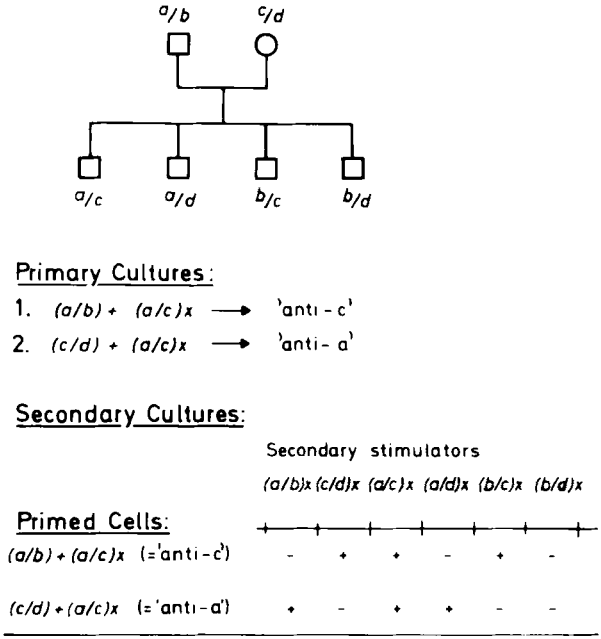

FIG. 7. Family haplotype priming. The primed cells (a/b) + $(a/c)_x$ (= "anti-c") react only with cells carrying the *c* haplotype, and (c/d) + $(a/c)_x$ (= "anti-a") react only with cells carrying the *a* haplotype.

al., 1975; Sheehy *et al.*, 1975), and, conversely, the response of haplotype-primed cells toward unrelated secondary stimulator cells is predictive of the intensity of the MLR between these cells (Sheehy *et al.*, 1975).

1. HLA-D and -DR Antigens

Investigations of the responses of primed cells toward panels of HLA-D/DR typed cells have shown that the responses of the primed cells may be grouped into clusters, and that the cells in these clusters usually share HLA-D/DR antigens (Fradelizi *et al.*, 1975; Bach *et al.*, 1976b; Bradley *et al.*, 1976; Hirschberg *et al.*, 1976; Thomsen *et al.*, 1976). If cells are primed toward a known HLA-D/DR disparity, the response of the primed cells in general correlate to the HLA-D types (Bach *et al.*, 1976b; Bradley *et al.*, 1976; Hirschberg *et al.*, 1976; Thomsen *et al.*, 1976) and the HLA-DR (Sasportes *et al.*, 1977a,b; Fradelizi *et al.*, 1978) of the secondary stimulators.

In many of these studies, however, no complete correlation was found between the response of primed cells and HLA-D/DR, and a

great number of unexpected positive responses were observed, when referring to HLA-D/DR.

Both HLA-D and -DR antigens have been suggested to be responsible for the major responses in secondary MLR, but primed cells may recognize determinants that are closely associated with the antigens presently defined in the HLA-D series but not in the DR series, and vice versa. These phenomena have been demonstrated by, e.g., Fradelizi *et al.* (1978), who showed that the response of cells primed toward a Dw3/DR3 HTC segregated with a *Dw4,DR3* haplotype in a family. Both Hartzman *et al.* (1978, 1979) and Reinsmoen *et al.* (1978b, 1979b) have demonstrated that haplotype-primed cells may recognize determinants that are closely correlated to either D or DR, or to both.

Accordingly, the secondary MLR seems to be governed by *HLA-D* region genes, the gene products of which are otherwise recognized as (*a*) HLA-D with homozygous typing cell technique; and (*b*) HLA-DR with serological technique, as discussed in Sections II,B and III,D.

2. *Non-HLA-D/DR Determinants*

In the *HLA-D* region, other genes than the *HLA-D* and *-DR* genes may code for determinants recognized in secondary MLR as suggested by many studies. A series of PLT-defined determinants seems to be coded by genes between *HLA-D/DR* and *GLO*, and these genes may be considered as belonging to the *HLA-D* region.

As discussed in Section XII,D, Mawas *et al.* (1978, 1980), Charmot *et al.* (1981), and Shaw *et al.* (1979, 1980a,b) have provided evidence from population and family studies favoring the existence of secondary MLR determinants controlled by genes centromeric to the *HLA-D/DR* locus. These determinants have been called SB antigens (Shaw *et al.*, 1980a,b) and follow the GLO determinants in some *GLO-HLA-D/DR* recombinants.

Besides these genes, the *HLA-D/DR* region may code for other alloantigens. Charmot *et al.* (1981) have extended their previous studies (Mawas *et al.*, 1978, 1980) and have suggested that, besides the SB antigens, a further complexity may exist in the *HLA-D/DR* region leading to the expression of three components coded by genes telomeric to a recombination between *HLA-D/DR* and *GLO*. This may give rise to four possible phenotypic traits coded by the *HLA-D/DR* region per haplotype.

There is evidence that determinants coded by genes situated telomeric to *HLA-D/DR* exist (Zier *et al.*, 1978). The determinants have been identified in one *B/D* recombinant family, and the genes may

tentatively be considered to belong to the *HLA-D/DR* region. Many other investigators have demonstrated that it is possible to generate PLT cells that recognize non-HLA-D/DR determinants, although the position of the genes coding for these determinants has not been established (e.g., Reinsmoen *et al.*, 1977a; Singal and Naipaul, 1977; Suciu-Foca *et al.*, 1977; Wank *et al.*, 1977, 1978a,b; Nunez-Roldan *et al.*, 1978; Sasportes *et al.*, 1978d; Reinsmoen *et al.*, 1979a; Zier *et al.*, 1980; Termijtelen *et al.*, 1980; Morling *et al.*, 1981d). Most of these determinants are believed to be coded by *HLA-D/DR* region genes.

B. Role of the HLA-A Region

The early studies of a few families including individuals in whom recombinations had taken place between *HLA-B,D* or *-A,B* (e.g., Mawas *et al.*, 1975; Sheehy *et al.*, 1975; Bradley *et al.*, 1976) seemed to indicate that genes in the *HLA-A* region played no role in secondary MLR. In a few cases, however, primed cells have been generated between sibs identical for all HLA determinants except the *HLA-A* region of one haplotype (Wank *et al.*, 1979). The response of these primed cells segregated with the *HLA-A* segment of the stimulator haplotype in the family in question.

These data suggest that genes situated telomeric to *HLA-B* and possibly in the vicinity of *HLA-A* may also play a role in the secondary MLR. In order to be able to explain the difficulties in demonstrating the determinants coded by these genes, the determinants may be assumed to have an extremely weak alloantigeneic capacity or the polymorphism must be very limited. The existance of such HLA-A-associated factors is supported by the observations that *HLA-A* region products may also stimulate, albeit weakly, in primary MLC (cf. Section III,G).

C. Non-HLA Determinants

It cannot be excluded that some of the above-mentioned reactions in secondary MLR may have been caused, in part, by non-HLA-coded determinants. There is some evidence that determinants closely associated with or identical to the blood group antigen Lewis may be recognized in secondary MLR, whereas a limited number of experiments have failed to demonstrate a role for ABO blood group antigens and the products of the *secretor* genes in secondary MLR (Singal *et al.*, 1981).

Neoplastic antigens may be demonstrated in both primary and secondary MLR. Viza *et al.* (1969) found that, in primary MLR, leukemic blast cells may stimulate autologous lymphocytes taken in remission.

Reinsmoen *et al.* (1978a) have shown that responder lymphocytes from healthy individuals may be primed with leukemic blast cells from HLA-identical sibs with acute myeloid leukemia. These primed cells are restimulated only by the leukemic cells, not by parental cells or by cells taken from the patient in remission.

In primary MLR, T cells may respond significantly to autologous non-T lymphocytes (Opelz *et al.*, 1975; Kurtz *et al.*, 1976). A similar phenomenon may be demonstrated in secondary MLR (Weksler and Kozak, 1977; Thorsby and Nousiainen, 1979; Zier *et al.*, 1979a), where (*a*) T cells may be primed toward autologous non-T lymphocytes to give an accelerated proliferative response to both autologous non-T cells as well as some allogeneic non-T cells; and (*b*) alloantigen primed cells may respond to autologous non-T cells. In these experiments, the kinetics in "autologous" secondary MLR was very similar to that of a conventional secondary MLR, and there is some evidence that the "autologous" secondary MLR may in some cases be restricted to determinants strongly associated with or identical to the HLA-D/DR antigens (Thorsby and Nousiainen, 1979).

VII. Primed Lymphocyte Typing (PLT)

Human histocompatibility antigens may presently be defined by means of (*a*) serological techniques (e.g., HLA-A, B, C, DR antigens); (*b*) cell-mediated lympholysis; (*c*) primary MLR with HTC technique (HLA-D antigens); and (*d*) PLT technique. Each of these techniques has advantages, and none of them are obsolete. The maximum information is achieved by combining the results obtained using all four principles.

The basic principle in both primary and secondary MLR is recognition and a subsequent T-lymphocyte proliferation. Thus, both HTC typing and the PLT are typing systems based on *in vitro* functions, which may have *in vivo* correlates. In HTC typing, the basic principle is a test for differences between the test cells and the reference cells (the HTCs), and the lack of response of the test cells is interpreted as similarity or even identity between test cells and reference cells. As discussed above, this conclusion may occasionally be wrong. With the PLT technique, antigenic similarities are recognized and followed by a positive reaction, and even in complex genetic systems it should be possible to generate PLT cells that recognize single determinants. Thus, the PLT system seems to be ideal for the identification of determinants that give rise to a proliferative response, and it is possible to establish batteries of PLT cells defining histocompatibility antigens.

The great potential of the PLT technique has not led to an international definition of PLT antigens. Attempts were made during the 7th International Histocompatibility Workshop (Bradley *et al.*, 1977a), where the correlation between PLT results and HLA-D was confirmed, but the reproducibility of the results was poor, and the PLT technique was not included in the 8th International Histocompatibility Workshop (1979/1980). In many laboratories, local batteries of PLT cells identifying different antigens have been established, and an international exchange of these PLT cells might be most fruitful.

Establishing a battery of PLT cells includes—besides the purely technical aspects—(*a*) generation of primed cells; (*b*) a screening procedure for the specificity of the primed cells; and (*c*) a subsequent generation or expansion of selected primed cells with the wanted specificities. Different approaches may be used, but the final goal is the generation of functionally "specific" reagents, whether these reagents are monoclonal or not. When such a battery of specific PLT cells has been established, a secondary PLT system with a high reproducibility of the antigen assignment may be established.

In the following sections, we shall discuss the various methods that can be used in order to establish a typing system for PLT-defined determinants and review the results obtained in humans.

VIII. Technical Aspects of PLT

This review is not meant to focus on the technical aspects of the human PLT, which has been described extensively elsewhere (e.g., Sheehy and Bach, 1976; Moen and Thorsby, 1978, 1979; DeWolf *et al.*, 1979a; Hartzman, 1979; Termijtelen and Bradley, 1979; Morling *et al.*, 1981a). Only two items will be discussed here: (*a*) the sources of variability in PLT; and (*b*) clonal expansion of primed cells.

A. Sources of Technical Variability

In the PLT system, the crucial point is to be able to perform a reproducible antigen assignment that is based on objective criteria. The PLT, like all other cellular techniques, has a number of sources of variability that cannot be neglected.

The sources of experimental variability in PLT include (*a*) the day-to-day variation in the priming phase (the "primary" PLT), especially concerning the yield, responsiveness, and specificity of the PLT cells; (*b*) the day-to-day variation in secondary PLT, which includes the systematic variabilities in both the general responding capacity of the PLT cells and the general stimulatory capacity of the secondary stimu-

lators; and (*c*) other sources of experimental variations. Part of the "rest variation" may be considered the "true" biological interaction between the PLT cell and the secondary stimulator.

For practical PLT, the generated PLT cells should be prescreened to confirm the expected specificity. Most of the technical variability in the secondary PLT originates from differences in (*a*) the general responding capacity of different PLT cells; and (*b*) the general stimulatory capacity of different secondary stimulators. The first of these points is the more important one and is generally recognized. The second point—the stimulator variability—is often of less importance and is neglected by most investigators. Neglect of the stimulator variability may be acceptable, for example, if selected stimulator cells from healthy individuals are used, and if some differences in stimulatory capacity is tolerated. If a panel of secondary stimulators includes lymphocytes with greater differences in the stimulatory capacity—for example, samples of both fresh and frozen lymphocytes, peripheral blood lymphocytes, spleen cells, or lymphocytes from patients with "autoimmune" diseases—it is not possible to obtain reproducible results without an effective neutralization of the stimulator variability. In such PLT experiments, the general stimulatory capacity of different secondary stimulators may vary up to a factor of 10 (Morling *et al.*, 1980a).

The data in Table VIII illustrate the sources of variability and give an example of how the variability may be reduced by a simple data treatment procedure that includes only a simple nonparametric procedure that compensates for differences in (*a*) the general responding capacity of different PLT cells; (*b*) the general stimulatory capacity of different secondary stimulators. This data treatment method and other methods as well are discussed in Sections IX,C and XII,C).

B. Production and Clonal Expansion of PLT Cells

For the immunogenetic investigations of PLT-defined determinants, it is of the utmost importance to be able to create "unlimited" amounts of primed cells with constant specificity, regardless of whether the PLT cells are generated by repeated set ups of conventional primary *in vitro* cultures or by bulk expansion *in vitro* of primed cells (e.g., Pappas *et al.*, 1979; Schendel *et al.*, 1978a, 1980), or produced by clonal expansions of primed cells (Bach *et al.*, 1979; Charmot *et al.*, 1980; Inouye *et al.*, 1980b; Malissen *et al.*, 1981; Pawelec *et al.*, 1980). Each of these methods has advantages and limitations, but it should be stressed that a PLT reagent that is prepared

TABLE VIII

REPRODUCIBILITY IN THE PLT. PAIRED COMPARISONS OF THE RESPONSES OF PLT CELLS AGAINST THE SAME SECONDARY STIMULATORS[a,b]

Priming	Sec. PLT	Comments on PLT cells from	No. of experiments	No. of PLT cell pairs	No. of paired observations	NMR[c]			CPM[c]	
						% Discrepancies	Kendall's *R*	Mean CV	Kendall's *R*	Mean CV
Same	Same	Same tubes	1	1	15	0	0.75	0.20	0.85	0.21
Same	Same	Different tubes	1	1	15	6.7	0.60	0.23	0.70	0.22
Different	Same	—	8	9	116	7.8	0.53	0.20	0.71	
Same	Different	Tested repeatedly against the same	10	33	165	9.1	0.58	0.21	0.46	0.39
Different	Different	five individuals	8	9	45	6.7	0.70	0.21	0.002	0.61

[a] From Morling *et al.* (1980a).

[b] Each pairwise comparison concerns primed cells involving the same primary responder and stimulator. A total of 35 different primary responder/stimulator combinations are included in the table.

[c] NMR, normalized median response; CV, coefficient of variation; cpm, counts per minute.

by repeated bulk primings may often be as valuable as a clonally expanded PLT-cell batch.

All ordinary uncloned PLT reagents most probably contain many different clones with partly or totally different specificities and "affinities," just as ordinary HLA antisera contain mixtures of many different antibodies even when they behave as good typing reagents. HLA antisera may be absorbed to become more specific, but this is not possible for PLT cells; the use of "suicide" techniques (Dutton and Mishell, 1967) to render PLT reagents more specific is not possible for practical purposes.

From a theoretical point of view, it would seem optimal for typing purposes to use cloned primed cells that are all the progeny of one single cell. In theory, cloning and the subsequent clonal expansion of primed cells might lead to the production of unlimited amounts of monospecific PLT cells.

The principle in clonal expansion is based on the observations of Morgan *et al.* (1976) and Ruscetti *et al.* (1977), who showed that supernatants from PHA-stimulated lymphocyte cultures can induce continuous proliferation of T lymphocytes. The supernatants with this property have been called conditioned medium (CM) and T-cell growth factor (TCGF) and are now called interleukin-2 or IL-2 (Mizel and Farrar, 1979). The cloning procedure may be performed by either limiting dilution or soft agar cloning with IL-2 as growth promotor. The technique has been applied on both mouse PLT (e.g., Peck *et al.*, 1977b; Fathman and Hengartner, 1978) and human PLT (e.g., Bach *et al.*, 1979; Charmot *et al.*, 1980; Malissen *et al.*, 1981; Pawelec *et al.*, 1980). The protocols for the production of IL-2 may be found in the above cited works as well as in other publications (Bonnard *et al.*, 1978; Inouye *et al.*, 1980a; *Immunological Reviews*, 1980, 1981). There is also some evidence that IL-2 may be produced by leukemic cell lines (Gillis and Watson, 1980), and hybridomas producing IL-2 may be established (Harwell *et al.*, 1980). Furthermore, IL-2 is being purified and biochemically identified (Gillis *et al.*, 1980; Mier and Gallo, 1980), which might lead to a biochemical production of IL-2.

In human PLT, the present technology has not yet allowed us to reach the final goal. Clones of *in vitro* primed human PLT cells have been established (Bach *et al.*, 1979), but it has not been possible to keep these cloned PLT cells in continuous cultures for more than a few months (Bach *et al.*, 1979; Charmot *et al.*, 1980; Malissen *et al.*, 1981; Pawelec *et al.*, 1980). Furthermore, monoclonality does not necessarily imply monospecificity, although some clones may be more specific than the original bulk PLT (Bach *et al.*, 1979; Charmot *et al.*,

1980; Inouye *et al.*, 1980b; Malissen *et al.*, 1981; Pawelec *et al.*, 1980). Therefore, a screening procedure must be performed in order to select clones of PLT cells with the wanted specificity, just as in conventional bulk PLT, if the PLT cells are to be used for typing purposes.

With the present technology, the cloning procedure may be a potential tool for the investigation of subpopulations of activated lymphocytes, including both their surface structures, secretion products, and functions, since small cell populations—or even single cells—may be studied extensively after clonal expansion.

IX. Data Reduction in PLT

A. Introduction

The basic phenomenon taking place in secondary MLR is blastogenesis or proliferation of T lymphocytes. This phenomenon is usually indicated by the cellular uptake of radioactive labeled thymidine. This allows a quantitative evaluation of the thymidine uptake and, thereby, a quantitative estimate of the blastogenesis or proliferation. The results are usually measured in arbitrarily defined units presented as counts per minute (cpm). For the demonstration of basic phenomena in the secondary MLR, the cpm themselves may be fully satisfactory for the interpretation of the observations. In most other cases, however, some kind of treatment of data is necessary because of the technical variability in secondary MLR as in all other cellular systems (cf. Section VIII,A). The aim of the data treatment is (*a*) to neutralize as much of the technical variability as possible; and (*b*) to present the data in a form that allows the investigator to interpret the data in an easy way. In essence, this procedure may be considered a *data reduction*, and this does not necessarily have to include complicated statistical procedures.

The data treatment procedure should be chosen in accordance with the purpose of the investigation, and no data treatment system is optimal for all kinds of investigations. On the other hand, several different data treatment methods may be well suited for a given type of investigation. For formal typing purposes, the quantitative measure (cpm) of proliferation should, if possible, be reduced to an assignment of the one of two possible reactions: a positive or negative reaction. In this situation, the definition of exact criteria for positive and negative reactions is most helpful, but can rarely be made. It is of great practical importance to select PLT cells that give a good discrimination between "positive" and "negative" reactions. It should be stressed that

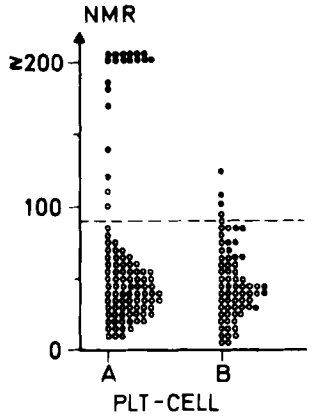

FIG. 8. Plot diagrams of a well discriminating primed lymphocyte typing (PLT) cell (A) and a poorly discriminating PLT cell (B). Both PLT cells were generated by homozygous typing cell (HTC) priming of HLA-D/DR heterozygous responder cells not carrying the HLA-D/DR antigen of priming HTC (A: Dw2/4 + Dw1/1_x; B: Dw6/9 + Dw5/5_x). Expected positive reactions (●) and expected negative reactions (○) based on the HLA-D/DR types. NMR, normalized median response.

no data reduction method can give meaningful result for PLT cells, which basically show poor discrimination (Fig. 8).

The description of the complex genetic and biological phenomena taking place by the restimulation of unselected PLT cells cannot be reduced to a simple question of a dichotomization of the responses of a given PLT cell. In such cases, the data analysis may include more complicated statistical methods in order to be able to decide, for example, whether or not two or more responses are quantitatively different from each other. In other kinds of investigations, the general pattern of reactivity of two or more test cells toward a panel of cells may be the object of investigation, and, in such cases, the dichotomization between positive and negative responses may give too little information compared, for example, to correlation tests.

This section deals mainly with data treatment of the results of typing experiments. Nearly every laboratory has its own way of treating PLT data, and we shall try to mention only some few basically different methods and discuss some of the pitfalls in data treatment from cellular experiments.

B. Distribution of PLT Data

1. Distribution of Counts per Minute

If parametric statistics are to be applied to PLT data, some important conditions must be considered: normality, mutual indepen-

dence, and variance homogeneity of the data. Owing to the Poisson distribution of the cpm data, the variance of the observed mean (cpm) is equal to the mean (cpm) of replicate investigations (e.g., triplicates). Accordingly, the variance will increase with increasing cpm, and parametric statistics cannot be applied to the "raw" data. Theoretically, the data should be logarithmically transformed, but other transformations, for example, square root transformation, may be equally (or even better) suited to establish an acceptable fit to the normal distribution and homogeneity of variance of data obtained from cellular typing (e.g., Franks and Bradley, 1977; Jensen *et al.*, 1977). If such transformation of the data is performed, parametric statistical analyses may be performed.

2. *Medians or Means of Replicate Cultures*

Before some of the different types of data treatment systems are presented, we should discuss the problems of replicate culture samples. In most cases, identical cultures are set up as replicates—most frequently in triplicate—owing to the considerable stochastic variation in MLR. Counts per minute are measured in all the replicate cultures, and only one figure (either the mean or the median value) is used to present the proliferation in that particular replicate. The value of the median has the advantage that it is less sensitive to outliers than is the mean. If parametric statistics is used, the data should be arithmetically transformed (e.g., log or square root) and the mean will be less sensitive to outliers than the raw counts. An automatic deletion of outliers may be performed in this case (e.g., outliers outside the 95% confidence interval, $\simeq$mean $\pm$ 2 SD, but it should be stressed that such procedures may be unwise, and that a high variance in a replicate or a severe departure from normality should encourage one to consider the cause of the phenomenon (Jensen *et al.*, 1977).

3. *Autologous Response*

The subtraction of the spontaneous response or the response toward autologous cells is often performed as a routine procedure as the first step in data treatment. From a theoretical point of view, this procedure seems reasonable, but in practical PLT testing the procedure decreases reproducibility by introducing a new source of variability rather than compensating for variability—at least when selected frozen and thawed PLT cells are used (Morling *et al.*, 1980a). A similar conclusion has been reached in HLA-D typing with the HTC technique (Ryder *et al.*, 1975).

4. *Biological Significance of the Distribution of PLT Data*

If PLT-cell combinations are selected for typing purposes, usually the responses of the PLT-cell may be divided into at least two groups representing positive and negative reactions, but frequently there may be more groups. The existence of these different groups is often blurred by the influence of the technical variability. Even if most of the technical variability is neutralized by a data treatment procedure, it may still be difficult to identify the individual groups. Figure 8 shows the normalized responses (cf. below) of a PLT cell typing for an HLA-D/DR associated determinant (Morling *et al.*, 1980c). The responses toward the specific restimulator, restimulators sharing HLA-D/DR antigens with the primary stimulator, or restimulators with antigens "cross-reacting" with those of the primary stimulator are usually ranked in this order above the central tendency of the distribution (Morling *et al.*, 1980a,b). This interpretation is naive but may be of didactic value when normalization and antigen assignment procedures are discussed.

C. Nonparametric Data Reduction Methods

For simplicity, we shall first consider a theoretical situation where all secondary stimulator cells have the same stimulatory capacity, and only the responses of different cells (e.g., PLT cells) need to be normalized.

1. *Normalization of PLT-Cell Responses*

All responses should be normalized according to a reference value. Theoretically, the value of the PLT-cell response toward the specific restimulator (i.e., the primary stimulator) should be used as the reference value, i.e., $AB_x + B_x$ [or if correction for "autologous" response is made: $(AB_x + B_x) - (AB_x + A_x)$; this point will be left out in the following discussion]. All other responses of the PLT cell in question (e.g., $AB_x + C_x$) are then expressed as a fraction or percentage of the reference value: cpm $(AB_x + C_x)$/cpm $(AB_x + B_x)$. In this case, the reference value is considered to be representative of a "positive" response. Other reference values that represent positive responses may be used. These include, for example, the maximum response of the PLT cell to the total number of stimulators or to a pool of allogeneic cells (Osoba and Falk, 1974). With these methods, the normalization is highly dependent on only *one* secondary MLR combination, and it is wise to include that particular combination in replicates.

The data may also be normalized according to the value of a certain fractile of all the responses of a given PLT cell. When choosing the

reference fractile, the expected distribution of the data should be considered. In the ideal situation with two clusters—positive and negative values—the reference fractile may represent either the negative or the positive values. In each case, the reference value should be chosen as close to the central tendency of that particular cluster, for example, the median of the cluster. This approach has been applied on data from both primary and secondary MLR. In HLA-D typing with HTCs in primary MLR, this kind of normalization is widely used, and the standard reference value is the 75 percentile (Ryder *et al.*, 1975), although, this approach was originally suggested with a reference value of 50% (Jørgensen *et al.*, 1973).

In PLT, data normalization according to the 90 percentile has been suggested when typing for HLA-D/DR-associated determinants (Sasportes *et al.*, 1978c). In this procedure, the assumption is made that at least 10% (in general approximately 20%) of the responses of a PLT cell may be considered positive, and the normalization is done according to a value representing a positive response. The normalization may also be done according to a value representing a negative response. In PLT typing for HLA-D/DR-associated determinants and other determinants of low frequency in the population, such a procedure may be applied by choosing the 50 percentile (= the median value) as the reference value (Morling *et al.*, 1980a). In this procedure, the assumption is made that less than 50% of the responses are positive.

2. *Stimulator-wise Normalization*

The varying stimulatory capacity of secondary stimulators is often neglected, but, as it may vary greatly in certain cases, it is wise to consider this point (see Section VIII,A). If the variability of the general stimulatory capacity of different secondary stimulators should be neutralized, this may be done by methods that are very similar to the responder-wise normalization procedures. In HLA-D typing, the double normalization procedure according to the 75 percentile both ways (Ryder *et al.*, 1975) is widely used, and a very similar approach may be used in PLT typing by a double normalization according to the 50 percentile both ways: the normalized median response (NMR) (Morling *et al.*, 1980a). We have chosen the NMR method (see Section XII,B) for the evaluation of PLT results for HLA-D/DR-associated determinants as well as other determinants because of its robustness and reproducibility compared to other methods (Morling *et al.*, 1980a). The method, however, can be used only if large PLT experiments are performed, i.e., experiments with more than 10 PLT cells and 10 stimula-

tors, preferably more. It should also be recalled that, for each PLT cell, the expected number of negative reactions should exceed that of expected positive responses; and the same condition counts for each stimulator.

If these demands are not fulfilled, the data reduction system breaks down and another reference fractile may be chosen ad hoc after a visual inspection of plot diagrams of the data. This is a very unsatisfactory approach, and all efforts should be made to respect the limitations of the data treatment system when the experiments are planned. However, if these precautions are taken, fixed criteria for positive and negative reactions may be established and the reproducibility of the NMR values is high in repeated secondary PLT experiments with different batches of PLT cells and secondary stimulators (Morling *et al.*, 1980a; cf. Sections. VIII,A and XII,B).

D. Parametric Data Analysis

The majority of these methods are based on the analysis of variance; accordingly, the data should be transformed (e.g., log or square root) in order to establish an acceptable fit to the normal distribution and homogeneity of variance (cf. above). These statisical methods include the *interaction index* method (Mickey *et al.*, 1975) based on the representation of two-way tables in terms of effects and interaction as treated in statistical analysis of variance. The *cluster analysis* (Piazza and Galfré, 1975) is widely used, and the basic principles will be summarized as originally proposed for MLC typing during the 6th Histocompatibility Workshop. The method may be used with minor modifications for PLT data: (*a*) the responses of each responder are divided into two groups (clusters) in such a way that the intergroup variance is maximal, while the intragroup variance is minimal; (*b*) the mean value of the upper cluster is used as reference value for a normalization of all responses of each responders; (*c*) on these "responder" normalized data values, a second stimulator-wise normalization is performed in a similar way. In PLT data, the second stimulator-wise normalization is often not performed. The cluster analysis normalization procedure, is, in robustness, comparable to the nonparametric SRR method (Ryder *et al.*, 1975) when applied to MLC data, but the cluster analysis is slightly more dependent on the number of cells included in the experiments than is the SRR method (Piazza and Galfré, 1975; Joint Report, 1975). Another example of normalization of PLT data is the method suggested by Rubinstein *et al.* (1977), where the PLT-cell responses are normalized according to the response toward the autologous cells, the stimulation index, cpm $(AB_x + C_x)$/cpm $(AB_x + A_x)$.

When the normalization procedure has been performed, the further treatment of data depends on the purpose of the investigations. If "assignment of antigens" is wanted, the distribution of the responses of each reagent may be investigated either by visual inspection of the data in order to try to identify what may be considered positive and negative responses based on poorly defined experience or intuition, or by some fixed criteria for positive and negative reactions. In most cases, this procedure will force one to introduce the "doubtful reaction" (or "gray area").

Statistical methods have been suggested to help to investigate whether the responses of a given cell can be separated into two or more groups, and whether two reactions may be considered significantly different. These methods include (*a*) multiple range testing based on the analysis of variance (Franks and Bradley, 1977), where it may be determined which of the results differ statistically significantly from each other and where no assumptions are made concerning the nature of distribution of the responses; (*b*) linear cluster analysis (Rubinstein *et al.*, 1977) or consecutive significance testing between group means of *two* clusters consecutively permutated by all possible combinations of responses (Mendell *et al.*, 1977); (*c*) grouping by testing for outliers by, for example, a kurtosis test (Mendell *et al.*, 1977) or a skewness test (Sheehy, 1978).

These considerations have mostly concerned the discrimination between the responses of one reagent. The comparisons of the patterns of reactions of different reagents may be done, for example, by comparing their capability to divide a test panel into two populations (positives and negatives). For the selection of typing reagents and for the definition of specificities, this is a valuable approach. Further information concerning other biological phenomena may be extracted by comparing the total reaction pattern of a reagent by performing a correlation test, either a parametric test on arithmetically transformed data or a nonparametric rank correlation test.

When the PLT technique is used for typing purposes, the repetition of experiments is the best (and in many cases only) way to reduce the effect of certain sources of error, such as errors in preparation of cell suspensions or erroneous triplicates. The statistical treatment of data from repeated experiments have been discussed by, for example, Franks and Bradley (1977) and Jensen *et al.* (1977), and we shall only mention that repeated investigations are the most powerful tool for the evaluation of the typing procedure, including the data treatment system (e.g., Franks and Bradley, 1977; Jensen *et al.*, 1977; Morling *et al.*, 1980a).

X. Generation and Selection of PLT Cells

An unequivocal demonstration of a PLT activating determinant can be made only by PLT cells that can dichotomize between test cells that do possess the determinant in question and cells that do not. PLT cells that give "intermediate" reactions toward certain test cells only suggest the sharing of one or more determinants between the primary stimulator cells and the test cells. The selection of highly discriminatory PLT cells is therefore of great importance.

The quality of PLT cells is the result of (*a*) the priming phase, which may include priming between cells differing for different numbers of determinants; and (*b*) a meticulous screening of the PLT cells generated. A careful a priori selection of the primary responder and stimulator cells according to the genetics of the secondary MLR considerably increases the chance of producing "specific" PLT cells because the influence of multiple determinants (see below) and cross-reactivity (see Section XII,C) can then be taken into account.

In this section we discuss the genetic concepts of the priming phase and the screening procedure of generated PLT cells.

A. Genetic Concepts of Priming

If lymphocytes from random unrelated individuals are used for priming, PLT cells may be generated, but these PLT cells are almost always polyspecific and cannot be used for typing purposes. The behavior of such PLT cells is comparable to that of multispecific anti-HLA-A, B, C antisera. The specificity of the PLT cells may be increased by priming against incompatibility of known alloantigens or by priming between cells sharing histocompatibility antigens, and it seems to be possible to generate PLT cells of almost any specificity one may desire by choosing the right combinations. However, before discussing the various approaches, it might be useful to present our concept of the genetics of the *D/DR* region.

1. Genetic Concept of the D/DR Region

It has previously been suggested that a single HLA haplotype controls a number of different MLR-stimulating determinants (Dupont *et al.*, 1973b; Bach *et al.*, 1975). A theoretical possibility based on this concept and taking into account the pronounced linkage disequilibrium within HLA is exemplified in Fig. 9, which illustrates some of the possible combinations of various alleles at four different loci, each of which is assumed to control an MLR determinant. At the top are

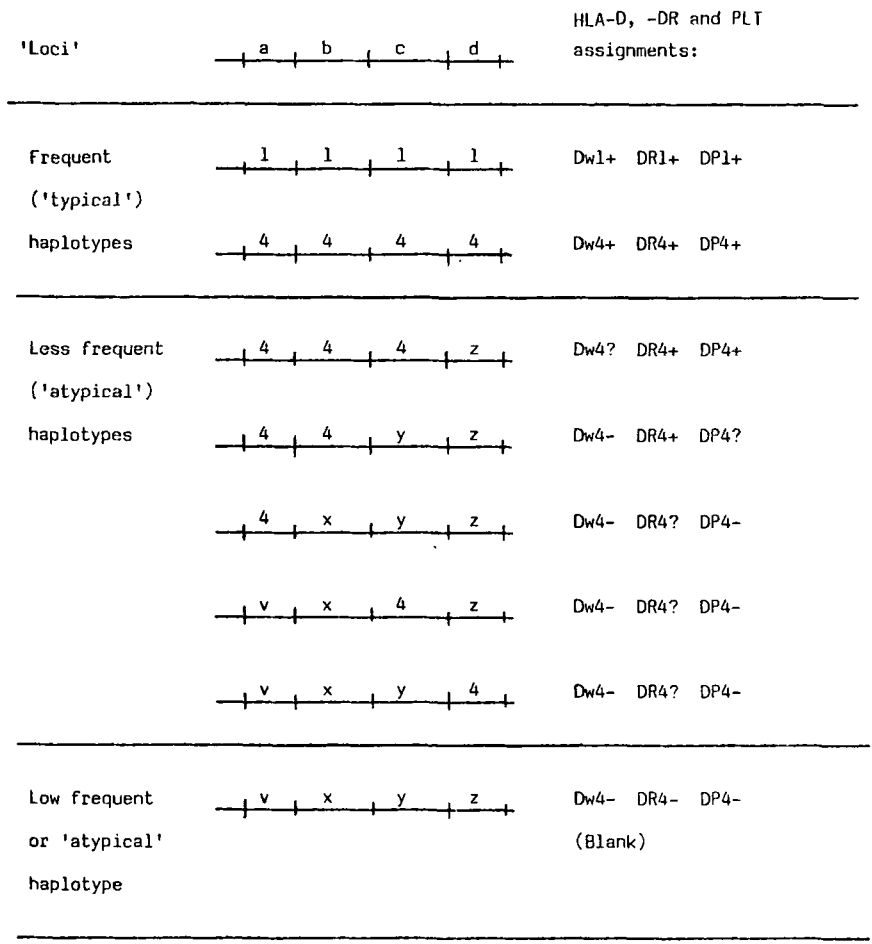

FIG. 9. Simplified theoretical model explaining the heterogeneity of the *HLA-D/DR* region. Four hypothetical loci, a, b, c, and d, are indicated. The symbols 1, 4, v, x, y, z, indicate different alleles at the various loci. A "typical" *Dw1,DR1,DP1* haplotype would be a $a_1b_1c_1d_1$, where a_1, b_1, c_1, and d_1 are assumed to be in positive linkage disequilibrium with each other. For further explanation, see text.

listed two "typical" haplotypes: $a_1b_1c_1d_1$ and $a_4b_4c_4d_4$; individuals homozygous for these would provide good HTCs for Dw1 and Dw4, respectively, and when used as primary stimulators in PLT, they might induce a good anti-"DP4" PLT reagent depending on the antigenic make-up of the responder. Cells from individuals who are heterozygous for the typical *D/DR/DP4* haplotype and a slightly atypical

haplotype ($a_4 b_4 c_4 d_z$) would clearly type as Dw4-positives by Dw4 HTCs, but would not behave as well when used as HTCs themselves, because the d_z determinant would induce some stimulation in cells possessing the typical D/DR/DP4 determinant. When used as priming cells, such heterozygotes could give rise to a PLT cell reacting most strongly with D/DR/DP4-positives (if the responder is D/DR/DP4-negative), but that may give (weak) extra reactions with D/DR/DP4-negative cells carrying a d_x antigen. If the same cell is used to prime a truly $a_4 b_4 c_4 d_4$ homozygous cell, an anti-d_z reagent may result.

With increasing complexity of the gametic combinations, cells arise that give different reaction patterns to HTCs, anti-DR antibodies, and PLT reagents. For example, cells possessing the $a_4 b_x c_y d_z$ are unlikely to give typing responses with Dw4 HTCs (provided the a determinants are not immunodominant compared to b, c, and d), and would probably not induce a strong response in anti-DP4 PLT cells because these contain a mixture of anti-a_4, -b_4, -c_4, and -d_4 cells; on the other hand, some anti-DR4 sera might contain anti-a_4 antibodies reacting with these cells. When cells with such "atypical" antigenic combinations are used to prime other cells, polyspecific PLT cells will be the most likely result; however, if the responding cells by chance (or by careful selection) also carry the b_x, c_y, and d_z determinants, a monospecific (or perhaps narrow) anti-DP4 may develop.

It should be stressed that this model is simplified and theoretical, although there is some evidence to support it (see Section I,C, II,B, III,C, and VI,A). However, it would explain several well-known phenomena. For example, that some HLA-D "blank" cells possess both certain well-defined DR antigens and the corresponding DP antigen, and that such cells when used as stimulators may induce the formation of PLT cells with corresponding DP specificity. The model also provides a good explanation for the discrepancies usually seen when comparing D and DR typing results between different ethnic groups, who may have as different gametic associations for these determinants as those seen for HLA-A and -B determinants.

Differences in the immunodominance of a, b, c, and d and in the degree of polymorphism at the corresponding loci and the possible existence of true *null* alleles (as known in mice: Klein *et al.*, 1981) might further complicate the situation.

We may note that the hypothetical a, b, c, and d loci in Fig. 9 may or may not correspond to the A_α, A_β, E_β, and E_α loci (the order being indifferent) in Fig. 4.

2. *Priming between Family Members*

Two family members may differ for minor parts of the HLA region: for example, one HLA haplotype or even only a small part of the HLA region if a genetic recombination has taken place (Fig. 3).

Most primings have been performed between family members differing for one HLA haplotype. In such cases, several determinants coded by the entire HLA haplotype may cause stimulation as suggested by Bach *et al.* (1975), and it may account for the low fraction of HLA-D specific haplotype-primed PLT cells (Bach *et al.*, 1976a). Haplotype-primed PLT cells, however, may be of great value in cases where determinants cannot be identified in any other cellular system, as in the case, for example, of DRw9 and DRw10 (Morling *et al.*, 1980d). The principle of haplotype priming may be modified if the HLA-types of the disparate haplotypes are taken into consideration (e.g., Zier *et al.*, 1980).

Priming toward the gene products of an entire HLA haplotype coding for D/DR determinants that are not concordantly assigned by the HLA-D and -DR techniques will favor the generation of PLT cells defining "uncommon" or "atypical" determinants. The few published reports have shown that such PLT cells may be "specific" for either the HLA-D or the HLA-DR antigen, or both -D and -DR (Hartzman *et al.*, 1978, 1979; Reinsmoen *et al.*, 1978b, 1979b).

Priming between individuals differing only for a segment of an HLA haplotype may seem ideal in order to create PLT-cell reagents typing for determinants coded by "new" series of alloantigens. Such priming has demonstrated the existence of non-HLA-D-coded alloantigens telomeric to HLA-D (Zier *et al.*, 1978; Wank *et al.*, 1979).

Priming between HLA-A,B,C,D/DR identical sibs differing for GLO owing to crossover between *HLA-D/DR* and *GLO* has made it possible to identify at least one determinant coded by genes centromeric to HLA-D/DR (Mawas *et al.*, 1978, 1980).

If determinants coded by genes outside the HLA region exist, such alloantigens may be demonstrated by PLT cells generated between HLA-identical sibs.

Although priming between individuals sharing most of the HLA genes seems optimal for the generation of PLT cells specific for single determinants, this approach is cumbersome because (*a*) genetic recombination in the HLA region is a rare event; and (*b*) even when crossover takes place, there is only a small chance of generating PLT cells if the sibs share the HLA-D/DR antigens (Mawas *et al.*, 1975;

Bradley *et al.*, 1976), most probably indicating that genes outside *HLA-D/DR* have a limited polymorphism and/or code for weakly stimulating determinants.

3. *Priming between Unrelated Individuals*

PLT cells generated between individuals differing for one HLA-D antigen will, in most cases, result in primed cells reacting with cells carrying the antigen of the primary stimulator, but the PLT cells will often give "extra" reactions (Bradley *et al.*, 1976; Fradelizi *et al.*, 1976; Hirschberg *et al.*, 1976). The specificity of the PLT cells may be increased dramatically if HTCs are used as stimulators or as both stimulators and responders (Hartzman *et al.*, 1977, 1979; Morling *et al.*, 1980b) when the specificity of the PLT cells is compared to HLA-D. If one accepts the concept that the *HLA-D* region includes two or more closely linked genes coding for alloantigens, then homozygous cells—performing as HTCs in primary MLR—are believed to be the cells that have the most common combination of determinants in the *D* region (Fig. 9). Accordingly, PLT cells generated toward HTCs should be primed toward a standard combination of *HLA-D* region-coded determinants.

Priming between individuals sharing HLA-D/DR antigens may lead to the production of primed cells. This indicates that the HLA-D/DR antigens are heterogeneous or that other determinants may influence the MLR. Such generated PLT cells may identify "new" determinants (Wank *et al.*, 1978b; Termijtelen *et al.*, 1980; Zier *et al.*, 1980; Morling *et al.*, 1981d), and a series of alloantigens coded by genes situated between *HLA-D* and *GLO* have been identified by PLT cells generated between HLA-A,B,C,D/DR identical individuals (Shaw *et al.*, 1979, 1980a,b).

B. Selection of PLT Cells

When PLT cells have been generated, "specific" cells with good discriminatory capacity should be selected. A cloning procedure may be performed in order to select "specific" clones because cloned PLT cells may be more "specific" than conventional PLT cells prepared as bulk PLT (Bach *et al.*, 1979; Charmot *et al.*, 1980; Inouye *et al.*, 1980b; Pawelec *et al.*, 1980).

The selection of PLT-cell reagents for immunogenetic investigations does not, in principle, differ from that in other systems, such as HLA serology and cellulogy. High priority should be given to the selection of "specific" and highly discriminatory reagents (Fig. 8). The

many approaches for screening will not be discussed, but the screening procedure should be performed on a panel that is representative for the population to be typed. Most investigators have experienced the phenomenon that haplotype-primed cells, which work beautifully in families, can only rarely be used as typing reagents for HLA-D/DR-associated determinants in a population (e.g., Bach *et al.*, 1976a).

XI. Antigen Assignment in PLT

When the PLT system is used for typing purposes, one of the most challenging procedures is the "assignment of antigens," which is the conclusion of the reactions in secondary PLT. Many factors affect the assignment procedure. The influence of technical factors has been discussed above, but one of the most important factors is the investigators' concepts of the genetics of MLR, because the MLR is most probably governed by a complex of genetic factors. The bias not only influences the interpretation of the results of secondary PLT, but it also plays a fundamental role during the creation of primary PLT cell combinations and the subsequent screening of the PLT cells generated. Owing to the genetic complexity of the MLR, many PLT cells may contain specificity toward determinants coded by more than one gene. This fact, however, should not keep one from trying to generate PLT cells that are operationally "monospecific" according to the present knowledge and to try to select, if possible, the PLT cells that have a good capacity to discriminate between positive and negative reactions. This is the safest way to reduce the bias in the final interpretation of the data and the best way to obtain reproducible results of antigen assignments.

Only few studies have dealt with the assignment procedure. During our attempts to establish a typing system for HLA-D/DR-associated PLT-defined determinants, we studied the assignment procedure (Morling *et al.*, 1980c). The "true" biological significance of the reactions of the PLT cells used in our studies is not known, but it was possible to establish a system that typed for determinants very closely associated with the antigens HLA-D/DR1-w8 (Morling *et al.*, 1981b). Objective criteria for the antigen assignment were established (Table IX), and the subjective assignments of antigens made blindly were compared to the objective assignments (Table X). Only six "strictly" positive and two "strictly" negative assignments were discrepant with the objective and the subjective criteria in 100 individuals. The polymorphism of the HLA-D/DR1-w8 antigens is thought to be codominant traits controlled by genes at one locus; accordingly, each individ-

TABLE IX
TRANSLATION OF PRIMED LYMPHOCYTE TYPING PLT CELL REACTIONS (NMR[a] VALUES) INTO NMR ASSIGNMENTS OF DP ANTIGENS[b]

PLT-cell reaction	Score
NMR > 90	+
70 ≤ NMR < 90	(+)
NMR < 70	−

Score(s)[c]	NMR assignment
+++	Positive
++(+)	
+(+)(+)	
++	
+(+)	
+	
++−	Equivocally positive
+(+)−	
(+)(+)(+)	
+−	
(+)(+)	
(+)	
+−−	Negative
(+)(+)−	
(+)−−	
−−−	
(+)−	
−−	
−	

[a] Normalized median response.
[b] From Morling *et al.* (1980c).
[c] Score(s) obtained with one or more PLT-cell reagents typing for the same DP antigen in one experiment. Three, two, and one plus or minus signs indicate that three, two, and one PLT-cell reagents were used, respectively.

ual should maximally possess two antigens. The investigations (Table X) indicated that the bias leading to the assignment of two antigens and avoiding triplets could not be severe, since the frequency of neglected "equivocally positive reactions" according to objective criteria was greater in the one-antigen group than in the two-antigen group.

In these experiments, fixed criteria were used for the assignment of positive and negative reactions and for the antigen assignments. This can be made in a meaningful way only if the PLT cells discriminate

TABLE X
ANALYSIS OF THE FINAL DP-ANTIGEN ASSIGNMENT PROCEDURE[a]

Final DP-antigen assignment based on[b]	No. of individuals		
	2 DP antigens	1 DP antigen	Total
Only positive NMR assignments	33	15	48
Positive and equivocally positive NMR assignments	13	0	13
Only equivocally positive NMR assignments	0	2	2
Exclusion of positive NMR assignments	3	3	6
Exclusion of equivocally positive NMR assignments	17	12	29
Inclusion of negative NMR assignments	2	0	2
	68	32	100

[a] From Morling *et al.* (1980c).
[b] The translation of normalized median response (NMR) values of PLT-cell reactions into NMR assignments (positive, equivocally positive, and negative) is shown.

well and if the design of the secondary PLT experiments allows a robust data treatment of data.

The quality control of the PLT system was easy in these experiments, since the aim was to establish a system typing for HLA-D/DR-associated determinants. The correlation coefficients between the expected assignment (according to HLA-D) and the observed assignment was 0.92, and the correlation coefficient to the observed reactions of single PLT-cell reactions (fixed criteria for positive and negative reactions) was 0.79. The discrepancy between these two values reflects the facts that (*a*) the antigen assignment was based on the reactions of at least two PLT-cell reagents typing for each specificity; and (*b*) some of the technical variabilities that may not be neutralized by the data treatment system may be compensated for by the investigator (Morling *et al.*, 1980c).

In other systems where there is no known reference, the quality of the assignment as well as the PLT experiments may be estimated by the reproducibility of repeated investigations.

XII. Typing with the PLT Test

A. INTRODUCTION

The principal role of the PLT test today is to allow the recognition, definition, and typing for known and "new" alloantigens that give rise

to lymphocyte activation. This is particularly true in man, where humoral antibodies cannot be obtained deliberately as they can in, for example, mice by immunizing highly selected donor–recipient combinations. The PLT test is excellent for the identification of single determinants even though a complex of alloantigens may exist in all individuals. This is true because primed cells may be generated in all available combinations, and, in selected cases, this may result in priming between individuals disparate for single determinants. Furthermore, the discriminatory capacity of PLT cells may be increased by cloning of PLT cells that may have been generated in combinations that were not optimal. In addition, cloned PLT cells represent a powerful tool for basic immunogenetic research of the possible existence of, for example, "hybrid" or "combinatorial" antigens and of cross-reactivity.

Accordingly, the value of the PLT test is obvious although it is possible to define human alloantigens by means of other techniques, as in MLC with the HTC technique (HLA-D) or with serology (HLA-DR). The PLT test may be of great help in defining "new" determinants most probably belonging to the series of HLA-D or -DR antigens, because good reagents defining these determinants are rare—especially with respect to the low-frequency HLA-D and -DR antigens. A few groups have established batteries of PLT cells typing for both "known" and "new" determinants.

In our laboratory, we have established a PLT system for (*a*) antigens closely associated with, or identical to, the majority of the known HLA-D and -DR antigens; (*b*) "new" DR determinants not recognized by available HTCs and "new" determinants not presently recognized with other techniques. In the early phase, much effort was put into the development of a PLT system that could define the known HLA-D/DR antigens, because we wanted to refine the technique during the phase where we had a fixed reference (the HLA-D and/or -DR types), and because we think that the cellular HLA-D typing with HTC technique will eventually be replaced by PLT. In this section, some of the results of our work concerning the establishing of the PLT system is discussed together with the results obtained by others and ourselves.

B. Typing for HLA-D/DR-Associated Antigens

Our earliest studies confirmed the observations of Mawas *et al.* (1975) and Sheehy *et al.* (1975) that determinants closely associated with, or identical to, HLA-D are responsible for the PLT-cell response (Bach *et al.*, 1976b). It was also shown that relatively specific PLT cells typing for Dw1-4 and Dw7 and Dw8 could be generated by

priming with HTCs (Thomsen *et al.*, 1976), and that these PLT cells could be used for the "HLA-D typing" of an individual who could not be HLA-D-typed with the HTC technique owing to unresponsiveness to a great number of allogeneic cells (Thomsen *et al.*, 1976, 1978). Therefore, we intensified our studies on the PLT and established a PLT system typing for determinants closely associated with, or identical to, the known and "new" HLA-D and -DR antigens.

1. Selection of PLT Cells

The conceptual approach was as follows.

1. For the generation of HLA-D-specific PLT cells, HTCs were used as primary stimulators and different cells not possessing the HLA-D antigen of the HTC were used as primary responders.
2. "HLA-DR specific" PLT cells and cells identifying "new" determinants possibly belonging to the HLA-D or -DR series were generated by family haplotype primings keeping one haplotype constant between the responder and stimulator.
3. The PLT cells obtained were screened with respect to a panel of all our HTCs, selected heterozygotes, and cells possessing no known HLA-D and -DR antigens ("blanks"). High priority was given to PLT cells giving a clear-cut discrimination between "expected" positives and "expected" negatives (Fig. 8). The "expectations" were based on the HLA-D and/or -DR typing results.

The generation and subsequent screening of PLT-cell combinations was continued until at least two PLT-cell combinations that discriminated well were found for each specificity (Morling *et al.*, 1980b,c). We found that less than 20% of the PLT-cell combinations with HTCs as primary stimulators could be considered satisfactory for typing purposes, while the rest—although giving in nearly all cases responses correlated to HLA-D—did not allow a clear-cut discrimination. The problem with these PLT cells was in almost all cases "extra-reactions" when referring to the corresponding HLA-D specificity. In some cases, HTCs were used both as primary responders and stimulators, but in general, this approach did not improve the specificity of the PLT cells. This is in contrast to the observation of Hartzmann *et al.* (1977, 1979). Another point that was of some help during the selection of primary responders, was to take into account the "cross-reactivity" phenomenon (see Section XII,C). In many cases, unexpected extra reactions toward cells carrying certain HLA-D types (e.g., Dw1 and Dw7) could be avoided by selecting primary responder cells possessing these antigens (i.e., Dw1 and Dw7). The significance of these "cross-reactions" is discussed in Section XII,C.

2. *Data Reduction*

During the initial screening phase, the results of secondary PLT experiments were expressed as the relative response (RR), i.e.,

$$RR\ (P_u;\ S_v) = 100 \times \frac{(P_u;\ S_v)}{\text{max. } (P_1 - P_m;\ S)}\ \%$$

where $(P_u;\ S_v)$ is the response (cpm) of PLT cell u (P_u) to the secondary stimulatory v (S_v) in a system with m PLT cells and n secondary stimulators. Both the specific restimulators, pools of allogeneic cells combined in different ways, and HTCs were incorporated as secondary stimulators. The RR method was acceptable for the screening procedures when frozen lymphocytes from healthy individuals were used, but appeared to be unsatisfactory when various sources of secondary stimulator cells were used, for example, spleen and peripheral blood lymphocytes and cells from nonhealthy individuals. Therefore, we included a stimulator-wise normalization and introduced the normalized median response (NMR) mentioned in Section IX,C (Morling *et al.*, 1980a).

The reference points for both of the normalization procedures are the median response and median stimulation because these values are close to the central tendency of the distribution:

$$NMR\ (P_u;\ S_v) = 50 \times \frac{MR\ (P_u;\ S_v)}{\text{median } [MR\ (P_1 - P_m;\ S_v)]}$$

where

$$MR\ (P_u;\ S_v) = 50 \times \frac{(P_u;\ S_v)}{\text{median } (P_u;\ S_1 - S_n)}$$

where $(P_u;\ S_v)$ is the response (cpm) of PLT cell u (P_u) to the secondary stimulator v (S_v) in a system with m PLT cells and n secondary stimulators. Median $(P_u;\ S_1 - S_n)$ is the median response of P_u to S_1, S_2 . . . S_n. If n is an odd number, the $(N + 1)/2$ smallest number is the median; and if n is an even number, the median is the average of the $N/2$ smallest number and the $(N/2) + 1$ smallest number (Table XI and Figs. 10 and 11).

This method has been found to be superior to the various parametric methods discussed in Section IX,D (Morling *et al.*, 1980a); in particular, the stimulator variability made the parametric methods less suitable.

The value of the NMR method was compared to that of the RR method and of the so-called RRM method in eight subsequent PLT

TABLE XI

DATA FROM ONE SECONDARY PLT EXPERIMENT[a,b]

PLT cell No.	Stimulator No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Median
	a. Median cpm of triplicates																
1	1316	654	1544	1104	126	3266	1080	1296	1540	1030	2954	1604	1122	1082	1918	5722	1306
2	656	142	556	408	92	3590	726	514	836	846	3660	418	966	846	694	3484	710
3	3052	5990	1694	1602	506	2490	1070	2936	1632	1120	2898	4394	3084	1820	1588	8354	2155
4	1962	4666	1656	1036	542	330	1462	2342	2986	1590	1558	4414	2786	1596	2304	6844	1809
5	292	266	404	326	662	412	270	550	816	3544	686	2918	812	672	878	3124	667
6	494	76	838	326	746	888	666	764	886	3512	1578	2028	1590	1108	1104	2964	887
7	564	237	380	384	424	98	654	738	498	756	1672	552	676	340	1012	1494	558
8	1100	448	706	506	1256	200	1462	1002	928	734	3900	1112	1714	554	2396	4123	1051
9	2557	94	2008	862	224	288	642	2906	1700	292	1106	70	4796	1212	2538	4182	1159
10	4534	228	2494	2550	376	738	1138	5121	1608	1326	1536	1138	5981	1420	3276	5692	1572
11	376	170	656	276	56	330	214	368	1058	292	330	160	324	2560	602	3280	330
12	328	388	1870	622	52	140	810	364	3294	1754	316	1170	882	2128	664	3128	737
13	314	36	148	214	48	142	140	374	272	222	288	164	1038	252	450	2418	237
14	266	310	380	280	96	262	478	474	630	412	1112	836	996	648	886	2940	476
15	1558	1014	1138	6420	220	1578	1244	6260	1518	1628	2880	2066	1958	5335	3144	9731	1793
16	1920	1271	1030	4426	194	194	1026	5820	1932	1266	1440	1854	3286	3652	1872	6138	1863
17	5298	834	5027	2058	1052	2568	2090	4533	4278	2266	4192	2590	5795	2742	8996	11470	3467
18	228	301	448	350	64	356	462	524	730	604	1104	454	1000	216	1791	2560	458

b. MR values

1	50	25	59	42	5	125	41	50	59	39	113	61	43	41	73	219
2	16	10	39	29	6	253	51	36	59	60	258	29	68	60	49	245
3	71	139	39	37	12	58	25	68	38	26	67	102	72	42	37	194
4	54	129	46	29	15	9	40	65	83	44	43	122	77	44	64	189
5	22	20	30	24	50	31	20	41	61	266	51	219	61	50	66	234
6	28	4	47	18	42	50	38	43	50	198	89	114	90	62	62	167
7	51	21	34	34	38	9	59	66	45	68	150	49	61	30	91	134
8	52	21	34	24	60	10	70	48	44	35	186	53	82	26	114	196
9	110	4	87	37	10	12	28	125	73	13	48	3	207	52	109	180
10	144	7	79	81	12	23	36	163	51	42	49	36	190	45	104	181
11	57	26	99	42	8	50	32	56	160	44	50	24	49	388	91	497
12	22	26	127	42	4	9	55	25	223	119	21	79	60	144	45	212
13	66	8	31	45	10	30	30	79	57	47	61	35	219	53	95	510
14	28	33	40	29	10	28	50	50	66	43	117	88	105	68	93	309
15	43	28	32	179	6	44	35	175	42	45	80	58	55	149	88	271
16	52	34	28	119	5	5	28	156	52	34	39	50	88	98	50	165
17	76	12	72	30	15	37	30	65	62	33	60	37	84	40	130	165
18	25	33	49	38	7	39	50	57	80	66	121	50	109	24	196	279
Median:	51	23	43	37	10	30	37	61	59	44	64	51	79	51	89	204

(*continued*)

TABLE XI (*Continued*)

PLT cell No.	Stimulator No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
							c. NMR values								
1	49	54	69	57	24	206	56	41	50	45	88	60	27	40	41
2	45	22	46	39	32	416	69	30	50	68	201	29	43	58	27
3	69	300	46	50	58	95	34	56	32	29	53	99	45	41	21
4	53	278	53	39	74	15	55	53	70	50	34	119	49	43	36
5	21	43	35	33	246	51	27	34	52	301	40	213	38	49	37
6	27	9	55	25	208	82	51	35	42	224	69	111	57	61	35
7	50	46	40	46	188	14	79	54	38	77	117	48	38	30	51
8	51	46	39	32	296	16	94	39	37	40	145	52	51	26	64
9	108	9	101	50	48	20	38	103	62	14	37	3	131	51	61
10	141	16	93	109	59	39	49	134	43	48	38	35	120	44	58
11	56	56	116	56	42	82	44	46	136	50	39	24	31	378	51
12	22	57	148	57	17	16	75	20	190	135	17	77	38	141	25
13	65	16	36	61	50	49	40	65	49	53	47	34	138	52	53
14	27	70	47	40	50	45	68	41	56	49	91	86	66	66	52
15	43	61	37	241	30	72	47	143	36	51	63	56	34	145	49
16	50	74	32	160	26	9	37	128	44	39	30	48	56	95	28
17	75	26	85	40	75	61	41	54	52	37	47	36	53	39	73
18	24	71	57	51	35	64	68	47	68	75	94	48	69	23	110

d. +, − Scoring[c]

1	−	−	−	−	−	+++	−	−	−	−	(+)	−	−	−	−
2	−	−	−	−	−	+++	−	−	−	−	+++	−	−	−	−
3	−	+++	−	−	−	+	−	−	−	−	−	+	−	−	−
4	−	+++	−	−	−?	−	−	−	−?	−	−	++	−	−	−
5	−	−	−	−	+++	−	−	−	−	+++	−	+++	−	−	−
6	−	−	−	−	+++	(+)	−	−	−	+++	−	++	−	−	−
7	−	−	−	−	+++	−	−?	−	−	−?	++	−	−	−	−
8	−	−	−	−	+++	−	+	−	−	−	+++	−	−	−	−
9	+	−	+	−	−	−	−	+	−	−	−	−	+++	−	−
10	+++	−	+	+	−	−	−	+++	−	−	−	−	++	−	−
11	−	−	++	−	−	(+)	−	−	+++	−	−	−	−	+++	−
12	−	−	+++	−	−	−	−?	−	+++	+++	−	−?	−	+++	−
13	−	−	−	−	−	−	−	−	−	−	−	−	+++	−	−
14	−	−?	−	−	−	−	−	−	−	−	+	(+)	−	−	−
15	−	−	−	+++	−	−?	−	+++	−	−	−	−	−	+++	−
16	−	−?	−	+++	−	−	−	++	−	−	−	−	−	+	−
17	−?	−	(+)	−	−?	−	−	−	−	−	−	−	−	−	−?
18	−	−?	−	−	−	−	−	−	−	−?	+	−	−	−	+

[a] From Morling *et al.* (1980a).

[b] The normalized median response (NMR) data reduction method is applied on a randomly selected secondary primed lymphocyte typing (PLT) experiment typing for HLA-D/DR-associated DP antigens. For each PLT cell, stimulator No. 16 was a homozygous typing cell (HTC) of the same HLA-D specificity as the primary stimulator. Stimulator-type normalization of the responses to the HTCs (stimulator No. 16) adds no information, and the values have been omitted in part c. Part d shows a translation of the values in part c into a scoring code that has been useful in interpreting the results.

[c] NMR scoring code: ≥130, +++; ≥110, ++; ≥90, +; ≥80, (+); ≥70, −?; <70, −.

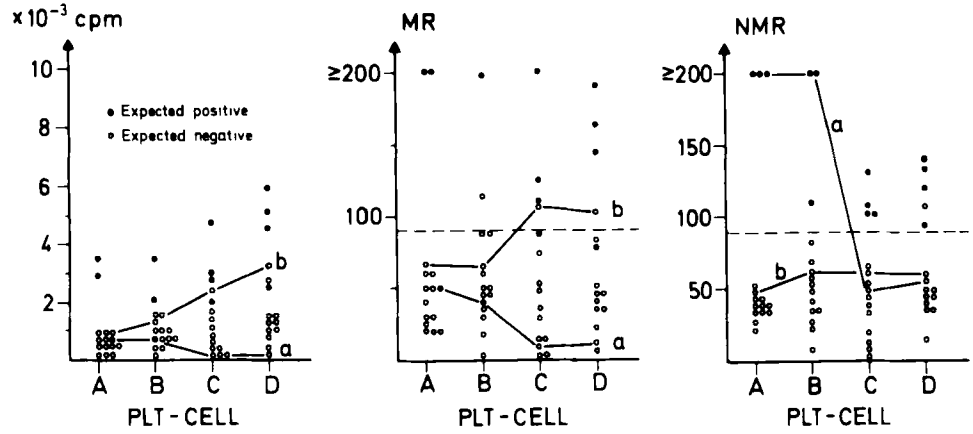

FIG. 10. Normalized median response (NMR). Plot diagrams of the responses of four different primed lymphocyte typing (PLT) cells (A, B, C, and D) toward 15 different secondary stimulators. The experiment included 18 PLT cells and 16 secondary stimulators (here the specific restimulators have been excluded), and the MR and NMR values were calculated on the basis of all these results. The lines connect the values of a weak stimulator *a* and a strong stimulator *b*. Positive reactions were defined as NMR ≥ 90.

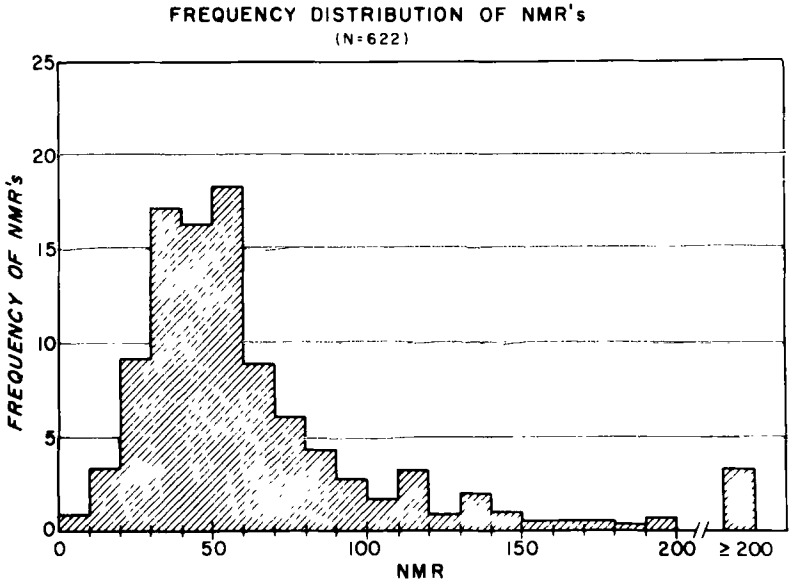

FIG. 11. Relative frequency distribution (%) of 622 normalized median response (NMR) values obtained with 35 different primed lymphocyte typing cells and 92 secondary stimulators in 13 different experiments. (From Morling *et al.*, 1980a.)

TABLE XII
THREE DIFFERENT NONPARAMETRIC DATA REDUCTION METHODS IN PRIMED LYMPHOCYTE TYPING (PLT)[a]

Data reduction method[b]	Correlation coefficient	
	Median	Range
RR	0.64	0.40–0.80
RRM	0.67	0.49–0.79
NMR	0.77	0.71–0.82

[a] Data from eight different secondary PLT experiments typing for HLA-D/DR-associated DP antigens (cf. text). The correlation coefficients were calculated from 2 × 2 tables comparing the HLA-D types with the observed reactions of 1334 PLT cell reactions.

[b] RR, relative response of PLT cells (cf. text); RRM, relative response of PLT and a further stimulator-type normalization according to the median (cf. text); NMR, normalized median response (cf. text).

experiments. The RRM includes a PLT-wise normalization as in the RR and also a stimulator-wise normalization as in the NMR. In these experiments, it was intended to type for HLA-D, and, accordingly, the reference was the HLA-D types of the secondary stimulators. The observed reactions were scored either positive or negative with a fixed cutoff for all methods, and the NMR method gave a very high correlation between observed and expected reactions (Table XII). The NMR method was also very robust from experiment to experiment, which is very important. Later experiments including PLT cells typing for "new" determinants have shown that the reproducibility of duplicate investigations with different batches of PLT cells and secondary stimulators is high (correlation coefficient $r = 0.75$).

3. *Correlation with HLA-D and -DR*

During the Eighth International Histocompatibility Workshop, a panel of 79 individuals were typed for HLA-D/DR-associated PLT-defined "DP" antigens, HLA-D, and HLA-DR antigens (Morling *et al.*, 1981b). Typing for DP antigens was performed with local HTC-primed and haplotype-primed PLT cells. DP typing was performed once or twice in case of doubtful reactions or when discrepancies between the DP/D/DR assignments were found. HLA-D typing was performed two to four times with all HTC included in the 8th Workshop, and thus the antigens Dw1-12 were defined ("Histocompatibility Testing, 1980"). HLA-DR typing was performed once or twice in case of DP/D/DR discrepancies with all sera included in the 8th Workshop, and the antigens DR1-w10 could be identified ("Histocompatibility

TABLE XIII: "DP" ANTIGEN (PLT), HLA-D AND

HLA-Dw, DR, and DP	DP/D/DR +++				DP/D/DR ++−				DP/D/DR +−+				DP/D/DR +−−				DP/D/DR −++			
	R	S	H	B	R	S	H	B	R	S	H	B	R	S	H	B	R	S	H	B
1	10	0	1	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
2	14	4	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
3	9	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	14	2	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
5	6	3	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	11	2	1	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
7	11	2	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
8	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	112				2				5				1				0			

[a] From Morling *et al.* (1981b).

[b] R = Random individuals (N = 49); S = selected individuals possessing "rare" HLA-D antigens (N = 8); H = donors of homozygous typing cells (N = 9); B (blanks) individuals previously found to possess fewer than two known HLA-D antigens (N = 13).

Testing, 1980"). Assignments of HLA-D, -DR, and -DP antigens were done independently.

This panel was, thus, extremely well defined in terms of HLA-D and -DR. The panel included (*a*) random individuals (R); (*b*) selected individuals (S) with "rare" HLA-D antigens; (*c*) donors of HTCs (H); and (*d*) individuals previously found to possess less than two known HLA-D antigens—"blanks" (B).

The antigens HLA-Dw1-8 and -DR1-w8 are significantly associated with each other (Histocompatibility Testing, 1977, 1980), and we studied the correlation between Dw/DR/DP1-8. Table XIII shows the assignments of DP, HLA-D, and -DR antigens. Discrepancies were found for all antigens, but the pooled data showed an extremely strong correlation between DP and both HLA-D (r = 0.95) and HLA-DR (r = 0.94). Table XIV shows the correlation coefficients between DP/D/DR in the four groups of individuals, and there is a complete fit in the HTCs and selected individuals, whereas the correlation is low in the group of blanks (B). This may indicate that the blanks carry "atypical" HLA-D/DR antigens. In the random (R) group, approximately 80% of the individuals were assigned to possess the "same" antigen with all three techniques, but this was the case in less than 50% of the blanks (Table XV).

-DR ANTIGEN ASSIGNMENTS IN 79 INDIVIDUALS[a,b]

DP/D/DR −+−				DP/D/DR −−+				DP/D/DR −−−				Correlation coefficients (r) for the total group (N = 79)		
R	S	H	B	R	S	H	B	R	S	H	B	DP/HLA-D	DP/HLA-DR	HLA-D/-DR
0	0	0	0	0	0	0	0	38	8	8	10	0.96	1.00	0.96
0	0	0	0	0	0	0	0	35	4	8	12	0.97	1.00	0.97
0	0	0	0	1	0	0	1	39	7	8	11	1.00	0.91	0.91
0	0	0	0	1	0	0	3	33	6	8	8	0.93	0.84	0.85
0	0	0	1	2	0	0	0	41	5	8	8	0.96	0.92	0.88
1	0	0	0	0	0	0	0	36	6	8	10	0.93	0.93	0.86
0	0	0	2	0	0	0	0	37	6	8	10	0.89	1.00	0.89
0	0	0	0	2	0	0	0	44	7	8	13	1.00	0.83	0.83
4				10				498				0.95	0.94	0.89

These data show that there is a remarkably strong correlation between the antigens HLA-D/DR1-w8 and that it is possible to establish a PLT system typing for determinants that are strongly associated with these HLA-D/DR antigens. The correlation between these DP antigens and HLA-D and -DR is even higher than that between HLA-D and -DR (Table XIII).

When discrepancies were seen—especially in blanks—HLA-DR was in general "broader" than DP, which in turn was broader than HLA-D. The discrepant results were reproducible with all three techniques with the presently available reagents; accordingly, it should be possible to "split" HLA-DR and HLA-DP into more "narrow" specificities.

The series of HLA-D/DR antigens most probably have as yet unidentified determinants. Such "new" MLR activating antigens—or closely associated determinants—may be identified and typed for with the PLT.

Reinsmoen *et al.* (1978b, 1979b) demonstrated that haplotype primed cells might be generated that specifically recognized a "new" HLA-D determinant, which later was established as Dw12 (Histocompatibility Testing, 1980). Cells possessing this "new" determinant were usually DR2 positive. The existence of DR2-positive cells that are either Dw2 or Dw12 is probably an example of cross-reaction of HLA-DR antigens, and the observation should encourage serologists to search for DR antisera specific for "Dw2/DR2" as well as "Dw12/DR2."

TABLE XIV
PAIRWISE CORRELATIONS BETWEEN THE ANTIGEN ASSIGNMENTS WITH THE PRIMED LYMPHOCYTE TYPING (DP), HLA-D, AND HLA-DR TECHNIQUES IN 79 INDIVIDUALS[a,b]

	Correlation coefficients (r)			
	R	S	H	B
DP/HLA-D	0.97	1.00	1.00	0.77
DP/HLA-DR	0.94	1.00	1.00	0.83
HLA-D/HLA-DR	0.92	1.00	1.00	0.61

[a] From Morling *et al.* (1981b).
[b] R = random (N = 49); S = selected (N = 8); H = homozygous typing cells (N = 9); B = blanks (N = 13).

Haplotype-primed cells that identify "new" serologically defined determinants may also be generated. Primed cells that type for a determinant closely associated with the later internationally established HLA-DRw10 antigen ("Histocompatibility Testing, 1980") were generated in two different unrelated families (Morling *et al.*, 1980d). The reactions of these PLT cells were in complete accord with the reactions of the antiserum "LTM" (Jakobsen and Svejgaard, 1979), which was the leading serum for the identification of DRw10 ("Histocompatibility Testing, 1980"). This demonstrated that serologically defined HLA-DR antigens—or determinants strongly associated with DR—may be recognized and identified with the PLT technique. The HLA-DRw10 antigen has not yet been identified with HTC technique, and it would be difficult to find HTCs for "DRw10" because the esti-

TABLE XV
OVERALL CORRELATIONS BETWEEN THE ANTIGEN ASSIGNMENTS WITH THE HLA-D, HLA-DR, AND PRIMED LYMPHOCYTE TYPING (DP) TECHNIQUES IN 79 INDIVIDUALS[a]

Assignments in each individual	Random (N = 49)		Selected (N = 8)		HTC (N = 9)		Blank (N = 13)		Total (N = 79)	
	N	%	N	%	N	%	N	%	N	%
DP = D = DR	39	79.6	8	100	9	100	6	46.2	62	78.5
DP = D ≠ DR	6	12.2	0	0	0	0	3	23.1	9	11.4
DP = DR ≠ D	3	6.1	0	0	0	0	3	23.1	6	7.6
DP ≠ D = DR	1	2.0	0	0	0	0	0	0	1	1.3
DP ≠ D ≠ DR	0	0	0	0	0	0	1	7.7	1	1.3

[a] From Morling *et al.* (1981b).

mated frequency of Caucasian individuals homozygous for DRw10 is approximately $(0.6\%)^2 = 3.6 \times 10^{-5}$.

Other PLT cells, specific for HLA-DR-associated determinants that have not been identified by other cellular techniques, may be generated; e.g., PLT cells typing for HLA-DRw9-associated determinants (own observation, unpublished).

The overall conclusion of these results is that it is possible to establish PLT systems typing for determinants strongly associated or identical to the known HLA-D and -DR antigens. However, occasionally there are reproducible differences in the HLA-D, -DR, and/or -DP antigen assignments, and these discrepancies are important for the unraveling of the D/DR region as discussed in Section VI.

4. Population Studies

Our first studies on 100 healthy unrelated random Danes (Morling *et al.*, 1980c) showed that the estimated sum of gene frequencies corresponding to definable DP antigens (Table XVI) was 0.92, indicating that approximately 16% of random individuals possessed undefined DP antigens. The Hardy–Weinberg fit was calculated based on the DP phenotype distribution, and the observed phenotype distribution did not differ significantly from the expected distribution. These data support our assumption that these DP determinants are codominant traits coded by genes at one locus or at extremely closely linked loci.

TABLE XVI
FREQUENCIES OF "DP" ANTIGENS AND GENES IN 100 RANDOM UNRELATED HEALTHY DANES[a]

DP antigen	No.	$N = 100$ Antigen frequencies	Gene frequencies[b]
1	24	24.0	0.122
2	27	27.0	0.146
3	25	25.0	0.134
4	28	28.0	0.149
5	16	16.0	0.087
6	23	23.0	0.126
7	20	20.0	0.106
8	9	9.0	0.045
Blank	1	1.0	0.085
			1.000

[a] Hardy–Weinberg fit: $\chi^2(28) = 26.43$ ($p > 0.05$).

[b] Gene frequencies were estimated by the gene-counting method of maximum likelihood.

C. Cross-Reactions of PLT Cells

The establishing of batteries of PLT cells typing for HLA-D-associated determinants is cumbersome since only 20–30% of the PLT-cell combinations generated by haplotype or HTC priming are reasonably "specific" when compared to HLA-D (Bach *et al.*, 1976a; Morling *et al.*, 1980b). The generation of PLT cells "monospecific" for determinants strongly associated with or identical to HLA-D antigens may be facilitated by systematic investigations of "cross-reactions" (cf. Section II,C). If a given PLT cell is generated in a combination differing for only one HLA-D/DR antigen (e.g., HTC-priming or haplotype priming), positive reactions are expected only if the secondary stimulator possesses the priming HLA-D/DR antigen. If a positive reaction is elicited by a secondary stimulator that does not possess the priming HLA-D/DR antigen, we consider this an unexpected or "extra" positive reaction. The term "cross-reactivity" is often (and not always correctly) applied to describe this phenomenon. Apart from immunologic cross-reactivity, "extra" positive reactions may be due to duo-, oligo-, or even polyspecific PLT reagents, but in general, it is difficult to distinguish these two possibilities from each other.

The discriminatory capacity of primed cells may vary from individual to individual (Zier *et al.*, 1979b; Morling *et al.*, 1980b) and from clone to clone in the same PLT combination (Bach *et al.*, 1979; Fathman and Hengartner, 1979), demonstrating that characteristics of the primed cell population are of utmost importance in the fine discrimination of the cross-reacting determinants.

In the PLT for DP antigens, many of the extra positive reactions are associated with HLA-D antigens. During our analysis of the correlation between DP antigens and HLA-D and -DR (Morling *et al.*, 1980b), we observed some possible cross-reactions between various HLA-D/DR/DP antigens (Fig. 12). It is of interest that some of these cross-reactions are also known from DR serology ("Histocompatibility Testing, 1977").

The precise biological background for the cross-reactions in PLT is unknown. In true immunologic cross-reactivity, the cross-reacting cells carry antigenic determinants that are so similar that allogeneic lymphocyte cross-activation takes place. The classical example of such cross-reactivity in serology is that between blood group A and B antigens, and the same explanation may hold for the cross-reactivity between the HLA-A2 and A28 antigens (see Section II,C). However, for the HLA system, it is possible that different molecules, although different for the epitopes defining the "fine" specificities, may share

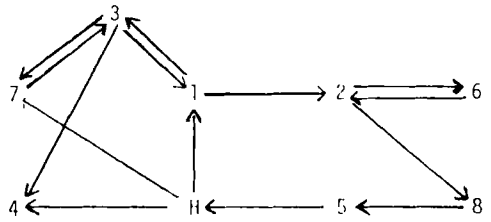

FIG. 12. Schematic representation of the observed cross-reactons in primed lymphocyte typing (PLT). 1 → 3 indicates that PLT cells primed with homozygous typing cells (HTCs) typing for HLA-Dw1 showed cross-reaction with a secondary stimulator (HTC) of the HLA-Dw3 cluster. (From Morling *et al.*, 1980b.)

identical alloantigens in other parts of the molecule. Such "identity" will be extremely difficult to distinguish from "similarity" (before biochemical analysis can be done). Accordingly, for practical purposes we consider immunologic cross-reactivity as being due to similarity of different epitopes or to identical epitopes. The epitopes may be located at homologous or different places on homologous molecules (i.e., molecules controlled by genes at the same locus) or on different molecules.

However, in an assay as complicated both technically and genetically as the PLT test, it is unlikely that all "extra reactions" are due to true cross-reactivity. If our concept of the D/DR region (Fig. 9) is correct, it is even likely that many extra reactions are due to oligospecificity rather than cross-reactivity. As discussed in Section XII,D, a "new" series of HLA-controlled PLT determinants have been defined, and it is quite possible that the extra reactions of some PLT cells are due to the presence of mixtures of PLT-cell clones, some of which are directed against D/DR antigens while others may be directed against other antigens, e.g., belonging to the "new" series. Some of the "new" determinants have been shown to be in linkage disequilibrium with HLA-D antigens, and this might explain why some of the observed extra reactions are HLA-D associated.

Antigenic determinants coded by other loci inside or outside HLA may also give rise to extra reactions, but, as none of these possible determinants are well identified, we cannot evaluate their significance.

The following example illustrates how difficult it may be to decide whether cross-reactivity or different determinants are involved, e.g., when discrepant results are seen for HLA-D, -DR, and DP typing. We observed one individual who gave typing responses to none of the Dw4-HTCs, reacted with approximately one-third of the anti-DR4 antisera, but stimulated all of four different HTC primed PLT cells typ-

ing for DP4. This individual was scored as Dw4-/DR4-/DP4+. However, these lymphocytes, when primed with Dw4-HTCs gave rise to a "narrow" anti-DP4 PLT cell reacting with all DP4 positives except the donor's own cells. Accordingly, this individual may possess a "new" private, low-frequency D/DR/DP antigen, which cross-reacts with DR4/DP4. However, these results could also be explained by assuming the presence of, say, an $a_v b_4 c_y d_z$ haplotype in this donor (cf. Fig. 9); "anti-b_4" reactivity would then be present in about a third of the anti-DR4 sera and in all our anti-DP4 PLT reagents. This donor would clearly react toward the a_4, c_4, and d_4 determinants in Dw4 HTCs: when primed with such HTCs, anti-a_4, -c_4, and -d_4 would develop, and the resulting PLT cells would then behave as an "anti-DP4" reagent.

Obviously, truly monoclonal PLT reagents would constitute a potentially very powerful tool in defining cross-reactivity, because such reagents may well be cross-reacting but cannot be polyspecific. However, it would still be difficult to distinguish between "similarity" and "identity."

D. Human PLT Alloantigens Belonging to "New" HLA Series

A number of investigators have reported results of PLT experiments that suggest the existence of human alloantigens not belonging to the determinants strongly associated with HLA-D/DR (cf. Section VI,A). It seems to be possible to generate PLT cells that recognize gene products of the *HLA-A* region (Wank *et al.*, 1979), of the *HLA-B-D* region (Zier *et al.*, 1978), and other HLA regions which have not been further identified (e.g., Reinsmoen *et al.*, 1977a, 1979a; Singal and Naipaul, 1977; Nunez-Roldan *et al.*, 1978; Sasportes *et al.*, 1978d; Wank *et al.*, 1978a,b; Zier *et al.*, 1980).

Human alloantigens belonging to a "new" series of antigens coded by genes between *HLA-D/DR* and *GLO* on chromosome No. 6 have been defined by three different approaches.

Shaw and co-workers (1979, 1980a,b) have established a series of five antigens called secondary B-cell antigens (SB) by priming allogeneic lymphocytes toward HLA-A,B,C,D,DR and MB phenotypically identical lymphocytes from unrelated individuals. These primings resulted in primed cells that defined a "new" series of antigens. The determinants could be detected both by the PLT technique and by the CML technique. The frequencies of the genes controlling the SB antigens ranged from 2% to 50% in the Caucasian population with approximately 25% "blanks." The SB determinants are different from the D,

DR antigens but one of the alleles, SB1, is significantly associated with DR3, indicating that the SB antigens belonging to the HLA system. They can be demonstrated only on B lymphocytes with the presently available technique. The SB antigens seem to induce only weak stimulation in primary MLR.

Mawas and co-workers (1978, 1980) have identified one determinant by generating haplotype-primed PLT cells between sibs identical by descent for HLA-A,B,C,D,DR but discrepant for GLO owing to a recombination. This determinant is coded by genes between *HLA-D/DR* and *GLO* and is closely associated or identical to SB1 (S. Shaw, personal communication).

Termijtelen and co-workers (1980) generated PLT cells that identify a determinant, PL3A, which is most probably identical to SB1 (S. Shaw, personal communication). This was done by priming D/DR3, W8 responder cells with stimulator cells most probably homozygous for D/DR3. The determinant is coded for by genes in the HLA region, and PL3A is associated with A1,B8,D/DR3. The antigen frequency of PL3A seems to be approximately 15% [calculated from the data of Termijtelen *et al.* (1980)]. The PL3A determinant has been shown to be responsible for a significant amount of the MLR between D/DR3 homozygous cells that differ for PL3A (Termijtelen and van Rood, 1981). The PL3A has not (yet) been demonstrated to be the target in CML (as has the SB1 antigen), but the relevant B lymphocyte-enriched suspensions have not been used as targets (Termijtelen and van Rood, 1981).

The determinants identified by Mawas *et al.* (1978, 1980); Shaw *et al.* (1980a,b), and Termijtelen *et al.* (1980) seem to belong to one segregating series (Table XVII). Studies in families with recombinations between *HLA-D* and *GLO* have shown that the determinants are coded by genes situated between *HLA-D/DR* and *GLO* (Mawas *et al.*, 1980; Shaw *et al.*, 1980a,b), i.e., centromeric to *D/DR*. A more precise localization has not yet been possible, and these determinants are likely to be coded by genes quite close to *HLA-D/DR* and thus to be located in the *HLA D/DR* region.

We have identified at least one determinant, and possibly more, that seems to be coded by non-*D/DR* genes (Morling *et al.*, 1981d). This was done by priming cells from a D/DR4,7-positive individual with cells from a D/DR4 HTC donor. The PLT cells type for a determinant provisionally called EP1, which has a frequency of approximately 25% in random Caucasians (Fig. 13). The determinant is most probably HLA-coded, although this has not been definitely proved. The frequency of EP1 is very low in our 19 HTCs defining Dw1-w8, and very high in individuals selected because they have fewer than two identi-

TABLE XVII

PRELIMINARY ESTIMATES OF FREQUENCIES OF THE GENES CONTROLLING SB, PL3A, GI$^+$ (= Faubert), AND EP1

N = 57 haplotypes		N = 89 individuals		N = 71 individuals		N = 87 individuals	
Determinant(s)	Gene frequency[a]	Determinant	Gene frequency[b]	Determinant	Gene frequency[c]	Determinant	Gene frequency[d]
SB1	0.12	*PL3A*	0.08	*GI*$^+$ (= Faubert)	0.10	*EP1*	0.14
SB2	0.09						
SB3	0.02						
SB4	0.50						
SB5	0.04						
Blank	0.25	*Blank*	0.92	*Blank*	0.90	*Blank*	0.86

[a] Data from Shaw *et al.* (1980b).
[b] Data from Ternijtelen *et al.* (1980).
[c] Data from Charmot *et al.* (1981).
[d] Data from Morling *et al.* (1981d).

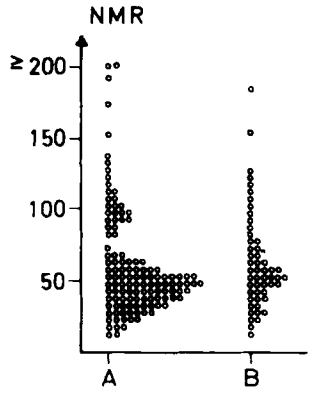

FIG. 13. Plot diagram of two primed lymphocyte typing (PLT) cells typing for EP1. The PLT cell L. M. /Priess$_x$ (A) was tested against 87 random, 16 HTCs, eight selected, and 12 blank individuals. The PLT cell Leif/Priess$_x$ (B) was tested against 30 random, 16 HTCs, five selected, and one blank individual. NMR, normalized median response.

fiable HLA-D antigens (Morling *et al.*, 1981d). This fact suggests that EP1 gives rise to a substantial amount of the reactivity in primary MLR and may interfere with the typing for HLA-D antigens with HTCs: if an HTC carries EP1 in addition to a well-known HLA-D antigen (e.g., Dw4), it would often stimulate individuals carrying this D antigen (i.e., Dw4-positives) and would be considered a poor typing cell. Indeed, our EP1-positive Dw4/4 HTC (Priess) is a poorly discriminating HTC. The role of EP1 in primary MLR has been further investigated in HLA-D phenotypically identical individuals by comparing the MLR between individuals sharing and differing for EP1. The MLR between unrelated individuals sharing HLA-D but discrepant for EP1 gives MLR that are approximately 30% higher than the MLR between HLA-D-identical individuals both sharing or lacking EP1 (N. Morling *et al.*, unpublished data). Other determinants that may belong to the same series as EP1 have been identified (N. Morling *et al.*, unpublished data), and we suspect that these determinants are closely related or identical to some of the SB antigens (Table XVII).

It is tempting to speculate that the SB series antigens are controlled by, say, the hypothetical *c* and/or *d* loci in Fig. 9, while the D/DR-associated DP antigens could be controlled by the *a* and/or *b* loci.

The very likely existence of this "new" series of HLA-controlled PLT antigens (Table XVII) may explain the rather poor predictive value of HLA-D typing for the results of MLR between unrelated individuals (see Section III,F).

XIII. Histocompatibility Testing with the Secondary MLR

Since the first reports on the secondary MLR, it has been suggested to take advantage of the accelerated response of secondary MLR when testing for histocompatibility in clinical transplantation (Sondel *et al.*, 1975). It has also been suggested to perform cellular typing for *HLA-D* region determinants in individuals who cannot be typed for HLA-D antigens with HTC technique because of lack of responsive capacity in MLR, e.g., in individuals suffering from T-cell deficiency or severe combined immunodeficiency (SCID). The PLT technique may be of special value in the selection of bone-marrow donors for such patients. Moreover, the shorter time necessary for the secondary MLR has lead to suggestions that this method may be applied in some cases of cadaver kidney transplantation.

A. The Secondary Stimulation Test

When lymphocytes from an individual are primed with a pool of lymphocytes from a large number of random unrelated individuals or from individuals with selected HLA-D antigens covering the known HLA-D antigens as well as blanks, the primed cells will respond to most other cells with an accelerated proliferative response (Sondel *et al.*, 1975). The response will be low only toward cells autologous to the primary responder and to cells carrying no antigens foreign to the primary responder. Thus, secondary MLR may be performed, and the result can be obtained within 24–48 hours. The MLR seems to be of major importance in graft survival, since a low MLR of the recipient against donor cells is associated with a good graft survival in kidney transplantation (Sachs *et al.*, 1977; Thomsen *et al.*, 1980). It would thus seem to be possible to apply this secondary stimulation test on clinical transplantation of necrodonor kidneys by generating pool-primed lymphocytes from the potential recipients and test these cells against stimulator lymphocytes from offered cadaver kidney donors as an *in vitro* histocompatibility test. However, we are not aware that this approach has been tried.

B. Typing of Cadaver Kidney Donors

HLA-D typing of cadaver kidney donors with HTCs is almost always impossible owing to lack of time. The shorter duration of the PLT test should make it possible to perform PLT in some cases. However, practical experience shows that such cases would be few. Moreover, the close correlation between HLA-DR and -D antigens is a good basis for the assumption that DR typing (which almost always

can be done within the time available) can substitute for HLA-D typing. Nevertheless, for experimental purposes, PLT studies of "new" antigens in donor-recipient pairs may be valuable.

C. Typing and Search for Bone Marrow Donors in Severe Combined Immunodeficiency

The allogeneic lymphocyte transformation in severely T-cell-deficient individuals, including patients with severe combined immunodeficiency, is abolished. These individuals cannot be HLA-D typed with the HTC technique because their cells cannot function as responder cells. If bone-marrow transplantation (BMT) is going to be performed, it should preferentially be done with an HLA-identical MLR-negative sibling as the donor, although other HLA-D compatible, MLC-negative donors may be used—especially if related (Dupont *et al.*, 1973a; L'Esperance *et al.*, 1975; O'Reilly *et al.*, 1977). If no HLA-identical sibling is available for BMT, the search for potential donors may be greatly facilitated by the PLT technique, even in patients with low lymphocyte counts of mainly B-lymphocyte type. The HLA-A, B, C, D, and DR typing of the patient and the family will in most cases give the genotype of the two HLA haplotypes of the patient. Family members who are DR compatible with the patient may then be tested in MLR with the patient in order to find a suitable donor. This procedure, however, cannot be used if the patient carries an undefined HLA-DR antigen. In such cases, haplotype-primed PLT cells typing for the "unknown" DR-associated determinant(s) may be generated in the family and tested in the family members. Individuals giving rise to high PLT responses may then be selected for further investigations as potential BMT donors.

XIV. Associations between PLT-Defined Determinants and Disease

The first observation of a possible association between an HLA factor and a disease, made by Amiel (1967), prompted a great number of investigations of associations between HLA and disease. A surprising number of associations have been found (for review, see, e.g., Dausset and Svejgaard, 1977). In most diseases, the strongest—and perhaps the primary—associations have been found to HLA-D/DR antigens. In this context, the PLT technique may offer a tool for the investigation of association to as yet undefined determinants, defined by haplotype-primed PLT cells generated in families with diseased individuals.

Some investigations of antigen frequencies of PLT-defined deter-

minants in different populations of patients have been published, but these investigations have mainly been of confirmatory character, as the patients have also been HLA-D/DR typed. These studies concerned juvenile rheumatoid arthritis (Morling *et al.*, 1981c), and insulin-dependent diabetes mellitus (IDDM; Platz *et al.* 1981). In IDDM, we found that the association with DR4 is stronger than that with Dw4 and that most DR4+/DW4− IDDM patients are DP4 positive (Platz *et al.*, 1981).

The PLT technique may prove to be a very useful tool for the investigation of HLA association in diseases where associations are found to more than one HLA antigen, as is the case in, for example, IDDM, which is associated with both HLA-D/DR3 and HLA-D/DR4.

The relative risk of IDDM in DR3-positive individuals is 4.3, and the RR in DR4-positives is 10.5. The RR in DR3/4 heterozygotes, however, is 44.4, indicating that the DR3/4 heterozygotes have a higher relative risk. These and other data argue against the three simplest genetic models, dominant, recessive, and intermediate, for the inheritance of IDDM (Platz *et al.*, 1981). Accordingly, more complicated models must be investigated.

The PLT technique may be of value for some of the possible hypotheses that may be tested.

1. It has been suggested (Svejgaard *et al.*, 1982) that association between IDDM and DR3/4 heterozygous individuals might be due to the effect of combinatorial (or hybrid) antigens created by gene products of both HLA haplotypes (see Section I,C). This hypothesis may be tested if PLT cells specific for such putative combinatorial determinants could be generated.
2. The HLA-D/DR3 and -4 antigens may not themselves be responsible for the association between IDDM and HLA, which could be due to a common determinant positively associated with both D/DR3 and D/DR4. As suggested by F. H. Bach (personal communication), monoclonal PLT-cells may prove to be useful in exploring this possibility.

The association between juvenile rheumatoid arthritis (JRA) and HLA-Dw5 and -8 was demonstrated both with the HTC technique and the PLT technique at a time when DRw8 could not be defined (Morling *et al.*, 1981c). The association to both HLA-D/DR5 and -8 may be secondary to a primary association to a determinant common to both D/DR5 and -8, and this hypothesis is supported by the observed cross-reaction in PLT between Dw5 and Dw8 (Morling *et al.*, 1980b).

Studies on the association between SB antigens have not yet been published, but such studies may give valuable new information concerning the associations between HLA and disease.

XV. Conclusion

The two areas within the immunogenetic field that presently attract most interest concern immunoglobulins and T-lymphocyte receptors on the one hand and the major histocompatibility complex (MHC) on the other. Of these two interrelated areas, that concerning the MHC is perhaps the most challenging, not only because of the extreme genetic polymorphism of these complexes, but also because of their pleiotropy, that is, the variety of characters they control. For example, the MHC of man, the HLA system, controls not only two types of transplantation alloantigens (the HLA-A, B, C and HLA-D/DR antigens), but also some components of the complement cascade, the susceptibility to a variety of diseases, and immune-response determinants that govern the cooperation of cells in thymus-dependent immune responses. The *HLA-D/DR* region is of special interest because it controls the early events of the immune response and seems to play a key role in transplantation. One of the methods that has proved to be useful in the exploration of the *D/DR* region of the HLA system is the so-called primed lymphocyte typing (PLT) test.

The PLT test has provided very valuable information concerning the genetics of human lymphocyte-activating alloantigens and is a powerful tool for further investigations of such antigens. The PLT test can be used as a cellular typing system for alloantigens, and in this context the PLT is potentially much more valuable than the typing with homozygous typing cells (HTCs) because the number of reagents defining "new" determinants in the PLT test theoretically is unlimited and also the development of new reagents does not require access to homozygous individuals.

The most important lymphocyte-activating determinants responsible for both primary and secondary MLR reside in the so-called *HLA-D/DR* region, which contains the genes controlling HLA-D antigens detected by HTCs and DR antigens detected by serology. The *HLA-D/DR* region is most probably homologous to the *I* region in the H-2 system of mice. The *I* region is known to contain a number of genes on at least four different loci, and, for this and other reasons, the *HLA-D/DR* region is very likely also to contain more than one locus. The alleles of these loci are assumed to be in quite strong linkage disequilibrium and, conceivably, "good" HTCs represent "common" combi-

nations of these alleles and thus type for the corresponding combinations of antigens rather than for single determinants.

On the basis of these assumptions, we have produced a battery of PLT reagents by priming selected responder cells with HTCs. This battery types for a number of PLT determinants that, in population studies, appear to be very closely associated both with the HLA-Dw1-w8 antigens and with the corresponding HLA-DR1-w8 antigens. These HLA-D/DR-associated PLT determinants have been named DP1-8 and behave as though controlled by alleles at one locus, that is, as one segregant series. Typing for DP antigens is at least as reliable and reproducible as HLA-D typing with HTCs and may eventually replace HLA-D typing. It has been possible to generate PLT reagents for HLA-DR antigens (e.g., DRw10), which are so rare that the corresponding HTCs are unlikely to be found without extraordinary efforts. Although the serological technique typing for HLA-DR and other *D/DR*-region-controlled antigens is attractive on the basis of its speed and relative simplicity, it is hampered by the fact that good antibodies are rare, and the serological identification of "new" *D/DR* region antigens is progressing only slowly. In this situation, the PLT test offers an important alternative in the unraveling of the *D/DR* region. Moreover, the PLT test will for a long time serve as an important reference point for the results obtained both with new serologic reagents and in other (e.g., biochemical) studies of this region and its biological function.

Although the HLA-D, HLA-DR, and DP antigens are very strongly associated, there are occasional, but reproducible and important, differences between the results obtained by the corresponding three techniques. These discrepancies are readily explained by assuming heterogeneity within the *D/DR* region and on the basis of the concept that the MLC test (including typing with HTCs) detects differences, while PLT and serologic typing would tend to reveal similarities, including cross-reactivity. Although HLA-D typing, as just mentioned, discloses common combinations of different *D/DR* region products, and the same may be true for PLT reagents typing for DP antigens, careful selection of primary responders and stimulators for PLT reagents makes it possible to detect the single determinants.

Thus, additional evidence supporting the existence of heterogeneity within the *D/DR* region has been obtained by the discovery of a set of "new" PLT-defined determinants provisionally called "SB" antigens, which seems to constitute a segregant series of human alloantigens. These traits were discovered by PLT reagents from highly selected combinations and seems to be coded by genes situated

between the *HLA-B* and *GLO* loci, most probably between *HLA-D/DR* and *GLO*. The determinants are different from known HLA antigens, but one of the determinants, SB1, is in positive linkage disequilibrium with HLA-D/DR3. Accordingly, these putative "SB" alloantigens may be considered to be part of the HLA system, and the mapping of the corresponding genes indicates that they belong to the *D/DR* region.

It is very likely that other determinants coded by genes in the HLA region may be identified with the PLT test. Thus, there is some evidence that determinants coded by genes situated (*a*) between *HLA-B* and *-D/DR* and (*b*) in the *HLA-A* region may be typed for with the PLT test.

There are also unconfirmed data suggesting that certain non-HLA factors, for example, the Lewis blood group antigen, may be recognized by PLT.

Thus, the PLT technique is a powerful tool for the further investigation of the genetics of human alloantigens, especially those controlled by the *HLA-D/DR* region, which are particularly interesting because they may be the human immune response determinants themselves.

An exciting achievement in the PLT field is the development of methods allowing clonal expansion of PLT cells that are all progeny of one single cell. Such monoclonal PLT reagents will be crucial in the exploration of the heterogeneity of the *D/DR* region and in studies of cross-reactivity between various PLT determinants. Such reagents may also be used to investigate the possible existence of so-called combinatorial or "hybrid" antigens controlled by genes in transposition, and to study various HLA and disease associations in more detail.

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ADVANCES IN IMMUNOLOGY, VOL. 32

Protein A of *Staphylococcus aureus* and Related Immunoglobulin Receptors Produced by Streptococci and Pneumococci

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I. Introduction

As part of his efforts to classify strains of staphylococci on the basis of serological tests, Verwey developed an extraction procedure to separate cellular constituents into five fractions (Verwey, 1940). One of these, designated fraction B, was highly antigenic and apparently type specific. Several years later, similar attempts by Jensen to classify these organisms by gel precipitation techniques led to his finding that *Staphylococcus aureus* strain Cowan I was particularly effective in stimulating an immune response in rabbits to a component that he called antigen A (Jensen, 1958a). However, in some preimmunized animals, Jensen detected what he considered to be normally occurring antibodies against this antigen. He subsequently found that all samples of normal human serum and several samples of colostrum and human milk also gave strong precipitin lines with the antigen extract (Jensen, 1958a, 1959). Although Verwey's fraction B was shown to be protein, Jensen's antigen A was classified as a polysaccharide (Jensen, 1958a,b, 1959). This assignment apparently was based on faulty chemical tests, for it was clearly demonstrated (Löfkvist and Sjöquist, 1962, 1963; Grov *et al.*, 1964) that Jensen's antigen A was a protein identical to the major component of Verwey's fraction B. In fact, when the protein from an extract prepared according to Jensen's method (Jensen, 1959; Löfkvist and Sjöquist, 1962) was purified by ion exchange chromatography (Grov *et al.*, 1964) it was shown to contain no sugar. To stress the protein structure and to clarify the ambiguous nomenclature, the name protein A (SpA) was adopted (Grov *et al.*, 1964; Oeding *et al.*, 1964). The continued search for type-specific antigens for serological classification and what appeared to be the ubiquitous presence of antibodies to SpA led to the introduction and widespread use of Wood strain 46 as a source of bacteria free of SpA (Haukenes, 1962) and as the immunogen of choice to produce antibodies to another major staphylococcal antigen, polysaccharide A. It should be noted that normally occurring antibodies to staphylococcal proteins are common; Espersen and Schiøtz (1981) have reported that all 263 normal human sera tested contained precipitating antibodies to 6–10 S. *aureus* antigens present in a standard preparation free of SpA, and that 10 colostrum samples also contained precipitating antibodies to 3 antigens in addition to those occurring in the corresponding maternal sera. The immune response to SpA is discussed further in Section V,F.

Interest in SpA as something other than a curiosity (and perhaps a nuisance to bacteriologists) is based in its rather unique reactivity with immunoglobulins (Igs) as defined by Forsgren and Sjöquist

(1966). In their experiments, they tested the ability of mixtures of SpA and either normal or myeloma IgG, or isolated Fab, Fc, or Fc′ fragments to inhibit the formation of precipitin lines against whole IgG in gel diffusion. They concluded that SpA reactivity is directed to the Fc region of IgG and classified the binding as a nonspecific "pseudo-immune" reaction as opposed to the specific binding of antigen at antibody Fab sites. These findings were consistent with results of other workers who demonstrated reactivity of several myeloma IgG proteins with SpA (Dammacco and Clausen, 1966; Kronvall, 1967), or the selective removal of IgG from normal or immune sera by several strains of *S. aureus* known to express SpA on the membrane (Lind, 1968; Lind and Mansa, 1968). Work by Forsgren and Forsum (1970) also established that the binding of fluorescent-labeled normal or immune IgG to most strains of *S. aureus* (Cohen *et al.*, 1961, 1963; Korn and Majorova, 1963; Bergman *et al.*, 1966; Lind, 1968; Lind and Mansa, 1968) was due not to an antigen–antibody reaction, but to binding through the Fc region to SpA on the surface of the bacteria.

These studies demonstrated the ability of SpA to behave as an Fc receptor, although more recent results suggest that it also can show minor binding activity at sites located in the Fab region of certain Igs. Furthermore, classes other than IgG can bind SpA and reactivity depends to a large extent on the species and subclass. Although SpA has been studied most extensively, other microorganisms, most notably streptococci, produce proteins that selectively bind IgG or other classes of Ig primarily through Fc binding sites. The production, properties, and applications of SpA and these related proteins will be discussed in detail.

II. Occurrence of Protein A

Once SpA had been classified as a cell wall protein that bound selectively to the Fc region of Ig molecules, most notably IgG, there were several studies aimed at determining its prevalence in a systematic way. Although early results (see Section I) suggested that SpA was common to staphylococci, it was not until around 1970 that a strict classification began to emerge. Since then several studies have appeared, the results of which are summarized in Table I.

Several hundred strains of staphylococci and related organisms have been tested for the production of cell surface and/or extracellular SpA. Techniques for detecting the cell-bound protein include agglutination of erythrocytes sensitized with SpA-reactive antibody (Sjöquist and Stålenheim, 1969; Kronvall, 1973a; Winblad and Ericson, 1973) or

TABLE I

PRODUCTION OF PROTEIN A BY DIFFERENT STRAINS OF *Staphylococcus* AND NON-*Staphylococcus* BACTERIA

Strain	Sources	Method[a]	No. positive/ no. tested	Comments	References
S. aureus	Human	a	692/700 (99%)	Data for extracellular SpA; all strains coagulase and DNase positive	Forsgren (1970)
S. aureus	Human	a	2/100 (2%)	All strains coagulase negative; 98 were DNase negative	Forsgren (1970)
S. aureus	Human	b	141/156 (90%)	Only cell-wall SpA determined; no correlation between SpA production, phage type or group, or other antigenic characteristics in 16 strains studied extensively; SpA production dependent on culture conditions	Kronvall *et al.* (1971)
S. epidermidis	Human	b	0/47 (0%)	All strains coagulase negative	Kronvall *et al.* (1971)
Non-*Staphylococcus*	Human	b	0/13 (0%)	*Escherichia coli* (3) *Pseudomonas* (1), *Proteus* (1), *Klebsiella pneumoniae* (1), *Shigella* B_3 (1), *Shigella* D (1), and *Serratia marcescens* (5) tested	Kronvall *et al.* (1971)
S. aureus	Human	c	302/455 (66%)	Studied correlation between SpA production, phage type, production of lipase and hemolysin, and drug sensitivity	Lind (1972)
S. aureus	Human	a	215/215 (100%)	19/215 (9%) produced only extracellular SpA	Kronvall *et al.* (1972)
S. aureus	Human	a	318/341 (93%)	6/318 produced only extracellular SpA. 52 methicillin-resistant strains produced only extracellular SpA or were nonproducers	Winblad and Ericson (1973)
S. aureus	Human and other species	d	—	567 strains tested; 92% of negative strains were gelatinase positive; no correlation of SpA production with lysozyme or lypase activity	Galinski (1973)

S. aureus	Human	a, d	49/56 (88%)	SpA determined in cell extracts	Lind (1974a)
S. aureus	Human	a	297/360 (83%)	Formalinized then sensitized sheep erythrocytes detected low levels of SpA produced by methicillin-resistant strains	Flandrois *et al.* (1975)
S. epidermidis	Human	a	0/157 (0%)	—	Flandrois *et al.* (1975)
S. aureus	Human	a	75/107 (70%)	94% of human and animal strains were coagulase- and SpA-positive; 4% were coagulase-positive and SpA-negative; 3% were coagulase-negative and SpA-positive	Bind *et al.* (1978)
S. aureus	Human	a, d	46/48 (95%)	Production of SpA dependent on the medium used	Flandrois *et al.* (1978)
S. aureus	Human	a, d	95/106 (90%)	Number of producing strains depended on culture conditions and ranged from 7 to 90%	Bornstein *et al.* (1980)
S. aureus	Bovine and other species	a, d	54/120 (45%)	100 strains from bovine mastitis, 20 from other strains; 78 typable strains also tested for other antigens	Marandon and Oeding (1967)
S. aureus	Bovine	d	58/173 (34%)	41% (38/92) from chronic mastitis and 25% (20/81) from healthy animals were positive	Oeding *et al.* (1971)
S. aureus	Bovine	a	30/30 (100%; acute mastitis) (46–58)/97 (48–60%; chronic mastitis)	Cell-bound SpA determined; number of producing strains depended on culture conditions	Kronvall *et al.* (1972)
S. epidermidis	Bovine	a	(1–7)/77 (1–9%)	Cell-bound SpA determined; number of producing strains dependent on culture conditions	Kronvall *et al.* (1972)

(*Continued*)

TABLE I (*Continued*)

Strain	Sources	Method[a]	No. positive/ no. tested	Comments	References
S. aureus	Several species	a	148/149 (99%)	—	Bind *et al.* (1978)
S. aureus	Milk	a	420/433 (97%)	—	Bind *et al.* (1978)
S. aureus	Various	a	0/570 (0%)	Strains from human (18) animal (125), and milk (427) were tested	Bind *et al.* (1978)
S. aureus	Various	c	142/143 (99%)	Cell-bound SpA; strains from human (72), dog (5), poultry (6), cow (55), and hare (5) were tested	Lachica *et al.* (1979)
S. hyicus	Various	c	0/34 (0%)	Strains from cow (32) and poultry (2) were tested; 17 cow strains were coagulase-positive	Lachica *et al.* (1979)
S. intermedius	Various	c	4/127 (3%)	Strains from dog (122) and pigeon (5) were tested	Lachica *et al.* (1979)
S. aureus	Various	a, d	38/100 (38%)	Number of producing strains depended on culture conditions and ranged from 2 to 38%	Bornstein *et al.* (1980)

[a] a, Agglutination of antibody-sensitized sheep erythrocytes; b, binding of ^{125}I-labeled human myeloma IgG_1 protein; c, binding of fluorescein-labeled normal human, rabbit, or swine IgG; d, gel diffusion.

binding of fluorescent-labeled (usually fluorescein) IgG (Lind *et al.*, 1970; Lind, 1972, 1973; Lachica *et al.*, 1979). Results from these determinations are essentially qualitative, but they are useful for rapidly screening large numbers of samples. Cowan strain I and Wood 46 are generally used as positive and negative controls, respectively. Kronvall and co-workers (1970b, 1971) used the binding of an ^{125}I-labeled myeloma IgG_1 protein to quantify the number of binding sites on the surface of *S. aureus* and showed that Cowan strain I expressed approximately 80,000 binding sites per organism (Kronvall *et al.*, 1970b). Extracellular SpA and SpA extracted from bacteria usually have been detected by gel diffusion by allowing supernatants to migrate into agar containing IgG. Formation of precipitin lines and comparison to standard preparations of purified SpA allowed estimation of the concentration (Forsgren, 1969; Lind, 1974; Sjöquist *et al.*, 1972a) analogous to the Mancini technique. Similarly, some workers used gel containing antisera raised against different strains of *S. aureus* (Marandon and Oeding, 1967; Oeding *et al.*, 1971). Several conclusions can be drawn from these studies.

1. Based on results so far, production of SpA is restricted to strains of *S. aureus*.
2. Generally (Table I) over 95% of the human strains of *S. aureus* produced SpA. Most strains contained the cell-bound product, but released significant amounts into the medium, and only extracellular SpA was produced by approximately 5% of the positive strains (see Section III). Wood 46, Cowan II, and strain 28 are examples of nonproducers.
3. Except for strains isolated from bovine chronic mastitis (approximately 50% producers), over 95% of the *S. aureus* strains isolated from several animal species were SpA positive.
4. Approximately 50% of methicillin-resistant strains of *S. aureus* are either nonproducers or give only extracellular SpA.
5. Production of SpA by *S. aureus* shows a strong correlation with coagulase and thermostable nuclease activity. However, since 57% of 30 SpA-negative strains of *S. hyicus* were coagulase positive and all 30 were themonuclease positive, SpA production may be the best of these criteria for purposes of identification (Lachica *et al.*, 1979; Sperber, 1976). There appears to be no correlation of SpA with lysozyme or lipase activity, and a negative relationship with gelatinase activity (Galiński, 1973).

It should be emphasized that there probably is a significant degree of overlap in the strains tested by the different groups represented in

Table I. Also the relative sensitivities of the detection methods are different, and some weak producers would be misclassified as nonproducers if a less sensitive method [e.g., gel diffusion (Flandrois *et al.*, 1980)] were used. In this regard, the hemagglutination test is 30 times more sensitive for extracellular SpA than for SpA heat-extracted from the cell surface (Sjöquist and Stålenheim, 1969; Forsgren, 1970) probably owing to loss of SpA activity at high temperature. Also strains producing only extracellular SpA would be missed in the cell-binding assays. In most of these studies, the Ig binding component on the cell surface has been classified as SpA on the basis of functional criteria alone, although in studies where gel diffusion was used the reactive component in cell extracts or supernatants gave a line of identity with a standard preparation of SpA (Lind, 1974a; Flandrois *et al.*, 1978; Bornstein *et al.*, 1980).

III. Production of Protein A

A. Growth and Storage of Bacteria

Staphylococcus aureus is a pathogen, and suitable precautions must be taken to prevent spills and formation of aerosols. Optimal growth conditions have been established, and two basic formulations, CCY broth (Arvidson *et al.*, 1971) and fortified Penassay broth (Kessler, 1975, 1976), are suitable for most strains including Cowan I. Detailed descriptions of the methods for culturing the bacteria in either small-scale or large-batch production are given by Kessler (1981). Several variations have been used (Löfkvist and Sjöquist, 1963; Forsgren and Sjöquist, 1966, 1969; Forsgren, 1969; Kronvall *et al.*, 1977; Landwall, 1978; Osland, 1981) in volumes ranging up to 500 liters (Löfkvist and Sjöquist, 1963; Forsgren and Sjöquist, 1966). Culture times have varied, but generally the organisms are collected after 12–18 hours at 37°C, cultures over 5 hours being performed in fermentors designed to supply sufficient aeration. From the routine batch cultures, 9–13 gm of cells per liter (wet weight) have been isolated (Kessler 1975, 1976, 1981) although up to 25 gm/liter have been reported (Forsgren and Sjöquist, 1966). *Staphylococcus aureus* also has been cultured on nutrient-enriched agar (Löfkvist and Sjöquist, 1962; Lind and Mansa, 1968, 1974a; Lind, 1974a; Mallinson *et al.*, 1976; Bornstein *et al.*, 1980; Osland, 1981) or commercial gelified broth (Flandrois *et al.*, 1978).

A typical growth curve for Cowan strain I along with the production

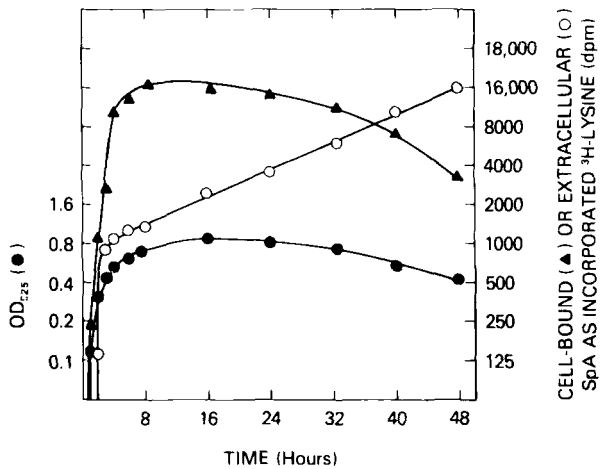

FIG. 1. Comparison of the growth curve (●) for Cowan strain I bacteria versus the production of cell-bound (▲) and extracellular protein A (○) measured as incorporation of [^{3}H]lysine. (Adapted from Movitz, 1976.)

of cell-bound and extracellular SpA are shown in Fig. 1 (Movitz, 1976). The decrease in cell-bound SpA due to cytolysis after extended culture times with the continued increase in extracellular SpA due to turnover of the cell wall during the stationary phase are evident.

After the organisms have been collected and washed, they can be stored frozen at −30° to −70°C (Forsgren and Sjöquist, 1966; Sjöquist *et al.*, 1972a; Kessler, 1981) or lyophilized (Kessler, 1981) without significant loss of SpA activity. Heat-killed bacteria treated with dilute solutions of formalin also retain activity (Lind and Mansa, 1968; Kronvall *et al.*, 1971; Jonsson and Krønvall, 1974; Kessler, 1975, 1976, 1981; Mallinson *et al.*, 1976) and are sold commercially as a precipitating agent for IgG or IgG-containing immune complexes. Kronvall *et al.* (1971) found that bacteria treated with 0.5 or 3% formalin for 3 hours or 30 minutes, respectively, retained 70% of the original IgG binding capacity, whereas bacteria incubated with 5% formalin for 24 hours retained only 25% activity. No depletion of IgG binding was detected after storage at 4°C for up to 6 days. In contrast, untreated bacteria stored at 4°C for 3 days in suspension or as a pellet lost approximately 50 and 85% SpA activity (Kronvall *et al.*, 1970b). Others also have shown that treatment with 5% formalin for up to 24 hours at room temperature (Lind and Mansa, 1968; Nickerson, *et al.*, 1969) destroyed or modified SpA specificity.

B. Effect of Culture Conditions on Production of Protein A

Lind (1974a) tested 10 strains of *S. aureus* in 7 different solid media and found that SpA production differed by over 20-fold. Similarly, when Bornstein *et al.* (1980) tested over 200 strains from man and animals on several different media there were marked differences in the number of SpA producers detected. Of the 150 animal strains 2–38% were positive, whereas 7–90% of the human strains were positive. Earlier (Winblad and Ericson, 1973) it had been shown that peptone in the medium was necessary for optimal production of SpA, and Flandrois *et al.* (1978) found that 48 human strains failed to produce detectable levels of SpA when cultivated in trypticase soya medium but 46 of them became producers after subculture in a second medium (gelified "oxoid broth No. 2"). By using a dialysis culture technique with S. *aureus* strain A676, which produces only extracellular SpA, yields of SpA up to 2 gm/liter have been achieved compared to 0.2–0.3 gm/liter under usual batch culture conditions (Landwall, 1978). The level of SpA produced by this method differed significantly using three different media. High concentrations (e.g., 7.5%) of sodium chloride can depress the production up to 90% (Nickerson *et al.*, 1969; Kronvall *et al.*, 1971; Lind, 1974a), and protopolasts in 24% sodium chloride failed to produce SpA (Movitz, 1976).

Kronvall *et al.* (1971) found no quantitative difference in cell-surface SpA when three producer strains were cultivated for 8–42 hours at 37°C. However, others (Movitz, 1976; Lindmark *et al.*, 1977; Kessler, 1981) have found that after the stationary phase has been reached (12–20 hours) production of SpA ceases and a significant amount of cell-bound SpA may be lost by autolysis (Fig. 1). Incubation temperature also may effect levels of cell-bound SpA (Kronvall *et al.*, 1971), since, compared to incubation at 37°C, Cowan Strain I organisms incubated at 20°C in Penassay broth displayed 50% less SpA on their surface, whereas those incubated at 41°C had 20% more. Using less quantitative microscopic methods with peroxidase-labeled IgG, Nickerson *et al.* (1969) found no significant difference in the amount of surface SpA on organisms incubated at 37°C or 44°C in CCY broth.

C. Production of Protein A by Mutant Strains of S. *aureus*

Strains of S. *aureus* can be distinguished on the basis of drug resistance as it relates to the production of SpA. Whereas neomycin-resistant strains generally produce large amounts of SpA (Lind, 1972), about

50% of methicillin-resistant strains are nonproducers or yield only extracellular SpA (see above; see also Lind, 1972; Linblad and Ericson, 1973). In addition to these naturally occurring organisms, several groups have studied SpA production by induced mutant forms of *S. aureus*.

Experiments carried out with Cowan strain I organisms devoid of cell walls (Forsgren, 1969) showed that, compared to the L form, the intact bacteria contained approximately 20-fold more SpA and 5–10 times more extracellular SpA was present in the culture medium. However, the levels found in the nonreverting L form (0.15 mg/300 mg dry cell weight) and its culture fluid (0.13–0.27 mg/liter) were still significant.

Nitrosoguanidine and ethylmethane sulfonate have been used to produce mutants of Cowan strain I deficient in SpA (Forsgren *et al.*, 1971). Sixty such mutants were isolated, and six classes were distinguished on the basis of production of cell-bound and extracellular SpA, nuclease, coagulase, α-hemolysin, and fibrinolysin activity, of utilization of mannitol, and of phage type. More than a third of the strains were negative for all activities, and two classes showed only loss or deficiency of SpA production and α-hemolysin. Some non-SpA-producing mutants reverted spontaneously and with high frequency to producers, and some nitrosoguanidine mutants reverted to producers when treated with ethylmethane sulfonate. Although the site of mutation was not determined, the properties of the mutant strains suggested that a common regulatory mechanism controlled the synthesis and/or release of several extracellular products, including SpA. Cohen and Sweeney (1979) studied modulation of SpA production in strains derived from the Mec^r strain A676 by treatment with ethidium bromide. As already mentioned, strain A676 is methicillin-resistant and produces only extracellular SpA (Lindmark *et al.*, 1977). It was found that methicillin resistance and SpA production were lost coordinately and restoration of the genetic determinant for methicillin resistance (*mec*) by transduction or transformation restored SpA production. Marked reduction of SpA production also correlated with loss of *mec* from two other strains. Since in some cases incorporation of determinants for methicillin resistance enhanced SpA production, the determinant could enhance or suppress SpA content depending on the specific determinant and the host strain.

Masuda *et al.* (1975) devised a simple method for isolating SpA-deficient mutants based on cosedimentation of SpA-producing bacteria with antibody-sensitized sheep erythrocytes. The deficient organisms present in the supernatant were free of cell-bound SpA, and those that

failed to give precipitation in agar gel diffusion also failed to produce the extracellular product. By using this technique, in two experiments 4 of 478 and 2 of 432 spontaneous mutants were present in the culture of *S. aureus* 248Beta H, a strain that produces mainly cell-bound SpA. After ultraviolet (UV) irradiation, 62 of 205 colonies were found to be nonproducers and an additional 6 were weak or doubtful. The technique has been used to isolate 6 mutant strains producing only extracellular SpA (Movitz *et al.*, 1979) for comparison with the properties of SpA produced by the parental Cowan I strain (see Section IV).

D. Localization of Protein A in *S. aureus*

From the beginning, results have demonstrated the presence of extracellular as well as cell-bound SpA and implied that SpA on the membrane had easy access to Ig molecules in the fluid phase or bound to cell-surface antigens in such a way that the Fc region could interact with SpA receptors. Indeed, studies have shown that SpA is located primarily on the surface of Cowan strain I and is linked covalently to the peptidoglycan portion of the membrane (Nickerson *et al.*, 1969; Sjöquist *et al.*, 1972b; Movitz, 1974). By scanning electron microscopy using cell-bound antigen–antibody complexes as indicators, SpA was found to be distributed uniformly over the surface of strains Cowan I, 209P, and FDA (Umeda *et al.*, 1980). Similar results have been obtained with ferritin- or fluorescein-labeled IgG (Lind, 1973). Based on dry weight, Cowan strain I bacteria and isolated cell walls contain 1.7% and 6.7% SpA, respectively (Sjöquist *et al.*, 1972b). Extraction of cell walls with trichloroacetic acid, detergents, or high concentrations of lithium chloride or by treatment with DNase did not release SpA, although digestion with trypsin released inactive SpA peptide fragments. SpA released from cells by lysosyme contained cell wall peptidoglycan fragments that could be cleaved by incubation of the lysozyme product with lysostaphin to give a more homogeneous product (Sjöquist *et al.*, 1972b).

Studies on biosynthesis have been carried out in strains that produce cell-bound as well as extracellular SpA (e.g., Cowan I) or only the extracellular product (A676). Results with Cowan I have shown that SpA and peptidoglycan chains are synthesized independently and that, after synthesis on the ribosomes, SpA is linked directly to the preexisting membrane-bound peptidoglycan moieties, apparently without going through a soluble cytoplasmic stage (Movitz, 1974). Thus inhibition of peptidoglycan synthesis by vancomycin does not eliminate production of SpA or incorporation into the cell wall. Since streptomycin, chlorpromazine, and puromycin inhibited synthesis of

cell wall and extracellular SpA, all SpA production appears to take place on the ribosomes.

Studies on the origin of extracellular SpA (Movitz, 1976; Lindmark *et al.*, 1977) have shown that strains Cowan I and 3695 secrete up to 9% of total SpA produced, while 90–95% is bound to cell wall and 1% is found in the cytoplasm. Low levels of cytoplasmic SpA in Cowan I also have been reported by Forsgren (1969). Exponentially growing bacteria release extracellular SpA directly after synthesis on ribosomes, and this product and cell wall SpA released by lysostaphin treatment of strains Cowan I, E2219, and 4972 are indistinguishable by disc-gel electrophoresis and the amino acid composition is the same (Movitz, 1976; Lindmark *et al.*, 1977). Since protoplasts from strains Cowan I and 3695 produced 97% extracellular and 1% cytoplasmic SpA under conditions where the total rate of production was the same as in intact bacteria, the cell wall is not necessary for *de novo* SpA synthesis. Furthermore, the continued release of low levels of SpA during the chase segment of pulse and chase experiments and after complete inhibiton of SpA synthesis by tetracycline suggests that a low (1.5%) but significant degree of secretion also is not dependent on protein synthesis (Movitz, 1976).

Although puromycin inhibited incorporation of SpA into the cell wall of Cowan I by up to 99%, secretion was significantly enhanced at the optimal dose of drug. There was no enhanced release from protoplasts of Cowan I or from strain A676, suggesting that this type of augmentation works only in strains that have cell wall SpA.

IV. Isolation and Properties of Protein A

A. Isolation

SpA has been isolated by heat extraction from Cowan strain I in boiling phosphate buffer followed by precipitation with acid or high salt (Löfkvist and Sjöquist, 1962; Forsgren and Sjöquist, 1969; Masuda *et al.*, 1975), ion exchange and gel filtration chromatography, isoelectric focusing, and/or polyacrylamide gel electrophoresis (Forsgren and Sjöquist, 1966, 1969; Forsgren, 1969). Lysozyme also has been used to release SpA from the surface of *S. aureus* (Forsgren, 1969; Sjöquist *et al.*, 1972b), but the yields are variable and the products generally are heterogeneous. They often contain peptidoglycan fragments and are up to 25- to 30-fold less active than extracellular SpA found in culture fluid. In contrast, SpA isolated after lysostaphin digestion (Sjöquist *et al.*, 1972a; Björk *et al.*, 1972) is homogeneous and fully active. In the

original procedure, after enzyme treatment and acidification to precipitate extraneous protein, SpA is precipitated in (neutral) 80% ammonium sulfate and purified by ion exchange chromatography followed by gel filtration (Sjöquist *et al.*, 1972a). The yield from 300 gm of bacteria was 450–500 mg, accounting for 50% of the total available SpA.

The purification procedure has been improved by using affinity chromatography on immobilized IgG (Hjelm *et al.*, 1972; Kronvall, 1973a; Rao *et al.*, 1977). Following lysostaphin treatment (and with or without the acid precipitation step), the supernatant containing SpA is applied to a column of human IgG coupled to Sepharose (Hjelm *et al.*, 1972) or porcine IgG bound to thiophosgene-activated polyacrylamide (Rao *et al.*, 1977). After washing with neutral buffer, homogeneous and fully active SpA is eluted with 0.1 *M* glycine, pH 3.0. The yield is approximately 75% and as little as 5 μg can be recovered quantitatively. Similarly, SpA has been isolated in one step from the culture fluid of Cowan strain I (Kronvall, 1973a; Movitz *et al.*, 1979), methicillin-resistant strains 5515 (Kronvall, 1973a) and A676 (Lindmark *et al.*, 1977) or from UV and spontaneous mutants of Cowan I (Movitz *et al.*, 1979) that produce high levels of only extracellular SpA. Strain A676 gave 33 mg/l culture fluid (Lindmark *et al.*, 1977). Kronvall's procedure was somewhat different from the others in that after batchwise absorption, the IgG-Sepharose was placed in a column, washed with PBS, and SpA eluted with 2–3 *M* potassium thiocyanate. The yield was 85%. Human plasma polymerized with ethylchloroformate also was tested but the isolated SpA was impure (Kronvall, 1973a). Radioactive SpA labeled *in vivo* with [^{3}H]leucine or [^{3}H]lysine and present in culture fluid (extracellular) or cell lysate (Movitz, 1976) and active fragments of SpA prepared by enzyme degradation (Hjelm *et al.*, 1975; Sjödahl, 1977a,b) have also been isolated by affinity chromatography.

B. Physicochemical Properties

The physicochemical properties of SpA prepared by lysostaphin treatment of Cowan strain I are summarized in Table II (Björk *et al.*, 1972; Sjöquist *et al.*, 1972a; Sjöholm, 1975a). The high frictional ratio (2.1) and intrinsic viscosity (29 ml/gm) compared to values normally obtained for globular proteins (1.1–1.25 and 3–4 mg/gm, respectively) suggest that SpA is a relatively elongated molecule. The molecular weight of 42,000 is the average of several determinations and is the generally accepted value. This compares to the molecular weight of 29,500 found in the most active fraction obtained after chromatography of heat-extracted material. The deleterious effects of heating at

TABLE II
PHYSICOCHEMICAL PROPERTIES OF PROTEIN A[a]

Property	Value[b]
Molecular weight	42,000
$A_{275}^{1\%}$	1.65[c]
Isoelectric point	5.1[d]
$s^{\circ}_{20,w}$ (S)	2.1
$D_{20,w}$ (cm^2/s)	4.3×10^{-7}
Stokes' radius (nm)	5.0
Frictional ratio (f/f min)	2.1
Intrinsic viscosity; η (ml/gm)	29
Huggins constant, K	0.66
Specific volume, $\bar{v}$ (ml/gm)	0.72
pK_a (4 equivalent tyrosines)	10.25[e]

[a] Cell-bound protein A was isolated by lysostaphin treatment of Cowan strain I bacteria.

[b] From Björk *et al.* (1972) unless indicated otherwise.

[c] From Sjöquist *et al.* (1972a) and Sjöquist (1973).

[d] From Lindmark *et al.* (1977).

[e] From Sjöholm (1975a).

100°C also were indicated by the formation of several active low-molecular-weight fragments when purified SpA from lysostaphin digestion was heated under similar conditions; the most active component had a molecular weight of 34,000. Others have reported molecular weight values ranging between 12,000 and 32,000, and sedimentation coefficients between 1.26 and 2.6 for SpA isolated by extraction (Löfkvist and Sjöquist, 1963; Grov, 1967; Forsgren, 1969) or enzyme digestion with pancreatic DNase that probably was contaminated with proteolytic emzyme(s) (Yoshida *et al.*, 1963). All four tyrosine residues of SpA are proteolytically equivalent and subject to perturbation by 20% polyethylene glycol or dimethyl sulfoxide (Sjöholm, 1975a). SpA is partially but reversibly denatured in 6 *M* guanidine hydrochloride and is partly intact even after heating at 80°C (Sjöholm, 1975a). Based on the circular dichroism (CD) spectrum (Sjöholm, 1975a), the conformation is intact over a pH range of 0.99–11.8.

Extracellular SpA produced by strain A676 has a molecular weight of 41,000 by gel chromatography under denaturing conditions or by equilibrium sedimentation, and $A_{275}^{1\%} = 1.46$. Like cell-bound SpA from Cowan I, the isoelectric point is 5.1 (Lindmark *et al.*, 1977).

The extracellular product of one spontaneous and five UV-induced mutants of Cowan strain I had molecular weights between 13,000 and 41,000 (Movitz *et al.*, 1979), and a comparison showed that extracellu-

lar SpA produced by strains Cowan I, E2219, or 4972 had approximately the same molecular weight and charge characteristics as their cell-bound counterparts (Movitz, 1976). In all these studies, estimation of the molecular weight by disc-gel electrophoresis gave erroneous high values (e.g., 55,000–56,000) compared to values obtained by chromatography or sedimentation (41,000–42,000).

C. Structure and Activity of Protein A

1. Structure of Protein A and Active Fragments

Several structural studies have been carried out on intact SpA isolated with lysostaphin (Sjöquist *et al.*, 1972a; Sjöholm, 1975a,b; Movitz, 1976) or on fragments prepared by treatment of cell-bound or free SpA with trypsin alone or in combination with lysostaphin (Hjelm *et al.*, 1975; Sjödahl 1976, 1977a,b; Deisenhofer *et al.*, 1978; Deisenhofer, 1981). Reactive fragments were isolated by affinity chromatography on immobilized IgG and separated by gel filtration and ion exchange chromatography, and the amino acid composition or sequence was determined. Table III shows the amino acid composition of SpA isolated from Cowan strain I (Sjöquist *et al.*, 1972a; Movitz, 1976; Lindmark *et al.*, 1977) strain 4972, and strain E2219 (Movitz, 1976) after lysostaphin digestion, and of the extracellular product of strain A676 (Lindmark *et al.*, 1977). In general, the data are in good agreement and indicate a close structural similarity between the true extracellular product from strain A676 and cell-bound SpA. The most striking difference is in the composition of the cell-wall product from strain 4972, which contains significantly fewer amino acids, particularly lysine, aspartic acid, and proline.

In addition to SpA from these readily available strains, products of a spontaneous mutant and 5 UV variants of Cowan strain I also have been analyzed (Movitz *et al.*, 1979). The results show that the composition, like the molecular weight, was variable. However, two of the UV mutants (UV-2 and UV-7) gave products with compositions essentially the same as that of SpA from the parent organism. None of the products analyzed so far contain tryptophan or cysteine, and except for one UV mutant (V-1) they all are immunochemically similar, since they gave lines of identity in double diffusion against a precipitating IgG and against rabbit antibodies to SpA prepared by lysostaphin digestion of Cowan strain I. The one UV mutant that behaved anomalously had a molecular weight of 13,000 and probably was monovalent. The CD spectrum (Sjöholm, 1975a) indicates that SpA is composed of 50% right-handed helical structure and 10–

TABLE III
AMINO ACID COMPOSITION OF PROTEIN A FROM DIFFERENT STRAINS OF *Staphylococcus aureus*

Amino acid	Strain and nearest number of residues					
	Cowan I[a]			4972[a]	E2219[a]	A676[b]
Lysine	52[c]	52[d]	53[e]	34[d]	53[d]	48[e]
Histidine	4	4	4	5	4	3
Arginine	4	5	4	5	5	4
Aspartic acid	80	82	83	67	78	82
Threonine	6	5	6	7	5	4
Serine	14	17	16	17	18	16
Glutamic acid	60	65	70	61	63	64
Proline	28	27	26	20	24	27
Glycine	28	30	30	17	27	22
Alanine	36	34	36	38	38	31
Valine	8	5	8	7	7	7
Methionine	3	2	3	4	4	3
Isoleucine	11	9	12	11	11	11
Leucine	28	27	28	28	29	27
Tyrosine	4	5	4	4	4	4
Phenylalanine	12	12	12	12	12	13
	378	381	395	337	382	366

[a] Isolated by lysostaphin treatment of bacteria.
[b] Extracellular protein A produced by a methicillin-resistant strain.
[c] From Sjöquist *et al.* (1972a).
[d] From Movitz (1976).
[e] From Lindmark *et al.* (1977).

20% β-structure and is consistent with the proposed extended shape. The amino acid and other chemical analyses also suggested that SpA is made up of repetitive units and that cell wall or extracellular SpA contains little or no sugar (<0.2%) and no phosphate groups (Sjöquist *et al.*, 1972a,b; Lindmark *et al.*, 1977). However, extracellular SpA from Cowan strain I or from mutants that produce only the extracellular product are somewhat different from cell-bound SpA freed by lysostaphin. They have free N-terminal alanine and C-terminal lysine residues (Lindmark *et al.*, 1977; Movitz *et al.*, 1979), whereas the alanine in the cell-wall product is blocked and the C terminus is highly resistant to carboxypeptidase cleavage (Sjöquist *et al.*, 1972a,b).

Trypsin digestion of strain Cowan I under optimal conditions for production of the maximum amount of IgG-binding fragments of SpA gave two major active components designated A and B (Hjelm

et al., 1975). Both had molecular weights between 6000 and 7000 and corresponded to two of the six fragments (designated II and IV) produced by trypsin treatment of purified SpA. Based on amino acid analysis and peptide mapping, the six fragments appeared to be overlapping sequences. Sjödahl (1976) showed that some of them probably constituted fragments of a third Fc binding region, fragment C. Fragments A, B, and C were monovalent since they did not agglutinate sensitized sheep erythrocytes, but did inhibit reaction between IgG and cells coated with intact SpA.

Since trypsin digestion of intact cells failed to release fragment C, controlled trypsinization followed by lysostaphin treatment was used to degrade cell-wall SpA sequentially to give the more accessible fragments, followed by the remaining components including the segment joining SpA to the cell-wall peptidoglycan (Sjödahl, 1977a,b). By further degrading and sequencing these individual fragments, a fourth active region designated fragment D was found and the primary structure of the complete Fc binding region of SpA shown in Fig. 2 was obtained (Sjödahl, 1977b). The four highly homologous Fc-binding regions are composed of approximately 60 amino acids each and are in the order D, A, B, C from the N terminus. In addition to the close homology among the regions there is also significant internal homology, and since the N terminus is blocked an additional piece probably precedes fragment D. A fifth region, designated fragment X, is composed of approximately 150 amino acids (Sjödahl, 1977a,b). This segment does not bind Fc and differs both in primary and secondary structure from the active fragments.

The arrangement of the units and attachment to the cell wall are shown in the diagram in Fig. 3. Since fragment C (and X) is not released from cell-bound SpA, it is shown as being bound at or near the cell membrane to region X, which in turn is joined to the peptidoglycan backbone. Although there are four highly homologous regions capable of binding IgG, only two of them have been found to be expressed in the intact molecule (Sjöquist *et al.*, 1972a; Langone *et al.*, 1978c), and SpA is considered to be functionally bivalent.

Fragment B has been used to prepare complexes with the Fc fragment of human IgG for use in crystallographic analysis. The complexes were composed of one Fc fragment and two fragment B units with a molecular weight of 57,200 (Deisenhofer *et al.*, 1978; Deisenhofer, 1981). Fragment B is cylindrical, has a length of 26 Å and diameter of 16 Å, and is composed of three parallel helical structures arranged in a triangular array with no apparent intramo-

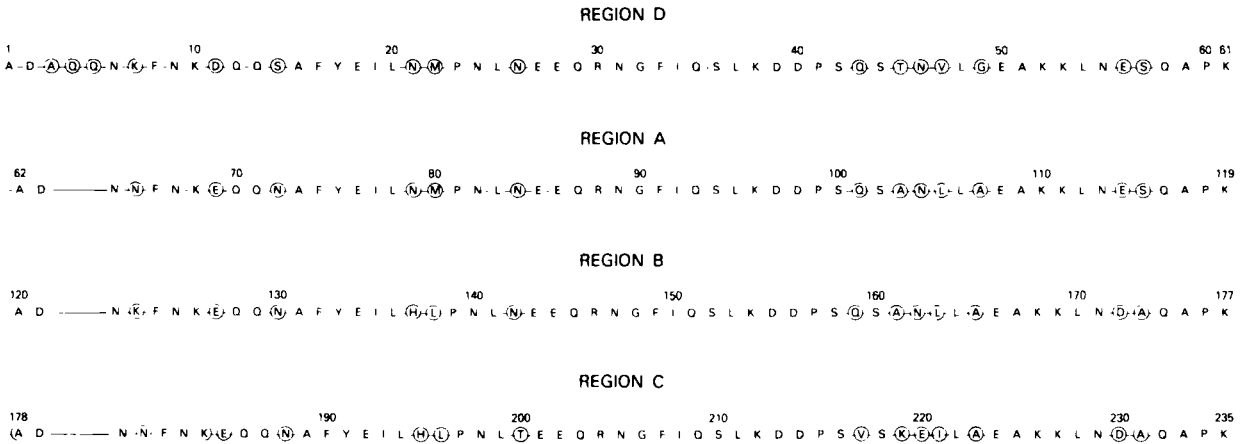

FIG. 2. Primary structure of the four Fc-binding regions (D, A, B, and C) of protein A. Circled amino acids indicate points of heteorgeneity, and the first seven residues in region C were not positively identified. (Adapted from Sjödahl, 1977b.) The one-letter amino acid nomenclature system is used (Dayhoff, 1969): A = Ala, B = Asx, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, X = unknown, Y = Tyr, Z = Glp or Glx.

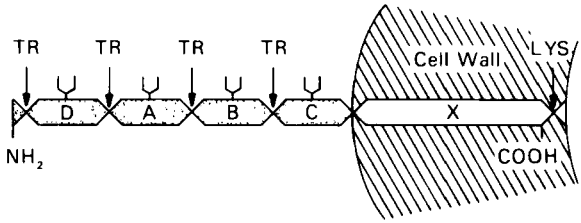

FIG. 3. Schematic diagram of the protein A molecule including the four Fc binding regions D, A, B, and C starting from the blocked N terminus and joined to region X, which is bound through the C terminus to the peptidoglycan portion of the cell membrane. The forks represent Fc binding sites, and LYS and TR indicate points of cleavage by lysostaphin and trypsin, respectively. Since region C is not released from bacteria by trypsin, it may be that region X, as shown here, is buried in the cell wall, making region C less accessible. (Adapted from Sjödahl, 1977a.)

lecular interaction. There is extensive contact between two of the helices and the C_H2 and C_H3 domains of Fc involving hydrophobic interactions, and a second type of interaction involving polar residues and the C_H3 domain only.

2. *Effects of Chemical Modification on Protein A Activity*

a. Modification of Protein A. Both SpA and IgG have been modified chemically to determine the role that specific amino acids may play in the binding reaction. When SpA was treated with acetylimidazole, 40 of 52 lysine residues were acetylated in addition to the 4 tyrosine phenolic hydroxyl groups (Sjöholm *et al.*, 1972). Since this reagent has been used selectively to label phenol groups in several proteins, including enzymes (Simpson *et al.*, 1963; Vallee and Riordan, 1970), the high level of incorporation into SpA may be related to the large excess used (two treatments with 60-fold molar excess). However, based on CD spectra, even this extensive labeling altered neither the gross conformation nor the local molecular environment of the aromatic amino acids, and reactivity with rabbit anti-SpA antibodies was not affected. Since 40% of the original SpA activity was retained and was increased to 60% by O-deacylation, tyrosine groups appear to be important for binding but probably are not solely responsible for full SpA activity.

This conclusion is consistent with results of other labeling experiments that involved tyrosine modifying reagents. Sjöholm *et al.* (1973) reported that nitration of all four tyrosines with tetranitromethane gave a product that reacted only weakly with human IgG or normal dog serum in gel diffusion and was 100-fold weaker than

unmodified SpA in agglutinating sensitized sheep erythrocytes. Reduction to amino groups restored about 40% of the original SpA activity, and both derivatives reacted as well as native SpA with anti-SpA serum. Based on the pK_a of the nitrotyrosine groups, compared to those of model compounds, it appears they were still located on the surface of the SpA molecule, and loss of activity must be attributed to some other factor(s) besides a gross conformational change. The bulk of the substituent groups or weakening of hydrogen bonding with Fc structures may be important.

The effect of nitration or reactivity of SpA with several species of IgG besides human or dog also has been tested by precipitation techniques that are 2–4 times more sensitive than those used routinely (Sloan and Butler, 1978). Neither bovine IgG_1 nor IgG_2 precipitated with SpA whereas swine, goat, guinea pig and rabbit IgG did, but to differing degrees. Nitrated SpA did not precipitate with goat or rabbit IgG, but did give a precipitin line with swine IgG. Reaction with guinea pig IgG was partially inhibited. By counterimmunoelectrophoresis all species precipitated with SpA, whereas only swine and guinea pig precipitated against the nitro derivative. From these experiments, it appears that the reactivity of nitrated SpA, like that of the parent molecule, depends on the species of IgG (see Section V), and weak reactions can be detected, provided sufficiently sensitive test systems are used.

In addition to 4 tyrosine residues, SpA has 4 histidine groups that are susceptible to iodination. Reaction under strictly controlled conditions introduced 3.5 atoms of iodine per molecule of SpA, but only 0.68 residue of monoiodotyrosine and 0.12 residue of diiodotyrosine were produced. The rest was incorporated into histidine (Sjöholm and Sjödin, 1974). The product lost about 70% of the original Fc reactivity, and since iodine is roughly as large as a benzene ring, steric hindrance involving modified histidine and/or tyrosine residues may be responsible. Similarly, Dorval *et al.* (1975) reported that introduction into tyrosine or histidine of more than one ^{125}I atom per SpA molecule resulted in loss of binding activity.

In contrast, SpA with > 1 atom of ^{125}I introduced through linkages at lysine residues is 80–90% reactive with IgG (Langone *et al.*, 1977a; Langone, 1978, 1980a). Similarly, reductive methylation introduces hydroxymethyl groups into lysine residues but does not affect the functional activity (Wilder *et al.*, 1979; Tack and Wilder, 1981). These results indicate a limited and perhaps insignificant role for lysine residues and is consistent with the results of Sjöholm *et al.* (1972), who found that labeling all accessible lysines reduced SpA activity by only

40% (see above). Several other functionally active derivatives incorporating fluorescent molecules, enzymes, radioactive nuclides, or other groups have been prepared for use primarily as tracers in immunoassay. They are discussed in Section VII. When ligands are introduced into tyrosine residues, labeling is generally limited to one tracer molecule per SpA. When lysine residues are labeled, the ratio is not so crucial for retention of functional activity.

b. Modification of IgG. Similar reactions have been used to modify lysine or the phenol ring of tyrosine residues in human (Kronvall *et al.*, 1970b; Stewart *et al.*, 1978) and rabbit (Stewart *et al.*, 1978) IgG. Acetylation with acetic anhydride rendered normal human IgG and three myeloma IgG proteins completely unreactive with SpA (Kronvall *et al.*, 1970d). Since the reagent is highly reactive and can acylate amino, sulfhydryl, phenol, and hydroxyl groups, it is difficult to determine the role of individual amino acids. However, carbamylation with potassium cyanate is more selective, reacting with lysine 6-amino groups and with sulfhydryl groups. Amidination (e.g., with ethylacetimidate) occurs at free amino groups, but unlike carbamylation does not alter the molecular charge. Only carbamylation had a significant effect, eliminating the ability of normal IgG and two of the myeloma proteins to precipitate with SpA. However, even though insoluble complexes were not formed they were still able to bind SpA since they inhibited precipitation of SpA by a normally precipitating Ig. These results suggest that sulfhydryl and/or lysine residues in IgG may be involved in a secondary reaction responsible for precipitation with SpA, but that the initial binding reaction is independent of this interaction. The mechanism of SpA–IgG precipitation is discussed in more detail in Section V,G. Results consistent with this hypothesis were obtained by Stewart *et al.* (1978), who tested the ability of modified human and rabbit IgG to inhibit the agglutination of sensitized sheep erythrocytes by SpA and therefore were considering the primary binding reaction, not the secondary precipitation process. They found that modification of 50–60% of the lysine residues by carbamylation or with fluorescamine did not alter the reactivity of either IgG. In addition, nitration of up to 18 or 19 tyrosine groups had no effect on inhibitory effectiveness, suggesting that tyrosine in IgG is not important in the binding step.

Skvaril *et al.* (1980) have used the interaction of chemically modified IgG with SpA as an indicator of the nativeness of the Fc region in γ-globulin preparations for clinical applications. They found that sulfonated human IgG, in which only subclasses 1, 2, and 4 could be de-

tected immunochemically, behaved like normal IgG in that all of it bound to SpA–Sepharose at neutral pH and was eluted at pH 2.8. In contrast, approximately 50% of each of these subclasses in IgG treated with β-propiolactone failed to bind. Identity of the modified amino acids and the degree of alkylation and acylation were not reported, but lysine, histidine, and cystine probably were involved. Although binding to SpA was affected, the same treatment does not alter Fab (i.e., antibody) activity or several Fc effector functions including metabolism, complement fixation, or transport of IgG across the placenta. Finally, reduction and alkylation of Fc fragments of human IgG yielding 0.8 cystine residue per mole did not inhibit reactivity with SpA. This indicates that the heavy-chain disulfide linkage is not required for SpA binding to Fc (Kronvall and Frommel, 1970) (see Section V), although it is necessary for complement fixation (Wiedermann *et al.*, 1964). Thus, while tyrosine or histidine residues are important in SpA [see also Wright *et al.* (1977) for supporting nuclear magnetic resonance (NMR) data], there is no convincing chemical evidence to support a role for specific amino acids in IgG even though changes in the CD spectrum of Fc–SpA complexes imply that aromatic amino acids are involved (Sjöholm, 1975b).

V. Reactivity with Immunoglobulins

A. Phylogeny

There were two major conclusions from the work of Forsgren and Sjöquist (1966) (Section I): the reaction of SpA with IgG was nonspecific in contrast to an immune reaction, and the binding site for SpA was located in the Fc region. This led the way for systematic and detailed studies into the structure and biological activity of SpA and the distribution of IgG reactivity among species.

Kronvall and co-workers (1970a, 1974) found that serum from all 65 mammalian species representing 7 classes and 30 orders of vertebrates were reactive. Sera from some species (37 of 65) precipitated SpA and also inhibited precipitation of a human myeloma IgG when preincubated with SpA, whereas many (28 of 65) failed to give precipitin lines but were effective inhibitors. Of the 72 species of birds tested, representing 15 of 28 orders, only two primitive species (*Rhea americana* and *Pterocnemia pennata*) were reactive. The reactive globulin was isolated by chromatography on Spehadex G-150 or G-200 and shown to have a molecular weight of approximately 185,000

and an electrophoretic mobility similar to that of chicken IgY (Kronvall *et al.*, 1974). None of the sera from five species of primitive fishes nor examples of amphibians (bullfrog) or reptiles (anaconda, crocodile, or snapping turtle) bound SpA (Kronvall *et al.*, 1970a). Based on these studies the authors speculated that the Fc structure of IgG involved in binding with SpA evolved as long ago as 200 million years (Kronvall *et al.*, 1974). Grov *et al.* (1970) and Goudswaard *et al.* (1978) also tested sera from 13 and 8 mammalian species, respectively, and found similar results.

Other workers (Lind and Mansa, 1968; Lind *et al.*, 1970) used immunoelectrophoresis to analyze sera from human, dog, swine, cow, and sheep after absorption with formalin-treated *S. aureus* Cowan strain I or with *S. aureus* Wood 46 as a negative control. They found specific absorption of essentially all IgG from human, swine, and dog serum, but only the slow migrating minor subclasses of cow and sheep IgG were reactive. These results were consistent with the relatively weak reactivity of goat and sheep IgG observed by Kronvall *et al.* (1970a), and also suggested that the nature of the subclass may be a determinant of SpA reactivity.

Although these results were essentially qualitative, the relative reactivity of IgG fractions from several species has been quantified on the basis of a competitive inhibition assay (Langone *et al.*, 1977a; Langone, 1978, 1980a). Rabbit IgG immobilized on polyacrylamide beads and known amounts of test IgG in the fluid phase compete for a limited amount of pure ^{125}I-labeled SpA. Inhibition of [^{125}I]SpA binding depends on the amount of IgG added, and dose response curves are obtained that permit estimation of inhibitory effectiveness with the amount of IgG required to inhibit binding by 50% under standard conditions chosen for comparison. Results for the 13 species tested are summarized in Table IV and show that reactivity differed by over a factor of 10^3 and was consistent with the earlier qualitative tests and the results discussed in the following sections. A similar approach was used later by Inganäs *et al.* (1980), who based their test on inhibition of human ^{125}I-labeled Fc binding to SpA-Sepharose. Results from the two tests correlated very well (Table IV). Evidence that some Igs from nonmammalian species may also bind SpA (Zikán *et al.*, 1980) is discussed in Section V,B,10.

These studies made it clear that although SpA activity was characteristic of IgG from most mammalian species, there were significant quantitative differences, and the studies led to a closer look at subclass specificity and a systematic examination of the reactivity with classes other than IgG from several species.

TABLE IV
INHIBITON OF ^{125}I-LABELED PROTEIN A BINDING TO IMMOBILIZED RABBIT IgG OR OF ^{125}I[IgE] OR [^{125}I]Fcγ BINDING TO PROTEIN A-SEPHAROSE BY IgG FROM DIFFERENT SPECIES[a]

Species	Inhibition of binding of		
	[^{125}I]SpA to rabbit IgG-polyacrylamide[b]	[^{125}I]Fcγ to SpA–Sepharose[c]	[^{125}I]IgE to SpA–Sepharose[c]
Human	0.06	2	7
Rabbit	0.05	3	>500
Guinea pig	0.06	2	35
Pig	0.14	7	10
Dog	0.29	11	3
Cow	3.0	245	>500
Mouse	4.5	310	>500
Horse	5.0	280	>500
Sheep	40	>500	>500
Goat	>100	>500	>500
Rat	>100	>500	335
Chicken	>100	ND[d]	ND
Rhesus monkey	ND	5	21

[a] Data given as micrograms of IgG required to inhibit binding by 50% under standard conditions established for each assay.
[b] Adapted from Langone (1978).
[c] Adapted from Inganäs *et al.* (1980).
[d] ND, not done.

B. IMMUNOGLOBULIN CLASSES, SUBCLASSES, AND FRAGMENTS FROM DIFFERENT SPECIES

1. General

The ability of Ig molecules to bind SpA has been studied by several workers usually using the gel precipitation techniques described earlier. This approach was developed into a quantitative test (Kronvall and Williams, 1969) using one-dimensional diffusion in agar gel incorporating SpA. In a related test, samples that failed to give precipitates could be shown to be reactive by their ability to inhibit precipitation of a myeloma IgG–SpA combination under standard conditions. In addition, absorption by SpA on intact bacteria or affinity chromatography using SpA–Sepharose has proved to be particularly useful, since procedures for bulk or stepwise elution of bound components permits quantitative as well as qualitative analysis and, in some cases, the relatively simple separation of Ig subclasses. The reactivity of immunoglobulin classes and subclasses

from the species that have been tested is discussed in the following sections.

2. *Human Immunoglobulins*

a. IgG. The early absorption experiments and the work of Forsgren and Sjöquist (1966) established that human IgG reacted efficiently with SpA. Following from this, SpA–Sepharose or intact bacteria have proved to be useful adsorbents for selectively removing >95% of IgG from human serum (Ankerst *et al.*, 1974; Chantler *et al.*, 1976; Langone *et al.*, 1979; Langone, 1980a). Kronvall and Williams (1969) were the first to determine that human IgG subclasses 1, 2, and 4 bound SpA, but reported that IgG_3 did not. They tested a total of 68 myeloma proteins; 16 of 41 IgG_1 proteins gave a precipitin line against SpA, and the remaining 25 gave positive inhibition tests. All 9 IgG_2 proteins also were reactive. Seven precipitated SpA, and the other two inhibited precipitation of the standard IgG_1–SpA mixture. Two of four IgG_4 proteins precipitated SpA, and the remaining two were inhibitors. Twelve of the 14 IgG_3 proteins failed to precipitate SpA directly or to inhibit, and the weak inhibition observed with the other two was attributed to the presence of low amounts of contaminating normal IgG. When binding of ^{125}I-labeled myeloma proteins to S. *aureus* was tested (Ankerst *et al.*, 1974), >96% of IgG_1, IgG_2, and IgG_4 were bound, but only 1.5% of the IgG_3. Just as myeloma proteins have contributed immensely to our understanding of Ig structure and certain aspects of biological activity, they have accounted for a good deal of our knowledge of SpA selectivity. Thus IgG_3 generally accounts for less than 5% of normal human IgG (Shur, 1972; Hjelm, 1975), and the techniques commonly used to test reactivity between SpA and components in whole serum were not sensitive enough to discriminate the subtle quantitative differences with human IgG subclasses.

Adsorption on SpA-Sepharose with single-step acid elution has been used to isolate IgG from serum (Hjelm *et al.*, 1972), and since binding depends on pH, gradient elution has been used to purify the individual subclasses IgG_1, IgG_2, and IgG_3 (Duhamel *et al.*, 1979). Although IgG_3 was found exclusively in the nonadsorbed fraction (see also Zola *et al.*, 1978) and is consistent with the reported failure of myeloma IgG_3 to bind SpA (Kronvall and Williams, 1969), Skvaril (1976) showed that up to 35% of the IgG_3 applied to SpA–Sepharose as Cohn fraction II or in whole serum was adsorbed at neutral pH and eluted under acid conditions. Other reports have also demonstrated trace amounts of IgG_3 along with sub-

classes 1, 2, and 4 in acid eluates when γ-globulin fractions were chromatographed this way (Van Kamp, 1979; Skvaril *et al.*, 1980). In these experiments care was taken to rechromatograph the eluted IgG_3 and demonstrate that binding was not due to nonspecific adsorption. Differences in the affinity of SpA binding to subclasses of human and guinea pig IgG also was indicated by elution of multiple peaks from SpA–Sepharose using a gradient of 1.4 to 2.7 *M* sodium thiocyanate (Endresen, 1979b). In view of these results, the original idea that IgG_3 does not react with SpA must be modified to account for the variable, but often significant, binding of polyclonal IgG_3. The structural basis for the persistent reactivity of a fraction of IgG_3 is not known, but may indicate the presence of further subclass diversity. The concept of using reactivity with SpA as a basis for detecting subclasses of IgM, IgA, and possibly IgE are discussed below.

Fragments and subfragments of IgG have been tested for reactivity with SpA to localize the active site in the Ig molecule. The data strongly indicate that the major reactivity resides in the Fc region. Several workers using precipitation with SpA, inhibition of precipitation in a standard gel diffusion system, or binding to immobilized SpA have shown that isolated IgG light chains (Forsgren and Sjöquist, 1966; Kronvall, 1967; Kronvall and Frommel, 1970), and the fragments Fab (Forsgren and Sjöquist, 1966; Kronvall, 1967; Kronvall and Frommel, 1970; Skvaril *et al.*, 1980), $F(ab')_2$ (Kronvall and Frommel, 1970; Skvaril *et al.*, 1980), Fc′ (Kronvall and Frommel, 1970), and pFc′ (Skvaril *et al.*, 1980) do not bind SpA. In each of these studies heavy chains and the intact Fc fragments were reactive, as well as Fc dimers resulting from plasmin digestion (Skvaril *et al.*, 1980). Consistent with these results, Kronvall (1967) showed that myeloma IgG proteins with specificity for streptolysin O bound SpA at the Fc region but retained antigen binding activity at the Fab sites.

By trypsin digestion of acidified Fc fragments, smaller subfragments shown in Fig. 4 were isolated with a molecular weight range 12,000–42,000. The low-molecular-weight piece did not bind SpA and may originate from the C_H3 region. All the reactive species contained intact Fc fragments (C_H2 and C_H3 domains) along with covalently bound peptides and exhibited other effector functions including complement fixation and binding of rheumatoid factor. The nonreactive subfragments fixed complement but did not bind rheumatoid factor, and they are probably derived from the C_H2 region. Thus, these results also strongly suggest that an intact Fc region is

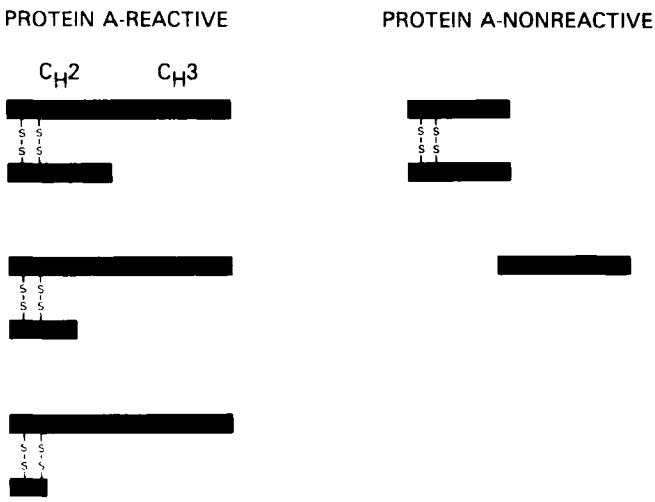

FIG. 4. Diagram representing Fc subfragments of human IgG obtained after trypsin digestion of acidified human Fc. Only those fragments with intact C_H2–C_H3 regions (i.e., at least one intact Fc chain) bind protein A. (Adapted from Endresen and Grov, 1976.)

necessary for SpA reactivity, and that intrachain rather than interchain interactions are important. This is consistent with the crystallographic data of Deisenhofer *et al.* (1978) discussed above that showed close association between fragment B of SpA and both the C_H2 and C_H3 regions of the Fc fragment.

Binding of some polyclonal IgE to SpA–Sepharose has been shown to occur through sites located in the Fab region (see below). Based on inhibition experiments using ^{125}I-labeled IgE and either whole IgG or IgG fragments as inhibitors, significant SpA activity also was shown to reside in the Fab region of the IgG molecule (Inganäs *et al.*, 1980; Inganäs, 1981). Thus, the $F(ab')_2$ fragments and intact IgG were equally effective inhibitors of [^{125}I]IgE binding, whereas Fc fragments were inactive. In contrast, when four myeloma IgG_1 proteins, their $F(ab')_2$ and Fc fragments were tested as inhibitors of [^{125}I]Fcγ binding to SpA–Sepharose, only the Fc fragments were active. The inhibition results with IgG from several species are summarized in Table IV as the amount of IgG required to inhibit the binding of [^{125}I]IgE or [^{125}I]Fcγ by 50% (Inganäs *et al.*, 1980). Except for the rabbit, IgG that showed strong Fc activity against [^{125}I]Fcγ also showed Fab activity against [^{125}I]IgE. Rabbit IgG behaved anomalously, showing strong Fc activity, but little or no Fab activity. Overall these results correlate very well with the

ability of different species of IgG to inhibit the binding of [^{125}I]SpA to immobilized rabbit IgG (Table IV), which we have used as a criterion of reactivity with SpA (Langone, 1978, 1980a).

b. IgM. Although the reaction between SpA and IgG has been studied most extensively, results have shown that other classes of human Ig also are reactive. The efforts of Harboe and Fölling (1974) to develop an immunoassay for idiotypic determinants on IgM using IgG antibodies and formalin-treated *S. aureus* to precipitate immune complexes, led to the finding that one of the ^{125}I-labeled monoclonal proteins, designated IgM(Se), bound to the bacteria in the absence of added antibody. This binding was inhibited by unlabeled IgM(Se), and inhibition by other sources of IgM was used as the basis for determining their reactivity. Monoclonal IgM(Ka) neither bound to *S. aureus* nor inhibited binding of [^{125}I]IgM(Se) and was used as a negative control. Of the 33 sera that contained over 30 mg IgM per milliliter, 11 inhibited binding between 88 and 96% and 22 failed to inhibit significantly under the assay conditions. One other sample gave questionable results. In comparison, approximately 20 times more IgG than cold IgM(Se) was required to give 50% inhibition, so inhibition by IgG in these and other sera was discounted. Polyclonal IgM isolated by repeated gel filtration of pooled or individual normal sera was approximately 50% as effective as IgM(Se) on a weight basis in the inhibition test, and the IgG-free macroglobulin fraction from 15 individual sera isolated by ultracentrifugation also inhibited significantly. Subsequent studies (Saltvedt and Harboe, 1976) corroborated these results using a second ^{125}I-labeled monoclonal protein [IgM(Ba)] that also bound to *S. aureus*.

These experiments (Harboe and Fölling, 1974) failed to detect reactivity between SpA and IgM(Se) in solution, and IgG was a poor inhibitor of [^{125}I]IgM(Se) binding, so the identity of SpA as the cell-surface receptor was still in question. However, Lind *et al.* (1975), using 15 of the original IgM monoclonal proteins, demonstrated that all 7 that inhibited [^{125}I]IgM(Se) binding reacted with SpA in gel diffusion and in an indirect hemagglutination test. All 8 that failed to inhibit binding of [^{125}I]IgM(Se) were negative in both tests. From these results, it was concluded (Harboe and Fölling, 1974; Saltvedt and Harboe, 1976) that a positive or negative reaction with SpA was not due to the presence of allotypes, but that two subclasses of human IgM were distinguished on the basis of SpA reactivity. One subclass, designated IgM_2, reacted with SpA; the other, IgM_1, did not.

Grov (1975a,b) tested the binding to SpA–Sepharose of IgM pro-

teins from sera of 7 patients with macroglobulinemia. Three of the proteins bound, and only these three gave positive reactions with SpA in gel diffusion. Two of the three proteins contained both SpA-reactive and nonreactive IgM (Grov, 1975a), as did three IgM preparations from different pools of normal human serum (Grov, 1975b). Antibodies to one reactive [IgM(AS)] and one nonreactive (IgM9628) IgM protein were prepared and used in gel diffusion and hemagglutination inhibition experiments to show that SpA-reactive IgM possessed a determinant(s) not present in the unreactive molecule. Thus absorption of the antisera to IgM(AS) on a column containing immobilized IgM9628 left antibodies that apparently were directed against SpA-reactive structures in IgM(AS). The Fab' fragments of these antibodies inhibited precipitation in gel and hemagglutination of tanned sheep cells sensitized with IgM(AS). Fab' fragments of the antibodies eluted from the immunosorbent column had no effect on these reactions. The reaction of IgM preparations from the normal sera also were inhibited by the Fab' fragments from specific anti-IgM(AS). Since both SpA-reactive and nonreactive IgM were present in the pooled normal sera, Grov (1975b) also concluded that two subclasses of IgM were differentiated on the basis of SpA reactivity. Brunda *et al.* (1977) also reported that up to 80% of an ^{125}I-labeled IgM myeloma protein used in their work bound to *S. aureus*.

Conflicting reports have appeared on the ability of IgM fragments to interact with SpA. However, the discrepancies may result from the use of different myeloma proteins or differences in the test systems used. Harboe and Fölling (1974) found that 7 S (reduced and alkylated or with intact disulfide bands) and monomeric Fab_u fragments of IgM(Se) failed to inhibit binding of [^{125}I]IgM(Se) to *S. aureus* in their assay (see above). Reduction and reassociation gave an active product. Treatment with propionic acid, potassium thiocyanate, or urea under denaturing conditions destroyed inhibitory activity and was taken as evidence that the tertiary Ig structure was an important determinant of reactivity with SpA. In contrast, the direct binding of fragments to SpA–Sepharose and inhibition of precipitation were used by Grov (1975a), who found that 7 S (reduced and alkylated or with intact disulfide bonds), Fcu, and $(Fc)_5u$ fragments of monoclonal IgM(AS), as well as the intact IgM protein treated with sodium thiocyanate still reacted with SpA, although in some cases the properties of the reactions in gel were modified. Like Harboe and Fölling, he found that Fab_u fragments did not react with SpA. Using a single monoclonal IgM, Ankerst *et al.* (1974) found that less than 3% of the intact molecules or the monomeric product bound to *S. aureus*. In principle, the

ability to detect direct binding of the fragments to SpA is more suitable than competitive inhibition assays, since the avidity of a polymeric molecule may be significantly higher than that of a monovalent fragment and give the false impression that a weakly reactive molecule is nonreactive. On this basis, Grov's conclusion that IgM activity resides in the Fc portion is probably correct. Similar enhancement in the affinity for SpA due to aggregation through immune complex formation has been reported for IgA (Harboe and Fölling, 1974). Immune aggregation and reactivity with SpA is discussed more fully in Section V,C.

Inganäs (1981) has also used competitive binding experiments to study the reactivity of human IgM with SpA. Inhibition curves were obtained by incubating differing amounts of monoclonal IgM proteins with SpA-reactive polyclonal [^{125}I]IgE or [^{125}I]Fcγ fragment in the presence of SpA–Sepharose. Although there was no significant inhibition of [^{125}I]Fc binding, all four IgM proteins inhibited the binding of [^{125}I]IgE, and three of them inhibited at least as well as polyclonal IgG. This reactivity appears to reflect SpA binding to the F(ab')$_2$ fragment of IgM related to the F(ab')$_2\epsilon$ interaction discussed below. From these results Inganäs has questioned the validity of using SpA reactivity as a basis for distinguishing IgM subclasses. However, it is not possible to compare directly these results and those described above, since the test systems were quite different. It may be that the four IgM proteins used by Inganäs would fall into the IgM_1 class as defined by Harboe and co-workers, while their proteins defined as IgM_1 would have to be tested for their ability to inhibit [^{125}I]IgE binding. It should be emphasized that Harboe and co-workers tested only a single dilution of myeloma sera, and so strict quantitation was lacking in their experiments. It is possible that weak reactivity could be detected if more concentrated preparations of purified proteins designated IgM_1 were tested.

We have found naturally occurring IgM antibodies to the folate derivatives methotrexate and folinic acid in normal sera of several species, including human (Borsos *et al.*, 1981a,b). Antibody class was determined by a hemolysis assay using sheep erythrocytes coated with the appropriate drug and by other physicochemical and immunochemical tests that also demonstrated the specificity of these antibodies. Up to 2000 molecules of IgM per cell (1.5×10^7 cells) did not bind detectable levels of [^{125}I]SpA directly but could be demonstrated easily by treating the IgM-sensitized cells with the IgG fraction of rabbit antibodies specific for human mu chains followed by [^{125}I]SpA. The concentration of complement-fixing IgM antibody in the serum

calculated from hemolysis data compared favorably with the values obtained by the indirect radioassay (Borsos *et al.*, 1981b). Absorption of sera with immobilized SpA (Langone *et al.*, 1979), especially as a preliminary to analysis for anti-rubella antibodies of classes other than IgG (Ankerst *et al.*, 1974; Chantler *et al.*, 1976) has shown little effect on the level of total serum IgM or IgA.

The human lymphoblastoid cell line LA173 has been shown to secrete IgM labeled with [^{3}H]leucine that binds specifically to *S. aureus* Cowan I but not to Wood strain 46, gives a typical precipitin curve with purified SpA and a line of identity in gel diffusion against SpA and anti-IgM (Howell-Saxton and Wettstein, 1978). IgM secreted by lymphoblastoid cell line RPMI 1788 gave a precipitin line with anti-IgM, but not with SpA. These workers also showed that intracellular 19 S IgM pentamers, 8 S (H_2L_2) monomeric HL subunits, and intermediates between 8 S and 19 S bound to SpA. These included glycosylated and nonglycosylated fragments and indicates that SpA-binding structures are incorporated into the IgM molecule at an early stage. Also, since saturation of bacteria with normal IgG inhibited binding of 8 S IgM monomers (and IgG) almost completely, but did not affect binding of 19 S pentamer, it appears that the intact IgM pentamer binds to SpA with a much higher avidity than the monomeric form or IgG.

Romagnani *et al.* (1980a) also demonstrated that a population of B lymphocytes (>30%) from human tonsil tissue is capable of forming rosettes with SpA-coated human erythrocytes, even after depletion of IgG-bearing cells. Incubation of these remaining cells with F(ab')$_2$ fragments of anti-human mu-, gamma-, or delta-chain antibodies reduced the number of rosettes, as did preincubation of the total lymphocyte population. The results suggested that SpA interacted with IgM and/or IgD on the cell membrane, in addition to IgG, although other possibilities, including the presence of reactive IgA or IgE on the cell surface, were not excluded.

In his studies on the isolation of cell-surface antigens using *S. aureus* as a precipitating agent, Kessler (1975, 1976, 1981) found, by polyacrylamide gel electrophoresis, bands corresponding to IgM as well as IgG solubilized from the surface of human peripheral blood lymphocytes. The presence of IgD or IgA could not be ruled out since they comigrated with IgM and IgG, respectively, under the assay conditions (Kessler, 1976).

c. IgA. The direct binding and competitive inhibition radioassays, and the gel precipitation techniques used to study the interaction of

IgG and IgM with SpA have been used to show that IgA from human colostrum, or polyclonal and monoclonal serum IgA, can bind SpA. McDowell *et al.* (1971b) were the first to suggest that colostrum IgA was reactive, and Grov (1976) later separated three such IgA preparations into SpA-reactive and nonreactive fractions by chromatography on SpA–Sepharose. The reactive proteins totaled 20–33% of total IgA, activity was localized in the Fc region since pepsin-digested samples failed to bind to the column, and there appeared to be no correlation between IgA subclass and SpA binding.

Reports (Harboe and Fölling, 1974; Saltvedt and Harboe, 1976) also showed that 2 of 7 myeloma IgA sera inhibited the binding of [^{125}I]IgM(Se) to *S. aureus* and the trimeric form of one reactive IgA inhibited even more effectively than cold IgM(Se), while the monomer was much less effective. Approximately 80% of trimeric [^{125}I]IgA bound to *S. aureus* and cold trimeric IgA and the two myeloma sera that inhibited [^{125}I]IgM(Se) binding were effective competitors. IgG and IgM(Se) failed to inhibit, suggesting that trimeric IgA has a higher affinity than the monomeric form or than IgM(Se) for SpA. Ankerst *et al.* (1974) found that only 4% of the IgA myeloma protein they tested bound to *S. aureus*, but the subclass was not specified.

When 54 myeloma IgA sera were tested in the inhibition assay against either [^{125}I]IgM(Se) or [^{125}I]IgM(Ba), all 5 IgA_2 proteins inhibited up to 92–98% using either tracer, whereas 48 of the 49 IgA_1 proteins failed to inhibit significantly in either system. Only one IgA_1 serum inhibited [^{125}I]IgM(Se) but not [^{125}I]IgM(Ba) (Saltvedt and Harboe, 1976). These results were not strictly quantitative, since relative reactivities were based only on dilutions of whole serum, but they established a principle, supported by other work (Patrick *et al.*, 1977; Brunda *et al.*, 1977; Virella *et al.*, 1978), that serum IgA_2 but not IgA_1 binds to SpA. For example, three IgA_2 myeloma proteins [two A2m(1) allotypes and one A2m(2) allotype] were bound to SpA–Sepharose and eluted under acid conditions, whereas three IgA_1 proteins did not bind (Patrick *et al.*, 1977). Similarly, Virella *et al.* (1978) found that three IgA_2 proteins bound SpA and six IgA_1 did not, and on the basis of SpA reactivity designated a protein as IgA_1 in spite of other serologic evidence suggesting that it belonged to the IgA_2 subclass. Polen *et al.* (1977) separated an IgA myeloma protein (subclass not given) into an SpA reactive and nonreactive fraction on SpA–Sepharose. The bound material was eluted with 3 *M* sodium thiocyanate and accounted for 30% of the total IgA. The nonreactive IgA was shown to be complexed with α_2-macroglobulin probably through Fc sites that blocked SpA

binding, since the dissociated IgA bound SpA. Inability of the pepsin-treated IgA to bind SpA suggested that reactivity was restricted to the Fc region.

It was shown (Van Kamp, 1979; Brunda *et al.*, 1979) that some IgA_1 as well as IgA_2 can bind to SpA. Pooled human serum was applied to a column of SpA–Sepharose (Van Kamp, 1979), and bound components eluted under acidic conditions. The acid eluate contained all four IgG subclasses (including a trace of IgG_3) and approximately 30% of the applied IgA. The IgA subclass distribution was the same in the bound and unbound fractions, indicating that there was no subclass selectivity, in agreement with the results obtained by Grov (1976) for colostrum IgA. Similarly, Brunda *et al.* (1979) used competitive inhibition and direct binding assays to show that 7 of 16 IgA_1 myeloma sera or purified IgA_1 proteins bound to SpA, while 1 of 4 IgA_2 proteins were reactive. Like several of the other studies, quantification often was limited, since only one dilution of whole serum was tested.

Direct binding experiments using *S. aureus* and several purified and ^{125}I-labeled proteins with a known content of monomers, dimers, trimers, and oligomers failed to show a correlation between degree of polymerization and SpA reactivity. Based on the inhibition pattern seen in heterologous competitive binding assays, the suggestion was made that in addition to a common receptor for IgG and IgA_1, there may be an additional receptor(s) that binds one or the other Ig specifically. However, since the IgA contains polymeric forms that might have different affinity for SpA compared to monomeric IgG (see above) this interpretation is highly speculative. In agreement with results already discussed, binding sites on one of the reactive IgA_1 proteins appeared to be localized in the Fc region, since ^{125}I-labeled $F(ab')_2$ fragments failed to bind to *S. aureus*.

Inganäs (1981) has also tested the ability of four oligmeric IgA_1 myeloma proteins and the $F(ab')_2$ fragments from two of them to inhibit the binding of [^{125}I]Fcγ or [^{125}I]IgE to SpA–Sepharose. Similar to his results with myeloma IgM (see above), he found little or no ability to inhibit [^{125}I]Fcγ, but strong (i.e., more effective than polyclonal IgG) to moderate inhibition of [^{125}I]IgE binding. Since the $F(ab')_{2\alpha}$ fragments inhibited about as effectively as the parent IgA_1 proteins, it appears that reactive sites in the Fab region of IgA, IgG, IgM, and IgE (see below) are detected using [^{125}I]IgE as the tracer. This is in addition to the classical Fcγ binding activity.

d. IgE. The SpA–IgE radioimmunoassay (Johansson and Inganäs, 1978; Inganäs *et al.*, 1980; Inganäs, 1981) was used originally (Johansson and Inganäs, 1978) to demonstrate that polyclonal, but not mono-

clonal, human IgE interacted with SpA. After serum was absorbed with SpA–Sepharose, bound IgE was detected by incubation with ^{125}I-labeled F(ab')$_2$ fragments of rabbit anti-IgE having D_{E1} and D_{E2} specificity. The [^{125}I]F(ab')$_2$ fragment was absorbed with IgG-Sepharose to remove anti-IgG activity and shown not to bind to SpA. There was a strong correlation between SpA binding activity and the level of polyclonal IgE in 78 serum samples determined by radioimmunoassay (Johansson and Inganäs, 1978). However, the degree of reactivity was low: approximately 6–9% of the total IgE in each sample was reactive compared to generally less than 0.1% for each of 9 myeloma IgE proteins. Similarly, we found that [^{125}I]SpA did not bind to one of these myeloma proteins (P.S.) that was immobilized to polyacrylamide beads nor were significant amounts absorbed by SpA–Sepharose when the IgE was added to human serum (Langone *et al.*, 1979). Kronvall *et al.* (1970c) also found that a myeloma IgE did not react with SpA by gel diffusion. ^{125}I-labeled polyclonal IgE bound to several strains (6 of 7) of SpA-containing *S. aureus* but showed only weak binding to 1 of 3 SpA-negative strains (Johansson and Inganäs, 1978).

Data indicate that the SpA-binding site in polyclonal IgE is localized in the Fab region. In the direct binding assay, F(ab')$_2$ but not Fcϵ fragments were absorbed by SpA–Sepharose as efficiently as the intact IgE molecule (Inganäs *et al.*, 1980). Furthermore, there was no detectable decrease in binding of whole IgE after heating at 56°C for 30 minutes, a treatment that destroys activity of the heat-sensitive D_{E2} antigenic determinant (Johansson and Inganäs, 1978).

Zola *et al.* (1978), using radioimmunoassay, found no depletion of IgE from human sera after passage through a column of SpA–Sepharose. However, the discrepancy may be due to the relatively low degree of binding (< 10%) of polyclonal IgE, which could easily be overlooked as insignificant or within the limits of precision of the assay. Over 98% of ^{125}I-labeled monoclonal IgE also was recovered in the unbound fraction.

e. IgD. The relatively small amount of data available has failed to demonstrate reactivity between human IgD and SpA. Kronvall *et al.* (1970a) found that an IgD myeloma protein did not react with purified SpA, based on gel diffusion experiments, and Ankerst *et al.* (1974) found that an ^{125}I-labeled myeloma IgD did not bind to *S. aureus*. Similarly Forsgren and Grubb (1979) showed that ^{125}I-labeled IgD did not bind to Cowan strain I, although there was significant binding to several other bacterial species, particularly *Neisseria catarrhalis* and *Haemophilus influenzae*, apparently through sites located mainly, but

not exclusively, in the C_H1 region of the IgD molecule. Others (Kessler, 1975; Romagnani *et al.*, 1980c) have only suggestive and inconclusive evidence that IgD on the lymphocyte surface may interact with SpA.

3. *Pig Immunoglobulins*

Procine IgG also binds to SpA–Sepharose and, like human and other species of IgG, has binding sites in the Fab as well as the Fc region. Papain, pepsin, and trypsin fragments have been tested, and the binding activity compared to whole serum IgG (Milon *et al.*, 1978; Endresen, 1979a,b; Zikán, 1980a) or to two fractions isolated by ion exchange chromatography and designated IgG_1 and IgG_2 (Zikán, 1980a). Up to 90–96% of intact porcine IgG binds to SpA–Sepharose (Milon *et al.*, 1978; Goudswaard, *et al.*, 1978; Endresen, 1979a; Zikán, 1980a), but the binding capacity of the adsorbent was only about 70% of the capacity for human IgG (Milon, *et al.*, 1978). A small percentage of IgG failed to bind even when rechromatographed. Since it has been shown with rabbit IgG that antibody-bound $C\bar{1}$ can inhibit the binding of SpA (Langone *et al.*, 1978a), it may be that the Fc region of the small fraction of unreactive material is blocked by steric hindrance or simply has undergone some degree of conformational change. In any case, at least 90% of both subclasses bound to SpA but gave different profiles when eluted with a gradient of magnesium chloride (Zikán, 1980a). Each class gave a heterogeneous elution pattern, perhaps owing to the ability of high concentrations of salt to disrupt intramolecular noncovalent bonds, giving a series of products with differing affinities for SpA.

The Fc fragment binds to a similar extent as whole IgG (Milon *et al.*, 1978; Zikán, 1980a), but Fc′ (Milon *et al.*, 1978; Endresen, 1979a; Zikán, 1980a), and smaller subfragments of Fc, including one that appears to represent the C_H3 domain (Endresen, 1979a), do not. Thus like human and other species of IgG, the intact C_H2–C_H3 linkage is required for optimal reactivity. Endresen (1979a) reported that only 5–15% of Fc isolated by papain digestion in the presence of cysteine bound to SpA, but this was probably because of overreaction with the enzyme, since an abnormally heterogeneous product with several low-molecular-weight cleavage products was obtained.

Fab and $F(ab')_2$ fragments also bound efficiently to SpA–Sepharose, but the binding site apparently is unrelated to that in the Fc fragment (Zikán, 1980a). Thus approximately half the Fab (40%) and $F(ab')_2$ (45–59%) fragments from whole IgG bound and could be eluted under acid conditions or with magnesium chloride. Similar results

were obtained for the Fab fragments from IgG_1 (46%) and IgG_2 (40%) (Zikán, 1980a), and Milon *et al.* (1978) reported that 50% of the Fab fragments from papain treatment and $F(ab')_2$ fragments from pepsin cleavage of whole IgG bound to SpA. Endresen (1979a,b) also found that Fab fragments were reactive, but the binding appears to be heterogeneous, since a diffuse pattern was observed with gradient elution from SpA–Sepharose (Endresen, 1979a; Zikán, 1980a). The binding sites on the Fc and Fab regions appear to be heterogeneous and independent, based on competitive adsorption experiments (Milon *et al.*, 1978) and the ability of Fc fragments to bind to SpA–Sepharose even after Fab sites have been saturated, and vice versa (Zikán, 1980a). Approximately 60% of porcine colostrum IgG also binds to SpA through sites located in the H chain (58% binding of purified fragments), but not through the L chain (no detectable binding) (Zikán, 1980a).

Also, like human and certain other species (see below), porcine IgM and IgA can bind SpA through sites in the Fab region (Milon *et al.*, 1978; Goudswaard *et al.*, 1978; Zikán, 1980b). Thus 40% of serum IgM and 47% of dimeric colostrum IgA bound to SpA–Sepharose. Reactivity of the intact immunoglobulin was accounted for by binding of tryptic or peptic Fab_u (43 and 56% binding) and peptic Fabα (37%) fragments, whereas only 6–12% of Fc5u fragments were reactive. SpA–Sepharose saturated with IgG, IgM, or IgA was still able to bind the other classes, although there was some degree of displacement of one class by another. Similar to results with Fab fragments of IgG, elution from SpA–Sepharose of whole IgM or IgA with a gradient of magnesium chloride gave several peaks indicating heterogeneity, possibly resulting from an effect on the conformation of the Ig. It is not clear why only about half the IgM and IgA bind to SpA. Although there is no immunochemical or other evidence to support the contention that subclasses are distinguished on the basis of reactivity with SpA, the possibility is viable and is reminiscent of a similar effort to distinguish two subclasses of human IgM on the same basis (Section V,B,2,b).

4. Rabbit Immunoglobulins

Aside from Endresen (1978), who found only 15% reactivity, most accounts agree that rabbit IgG binds efficiently to SpA and essentially all can be removed from whole serum by absorption with SpA–Sepharose (Goding, 1976; Langone *et al.*, 1978b; Miller and Stone, 1978; Boyle and Langone, 1979, 1980; Langone 1980a; Zikán, 1980a). Although the small amount of IgG that reportedly does not bind (Goding, 1976) may suggest the presence of subclasses, there is no evi-

dence to support this contention. There are at least three more likely explanations. One derives from the finding by Miller and Stone (1978) that a protease inhibitor must be added to the buffer when IgG is isolated from whole serum to prevent partial digestion of SpA from the column matrix by serum enzymes. Since most workers have not taken this precaution, it may be that a small amount of active SpA cleavage fragments bind to the Fc region of IgG and prevent adsorption to the column. Also, as described above, there is always the possibility that failure to bind SpA results from loss of activity during isolation or the presence of serum components bound to the Fc region that prevent SpA binding through steric hindrance. Finally, although the binding constant for rabbit IgG–SpA binding is high (Section V,C,1), equilibration would result in a trace of unbound IgG. This could be tested by rechromatography of the unbound fraction. In any case, rabbit IgG isolated by affinity chromatography retains full antibody activity and antigenic properties and is free of ribonuclease, unlike IgG prepared by salt precipitation and ion exchange chromatography (Miller and Stone, 1978).

Most of the reports indicate that binding activity in rabbit IgG is localized primarily in the Fc region, although a trace of activity is associated with the Fab part of the molecule perhaps outside the antigen combining site (Forsgren and Sjöquist, 1967; Goding, 1976; Stewart *et al.*, 1978; Endresen, 1979b; Zikán, 1980a). Stewart *et al.* (1978) used inhibition of SpA hemagglutinating activity against sensitized sheep erythrocytes to test the relative ability of IgG fragments to bind SpA and found the Fc fragment from pepsin digestion to be as effective as intact IgG. Plasmin digestion gave fragment Facb incorporating the C_H2 domain, together with $F(ab')_2$ and pFc′ including the C_H3 domain, These fragments were unreactive, and similar to other species of IgG, and the results of Deisenhofer *et al.* (1978) suggest that covalently bound C_H2–C_H3 units are required for reactivity of rabbit IgG with SpA. Consistent with this interpretation, unfractionated plasmin digests containing associated Facb and pFc′ fragments but with cleaved gamma chains, had lost binding activity. Pepsin and trypsin fragments $F(ab')_2$, Fab, pFc′, and tFc′ also were unreactive by hemagglutination inhibition (Stewart *et al.*, 1978) and in gel diffusion tests (Forsgren and Sjöquist, 1967), or only low levels [e.g., 2–3% of $F(ab')_2$] bound to SpA–Sepharose (Endresen, 1978; Zikán, 1980a). Lancet, *et al.* (1978) used spectroscopic methods to study the interaction between SpA and fragments of rabbit IgG, and they too found that pFc′ and Facb were unreactive, suggesting that binding activity required intact C_H2–C_H3 domains.

Evidence so far indicates that rabbit IgM does not bind SpA (Langone *et al.*, 1978b; Boyle and Langone, 1979, 1980). When rabbit antisera to the guinea pig hepatoma cells, line 1 or line 10, were absorbed with SpA–Sepharose, IgM cytolytic activity was not affected, whereas all IgG activity was absorbed. In contrast, IgM binds to the lectin concanavalin A (Con A), so IgM antibody activity is absorbed by Con A–Sepharose but IgG is not (Langone *et al.*, 1977b, 1978b; Boyle and Langone, 1979). This selectivity led to the development of a simple absorption procedure to give whole serum free of IgG or IgM antibodies (Boyle and Langone, 1980), and, under conditions where essentially all IgG was absorbed, there was no significant absorption of IgM (Boyle and Langone, 1980). Although it does not bind SpA directly, cell-bound IgM can be quantified by adding an anti-rabbit IgM antibody that binds [^{125}I]SpA (Boyle and Langone, 1979; Borsos *et al.*, 1981a,b).

5. *Guinea Pig Immunoglobulins*

Both guinea pig subclasses IgG_1 and IgG_2 bind to SpA (Forsgren, 1968; Stålenheim and Malmheden-Eriksson, 1971; Grov, 1973; Endresen, 1979b), but IgM does not (Forsgren, 1968). Based on gradient elution from SpA–Sepharose, IgG_2 appears to have a higher affinity, and although binding activity is located primarily in the Fc region, Fab activity also has been detected. Thus low but significant amounts of $F(ab')_2$ fragments isolated from guinea pig antibodies to egg albumin (Grov, 1973; Endresen, 1979b) and measles virus (Endresen, 1979b) by affinity chromatography bound to SpA–Sepharose, suggesting that Fab activity is located outside the antigen-binding site. Forsgren (1968) found that heavy chains and Fc fragments, but not light chains, of both classes inhibited precipitation of IgG by SpA in gel diffusion, but that Fab and $F(ab')_2$ were inactive. However, the test may be too insensitive to detect weak reactivity.

Attempts to localize the SpA binding site in the Fc region have also been carried out using fragments of Fc from IgG_2, and like results from other species suggest that an intact C_H2–C_H3 unit is required (Endresen and Grov, 1978). Papain and trypsin digestion of Fc fragment gave several subfragments that were separated by chromatography and tested for binding to SpA–Sepharose. The proposed structures and molecular weights of these fragments are shown in Fig. 5. Only those fragments with at least one intact Fc chain showed a high level of reactivity (65–80% binding). The other fragments were poor reactors or nonreactors (0–15%).

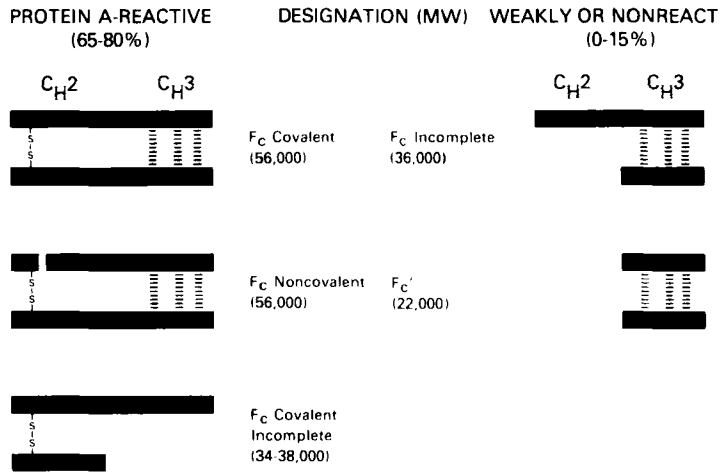

FIG. 5. Representation and designation of papain- and trypsin-derived Fc subfragments of guinea pig IgG_2 and their reactivity with protein A. As with human Fc subfragments (see Fig. 4), at least one intact Fc chain with covalently linked C_H2–C_H3 units is required for protein A reactivity. (Adapted from Endresen and Grov, 1978.)

6. *Mouse Immunoglobulins*

All four mouse IgG subclasses, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3, bind to SpA, although they bind with different affinities. Originally, Kronvall *et al.* (1970c) absorbed normal mouse serum and several myeloma proteins with Cowan strain I bacteria and determined the class or subclass of Ig remaining in the supernatant by immunoelectrophoresis. All three IgG_{2a}, two IgG_{2b}, and three IgG_3 myeloma proteins reacted with SpA, whereas all four IgG_1 proteins and the single IgA and IgM proteins tested did not. Although the reason why Kronvall *et al.* (1970c) failed to detect reactivity of their IgG_1 proteins is not clear, there now is ample evidence to show that polyclonal and monoclonal IgG_1 as well as the other subclasses do bind SpA (Mitchell *et al.*, 1977a,b; Goding, 1978; MacKenzie *et al.*, 1978b; Ey *et al.*, 1978; Chalon *et al.*, 1979). Thus chromatography of serum from parasite-infected mice gave all four IgG subclasses including relatively large amounts of IgG_1 in the acid-eluate (Mitchell *et al.*, 1977a,b; MacKenzie *et al.*, 1978b). Since then, gradient elution of bound Igs has given a better insight into the relative affinity of the subclasses for SpA and, like human (Section V,B,2) and goat (Section V,B,8) IgG, led to reasonably straightforward procedures for purification of the subclasses, a goal that is difficult to achieve by routine chromatographic methods (MacKenzie *et al.*, 1978b; Ey *et al.*, 1978; Chalon *et al.*, 1979). Mac-

Kenzie, *et al.* (1978b) tested the reactivity of several ^{125}I-labeled monoclonal IgG proteins and found that over 95% of each of three IgG_{2a} and IgG_{2b} proteins, and at least 70% of the three IgG_1 and IgG_3 proteins bound to SpA–Sepharose. Consistent with the results of Kronvall *et al.* (1970c), none of five myeloma IgA proteins bound.

When mouse serum (Ey *et al.*, 1978; Chalon *et al.*, 1979) or ascites fluid (Chalon *et al.*, 1979) was applied to a column of SpA–Sepharose at pH 7.0, the effluent was followed by a second peak consisting of almost pure IgG_1. Subsequent elution with a gradient of sodium thiocyanate (MacKenzie *et al.*, 1978b; Chalon *et al.*, 1979) gave some additional IgG_1 followed by IgG_{2a}, IgG_{2b}, and finally IgG_3. A rapid changeover from buffer gave all IgG_1 in a single peak. MacKenzie *et al.* (1978a) found a fairly clear-cut separation between IgG_1 and the two IgG_2 subclasses, which eluted at 0.5 *M* and between 1.5 and 2.0 *M* thiocyanate, respectively. Binding affinity of mouse subclasses also depends on pH (Ey *et al.*, 1978). When serum was applied to SpA–Sepharose at pH 8.0, all subclasses including IgG_1 were bound and eluted sequentially as the pH was lowered. Pure IgG_1 eluted at pH 6–7, IgG_{2a} at pH 4.5–5.0, and IgG_{2b} at pH 3.5–4.0. The behavior of IgG_3 (the minor subclass) was not discussed, but presumably it too binds.

Binding activity is concentrated in the Fc region of mouse IgG since Kronvall *et al.* (1970c) did not detect activity of Fab fragments in their system and Chalon *et al.* (1979) and MacKenzie *et al.* (1978b) recovered over 90% of $F(ab')_2$ fragments in the effluent peak from SpA–Sepharose.

Most reports suggest that little, if any, polyclonal IgM (Mitchell *et al.*, 1977a; Ey *et al.*, 1978; Chalon *et al.*, 1979), or IgE (Ey *et al.*, 1978), or polyclonal or monoclonal IgA (Ey *et al.*, 1978; MacKenzie *et al.*, 1978b; Chalon *et al.*, 1979) binds SpA. However, MacKenzie *et al.* (1978a) tested five IgM myeloma proteins and found that 89% of one of them (HPC-76) bound to SpA–Sepharose and the binding site probably involved the C_H2 region.

7. *Rat Immunoglobulins*

Of the four rat IgG subclasses, IgG_{2c} and IgG_1 interact most strongly with SpA (Medgyesi *et al.*, 1978; Rousseaux *et al.*, 1981) and IgG_{2b} shows weak reactivity (Rousseaux *et al.*, 1981). Three tests—precipitation against SpA in gel diffusion, binding of Ig to Cowan strain I or SpA–Sepharose, and binding of SpA to immobilized Ig—were used to determine reactivity on the basis of a simple binding reaction or on the ability to precipitate. In addition to subclasses in normal serum,

Medgyesi *et al.* (1978) tested three monoclonal IgG_1, two IgG_{2a}, and two IgG_{2c} proteins. All IgG_1 and IgG_{2c} samples were reactive in at least one of the tests, whereas IgG_{2a} and IgG_{2b} were always unreactive. These findings are consistent with results of others (Kronvall *et al.*, 1970a; Steele *et al.*, 1974; Langone, 1978; Zola *et al.*, 1978), who found either weak reactivity of purified rat IgG (e.g., Table I) or IgG in whole serum. By using monoclonal Igs and gradient pH or sodium thiocyanate elution, the order of decreasing binding affinity to SpA–Sepharose was shown to be IgG_{2c}, IgG_1, IgG_{2b}. IgG_{2a} was unreactive, and the weak binding of IgG_{2b} was observed at relatively high pH (8.0–9.0) (Rousseaux *et al.*, 1981). Even monoclonal proteins showed heterogeneous binding, probably as a result of partial proteolysis of the heavy chains, and pure IgG_1 and IgG_{2c} could be isolated by gradient elution at pH 7.0 or 4.0–3.0, respectively.

At least some polyclonal IgM is reactive, since 15–25% of total serum IgM was absorbed by Cowan strain I bacteria (Medgyesi *et al.*, 1978). Two of three monoclonal IgM proteins and an IgA protein also bound SpA, and, like human monoclonal IgE, a mouse monoclonal IgE protein was unreactive (Medgyesi *et al.*, 1978).

8. Goat, Sheep, and Cow Immunoglobulins

Like human and mouse subclasses, goat IgG_1 and IgG_2 binding to SpA–Sepharose is pH-dependent (Delacroix and Vaerman, 1979; Duhamel *et al.*, 1980). When whole serum was applied to the column at pH 9.1 and eluted at this pH, the effluent peak contained non-Ig and most of the IgM and IgG_1, whereas a second peak was essentially pure IgG_1, containing only 5–6% of the total IgM and IgA plus albumin. Pure IgG_2 was eluted at pH 5.9, and both subclasses were obtained in 70% yield (Delacroix and Vaerman, 1979). Contaminants could be removed by chromatography on G-200 before application to SpA–Sepharose. Duhamel *et al.* applied serum at pH 7.1 and eluted IgG_1 at pH 6.7 and IgG_2 at pH 5.8. Elution at a lower pH suggests that IgG_2 has a higher affinity for SpA than does IgG_1.

Goudswaard *et al.* (1978) also tested the binding of sheep subclasses to SpA–Sepharose. When serum was applied to the column at pH 7.5, 76% of the IgG_1 eluted in the void volume and only 2% was eluted with 0.1 *M* glycine at pH 2.5. In contrast, approximately 6% of IgG_2 eluted at pH 7.5, and 33% with acid. Thus, like goat subclasses, sheep IgG_2 has a much higher affinity than IgG_1 for SpA. These results are consistent with the earlier findings of Lind *et al.* (1970), who showed that fluorescein-labeled sheep IgG_2, but not IgG_1, was ab-

sorbed by Cowan strain I bacteria, and with similar experiments of Kessler (1976), who also found selective absorption of IgG_2.

Similarly, cow IgG_2 binds efficiently to SpA, whereas little or no IgG_1 is reactive (Goudswaard *et al.*, 1978). Serum or purified IgG_1 and IgG_2 were applied to a column of SpA–Sepharose at pH 7.5, and bound subclasses were eluted with acid and quantified by radial immunodiffusion (RID) or as total protein by the Lowry technique. From whole serum, only 7% of IgG_1 was bound and eluted, whereas 65% of IgG_2 was isolated in the acid wash based on RID. When pure subclasses isolated by ion exchange chromatography were tested, 90–94% of IgG_1 was detected in the effluent peak by both methods and only 2–4% was detected in the acid eluate. By RID, 60% of the applied IgG_2 was bound and eluted with acid, compared to 96% based on protein determination, and only 4% was found in the effluent peak. The discrepancy between the protein and immunochemical analyses may result from aggregation of acid-treated Ig, which could give a false low value by RID. This may also apply to the rather low value obtained for sheep IgG_2 after acid elution.

9. *Dog Immunoglobulins*

Canine IgG, IgM, and IgA all bind SpA (Lind *et al.*, 1970; Goudswaard *et al.*, 1978; Warr and Hart, 1979). When normal dog serum was applied to SpA–Sepharose at pH 8.0, >99% of the IgG and up to 90% of the IgM was absorbed (Warr and Hart, 1979). All four IgG subclasses were bound, and gradient pH or sodium thiocyanate elution failed to separate IgG and IgM. However, pure IgG (subclass mixture) and IgM could be obtained after acid elution and gel chromatography in yields of 60% and 26%, respectively. Goudswaard *et al.* (1978) found that between 36 and 47% polyclonal IgG, IgM and IgA as well as monoclonal IgM and IgA were bound to SpA–Sepharose at pH 7.5. Although bound IgM had no effect on IgG binding, the presence of IgG bound to the column or in a mixture with IgM, led to more efficient binding of IgM, suggesting that the IgM may be interacting in some way with the IgG. The higher binding observed by Warr and Hart, (1979) may be due to their use of a higher pH, a factor that already has been discussed and shown to be important for enhanced binding of human, mouse, and goat IgG subclasses.

IgM and IgG solubilized from the surface of canine lymphocytes after labeling with ^{125}I also bound to SpA–Sepharose, and IgA has been isolated from milk by affinity chromatography although there are no details of the latter results (Warr and Hart, 1979). In none of this work

was the binding site on the Ig molecule localized. However, based on a comparison with other species discussed above, reactivity at Fc sites would be expected to predominate.

10. Immunoglobulins of Other Species

Twenty to seventy percent of all four horse IgG subclasses, IgGa, IgGb, IgGc, and IgG(T) binds to SpA–Sepharose, but IgM and IgA do not bind significantly (Goudswaard *et al.*, 1978). Some but not all cat IgG, IgM, and IgA is active, but quantitative results were not obtained (Goudswaard *et al.*, 1978). Two 7 S Igs have been isolated from serum of the echidna, a monotreme mammal, by chromatography on SpA–Sepharose followed by ion exchange chromatography (Marchalonis *et al.*, 1978). On the basis of its slower electrophoretic mobility, the major component was designated IgG_2 and the minor component was designated IgG_1, although they may in fact represent different classes.

Zikán *et al.* (1980) reported that small amounts of nonmammalian serum IgM of chicken (3%), clawed toad (12%), and carp (13%) bound to SpA–Sepharose at pH 7.4, but not to Sepharose alone. When purified carp IgM was tested, 18% of the intact molecule bound to SpA, whereas 41% of the tryptic Fab fragments and only 8% of the Fc4 fragments bound. Although this was interpreted to mean that the binding activity was localized in the Fab region, the quantitative difference between whole IgM and Fab reactivity raises questions about the significance of these results, especially since the possibility that binding is due to Fab antibody activity against SpA was not dealt with satisfactorally.

C. Affinity and Kinetics of Protein A–IgG Binding

1. General Properties of the Reaction

Binding affinity has been determined for the reaction between intact human or rabbit IgG and SpA, and for purified rabbit Fc fragments and monovalent fragment B. The results are summarized in Table V.

The K_D value of approximately 2×10^{-8} *M* for human IgG was based on a Scatchard plot of binding data for an ^{125}I-labeled myeloma IgG_1 protein and Cowan strain I bacteria (Kronvall *et al.*, 1970b; Myhre and Kronvall, 1980a). The same approach was used with ^{125}I-labeled normal rabbit IgG. Two of these values (Jonsson and Kronvall, 1974; Kessler, 1975) (2×10^{-8} and 5×10^{-9} *M*), agreed well with the constant (7.9×10^{-9} *M*) obtained for the opposite reaction, that is, binding of ^{125}I-labeled protein A to immobilized IgG (Sandor and

TABLE V
AFFINITY OF PROTEIN A–IgG BINDING

Species of IgG	Method[a]	K_D (M)	References
Human myeloma IgG_1	a	2.2×10^{-8}	Kronvall *et al.* (1970b)
	a	2.0×10^{-8}	Myhre and Kronvall (1980a)
Rabbit	a	2×10^{-8}	Jonsson and Kronvall (1974)
Rabbit	a	5×10^{-9}	Kessler (1975)
Rabbit	a	1×10^{-10}	O'Keefe and Bennett (1980)
Rabbit	b	7.9×10^{-9}	Sandor and Langone (1981)
Rabbit Fc[b]	c	3.3×10^{-7}	Lancet *et al.* (1978)

[a] a, Binding of ^{125}I-labeled IgG to Cowan strain I bacteria; b, binding of ^{125}I-labeled protein A to rabbit IgG immobilized on polyacrylamide beads; c, fluorescence quenching.

[b] Binding between rabbit Fc fragments and monovalent fragment B of protein A.

Langone, 1981), and are similar to the K_D for a typical antigen–antibody reaction. Fluorescence quenching measurements of the fluid phase reaction between rabbit Fc and fragment B gave the highest K_D (approximately 3×10^{-7} M), but the reaction between Fc and intact SpA could not be studied, since fluorescence titration gave small changes and variable results (Lancet *et al.*, 1978). In related studies Mihăescu *et al* (1978) reported that the affinity of rabbit IgG for cell-surface antigen is enhanced up to 1600 times by complex formation with SpA. Similar enhancement in binding activity is discussed in the following section.

Binding of ^{125}I-labeled SpA to insoluble rabbit IgG alone (Langone *et al.*, 1977a) or in immune complexes (Dorval *et al.*, 1975; Langone, 1978) at temperatures ranging from 4° to 37°C is quite fast, equilibrium being reached in <30 minutes, although a longer time may be required if less than excess amounts of [^{125}I]SpA are used.

Although binding of fluid-phase rabbit IgG to Cowan strain I in the absence of antigen also is extremely rapid, in some cases taking 1 minute or less for maximum uptake at 20–25°C (Kronvall *et al.*, 1970b; Kessler, 1975; Brunda *et al.*, 1977; Ying and Guillemin, 1979; Rebar *et al.*, 1980; Ying, 1981), Kessler (1975) reported that absorption of immune complexes occurs even faster. Mottolese *et al.* (1980) found that 20 minutes was required for complete absorption of complexes between alkaline phosphatase-labeled BSA and rabbit anti-BSA, but this relatively slow reaction may be attributed to modified reactivity between the antibody and enzyme-labeled antigen. The effect of temperatures between 4° and 50°C on maximum binding and the kinetics

of IgG absorption are minimal (Kronvall *et al.*, 1970b; Kessler, 1975; Rebar *et al.*, 1980), and the capacity for antigen is essentially the same for fluid-phase antibodies and antibodies that are first bound to bacteria (Frohman *et al.*, 1979; O'Keefe and Bennett, 1980), although a reproducible twofold increase in affinity for antigen has been reported for absorbed vs fluid-phase IgG (O'Keefe and Bennett, 1980).

The kinetics of antigen binding to antibody that is preadsorbed to bacteria is significantly slower than reaction between fluid-phase antigen and antibody, taking up to twice as long to reach equilibrium (Frohman *et al.*, 1979; O'Keefe and Bennett, 1980). These results are not surprising for a two-phase reaction compared to a reaction between components in solution.

Dissociation of IgG or antigen–antibody complexes from *S. aureus* depends on time, temperature, and pH (Kessler, 1975; O'Keefe and Bennett, 1980). In neutral buffers at 4°C, there is little or no dissociation (<5%), and only small losses occur at temperatures up to 37°C. However, in the presence of competing excess IgG or whole serum, 75–90% dissociation occurs at 37°C after 30–60 minutes. Losses are less at 4°C, and rabbit IgG is more efficient than human IgG in displacing bound rabbit IgG (O'Keefe and Bennett, 1980). Under conditions where there is negligible displacement of rabbit IgG at pH 7 or 9, approximately 75% is released at pH 5 (O'Keefe and Bennett, 1980). Either adsorbed IgG alone or immune complexes can be eluted from the bacteria with a variety of reagents including detergents, urea, high-molarity neutral salts, as well as low pH (Kessler, 1975; MacSween and Eastwood, 1978, 1981; Natali *et al.*, 1980). Significant loss of antigenic activity can occur when complexes are eluted with guanidine hydrochloride, magnesium chloride, or sodium thiocyanate (MacSween and Eastwood, 1978, 1981). Elution with 0.1 *M* lithium diiodosalicylate gives high yields of active components and may be the reagent of choice for dissociating adsorbed antigens and antibodies for preparative purposes.

Because of the high binding constant, the fast kinetics, and the ability of SpA to bind a high proportion of IgG from several species, heat-killed and formalinized Cowan strain I bacteria are widely used as a precipitating agent in immunoassay and are commercially available from several sources (see Section VII).

2. *Effect of Immune Complex Formation on Protein A Reactivity with IgG*

The data summarized in Table IV and discussed in the preceding sections clearly show that the affinity of SpA for IgG varies over a

wide range. Whereas human and rabbit IgG bind with high affinity, goat, sheep, rat, and chicken IgG show weak reactivity. However, there is ample evidence to indicate that immune complex formation can markedly enhance the binding affinity between SpA and certain poorly reactive IgG and can even significantly enhance the reactivity with rabbit IgG. Thus goat (Boyle and Langone, 1979; Langone, 1980c), sheep (Langone, 1980c), and rat (Dorval *et al.*, 1975) antibodies could be used in immunoassay of cellular antigens with ^{125}I-labeled SpA as the tracer, since SpA bound efficiently to these antibodies once they were complexed to insoluble antigen. Similarly, the results of Kessler (1975), who showed that immune complexes made with rabbit antibodies were absorbed more rapidly than free IgG by Cowan strain I and were difficult to dissociate from the bacteria by addition of fresh IgG, also suggested that immune complex formation enhanced the affinity of SpA for rabbit IgG.

We have used ^{125}I-labeled SpA to quantify the effect of immune binding on the reactivity between SpA and goat antibodies to a myeloma IgE protein (P.S.) and a monovalent hapten, methotrexate, that were covalently linked to polyacrylamide beads (Langone, 1980b,c). There was at least a 300-fold increase in the reactivity of goat anti-IgE bound to insoluble antigen compared to fluid-phase or immobilized IgG in the absence of antigen. Immune complexes prepared in solution over a wide range from antigen to antibody excess also failed to show detectable reactivity with SpA in an inhibition assay (Langone, 1980c). These results suggest that immobilization of the antigen–antibody complex itself has a dramatic effect on the affinity of the binding reaction between SpA and IgG. This is consistent with the theoretical treatment of ligand–receptor interactions made by DeLisi (1981a,b) that predicts the possibility of such an enhancement by virtue of having an immobilized reactant. Even though goat IgG_2 reportedly can bind to SpA–Sepharose (Section V,B,8), there was no significant binding of SpA to goat IgG under the conditions of the experiment. In fact, even in the presence of 30 times the amount of irrelevant IgG, [^{125}I]SpA selectively bound to antibody complexed to immobilized IgE (Langone, 1980b).

Results with immobilized methotrexate and goat antibodies demonstrated that maximum binding of [^{125}I]SpA was a function of the concentration of both methotrexate beads and antibody, and that the complexes formed under optimal conditions consisted of two molecules of IgG per molecule of SpA. These results suggest that immune aggregation is necessary for optimal binding of SpA to insoluble immune complexes, at least in the case of monovalent antigen, and along with other

evidence is consistent with antigen density being a determining factor not only for this augmentation (Langone, 1980b), but also for other functions, such as complement binding and activation (Borsos *et al.*, 1981a,b).

Since SpA binds at Fc sites encompassing portions of the C_H2-C_H3 domains of IgG, and immune binding at Fab sites can enhance the affinity of the SpA–IgG reaction, a natural question is whether immune aggregation is sufficient or whether a conformational change induced in Fc by antigen binding also plays a role in altering the dynamics of the SpA–IgG reaction. Several other functions, including complement fixation, placental transport, and binding to Fc-receptor-bearing cells, also are localized in the Fc region, and the mechanism of how immune binding ultimately results in expression of various biological activities is an important question that still has not been answered satisfactorily (Metzger, 1974, 1978). Results of studies with SpA are sometimes in apparent conflict, but offer some rather interesting approaches and insights into the general problem as well as the specific details of the SpA–IgG–antigen reaction.

We have found (Sandor and Langone, 1981) that the enhanced ability of affinity-purified rabbit IgG antibodies to human serum albumin to interact with [^{125}I]SpA depends on the degree of antigen-induced aggregation. Maximum reactivity, measured as inhibition of [^{125}I]SpA binding to immobilized IgG was produced by complexes prepared at equivalence (i.e., under conditions of maximum aggregation), whereas the reactivity of complexes prepared in antigen or antibody excess was less (Fig. 6). If an antigen-induced comformational change played a dominant role, then complexes prepared in antigen excess would be expected to be the most effective inhibitors (Metzger, 1978). Consistent with these results, there was no difference in reactivity with SpA between rabbit anti-methotrexate alone, at equivalence, or in the presence of up to 1000-fold excess of methotrexate (Sandor and Langone, 1981).

Similarly, Wright *et al.* (1978) used a fluorescence technique to study the effects of antigen on the binding of monovalent fragment B of SpA to the Fc region of rabbit antibodies and could detect no evidence for allosteric changes.

In contrast, Moţa *et al.* (1981) reported that SpA–IgG–antigen complexes, but not complexes made in the absence of antigen, were able to bind to SpA–Sepharose, and they suggested that expression of latent Fc binding sites for SpA were the result of a conformational change induced by antigen. Since IgG–SpA complexes without antigen bound fragment B (but not intact SpA), latent sites may exist that

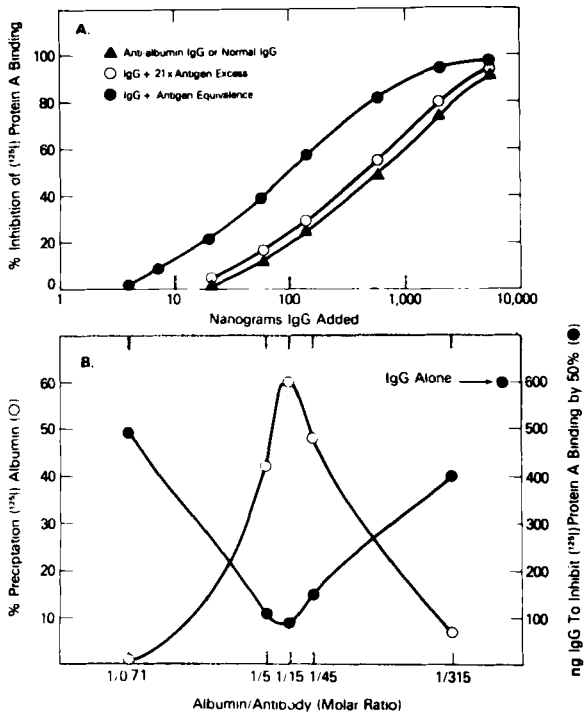

FIG. 6. (A) Inhibition of ^{125}I-labeled protein A binding to normal rabbit IgG by affinity-purified rabbit IgG anti-human albumin or normal rabbit IgG alone (▲); anti-albumin plus 21-fold antigen excess (○); anti-albumin plus antigen equivalence (●). (B) Precipitin curve (○) for the albumin–anti-albumin reaction compared to the ability of immune complexes prepared with different ratios of albumin and purified IgG antibodies to inhibit ^{125}I-labeled protein A binding to immobilized rabbit IgG. The inhibition data are plotted as the amount of IgG in immune complexes required to inhibit binding by 50% and were obtained from curves like those in (A). Equivalence is at an albumin to antibody ratio of 1 to 15. (From Sandor and Langone, 1981.)

were protected from further reaction with whole SpA by steric hindrance. Similarly, although chicken IgG does not bind SpA (Table IV), it was reported (Barkas and Watson, 1979) that immune complexes between ^{125}I-labeled acetylcholine receptor and chicken antibodies bound to *S. aureus* and to SpA–Sepharose. However, the authors' conclusion that an antigen-induced conformational change was responsible is not supported by the data, since, compared to 16% specific binding to SpA in antibody excess, there was only 3% binding in antigen excess.

Aggregation of antibodies by antigen appears to play a major role in the enhancement of IgG reactivity with SpA, whereas additional evi-

dence is necessary before the role of conformational changes can finally be resolved.

D. Reactivity with Other Proteins

As discussed in Section V,C, a complex between α_2-macroglobulin and a human myeloma IgA did not bind to SpA–Sepharose, although after dissociation in 8 *M* urea and separation by chromatography the free IgA protein did react (Dalen *et al.*, 1977) and free α_2-macroglobulin did not.

Since β_2-microglobulin is structurally similar to portions of IgG_1 light and heavy chains (Peterson *et al.*, 1972; Smithies and Poulik, 1972), reactivity of this protein also was tested. Both reports indicated no reactivity, since ^{125}I-labeled β_2-microglobulin mixed with human serum and applied to a column of SpA–Sepharose was found only in the effluent, not in the IgG fraction eluted at low pH (Ochi *et al.*, 1977), and it failed to inhibit SpA-induced agglutination of sensitized sheep erythrocytes (Stewart *et al.*, 1978).

Binding to 11 strains of *S. aureus* of ^{125}I-labeled fibrinogen or fragments isolated after plasmin or cyanogen bromide treatment also has been tested (Runehagen *et al.*, 1981). Compared to a fibrinogen-negative strain of *E. coli*, there was reproducible but very low ($\leq 10\%$) binding of fibrinogen fragment X, N-terminal fragment E, and C-terminal fragment D, and no significant binding of cyanogen bromide fragments N-DSK (CCD-153-1), Hol-DSK (CCD-153-5), and Hi2-DSK.

Björck and Kronvall (1981) absorbed human serum with *S. aureus* strain Cowan I, labeled adsorbed proteins (as well as bacterial membrane components) with ^{125}I, and analyzed the solubilized products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Five peaks were observed, two of them corresponding to H and L chains of IgG. The other three had molecular weights of >70,000, 70,000, and 35,000 and collectively accounted for a small percentage of radioactivity associated with the IgG peaks. Since the same pattern was observed with SpA–Sepharose, the three minor unidentified peaks apparently are due to serum components adsorbed specifically by SpA.

E. Activity Similar to Protein A in Other Strains of Staphylococci

Of 11 species of staphylococci representing 20 strains, 3 gave precipitin lines in double diffusion against normal human serum: *S. capitis*, *S. warnerei*, and *S. xylosus* (Osland, 1981). All strains of *S. ca-*

pitis also gave a reaction with serum from 8 other animal species. The reaction apparently was not due to SpA, since the active component was localized in the effluent peak from serum chromatographed on SpA–Sepharose; and it is not an immune reaction, since serum activity was in the albumin peak eluted from a Sephadex G-200 column. Also there was a line of identity with the various species of bacteria, but not against Cowan strain I. The active component has not been characterized; it is produced by bacteria cultured in brain heart infusion agar, but not in nutrient broth agar.

F. Antibodies to Protein A and Protein A–IgG

Antibodies to SpA have been produced in rabbits (Oeding, 1957; Löfkvist and Sjöquist, 1963; Oeding *et al.*, 1964; Grov *et al.*, 1964, 1970; Forsgren and Sjöquist, 1967, 1969; Grov, 1967; Lind, 1968; Lind and Mansa, 1968, 1974a,b; McDowell *et al.*, 1971a; Sjöholm *et al.*, 1973; Biguzzi, 1979; Rhodes and Lind, 1980), guinea pigs (Grov *et al.*, 1970; McDowell *et al.*, 1971b,c; Rhodes and Lind, 1980), mice (Rhodes and Lind, 1980), and chickens (Biguzzi, 1979) immunized with formalin-fixed Cowan strain I bacteria or purified SpA. The IgG antibodies have Fab antigen binding sites specific for SpA in addition to the nonimmune Fc binding activity (except for chicken). Rabbit antibodies gave a typical immune precipitation reaction with SpA, unlike normal rabbit IgG (and IgG from certain other species), which gives extremely weak or no precipitation resulting from the Fc-SpA interaction. The mechanism of the precipitation reaction and properties required of the reagents are discussed in Section V,G.

The spectral studies discussed in Section IV suggested that IgG–SpA binding induced significant conformational changes in both components, and it is reasonable to believe that changes in the Fc region are involved, since this is the principal site of SpA binding activity. Immunochemical evidence based on the specificity of antibodies produced against IgG determinants expressed in complexes between SpA and IgG are consistent with these observations (Lind, 1974a,b; Lind and Mansa, 1974a). Lind (1974b) found that rabbits immunized with purified SpA or SpA-producing strains of *S. aureus* gave antibodies against hidden determinants of autologous IgG that were exposed through binding to IgG *in vivo*. Rabbits immunized with SpA-positive strains Cowan I, 4972, or E2400 (that gave 155, 200, or 3 μg of SpA per 10^{10} cells) all produced these antibodies, whereas rabbits immunized with SpA-negative strains Wood 46 and 1433 did not, even though the antibody response to the total array of staphylococcal antigens was not significantly different for all five strains. The primary re-

sponse was IgM followed by a secondary response that was mainly IgG.

An indirect hemagglutination inhibition test using sheep erythrocytes sensitized with subagglutinating levels of rabbit IgG and SpA as the antigen was used to determine that antibody specificity was directed to determinants located in the Fc region of autologous IgG. Thus, intact rabbit IgG or the Fc fragment only in combination with SpA effectively inhibited agglutination by immune sera. SpA, IgG, or Fc alone, or unrelated antigen–antibody complexes, were ineffective. Some sera from nonimmune animals, especially swine, dog, human, guinea pig, and mouse, showed significant levels of antibody to modified IgG, and in a retrospective study (Lind, 1974b) all antisera to SpA-producing strains of *S. aureus* were positive whereas antisera to streptococci, gonococci, and SpA-negative strains were negative. The possible connection between antibody activity in $F(ab')_2$ fragments directed against hidden determinants in soluble SpA–IgG complexes and the precipitation reaction called the "star phenomenon" is discussed in Section V,G.

It was also shown (Lind, 1974a; Lind and Mansa, 1974a) that rabbits immunized with formalinized Cowan strain I bacteria that either had been grown in culture containing serum or grown in serumless medium and then treated with whole human, swine, horse, sheep, or cow serum produced antibodies against the particular species of IgG. Antibodies against human Fab, Fc, and both kappa and lamda chains were produced, whereas immunization with bacteria treated with the Fc fragment of a human myeloma IgG gave antibodies specific for the heavy chain. Although this may be a useful procedure for preparation of antibodies to IgG that circumvents the need for antigen purification, antibodies to a host of staphylococcal antigens also are produced, and limit its value. One important point that should be emphasized is the ability of organisms cultured in medium containing serum to bind 10–15% (corresponding to 8000–12,000 sites on the cell membrane of Cowan strain I) of the maximum amount of IgG adsorbed under optimal conditions, and that SpA purified from such organisms may be contaminated with IgG. Thus Lind (1974a) showed that even highly purified SpA from Cowan strain I grown in horse serum agar induced rabbit antibodies that reacted specifically with equine IgG.

G. Mechanism of Protein A–Ig Precipitation: the "Star" Phenomenon

Many of the structure–activity relationships established so far were based on precipitation reactions in gels to determine reactivity of in-

tact Igs or Ig fragments with SpA. Igs fall into two general catagories —precipitating and nonprecipitating. Reactivity of a nonprecipitating Ig can be detected by reactions like inhibition of indirect hemagglutination or inhibition of precipitating standard mixtures of IgG and SpA that are based on the primary binding reaction with SpA. The recognition of these two types of activity, and the observation that chemical modification of precipitating myeloma IgG by carbamylation (Section IV,C,2,b) converted it to a nonprecipitating form, led Kronvall *et al.* (1970d) to postulate a two-step mechanism for the precipitation reaction:

$$\text{SpA} + \text{IgG} \underset{}{\overset{K_1}{\rightleftharpoons}} \underset{\text{(soluble)}}{\text{SpA–IgG}} \xrightarrow{K_2} \underset{\text{(precipitate)}}{\text{[SpA–IgG]}}$$

where K_1 is taken as the K_D value (Table V). The primary step involves an SpA–Fc interaction, and the precipitation step, for which K_2 values have not been determined, involves another activity, perhaps localized in Fab sites. Results to date are consistent with this interpretation, including calorimetric measurements (Sjöquist and Wadsö, 1971) that showed the primary reaction between Fc of a human myeloma IgG_1 and SpA to be highly exothermic.

When the reactions in gels were examined more closely, an unusual type of precipitation reaction called the "star" phenomenon was discovered (Kronvall and Williams, 1971). This is a three-component reaction in which a starlike three-armed precipitation pattern was produced by the interaction of SpA, an IgG that formed only soluble complexes with SpA (e.g., rabbit, sheep, or cow), and the $F(ab')_2$ fragments of either rabbit IgG anti-SpA or a species having normally precipitating IgG (e.g., human, guinea pig, or pig). The reagents were applied in three separate wells, and star formation occurred only when all the components were present. In place of IgG that forms soluble complexes, some other Igs, e.g., human IgM and IgA (Grov, 1975a; Dalen *et al.*, 1977), could be used. When the $F(ab')_2$ fragments and the nonprecipitating IgG (or whole serum) were mixed in one well and run against SpA, a usual precipitin line was produced. This is designated direct coprecipitation. The mechanism of the star phenomenon has been studied (Kronvall and Williams, 1971; Lind, 1974b; Lind and Mansa, 1974b; Grov and Endresen, 1976; Endresen, 1979b), and the latest results (Grov and Endresen, 1976; Endresen, 1979b) agree that Fc activity in the nonprecipitating IgG is necessary, although it still is not absolutely clear whether the precipitation requires an $F(ab')_2$ component with antibody activity directed against SpA. Thus Endresen and Grov (1976) found that only the minor (<5%) SpA-reactive

$F(ab')_2$ from normal precipitating IgG, isolated by affinity chromatography on SpA–Sepharose, could replace $F(ab')_2$ fragments isolated from immune sera prepared against SpA. In addition to the unbound $(Fab')_2$ fragments, monovalent Fab' also were ineffective. Even rabbit IgG from anti-SpA serum yielded only a small amount of SpA-reactive $F(ab')_2$ with antibody activity, and only these affinity-purified fragments were able to participate in "star" formation. However $F(ab')_2$ fragments of affinity-purified guinea pig IgG antibodies against egg albumin and measles virus can participate in the "star" reaction, suggesting that antibody activity against SpA is not a requirement.

This is in contrast to the hypothesis of Lind (Lind, 1974b; Lind and Mansa, 1974b), who, on the basis of detection of antibodies to SpA-modified autologous IgG (Section V,F), suggested that the "star" phenomenon required $F(ab')_2$ activity against hidden determinants that were exposed when SpA and nonprecipitating IgG interacted in the gel. This mechanism has been questioned, since the SpA nonreactive $F(ab')_2$ fragments of rabbit anti-SpA failed to precipitate with SpA even after mixing with human Fc fragments in place of soluble IgG under conditions where SpA-reactive $F(ab')_2$, Fc, and SpA gave a star reaction (Grov and Endresen, 1976). One difficulty with these efforts to dismiss the Lind hypothesis is their use of Fc fragments in the precipitation reaction, since others have reported that no star is formed when Fc is used in place of IgG. It would have been better if Grov and Endresen had used the typical nonprecipitating intact IgG, although the discrepancy with the other reports may simply be due to the earlier workers using insufficient amounts of Fc in the test.

In light of these results it should be emphasized that, even though a small fraction of $F(ab')_2$ activity in normal IgG resembles antibody activity against SpA that may in fact result from exposure to staphylococcal antigens, the results are still rather convincing that SpA binding activity in some Ig is localized in the Fab region outside the antibody combining site (Section V,B).

H. Some General Conclusions

Reactivity with SpA appears to be restricted to Igs and depends on a number of factors including species, class and subclass of Ig, and pH. Reactivity can be detected by several methods that rely only on the primary binding reaction or that involve a more complex precipitation reaction.

The problem in sorting out the interactions with Ig arises from the ability of SpA to display three distinct types of binding activity: (*a*) the primary reaction with IgG and other classes of Ig at sites located in the

Fc region; (*b*) a similar but apparently weaker binding localized in the Fab region outside the antigen combining site; and (*c*) a typical antigen–antibody reaction involving Fab combining sites. Human polyclonal IgE seems to be the exception in that activity is restricted to Fab sites and is limited to approximately 6% of total IgE. Although arguments have been made against the active IgE behaving as antibody, the binding site has not been localized. In this and all cases where minor Fab activity has been reported but not fully characterized, the possibility always exists that antibodies produced after infection with *S. aureus* or other immune stimuli may be involved.

Although reactivity at Fab sites is quantitatively a minor reaction compared to Fc binding, it appears to be a necessary ingredient in precipitation reactions like the "star" phenomenon and direct coprecipitation.

For most practical purposes, the only significant reaction is between SpA and IgG. Thus, affinity chromatography on SpA–Sepharose is the preferred method of isolating IgG and immune complexes from serum of several species, and removing Fc-containing fragments from Fab and $F(ab')_2$ preparations. The use of SpA in immunoassay of antigens and immune complexes is an important application that is discussed in Section VII.

VI. Biological Activity of Protein A and Protein A–IgG Complexes

A. Fluid-Phase Complexes

SpA can bind to IgG antibody at Fc sites, even when the antigen combining sites are occupied. The antibody can be in solution or insoluble by virtue of binding to antigen either on a cell membrane or coupled covalently to a solid support. A good deal of our understanding of how SpA can induce biological effects that often are characteristic of antigen–antibody complexes has come through studies involving SpA and hemolytically active rabbit IgG (Langone *et al.*, 1978a,c).

1. Effects on Antibody and Complement

It was known for some time that SpA added to human (Kronvall and Gewurz, 1970; Stålenheim, 1971), guinea pig (Sjöquist and Stålenheim, 1969; Kronvall and Gewurz, 1970; Stålenheim, 1971), rabbit (Stålenheim, 1971), dog or pig (Kronvall and Gewurz, 1970) serum caused weak to marked depletion of complement activity depending on the species. Mixtures of SpA and human normal (Sjöquist and Stålenheim, 1969; Stålenheim *et al.*, 1973) or myeloma IgG or Fc frag-

ments (Stålenheim and Sjöquist, 1970; Stålenheim *et al.*, 1973), or normal guinea pig IgG (Sjöquist and Stålenheim, 1969) aggregated by SpA had a similar effect. Complexes between SpA and both guinea pig subclasses IgG_1 and IgG_2 fixed complement, whereas antigen–antibody complexes involving IgG_1 did not (Stålenheim and Malmheden-Eriksson, 1971; Stålenheim and Castensson, 1971). Although SpA can activate both complement pathways (Stålenheim, *et al.*, 1973; Sveen and Grov, 1978), a good deal of evidence indicates that at least in some cases SpA activates the classical pathway primarily with only indirect involvment of the alternative pathway (Kronvall and Gewurz, 1970; Peterson *et al.*, 1977; Verhoef *et al.*, 1977a; Verbrugh *et al.*, 1979).

In these experiments, complexes were not isolated or characterized, but rather dose-response curves were obtained by adding increasing amounts of SpA or SpA–IgG mixtures to serum and then measuring residual whole complement activity (Sjöquist and Stålenheim, 1969; Kronvall and Gewurz, 1970; Stålenheim *et al.*, 1973) or the titers of individual complement components (Kronvall and Gewurz, 1970; Stålenheim *et al.*, 1973). When SpA was added to whole serum, titers of the nine components except for C7, which was not determined, decreased from 30% (C8) to 85% (C3) and optimal inhibition occurred in IgG excess (Stålenheim *et al.*, 1973). Preformed complexes between SpA and the Fc fragment of a complement-fixing myeloma IgG_1 prepared under conditions for maximum activity decreased the whole complement titer by 64% and the titers of the individual components from 4% (C4) to 99% (C3). Similar depletion of whole human complement and activity of the early-acting components was reported by Kronvall and Gewurz (1970). SpA also inhibited binding of C1 to either heat-aggregated IgG or to the IgG_1 myeloma protein, but had no effect on the binding of C1 to IgG_3 (Stålenheim *et al.*, 1973). This result is consistent with the relatively low reactivity of SpA with human IgG_3 compared to the other subclasses (Section V,B,2). We also found that SpA inhibited binding of purified guinea pig C1 to anti-Forssman IgG bound to sheep erythrocytes, and, conversely, C1 bound to antibody-sensitized cells inhibited the binding of SpA (Langone *et al.*, 1978a). Others found that monovalent fragment B did not inhibit binding of C1 to human IgG (Lancet *et al.*, 1978). Together the results are consistent with distinct binding sites for C1 and SpA localized in the C_H2 domain and the C_H2–C_H3 interface, respectively, but close enough so that intact SpA, but not the smaller fragment B, can inhibit C1 binding by steric hindrance.

Depletion of complement activity depended on the amount of SpA

relative to IgG. Thus, as the dose of SpA added to serum increased, there was a maximum level of complement activation that decreased at high concentrations of SpA (Sjöquist and Stålenheim, 1969; Kronvall and Gewurz, 1970; Stålenheim *et al.*, 1973). In light of the results just discussed, the effect was interpreted to mean that high concentrations of SpA were inhibiting C1 binding. However, the whole process of complement activation by IgG–SpA, and the dose-dependent effect of SpA on IgG activity was clarified by experiments designed to examine the effect of SpA on antibody activity (Langone *et al.*, 1978a,c). The test system included sheep erythrocytes, hemolytically active rabbit anti-Forssman IgG antibody, and guinea pig complement. It offers the distinct advantages of easy quantification and functional classification of antibody activity on the basis of the slope of the titration curve (Borsos and Rapp, 1965; Rapp and Borsos, 1970). IgM antibodies or IgM-like complexes give a slope of 1, and IgG a slope of 2 or greater, when the average number of lytic sites per cell (z) derived from hemolysis data is plotted on a log–log scale against relative antibody concentration.

These experiments demonstrated that SpA and IgG formed complexes that behaved functionally like IgM in the way they interacted with whole complement or purified C1. Complexes also were formed at relatively high or low doses of SpA that behaved like authentic IgG, but with lower titer. Typical titration curves showing the spectrum of activities, including the IgM-like complexes, are shown in Fig. 7. Since only a single IgM molecule or IgM-like complex is required to fix C1 and lyse the cell, whereas approximately 800–1000 IgGs are required (Humphrey, 1967) to form a necessary doublet (Cohen, 1968), the IgM-like complexes are far more efficient than IgG alone or IgG–SpA complexes that behave like authentic IgG with lower titer. The behavior of these complexes accounts for the earlier effects on complement activity and offer a reasonable explanation for the role of SpA in several related biological activities both *in vitro* and *in vivo* that are associated with antibody–complement interactions and are discussed in the following sections.

The IgM-like complexes also behaved like authentic IgM in that hemolytic activity was inhibited by Con A (Langone *et al.*, 1978a), suggesting that glucose or mannose residues may be exposed on SpA binding. IgG not directed against Forssman antigen can participate in their formation (Langone *et al.*, 1978c), and complex formation enhances antibody affinity significantly, probably owing to multipoint attachment (Kessler, 1975; Langone, 1978c; McDougal *et al.*, 1979; Mihăescu *et al.*, 1979). Based on the available information there ap-

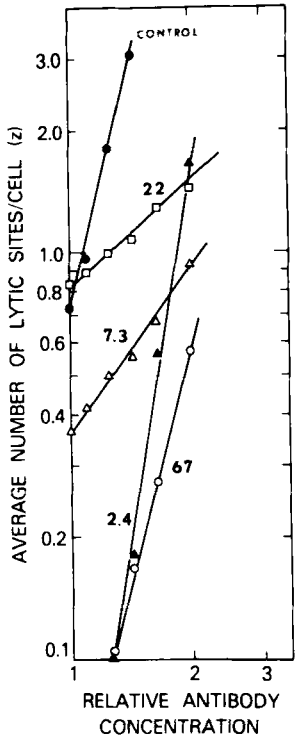

FIG. 7. Titration curves for rabbit anti-Forssman IgG alone (●) or for mixtures prepared with 2.4 (▲), 7.3 (△), 22 (□), or 67 (○) μg of protein A per milliliter. Hemolytic activity was determined against sheep erythrocytes using guinea pig serum as a source of complement. (After Langone *et al.*, 1978c.)

pears to be no correlation between the ability of a subclass of IgG to fix complement and to bind SpA in the case of mouse (Kronvall *et al.*, 1970c), rat (Medgyesi *et al.*, 1978), sheep Goding, 1978), and guinea pig (Stålenheim and Malmheden-Eriksson, 1971).

2. *Composition and Activity of Protein A–IgG Complexes*

Based solely on hemolysis data and the amounts of reagents required to give IgM-like activity, the empirical formula of the IgM-like complexes was shown to be $[(IgG)_2SpA]_n$ (Langone *et al.*, 1978a). Further studies were carried out to establish the molecular formula of this and other complexes formed when different molar ratios of IgG and SpA were prepared and separated by ultracentrifugation (Langone *et al.*, 1978c). At the appropriate ratio, IgM-like complexes were formed almost exclusively, and with the empirical formula and molecular

weight based on standards, the molecular formula was shown to be $[(IgG)_2SpA]_2$. The functional IgM activity was stable for at least 4 days at 4°C and 1 day at 37°C. Other complexes, probably IgG–SpA and $(IgG)_3SpA_2$, also were formed at relatively high levels of SpA or IgG, and these may also fix complement.

In contrast to these results, Moţa *et al.* (1978) reported that rabbit IgG and SpA formed only one complex at IgG to SpA ratios from 2:1 to 40:1 with the molecular composition $(IgG)_2SpA$, apparently corresponding to the IgM-like complex discussed above. However, the formula was based on a molecular weight of 350,000 obtained by combination of sedimentation data and the diffusion coefficient obtained by chromatography. The formula was later revised to the correct one, based on a molecular weight of approximately 690,000 obtained by gel filtration or 600,000 by equilibrium sedimentation, and the production of complexes with formula IgG–SpA in the presence of high levels of SpA also was corroborated (Gheţie and Moţa, 1980). As suggested earlier (Langone *et al.*, 1978c), the ability to incorporate IgG with different specificities into the complexes led to the simple preparation of multivalant antibodies with dual specificity (Mandache *et al.*, 1980; Gheţie and Moţa, 1980).

In similar studies, Lancet *et al.* (1978) showed by fluorescence quenching and light-scattering techniques that rabbit Fc had 2 binding sites for monovalent fragment B, and that complexes were formed with 2–4 IgG molecules or Fc fragments bridged by SpA. Two sites on the same IgG or Fc apparently can each accommodate the relatively small fragment B units (MW 7000), whereas one IgG is able to bind only 1 intact SpA. This is consistent with the crystallographic data (Deisenhofer *et al.*, 1978; Deisenhofer, 1981) discussed in Section IV,C, and probably explains why bivalent complexes, $IgG(SpA)_2$, have not been found.

B. Effects of Protein A *in Vitro*

1. Phagocytosis

a. Protein A–IgG Complexes. Human polymorphonuclear (PMN) leukocytes rapidly ingest and degrade insoluble complexes prepared with human IgG and SpA (Hällgren and Stålenheim, 1976, 1978) and white blood cells release histamine in the presence of SpA (Martin and White, 1969). Complexes with ^{125}I-labeled IgG were used to show that uptake depended on the concentration of neutrophils, the nature of the complexes, incubation time, and the presence of serum factors. Similar to complement activation dis-

cussed in the preceding section, the degree of phagocytosis depended on the dose of SpA and was optimal for precipitates formed at equivalence. Release of myeloperoxidase correlated with phagocytosis. Although up to 50% of the complexes were taken up by cells in serum-free medium, the presence of human serum resulted in more rapid and enhanced ingestion either when the complexes, serum, and neutrophils were mixed or when complexes were first treated with serum and then washed before exposure to the cells (Hällgren and Stålenheim, 1976). Complement probably plays an important role in the enhancement, but other factors also are involved, since heat-inactivated serum still had a significant opsonizing effect (Hällgren and Stålenheim, 1978). Purified polyclonal IgA and an IgA- and IgM-containing fraction isolated by affinity chromatography on SpA–Sepharose also showed enhancing activity, and it was postulated that IgA and IgM act as opsonins by interacting directly with SpA–IgG complexes. However, the mechanism is not entirely clear and it may be that the heat-stable opsonins are antibodies with specificity against hidden determinants exposed in IgG when SpA binds (see Section V,F). Alternatively, SpA-reactive fractions of IgA or IgM that also bind to SpA through Fc sites may contribute to the enhancement, since it has been shown that binding of one class of Ig to SpA does not necessarily prevent binding of another class (Section V,B). It is also important that binding of SpA at Fc sites in IgG does not block binding to Fc receptors on the neutrophils. For this reason, ^{125}I-labeled SpA could be used to detect binding to neutrophils of IgG antibodies in serum from patients with autoimmune or isoimmune neutropenia (McCallister *et al.*, 1979). Furthermore, insoluble complexes formed by adding SpA to normal guinea pig or mouse serum were ingested and degraded by homologous peritoneal macrophages to the same extent as insoluble immune complexes between HSA and guinea pig or mouse antibodies (Rhodes and Lind, 1980). In this example, Fc and Fab (i.e., antibody) binding results in equally effective opsonic activity. Rabbit serum formed only soluble complexes with SpA, and these were not ingested, whereas immune precipitates between SpA and rabbit (or guinea pig or mouse) anti-SpA were rapidly ingested and degraded. Heczko *et al.* (1974) found somewhat discrepant results in that 50% of ^{125}I-labeled SpA added to normal rabbit serum was associated with macrophages. However, it is difficult to assess the significance, since it was not demonstrated whether soluble SpA–IgG complexes or precipitates were present nor whether the complexes were actually ingested or simply localized on the cell membrane.

b. Bacteria. Heat-labile serum factors play an important role in opsonization and phagocytosis of bacteria, and several studies have shown that cell-bound and soluble SpA inhibited phagocytosis of *S. aureus* primarily by forming complexes with IgG that suppress the opsonizing ability of complement (Dossett *et al.*, 1969; Forsgren and Quie, 1974; Peterson *et al.*, 1977; Verhoef *et al.*, 1977a,b; Verbrugh *et al.*, 1979; Musher *et al.*, 1981). For example, up to 98% of *S. aureus* or *E. coli* were phagocytozed by human PMN leukocytes in 2–4% serum, whereas phagocytosis and complement activity were inhibited almost completely by 100 μg of SpA per milliliter (Forsgren and Quie, 1974). Heat-inactivated serum failed to opsonize bacteria, and, consistent with the effect of SpA on formation of complement-fixing complexes with rabbit IgG (Section VI,A), there was minimal inhibition of complement activity and phagocytosis at high concentrations of SpA (e.g., 500 μg/ml).

Experiments using several strains of *S. aureus* (including Cowan strain I and Wood 46) that produced differing amounts of SpA and human or rabbit PMN leukocytes plus homologous serum showed a direct correlation between inhibition of opsonization and phagocytosis, and levels of SpA (Dossett *et al.*, 1969; Verhoef *et al.*, 1977a; Peterson *et al.*, 1977). Most reports agree that abrogation of these activities depended primarily on the binding of SpA to IgG Fc sites (Dossett *et al.*, 1969; Verhoef *et al.*, 1977b; Musher *et al.*, 1981) to give complexes that inhibit the classical complement pathway (Verhoef *et al.*, 1977a; Peterson *et al.*, 1977; Verbrugh *et al.*, 1979). Although the alternative pathway may also be involved, it is of secondary importance (Verbrugh *et al.*, 1979). Furthermore, when IgG-deficient serum was used as a source of opsonic activity, some phagocytosis still occurred and SpA-rich strains of *S. aureus* were phagocytozed *more* efficiently than SpA-deficient strains, suggesting that, in the absence of IgG, cell-bound SpA can activate complement directly and promote opsonization (Peterson *et al.*, 1977; Verhoef *et al.*, 1977a,b).

The situation is somewhat complex, and it appears that the mechanism and magnitude of the SpA effect depends on several factors including the presence of soluble or cell-bound SpA and the presence or absence of IgG in the reaction medium. Taken together, the results have been interpreted to mean that human PMN leukocytes possess two types of receptors for opsonized *S. aureus*, one for complement and one for Fc, the latter being particularly important where SpA and heat-inactivated serum are used (Verhoef *et al.*, 1977b). Furthermore it has been reported that soluble SpA–IgG aggregates inhibited the ability of neutrophils to respond to a lymphokine (neutrophil migra-

tion inhibition factor) produced by T lymphocytes in response to SpA or mitogens (Weisbart *et al.*, 1979). This may mean that other inhibitory effects against lymphocyte mediators of phagocytic function also can operate (Colburn *et al.*, 1980).

If SpA plays a role in the virulence of certain strains of S. *aureus*, conditions *in vitro* that best mimic conditions *in vivo*, namely accessibility of bacteria to a concentrated pool of IgG and complement, suggest that inhibition of opsonization and phagocytosis, perhaps in conjunction with depression of the chemotactic response, due primarily to the SpA–IgG–complement interaction, would be important. This is especially true since nonimmune IgG, and not specific antibodies, would suffice. The effects of SpA *in vivo* are discussed in more detail in Section VI,C.

2. *Chemotaxis*

Conflicting results concerning the chemotatic activity of SpA have been reported. Some workers found that human (Harvey *et al.*, 1970) and rabbit (Sveen and Grov, 1978) PMN leukocytes migrated *in vitro* and *in vivo* in response to complexes between SpA and (presumably) IgG in normal serum or to immune complexes involving SpA and rabbit anti-SpA antibodies (Sveen and Grov, 1978). Complexes formed *in vitro* with microgram amounts of SpA and serum gave optimal activity, whereas SpA or serum alone were inactive. Complement was required since there was no chemotactic activity in heat-inactivated serum indicating that SpA would be a cytotaxigen rather that a cytotaxin. Both the classical and alternative pathways appeared to be involved, since there was reduced activity with C4-deficient serum; and C5a was considered to be the mediator, since there was no chemotaxis when C5-deficient serum was used (Sveen and Grov, 1978).

In contrast to the results of Harvey *et al.* (1972), Musher *et al.* (1981) found that SpA at approximately the same concentrations suppressed chemotactic activity of human PMN cells. The inhibition required serum and probably involved insoluble complexes between IgG and SpA, since absorption of serum with SpA–Sepharose or removal of precipitates by centrifugation restored chemotactic activity. It is not obvious why conflicting results have been obtained, although the earlier workers used heat-extracted SpA, which is known to be heterogeneous and contains smaller active fragments that can give soluble SpA–IgG complexes (see Section IV,A). Since Musher *et al.* (1981) used a better quality of SpA that gave insoluble complexes and inhibited chemotaxis, the discrepancy may be due to differences in activity depending on the physical state and/or composition of the SpA–IgG

products. More work is necessary to resolve the differences, although inhibition of chemotaxis would be more consistent with inhibition of phagocytosis of *S. aureus* by SpA discussed in the preceding section.

3. *Platelet Injury*

Hawiger *et al.* (1979) reported that lyophilized Cowan strain I bacteria incubated in human platelet-rich plasma (PRP) caused platelet aggregation and release of [^{3}H]serotonin. Platelet injury was attributed to membrane SpA interacting with IgG because Fc fragments or an excess of free SpA blocked aggregation, and trypsinization of bacteria to remove SpA abolished aggregation and reduced [^{3}H]serotonin release. Both effects did not require complement since they were unaffected by preincubation of bacteria in plasma decomplemented with cobra venom factor, zymosan or EDTA before addition to platelets.

However, the role of SpA, at least in the aggregation reaction, has been questioned by Pfueller and Cosgrove (1980), who found that lyophilized Cowan strain I (also used by Hawiger *et al.*) did cause low but variable platelet aggregation and release of [^{14}C]serotonin, but that formalinized bacteria with the same binding capacity for human IgG were inactive even when preincubated with IgG before exposure to PRP. In contrast, formalinized bacteria preincubated with fibrinogen either in the presence or the absence of IgG gave equivalent platelet aggregation and serotonin release. Since similar effects were observed with lyophilized bacteria, platelet injury may be due to an effect on the coagulation system in which fibrinogen, but not SpA, plays an important role. This is consistent with the observation that lyophilized, but not formalinized, bacteria produced fibrin strands in plasma under the assay conditions and that fibrinogen was necessary for platelet aggregation by IgG–latex or antigen–antibody aggregates (Pfueller and Cosgrove, 1980).

In contrast to cell-bound SpA, the soluble protein is free to form a variety of complexes with IgG (see Section VI,A,2) that may be responsible for release of serotonin. Thus SpA added to human (Hawiger *et al.*, 1979) or rabbit (Hawiger *et al.*, 1972) PRP does not agglutinate platelets but does cause a dose-dependent release of [^{3}H]serotonin. In the human system, free IgG, or [^{125}I]SpA alone or mixed with $F(ab')_2$ fragments did not bind to platelets, whereas mixtures with whole IgG or Fc fragments did. [^{3}H]Serotonin release was induced by SpA–IgG complexes bound to platelets in the absence of plasma proteins (e.g., fibrinogen), and the degree of release paralleled binding of the complexes.

Similarly, in the rabbit system (Hawiger *et al.*, 1972) SpA alone was

ineffective and required IgG. In addition, a functioning complement system was necessary to induce release of [^{3}H]serotonin. There was an optimal concentration of SpA for maximum release, and the dose-response curve paralleled the curve for complement consumption in a way that is very similar to the dependence of complement-fixing IgM-like complexes on the concentration of SpA (Section VI,A). With rabbit platelets, release of bioactive amines appears to be a two-step process: binding of SpA–IgG complexes to Fc receptors on the platelet membrane and activation of serotonin release involving interaction with complement.

The SpA–IgG Fc interaction does not interfere with binding of the complexes to platelet Fc receptors through the newly exposed sites. Since the two binding reactions can occur simultaneously, [^{125}I]SpA has been used to detect IgG antiplatelet antibodies or IgG involved in immune complexes that bind to platelets (Kekomäki, 1977; Kekomäki and Penttinen, 1979; Kekomäki *et al.*, 1980).

4. Protein A and Viruses

Soluble SpA neutralized infectious complexes between vaccinia or herpes simplex virus and specific antiviral IgG antibodies (Austin and Daniels, 1974, 1975). It also inhibited complement-dependent or antibody-dependent cell-mediated cytotoxicity (ADCC) against rabbit kidney cells or human fibroblasts infected with herpes simplex virus and sensitized with antiviral IgG (Austin and Daniels 1975, 1976). When SpA was added to human serum, it markedly depressed complement-mediated neutralization of virus–IgG complexes, probably by forming active aggregates with IgG that depleted complement activity (Austin and Daniels, 1975). Similarly, virus neutralization and inhibition of cytotoxcity can be explained by interaction of SpA with the Fc region of IgG antibodies bound to intact virus or to viral antigens expressed on the membrane of infected cells to sterically hinder C1 fixation or prevent the cytotoxic activity of mononuclear leukocytes in ADCC.

Austin and Daniels (1978) also found that adherence of Cowan strain I bacteria to rabbit kidney cells infected with influenza virus was enhanced fivefold when the cells were sensitized with rabbit anti-influenza serum. The binding involves an SpA–antibody Fc interaction, since the SpA-negative strain Wood 46 showed no significant adherence, and Cowan strain I did not adhere to infected cells treated with nonimmune serum or to uninfected cells incubated with anti-influenza serum. As above, soluble SpA inhibited complement-mediated lysis of antibody-coated virus-infected cells. The authors

speculated that *S. aureus* infections associated with influenza or other viruses and localized to sites of viral infection may involve SpA binding to IgG antibody on virus-infected cells. Although it is an interesting proposal, as yet there is no direct *in vivo* evidence to support such a role for SpA. Furthermore, by interfering with immune mechanisms responsible for destruction of virus-infected cells, it is conceivable that SpA on or released by bacteria could lead to a more severe infection.

5. *Protein A and Lymphocytes*

a. Mitogenic Activity: B Cells vs T Cells. In addition to the effects discussed already, SpA has been shown to activate lymphocytes *in vitro*. Since the first observations (Rodey *et al.*, 1972; Forsgren *et al.*, 1976), several studies have shown that mitogenic activity of SpA for B or T cells and the detailed nature of the response depended on several factors including whether the SpA was soluble or insoluble, the dose of SpA, the presence of serum factors, the role of helper or suppressor cells, the species and tissue source of lymphocytes, whether fetal or adult lymphocytes were used, and the presence of disease states such as immune deficiency or leukemia.

Since this aspect of SpA activity has been studied intensively for only 3 or 4 years, and, since several factors can influence the response of lymphocytes to SpA, it is not surprising that different and sometimes contradictory results have been obtained by workers using different sources of SpA, different methods of isolating and characterizing a lymphocyte subpopulation with different degrees of cell heterogeneity, and different techniques to assess cell response.

Table VI summarizes the results obtained by different groups that have studied the specificity of the mitogenic activity of soluble and insoluble SpA for isolated B and T cells from different species and tissue sources. In addition to human, mouse, hamster, and rabbit cells, soluble SpA is also mitogenic for unfractionated canine peripheral blood lymphocytes (Betton *et al.*, 1980). There is a good deal of disagreement over the specificity of soluble SpA. Although several of the factors listed above could contribute to the different results, one principal likely cause is the presence or the absence of serum in the culture medium. Thus Forsgren *et al.* (1976), who used serum-free medium, reported that soluble SpA was inactive against either peripheral blood T or B cells, whereas Turunen (1979), using cord and fetal lymphocytes, found that SpA was active in short-term culture, but only in the presence of serum. However, after culturing for 5 days even without serum, soluble SpA was mitogenic for both B and T

TABLE VI

MITOGENIC SPECIFICITY ATTRIBUTED TO PROTEIN A

Source	B-cell activity	T-cell activity	B- and T-cell activity
		A. Soluble SpA	
Human	Romagnani *et al.* (1978)[a,b] Kuritani *et al.* (1980)[c,d] Muraguchi *et al.* (1980)[b,c] Dosch *et al.* (1980)[b,c] Kasahara *et al.* (1980)[a–c]	Rodey *et al.* (1972)[c] Romagnani *et al.* (1978)[b,c] Romagnani *et al.* (1980a)[a,b] Guglielmi and Preud'homme (1980)[c] Farcet *et al.* (1980)[c] Schuurman *et al.* (1980)[c] Sumiya *et al.* (1980)[c] Kasahara *et al.* (1980)[a–c]	Sakane and Green (1978)[b–d] Ringden and Rynnel-Dagöö (1978)[c,e] Ringden *et al.* (1979)[c,e] Turunen (1979)[d,h]
Mouse	Möller and Landwall (1977)[e]	Nakao *et al.* (1980)[e,g]	Kanno *et al.* (1980)[e]
Hamster	Duthu *et al.* (1980)[e,f]		
Rabbit		Boros *et al.* (1980)[c,e,g]	
		B. Insoluble SpA	
Human	Forsgren *et al.* (1976)[c] Romagnani *et al.* (1978)[a,c] Vogt *et al.* (1979)[c,d] Kasahara *et al.* (1979)[a,c] Sirianni *et al.* (1979)[c] Räsänen *et al.* (1980)[c,d] Schuurman *et al.* (1980)[c] Sumiya *et al.* (1980)[c] Romagnani *et al.* (1988a)[a] Kasahara *et al.* (1980)[a,c]	Vogt *et al.* (1979)[b–d] Räsänen *et al.* (1980)[b–d]	
Mouse	Möller and Landwall (1977)[e] Nakao *et al.* (1980)[e,g]		
Rabbit		Boros *et al.* (1980)[c,e]	

[a] Tonsillar lymphocytes. [b] B- or T-cell helper activity is an absolute requirement or enhances response. [c] Adult peripheral blood lymphocytes. [d] Cord blood lymphocytes. [e] Spleen cells. [f] Bone marrow cells. [g] Thymocytes. [h] Fetal lymphocytes.

cells. Overall, it appears that fluid-phase SpA is more active against T cells but, depending on conditions, may activate B cells with the possibility of helper-cell involvement for optimal activity. In fact, if the behavior of lymphocytes from patients with chronic lymphocytic leukemia (Guglielmi and Preud'homme, 1980) or immune deficiency disease (Rodey *et al.*, 1972; Schuurman *et al.*, 1980; Dosch *et al.*, 1980) is taken as a true indicator, then soluble SpA is a T-cell mitogen that can activate B cells only with helper-T-cell activity. In several cases where soluble SpA was mitogenic for T cells (Table VI), it was at least as potent as other T-cell-active agents like Con A, phytohemagglutinin, and pokeweed mitogen.

Several of the reports listed in Table VI conclude that some type of helper-cell involvement or cell–cell contact is necessary for mitogenic activity of SpA. In this regard, either soluble SpA or Cowan strain I bacteria have been used to grow human T-lymphocyte colonies in semisolid agar culture, but colonies formed only on the top or bottom of the agar, presumably where sufficient cell–cell contact could occur (Farcet *et al.*, 1980). B-cell colonies could be grown by this procedure if the cells were first cultured with irradiated T cells (Muraguchi *et al.*, 1980). Unfractionated lymphocytes also form colonies if presensitized and cultured in the presence of soluble SpA (Shibasaki *et al.*, 1978).

In contrast to soluble SpA, there is general agreement that insoluble SpA on intact bacteria or bound covalently to Sepharose or Sephadex particles is primarily a T-cell-independent B-cell mitogen in the human and mouse. Two reports of T-cell mitogenic activity (Vogt *et al.*, 1979; Räsänen *et al.*, 1980) agree that helper cells are required. One should be cautious when using immobilized SpA in these experiments, since shedding or leakage of SpA from the bacteria (Schuurman *et al.*, 1980) or SpA–Sepharose (Sumiya *et al.*, 1980) has been reported. In this case, T-cell activity by soluble SpA could be falsely attributed to the immobilized protein.

The mechanism of SpA mitogenic activity has not been established, although a good guess would assume possible implication of IgG–SpA complexes (Forsgren *et al.*, 1976), especially since SpA enhances the binding of IgG to Fc on lymphocytes and macrophages (Sulica *et al.*, 1979) and can induce changes in the aggregation of Fc receptors on the surface of lymphoid cells (Gheţie *et al.*, 1974a). However, it appears that an effect other than the SpA–Fcγ interaction is responsible both for mitogenic activity (Nakao *et al.*, 1980) and polyclonal B-cell activation discussed in the following section. In fact Kuritani *et al.* (1980) and Schuurman *et al.* (1980) have shown that precursor cells for

SpA-induced proliferation were IgM and complement-receptor positive and IgD positive or negative, and Romagnani *et al.* (1980c) found that depletion of IgM- and/or IgD-bearing cells resulted in a greater reduction in mitogenic response than depletion of IgG-bearing cells. It has been claimed (Nakao *et al.*, 1980; Schuurman *et al.*, 1980) that mitogenic activity depends on a cell-surface receptor other than Ig.

There are two final points. Although mitogenic effects in solution and with SpA–Sepharose or SpA–Sephadex leave no doubt as to the identity of the active agent, it should be emphasized that many strains of *S. aureus* and other bacteria, regardless of SpA content, can activate lymphocytes to some degree (Räsänen *et al.*, 1978, 1980). Also, whereas intact SpA clearly activates lymphocytes, tryptic digests of SpA that presumably contain monovalent IgG-reactive fragments or other molecular species with exposed binding sites, are not mitogenic and abrogated the activation of mouse spleen cells induced by Con A, lipopolysaccharide, or undigested SpA (Cowan *et al.*, 1979c).

b. Polyclonal B-Cell Activation. Both soluble and insoluble SpA also are polyclonal B-cell activators. Muraguchi *et al.* (1980) and Gausset *et al.* (1980) found that soluble SpA stimulated the production and secretion of IgM, IgG, and IgA by human PBM cells. The three classes appeared sequentially over 3–5 days of culture (Muraguchi *et al.*, 1980), and, under optimal conditions for IgM and IgG, IgA production was inhibited (Gausset *et al.*, 1980). Lipsky (1980) found that IgM and IgG secretion depended on the dose of SpA; low concentrations (≤ 1 μg/ml) stimulated production, whereas higher doses (≥ 5 μg/ml) were suppressive. Studies with different cell populations suggested that soluble SpA is a T-cell-dependent suppressor T-cell-regulated B-cell activator, but, compared to pokeweed mitogen, which acted on mature B cells, SpA affected a population of less mature cells. In addition, SpA stimulated 5–10 times fewer cells than pokeweed mitogen for production of equivalent levels of Ig (Gausset *et al.*, 1980). Earlier, Ringdén and Rynnel-Dagöö (1978) reported that soluble SpA induced Ig synthesis and secretion in human spleen cells but not in peripheral blood cells, although the basis for their conflicting results is not clear.

SpA-containing bacteria also stimulate production and release of Ig from human peripheral blood lymphocytes (Ringdén *et al.*, 1977; Romagnani *et al.*, 1980c; Räsänen *et al.*, 1980; Pryjma *et al.*, 1980a,b; Sjöberg and Kurnick, 1980), cord blood (Räsänen *et al.*, 1980; Ruuskanen *et al.*, 1980), and spleen (Ringdén *et al.*, 1977). SpA–Sepharose also stimulates Ig production in peripheral blood and spleen cells (Ringdén and Dagöö, 1978) proving that at least some of the activity

attributed to the bacteria is due to SpA. This is important, since several groups (Ringdén *et al.*, 1977; Ringdén and Dagöö, 1978; Räsänen *et al.*, 1980; Pryjma *et al.*, 1980a,b; Sjöberg and Kurnick, 1980) have shown that many bacteria, including Wood 46, also can activate Ig synthesis. In contrast to Cowan strain I, pokeweed mitogen stimulated only adult blood cells and was T-cell-dependent. Although Ringdén *et al.* (1977) suggested that T cells may cooperate weakly in B-cell activation, most of the results (Romagnani *et al.*, 1980c; Ruuskanen *et al.*, 1980; Pryjma *et al.*, 1980a,b) agree that polyclonal B-cell activation by immobilized SpA is T-cell-independent. Although the same classes of Ig have not been detected uniformly, reports have demonstrated cytoplasmic and secreted IgM (Ringdén *et al.*, 1977), IgM and IgG (Romagnani *et al.*, 1980a), IgM and IgA (Ruuskanen *et al.*, 1980) IgM, IgG, and IgA (Pryjma *et al.*, 1980a), and all five classes with relatively less IgD and IgE (Pryjma *et al.*, 1980b).

SpA also has been shown to be a polyclonal T-cell-independent B-cell activator for mouse spleen cells (Möller and Landwall, 1977; Sjödahl and Möller, 1979). The results indicated that Fc binding activity is not involved, since activation took place in serum-free medium and was neither depressed by blocking SpA with human serum or IgG nor enhanced by pretreating lymphocytes with anti-Ig serum (Möller and Landwall, 1977). Furthermore, since monomeric fragment B or dimeric fragment A–B (Section IV,C,1) did not activate spleen cells, simply binding or cross-linking IgG through the Fc region is not sufficient to stimulate Ig production and secretion.

c. Interferon Production and Effects on Cytotoxicity. SpA stimulates production of type II interferon in Fc receptor-positive, T-cell-depleted preparations of human lymphocytes (Catalona *et al.*, 1981; Ratliff *et al.*, 1981), and supernatants from SpA-treated effector cells augmented natural killer (NK) activity and antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* (Catalona *et al.*, 1981; Ratliff *et al.*, 1981; Kasahara *et al.*, 1981). Interferon activity appeared after 8 hours in culture; peak activity, corresponding to augmentation of cytotoxicity, occurred after 24 hours. The kinetics of interferon production probably explains why others found augmentation of NK activity only after long-term (18 hours) culture, but not after short-term (4 hours) culture (Kay *et al.*, 1977; Vessella *et al.*, 1978; Pape *et al.*, 1979), and inhibition of ADCC in the 4-hour assay (Vessella *et al.*, 1978). Enhancement of cytotoxicity in the presence of SpA observed by other workers (Sulica *et al.*, 1976; Cowan *et al.*, 1979a) may also be due to stimulation of interferon production.

Sato *et al.* (1979) found that cell-dependent rabbit antibodies against a methylcholanthrene-induced rat fibrosarcoma were retained on a column of SpA–Sepharose, suggesting that the antibodies were of the IgG class with intact Fc. Similarly, when anti-effector cell antiserum that inhibited ADCC was chromatographed, the bound fraction was shown to be responsible for the inhibition. Since $F(ab')_2$ fragments of the retained Ig were not effective, inhibition of ADCC appears to depend on blockage of Fc receptors by IgG anti-effector serum.

Furthermore, Biguzzi (1979) found that affinity-purified chicken IgG anti-SpA or $F(ab')_2$ fragments of rabbit anti-SpA inhibited rosette formation of sensitizeed erythrocytes by a human PBM subpopulation carrying Fc receptors for IgG, suggesting a possible structural similarity between SpA and the Fc receptors. Also Crabtree (1980) has shown that a human promyelocytic leukemia cell line (HL-60) carries two types of receptors, one of which binds an SpA–IgG_1 complex.

d. Cell Separation. The interaction between SpA and IgG either as a component of the cell membrane or bound to cell-surface antigen (Gheţie *et al.*, 1974a) has been used to develop several related techniques to isolate lymphocyte subpopulations and other classes of cells. There are three basic solid-phase modifications in which components are adsorbed to plastic surfaces (Gheţie and Sjöquist, 1975; Nash, 1976; Bundesen and Gordon, 1979), SpA-Sepharose (Milon *et al.*, 1978; Gheţie *et al.*, 1978; Baxi *et al.*, 1980), or magnetic microspheres containing SpA (Widder *et al.*, 1979). The differences are mainly technical and essentially involve binding of SpA directly, or indirectly through a sandwich arrangement, to antibody directed against specific lymphocyte surface antigenic markers or, to separate B cells, to antibodies bound to Ig on the B-cell membrane. Cells were recovered mechanically, by incubation with SpA-reactive IgG (Gheţie *et al.*, 1978; Bundesen and Gordon, 1979) or by treatment with lysostaphin or ammonium chloride when SpA-bearing bacteria or erythrocytes were used as the immunosorbent (Gheţie and Sjöquist, 1975). In each case recovery of highly purified cells was generally 85–100% with no significant loss of viability or functional activity. In one of the procedures (Targan and Jondal, 1978) separation of Fc-bearing lymphocytes was achieved by adsorption to immune complexes immobilized on plastic followed by competitive elution with fluid-phase SpA.

Lymphoid cells that carry surface IgG also form rosettes with *S. aureus* or SpA-coated eythyrocytes that can be separated by density gradient centrifugation (Gheţie, *et al.*, 1974a–d, 1975).

C. Effects *in Vivo*

1. Protein A and Protein A–IgG Complexes

The ability of SpA to bind to the Fc region of IgG not only can result in complement activation and other effects *in vitro*, but also can produce effects *in vivo* that resemble immune reactions brought about by antigen–antibody complexes. Some of them appear to involve complement activated by SpA–IgG complexes in solution or on the cell membrane, or other mechanisms discussed in Section VI,B.

SpA administered intradermally to normal human subjects in doses as low as 10 μg produced wheal and erythema reactions detected after 30 minutes and a later reaction with maximum intensity after 24–48 hours (Martin *et al.*, 1967). Similarly, as little as 10 μg of SpA given intradermally to guinea pigs resulted in a significant anaphylaxis-like reaction, and 300–400 μg gave a hemorrhagic Arthus reaction with maximum intensity at 12–24 hours (Gustafson *et al.*, 1968). Guinea pigs given 500–1000 μg intracardially suffered fatal anaphylactic shock that could be prevented by prior administration of antihistamine. Heczko *et al.* (1973) also found that guinea pigs sensitized with as little as 50 μg of SpA in complete Freund's adjuvant gave a delayed reaction that was maximal at 24 hours, whereas the same dose given in incomplete adjuvant or in saline gave an Arthus reaction that was maximal at 6 hours. A second similar sensitization in any of the three media gave a delayed hypersensitivity reaction as well as an early reaction. Sensitivity could be transferred to normal animals by lymphoid cells but not by serum.

In contrast to man and guinea pig, other species with IgG that does not precipitate SpA failed to give hypersensitivity reactions when given SpA alone. Thus rabbits given a single injection of up to 6 mg of SpA and mice and rats given 10–500 μg showed neither early onset nor delayed reactions (Gustafsen *et al.*, 1967, 1968; Kronvall *et al.*, 1970c; Masuda and Kondo, 1979), nor was there any sign of kidney lesions in mice given up to five 10-μg doses per day for 8 days (Kronvall *et al.*, 1970c). However, in rabbits, a direct Arthus reaction was produced in 5–10 hours by administration of 120–180 mg of human IgG intravenously followed in 10–15 minutes by 0.025–1.5 mg of SpA intradermally, and hemorrhagic necrosis was evident 18–24 hours after intradermal injections of preformed precipitates between human IgG and SpA (Gustafson *et al.*, 1967). As with guinea pigs, rabbits sensitized with as little as 50 μg of SpA in complete Freund's adjuvant gave early and delayed hy-

persensitivity reactions when challenged with SpA after 10 days. Sensitization with SpA in saline was ineffective. If a booster injection was given before testing, the early reaction was enhanced and the delayed reaction was less pronounced (Heczko *et al.*, 1973). Delayed hypersensitivity could be transferred with lymph node or spleen cells to normal recipients with strong skin reactions detectable after 48 hours. Immediate hypersensitivity reactions probably result from a SpA–Fc interaction, whereas delayed reactions observed with the use of adjuvant or after resensitization may involve interaction in dermis with antibodies to SpA or perhaps antibodies against hidden determinants of autologous IgG (see Section V,F). Soluble complexes between rabbit IgG and ^{125}I-labeled SpA were cleared rapidly from the circulation of rabbits by liver and spleen, and up to 49% of labeled complexes were taken up *in vitro* by saline-perfused rabbit liver during one passage (Hällgren *et al.*, 1977).

Mice infected with staphylococci show a delayed hypersensitivity reaction, and several cell wall fractions including SpA have been tested to determine which components are responsible (Easman and Glynn, 1978; Tribble and Bolen, 1978, 1979; Bolen and Tribble, 1979). In mice primed with cyclophosphamide and given a single injection of straphylococci, challenge with SpA 12 days later give no delayed footpad swelling (Easmon and Glynn, 1978). However, in mice given repeated injections over several days, there were two peaks of delayed hypersensitivity, one occurring after 3 days, the second after 7 days (Bolen and Tribble, 1979, 1981; Tribble and Bolen, 1979). The first peak did not depend on SpA, but the second response depended on several cell-wall components, including SpA. Furthermore, SpA injected into mice immunized with sheep erythrocytes augmented or inhibited delayed hypersensitivity depending on the dose, perhaps by binding to the Fc region of IgG involved in the formation of active immune complexes (Cowan *et al.*, 1979a,b).

2. *Protein A and Pathogenicity of S. aureus*

Even before the scope of SpA activity on components of the humoral and cellular immune systems was fully appreciated, there was speculation that SpA may be an important factor in the pathogenicity of *S. aureus* and the inability of antibacterial antibodies to provide an effective deterrent to bacterial infection (Forsgren, 1970; Mudd, 1971). However, those attempts at determining the effect of SpA on the *in vivo* response to *S. aureus* have failed to demonstrate a dominant or highly significant role for SpA.

Forsgren (1972, 1973) compared the pathogenicity of Cowan strain I, chemically induced mutant strains deficient in SpA and/or other types of activity, and revertant strains (Forsgren *et al.*, 1971; see Section III,C) in mice by measuring the relative LD_{50} dose 10 days after the organisms were given intravenously. Effects in local infections also were assessed by counting viable bacteria at the site of injection after subcutaneous administration in cotton dust. Both sets of results showed no difference between Cowan strain I, strains lacking SpA and α-hemolysin, strains lacking SpA and coagulase, or revertant strains. Strains that were α-hemolysin positive but negative for SpA, nuclease, coagulase, fibrinolysin, mannitol utilization, and phage type pattern, or strains that were nuclease positive but deficient in the other properties, were significantly less virulent than Cowan strain I.

Similarly, in guinea pigs injected intracardially, Cowan strain I and a mutant strain deficient only in SpA had essentially the same LD_{50}, whereas the LD_{50} dose of a mutant strain deficient in all the above-mentioned properties, including SpA, was 40 times higher (Forsgren, 1972).

Gross *et al.* (1978) compared the clearance of an SpA-positive strain (FDA-209) and a nonproducing strain (Wood 46) of *S. aureus* from the lungs of mice given a 30-minute aerosol inoculation. Each mouse received the same initial deposit of organisms, and there was no significant difference in the rate of clearance over 4 hours. Hsieh *et al.* (1978) performed similar experiments with strain 566 as the SpA-producer, except they also estimated IgG on the surface of bacteria at different time points. Consistent with the results of Gross *et al.* (1978), they found no difference in the rate of lung clearance even though 25–40% of strain 566 organisms had IgG on the surface, whereas there was no significant amount of IgG associated with Wood 46. It would therefore appear that SpA or high levels of homologous IgG on staphylococci do not affect bacterial clearance by alveolar macrophages, the main route by which *S. aureus* is eliminated by the lung.

These results are not necessarily at odds with observations *in vitro*, since complement activation by SpA–IgG complexes is believed to play an important role in inhibition of phagocytosis by PMN leukocytes, while the *in vivo* results (Gross *et al.*, 1978) showed that complement probably is not involved in a major way and macrophages are the primary cells involved. Perhaps closer to the *in vitro* observations, Masuda and Kondo (1979) injected mice intravenously with a sublethal dose of a SpA-producing strain (βH)

or a nonproducing mutant strain (PN1) and after 1 week gave 250 μg of SpA intravenously. There was a marked increase in PMN leukocytes and C3 levels in both sets of animals after inoculation. However, there was a subsequent decline after SpA was administered, and there were several fatalities due to shock. Since the mutant does not give rise to anti-SpA antibodies, anaphylaxis in mice treated with this strain may well involve SpA interacting with IgG antibodies to other staphylococcal antigens leading to complement activation. A specific anti-SpA reaction may also be involved with the animals given the SpA-positive strain, consistent with the higher number of deaths in that group.

D. Protein A in Cancer Therapy

Serum-blocking activity associated with anti-tumor IgG antibodies, perhaps involved in immune complexes with antigen shed by tumor cells, may be responsible in part for the inability of the host to mount an effective immune response to his tumor. Steele *et al.* (1974) tested the effects of absorbing serum of rats carrying polyoma virus-induced sarcomas or chemically induced colon carcinomas with SpA-bearing staphylococci on anti-tumor activity *in vitro*. They found that absorption with Cowan strain I, but not with SpA-negative Wood 46 bacteria abrogated the ability of the serum to block specific lymphocyte- or complement-mediated cytotoxicity. Although absorption removed 20–30% of total IgG, significant levels of cytotoxic antibodies remained. This suggests that the latter represent a class or subclass of rat Ig that is unreactive with SpA, or that, if immune complexes are involved in blocking activity, they may have a higher affinity for SpA compared to free IgG and are absorbed selectively, leaving residual active antibody. Addition of SpA-absorbed serum to a sample of unabsorbed homologous serum also abrogated blocking activity but had no effect on unabsorbed serum from animals bearing a heterologous tumor.

After these results, extracorporeal perfusion of plasma from a patient with metastatic colon carcinoma (Bansal *et al.*, 1978a) or dogs with a variety of spontaneous cancers (Bansal *et al.*, 1978b; Terman *et al.*, 1980a,b; Terman, 1981) over SpA-containing adsorbents was shown to induce significant tumor regression and to affect several immunological activities in *in vitro* tests. Plasma separated from formed elements was filtered through a column of formalinized Cowan strain I bacteria, and the absorbed plasma recombined with cells and circulated back into the host. The patient with colon cancer was treated 20 times over 5 months with clearly beneficial results. There was a gen-

eral improvement in his condition, a reduction in tumor size, and histological changes consistent with specific tumor destruction. After each treatment there was a significant reduction in IgG levels but a minimal effect on IgM and IgA. This effect was transient, and Ig levels rebounded over preperfusion levels before returning to normal. There were several other effects including a transient increase in the population of Ig-bearing lymphocytes and a sharp decrease in rosette-forming cells that persisted for 7–10 days after perfusion, a decrease in serum-blocking activity and immune complexes, and a short-lived appearance of complement-dependent cytotoxic activity.

Similar beneficial results were obtained in dogs with mammary adenocarcinoma (Terman *et al.*, 1980a,b), squamous cell carcinoma, hemangiopericytoma, and fibrosarcoma (Terman, 1981). In these experiments it was shown that perfusion of tumor-bearing sera over strain 46 bacteria was ineffective, that SpA was not dissociated from the active adsorbent during perfusion, and that perfusion over SpA adsorbed to collodion charcoal was as effective as using Cowan strain I bacteria. These results strongly suggest that SpA is the active component. In addition, perfusion followed by treatment with cytosine arabinoside had a synergistic effect resulting in more rapid and severe tumor necrosis than observed with perfusion treatment alone. Drug treatment alone was ineffective.

A clear response generally was observed within 4 hours after perfusion, with progressive necrosis and often healing of large areas of ulceration within 1–3 weeks (Terman *et al.*, 1980a,b; Terman, 1981). Tissue damage was restricted to the tumor and the early stages of necrosis were associated with a humoral response with inflammatory cells, mainly neutrophils, appearing only after 48 hours. Immunofluorescence showed significant levels of IgG and C3 deposited on tumor cells after perfusion and localized at sites of membrane damage (Terman *et al.*, 1980a; Terman, 1981). There was little or no IgM detected on the cells before or after perfusion, and, as with the human subject, serum IgG levels decreased significantly after perfusion but rebounded within 72 hours to levels that often exceeded preperfusion levels concomitant with an increase in circulating anti-tumor antibodies (Terman, 1981). C3 levels also decreased and remained low for 48 hours, whereas levels of immune complexes increased significantly, both effects possibly owing to the increase in complement-fixing IgG antibodies that appeared to be associated with the successful anti-tumor response.

Although plasma perfusion over SpA-active adsorbents is a promising experimental approach to cancer treatment, especially in conjunc-

tion with chemotherapy, it still is in the early stages of development. Long-term effectiveness and clinical application to different types and stages of human cancer have yet to be tested.

VII. Analytical Applications of Protein A

SpA has become an extremely important immunochemical reagent beyond its ability to isolate IgG or IgG subclasses discussed in Section V,B. Some of the earlier applications have been reviewed by Goding (1978). Table VII lists labeled SpA derivatives that have been used in several methods including immunoassay of lymphocyte or tumor cell-surface antigens (Welsh *et al.*, 1975; Biberfeld *et al.*, 1975; Dorval *et al.*, 1975; Ades *et al.*, 1976; Langone *et al.*, 1977a; Zeltzer and Seeger, 1977; Birchall *et al.*, 1977; Brown *et al.*, 1977; Goding, 1978; Wilder *et al.*, 1979; Langone 1980a), viral antigens and antibodies (Cleveland *et al.*, 1979; Moar *et al.*, 1979; Madore and Baumgarten, 1979; Columbatti and Hilgers, 1979; Butler *et al.*, 1979; Potgieter *et al.*, 1980), assay of antigen-specific IgG antibodies in serum (Hamilton *et al.*, 1979; Hamilton and Adkinson, 1980) or monoclonal antibodies produced by hybridomas (Brown *et al.*, 1979; Buchanan *et al.*, 1981), or for quantification of antigens in physiological fluids by competitive radio- and enzyme-immunoassay procedures (for a review see Langone, 1980a; Gee and Langone, 1981). Derivatives with fluorescent, ferritin, hemocyanin, or gold particle labels (Table VII) also offer advantages over labeled antibodies as cytochemical markers.

Since these reagents are used routinely and additional applications are constantly appearing, the references given here are only representative and are not intended to constitute a comprehensive survey. However, each variation relies on the specific binding of SpA to the Fc region of IgG, and since the principles are essentially the same with only technical differences, these papers should serve as useful guides for the development of new assays. One distinct advantage of radiolabeled or enzyme-labeled SpA in competition immunoassays is the ability to use a single tracer molecule regardless of the nature of the antigen or specificity of the antibody. This has allowed the development of immunoassays for some drugs and prostaglandin derivatives that were unstable to conventional labeling conditions and for which classical radioimmunoassays could not be developed (Langone and Levine, 1979; Levine *et al.*, 1979; Langone, 1980a,d; Gee and Langone, 1981).

Heat-killed formalin-fixed Cowan strain I bacteria are sold by sev-

TABLE VII
LABELED DERIVATIVES OF PROTEIN A FOR USE IN IMMUNOASSAY

Protein A labeled with	References
I. Radionuclides	
A. ^{125}I	
1. Chloramine-T method	Dorval *et al.* (1974, 1975), Welsh *et al.* (1975)
2. Lactoperoxidase method	Biberfeld *et al.* (1975)
3. *N*-Succinimidyl-3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent)	Langone *et al.* (1977a), Langone (1980a)
B. ^{131}I (chloramine-T method)	Zeltzer and Seeger (1977)
C. ^{3}H	
1. [^{3}H]Formaldehyde (reductive methylation)	Wilder *et al.* (1979)
2. [^{3}H]Acetic anhydride (acetylation)	Lambden and Watt (1978)
D. [^{14}C]Acetic anhydride (acetylation)	Dorval *et al.* (1974)
E. ^{32}P-labeled Cowan strain I bacteria	Farrell *et al.* (1975)
II. Enzymes	
A. Horseradish peroxidase (type VI)	
1. Periodate method	Dubois-Dalcq *et al.* (1977), Falini *et al.* (1980), Nygren and Hansson (1981)
2. Glutaraldehyde	Nygren and Hansson (1981)
3. Benzoquinone	Nygren and Hansson (1981)
4. *N*-Succinimidyl-3-(2-pyridyldithio) propionate	Pain and Surolia (1981), Surolia and Pain (1981)
B. Alkaline phosphatase (type VII) (glutaraldehyde method)	Engvall (1978), Buchanan *et al.* (1981)
C. Invertase (glutaraldehyde method)	Pain and Surolia (1979), Surolia and Pain (1981)
III. Fluorescent tracer	
A. Fluorescein isothiocyanate	Gheţie *et al.* (1974), Dorval *et al.* (1974), Biberfeld *et al.* (1975), Ades *et al.* (1976)
B. Fluorescein-labeled Cowan strain I bacteria	Gheţie *et al.* (1974)
IV. Other tracers (for microscopy)	
A. Ferritin	Templeton *et al.* (1978)
B. Hemocyanin	Miller *et al.* (1980)
C. Gold particles	Roth *et al.* (1978, 1980, 1981), Batten and Hopkins (1979)
D. Iron-containing magnetic albumin microspheres	Widder *et al.* (1981)

eral manufacturers and offer an attractive alternative to double antibody precipitation or other methods of separating labeled antigens from antigen–antibody complexes in immunoassay. This procedure was developed by Jonsson and Kronvall (1974) for radioimmunoassay of α-fetoprotein and subsequently used in assays for albumin (Gupta and Morton, 1979; Mottolese *et al.*, 1980), epidermal growth factor (O'Keefe and Bennett, 1980), ferritin (Gauldie *et al.*, 1980), digoxin (O'Keefe and Bennett, 1980; Gauldie *et al.*, 1980), toxins (Miller *et al.*, 1978; Shaffer *et al.*, 1979), hormones (Ying and Guillemin, 1979; Frohman *et al.*, 1979; Mitchell *et al.*, 1980; Rebar *et al.*, 1980; Ying, 1981), autoantibodies to human albumin (Mihăescu *et al.*, 1981), and antibodies to viruses (Jahrling *et al.*, 1978; Soergel *et al.*, 1978). Precipitation with solid-phase SpA also is an extremely useful way to isolate immune complexes from serum (Chenais *et al.*, 1977; Tucker *et al.*, 1978; McDougal *et al.*, 1979; Bauer *et al.*, 1980; Maidment *et al.*, 1981; Virella *et al.*, 1981), as well as from solubilized cell membranes or other sources (Kessler, 1975, 1976; Cullen and Schwartz, 1976; Brunda *et al.*, 1977; MacSween and Eastwood, 1978, 1981; Pischel and Little, 1980; Lambris and Paramichail, 1980; Pearson and Anderson, 1980; McKay and DiMaio, 1981; McKay, 1981).

The plaque assay developed by Gronowicz *et al.* (1976) allows detection of Ig secreted by lymphocytes in culture. Erythrocytes coated with SpA bind the secreted Ig, and hemolytic plaque formation occurs in the presence of a second anti-Ig and complement. Variations and several applications have been reported (Lanzavecchia *et al.*, 1979; Smith and Hammerström, 1979; Hammerström *et al.*, 1979, 1980; Burns and Pike, 1981; Van Oudenaren *et al.*, 1981). Similarly, erythrocytes coated with SpA form rosettes with IgG-coated cells and can be used to detect and quantify IgG on lymphoid cells (Gheţie *et al.*, 1974c).

VIII. Immunoglobulin Receptors Produced by Streptococci

A. Introduction

The same techniques used to characterize SpA have been used to show that certain strains and types of streptococci carry surface receptors for Ig. Although unidentified reactive serum components have been reported (Björck and Kronvall, 1981), there are five defined specificities associated with steptococci, each with a distinct receptor.

1. Binding of aggregated β_2-microglobulin by most A, C, and G strains (Kronvall *et al.*, 1978).

2. Binding of fibrinogen and fibrinogen fragments by A, C, and G strains (Runehagen *et al.*, 1981) with a binding pattern similar to that of *S. aureus* (see Section V,D)
3. Binding of serum albumin by group C and G streptococci (Kronvall *et al.*, 1979; Myhre and Kronvall, 1980b) with approximately 80,000 sites per bacterium and a K_D value of approximately 10^{-7} *M*
4. Binding of haptoglobulin by strains carrying T4 antigen (Köhler and Prokop, 1978)
5. Nonimmune binding primarily of strains A, C, and G to the Fc region of Ig. This reaction is analogous to the reaction between SpA and Fc, but the evidence suggests that different binding sites on the Ig molecule are involved. This is the reaction that is discussed in the following sections.

B. Reactivity with Human Immunoglobulin Classes, Subclasses, and Fragments

1. IgG

a. Specificity. Kronvall (1973b) was the first to report that 15 of 57 A, C, and G strains agglutinated IgG-sensitized sheep erythrocytes and 25 of them produced soluble factors that had similar nonimmune Fc binding activity. Several human myeloma IgG proteins were used to test specificity by their ability to inhibit binding of ^{125}I-labeled polyclonal IgG to bacteria, and all four subclasses were reactive including two IgG_3 proteins; one reacted with a group A and both with a group G streptococcus, but neither reacted with *S. aureus* Cowan strain I. Christensen and Oxelius (1974) found similar results with A, B, C, and G strains. Myhre and Kronvall (1980c) did extensive studies with polyclonal IgG and 19 myeloma proteins and corroborated the reactivity of groups A, C, and G with all four subclasses, and showed that groups C and G bound twice as much IgG as group A bacteria. The four subclasses also inhibited agglutination of sensitized erythrocytes by group A, C, and G bacteria (Christensen *et al.*, 1979a). These results suggest that IgG_3 activity may distinguish between the streptococcal receptor and SpA, although it should be remembered that recent findings indicate that a fraction of polyclonal IgG_3 may bind SpA (Section V,B,2,a).

When sera from 14 different species were tested for their relative ability to inhibit binding of [^{125}I]IgG to either Cowan strain I or to a group A or group G bacterium, the inhibition patterns were different, lending further support to the concept that the receptors are different (Myhre and Kronvall, 1980b). In fact this test was used to

differentiate four types of receptors, based on inhibition patterns (Myhre and Kronvall, 1977; Myhre *et al.*, 1979). The SpA receptor is classified as type I, the receptor characteristic of group A streptococci is type II, and groups C and G carry a common or related receptor designated type III. Class IV Fc receptors are found only in bovine group G β-hemolytic streptococci. Another class, or perhaps a subclass of the SpA (type I) receptor is found only in strains of equine *Streptococcus zooepidemicus* (Myhre and Kronvall, 1980c). It shows the same subclass specificity as SpA, binding human IgG_1, IgG_2, and IgG_4 and bovine IgG_2, but not IgG_1.

Christensen and Kronvall (1974) reported that in addition to group A, C, and G activity, groups B and D and heat extracts of these strains agglutinated sensitized sheep erythrocytes. In contrast, Freimer *et al.* (1979) also used a hemagglutination test with purified human Fc and erythrocytes coated with anti-Rh antibodies and found that A, C, and G strains were active but groups B and D were not, and the Kronvall group later found (Myhre and Kronvall, 1977) that strains A, C, and G were able to bind ^{125}I-labeled IgG, but groups D and B did not. However, the conflict is still not resolved, since uptake of ^{125}I-labeled polyclonal or monoclonal IgG also was tested by a second group with strains A, B, C, and G, and all four were active (Christensen and Oxelius, 1974). At present it is agreed that nonimmune Fc activity is found in strains A, C, and G, but agreement on group B is not unanimous.

Furthermore, hot acid (Lancefield) extracts of group A type 15 streptococci have been shown to precipitate IgG from human serum (Schalén *et al.*, 1978), and IgG myeloma proteins bound to group A bacteria have been visualized by electron microscopy using ferritin-labeled reagents (Christensen *et al.*, 1976b).

Similarly to the reaction between SpA and IgG, the primary binding activity for receptors on streptococci is localized in the Fc region of the IgG molecule. Christensen *et al.* (1976a) showed that on a molar basis Fc fragments and intact IgG were equally effective inhibitors of ^{125}I-labeled myeloma IgG binding. There was a trace of activity associated with $F(ab')_2$ fragments, but Fab and Fc′ fragments were completely inactive. Similarly Fc but not Fab fragments inhibited hemagglutination of sensitized human red cells by Lancefield extracts of group A, C, and G streptococci (Christensen *et al.*, 1979a), and only Fc fragments were able to displace the precipitin line formed between human IgG and the Fc receptor present in alkaline extracts of group A type 15 streptococci (Christensen *et al.*, 1979b). Myhre and Kronvall (1980a) also found that ^{125}I-labeled Fc fragments from polyclonal or monoclonal IgG bound to group A and

G bacteria along with low but significant (7%) binding of polyclonal Fab fragments. Fab fragments from two myeloma IgG_1 proteins showed a high level of binding (61 and 21%) to a group G bacterium that was inhibited by polyclonal IgG but not by albumin or the other proteins that can bind to streptococci (Section IX,A).

These results are in general agreement with the original work of Kronvall (1973b), who found that reactivity of two IgG_1 proteins was localized in the heavy chains. His finding that Fc fragments were not active probably resulted from overreaction with papain since only 6% of the Fc preparation bound to Cowan strain I bacteria compared to undigested IgG. Although relatively little information is available compared to SpA, the fact that Fc′ fragments do not bind to the streptococcal receptor suggests that the C_H2 portion of IgG and perhaps an intact C_H2–C_H3 domain is required for binding.

Antibacterial antibodies bound to SpA-containing S. *aureus* through Fc sites retain their ability to bind antigen. These immobilized antibodies have been used in a simple agglutination procedure for grouping streptococci (Christensen *et al.*, 1973; Edwards and Larson, 1974; Rosner, 1977; Leland *et al.*, 1978; Prakash *et al.*, 1980), pneumococci (Kronvall, 1973c), and gonococcal antigens (Danielsson and Kronvall, 1974).

b. Aggregated IgG. The ability to bind aggregated IgG in the presence of fresh human serum is characteristic of only a few types of group A streptococci (Christensen *et al.*, 1977b, 1978), principally type 12 (Christensen *et al.*, 1981). All 52 such strains showed binding activity regardless of whether they were nephritogenic or not, whereas none of 24 type 49 strains were active and only 6 of 30 strains other than type 12 or 49 failed to bind (Christensen *et al.*, 1981). None of the group B, C, D; E, G, L, M, and N strains tested showed significant binding (Christensen *et al.*, 1978).

The early-acting complement components C1 and C4 are required because heated serum is ineffective, but activity is restored upon addition of purified C1q or C1. The alternative pathway does not appear to be involved (Christensen *et al.*, 1977b), and it is highly doubtful that the Fc–IgG interaction plays a role, since those types with reduced uptake of aggregates in serum often had similar or even enhanced ability to bind Fc (Christensen *et al.*, 1976b, 1977b).

2. *IgA*

Group A type M4 streptococci bind ^{125}I-labeled myeloma IgA proteins (Christensen and Oxelius, 1975; Schalén, 1980) whereas B, C, G, and D strains are inactive (Christensen and Oxelius, 1975). Both

IgA subclasses bind, and the reaction is specific since unlabeled polyclonal IgA or myeloma IgA_1, but not polyclonal IgG or myeloma IgG_1, inhibited binding. Similarly, IgA did not inhibit binding of ^{125}I-labeled IgG, suggesting that there may be different receptors for each class. This conclusion is supported by evidence discussed in Section IX,D. The specificity probably accounts for the incorrect conclusion that IgA did not bind based on the inability of cold IgA myeloma proteins to inhibit binding of ^{125}I-labeled IgG to group A strains (Kronvall, 1973b) or to inhibit agglutination of IgG-sensitized erythrocytes by streptococci (Christensen *et al.*, 1979a). Like the SpA- and strep-IgG interactions, binding activity is localized primarily in the Fc region (Schalén, 1980).

3. *IgD*

^{125}I-labeled myeloma IgD binds to group A, C, and G streptococci but more efficiently to *N. catarrhalis* and *H. influenzae* (Forsgren and Grubb, 1979). Studies with the latter two bacteria and using labeled IgD or IgD fragments in direct binding experiments or in inhibition tests suggest that activity is found primarily, but not exclusively, in the C_H1 region. Earlier Kronvall (1973b) reported that a single myeloma IgD failed to inhibit binding of ^{125}I-labeled IgG to streptococci, but this probably reflects heterogeneity in receptor specificity as discussed above.

4. *IgM and IgE*

Polyclonal (Christensen *et al.*, 1976a) or myeloma IgM proteins (Kronvall, 1973b) failed to inhibit binding of ^{125}I-labeled IgG to group A, C, and G streptococci or to inhibit bacterial agglutination of sensitized human erythrocytes (Christensen *et al.*, 1979a). Similarly, the single IgE myeloma protein tested (Kronvall, 1973b) did not inhibit [^{125}I]IgG binding. Although these results were negative, direct binding experiments preferably with radiolabeled IgM and IgE must be carried out before reactivity can definitely be ruled out.

C. Other Species of Immunoglobulin

In addition to human IgG, IgA, and IgD, rabbit, bovine, and mouse IgG have been shown to bind to certain strains of streptococci. Christensen *et al.* (1977a) found that all IgG could be absorbed by incubation of rabbit serum with streptococcol group A type M1 or M56 bacteria, and antibodies were absorbed regardless of antigen specificity.

Human and bovine group G streptococci show different binding

patterns for human and bovine IgG. Seventeen of twenty human strains bound both bovine IgG_1 and IgG_2 as well as human IgG whereas 13 of 16 bovine strains reacted with human IgG and failed to bind either bovine subclass (Myhre *et al.*, 1979). This is in contrast to SpA, which primarily binds bovine IgG_2 in addition to human subclasses (Sections V,B,2 and V,B,8). Inhibition studies described above using a panel of animal sera to determine relative reactivities indicated that both species of IgG bound to the same receptor. As already mentioned, *Streptococcus zooepidemicus* strains show SpA-like specificity binding human IgG_1, IgG_2, and IgG_4 in addition to bovine IgG_2 but not IgG_1 (Myhre and Kronvall, 1980c). Mouse IgG also binds through Fc sites to certain strains of streptococci (Myhre and Kronvall, 1980d).

D. Properties of Streptococcal Receptors

Most of the studies have been carried out with intact streptococci, although some attempts have been made to isolate and characterize receptors using techniques similar to those used with SpA. The IgG Fc receptor from *Streptococcus azgazardah* (group C) was purified 30-fold by passing a phage lysate over human IgG–Sepharose and eluting with 3 *M* potassium thiocyanate followed by gel filtration to give pure receptor protein (Christensen *et al.*, 1976a). The molecular weight was 60,000, and it was sensitive to trypsin and heating at 95°C for 10 minutes at pH 2, the conditions normally used to extract M protein. This receptor was similar to the active material isolated from group A type 56 bacteria by heating at 120°C for 30 minutes. The difference in heat stability suggests that the two receptors are different. An Fc receptor with a molecular weight of approximately 100,000 was isolated from *Streptococcus pyogenes* by a similar hot-acid extraction (Havliček, 1978), and there was no correlation between occurrence of Fc-reactive material and M protein. Similarly, others have shown that the Fc receptor(s) for human IgG from group A type 15 (Christensen *et al.*, 1979b) or type 4 (Schalén *et al.*, 1980) bacteria is different from M protein, peptidoglycan, group-specific carbohydrate, and lipoteichoic acid. Furthermore, the receptors for human IgA and IgG on the same group A type 4 bacterium have been separated by electrophoresis (Schalén *et al.*, 1980). They had similar electrophoretic mobility, but addition of IgA resulted in a change in the mobility of only the IgA receptor.

Analysis of the Scatchard plot for the binding of a ^{125}I-labeled human IgG_1 myeloma protein to *Streptococcus* A or G bacteria gave K_D values of 1.1×10^{-8} and 1.3×10^{-8} *M*, respectively (Myhre and

Kronvall, 1980a), which are similar to K_Ds obtained for the binding of IgG to SpA or of ^{125}I-labeled SpA to immobilized rabbit IgG (Section V,C; Table V).

The streptococcal Fc receptors are relatively heat stable with no significant reduction in IgG or IgA binding capacity or agglutinating ability after heating at temperatures up to 60°C for 30 minutes (Christensen and Kronvall, 1974; Christensen and Oxelius, 1974, 1975; Christensen *et al.*, 1977a) or 80°C for 5 minutes (Myhre and Kronvall, 1980a,c). Heating at 100°C for up to 15 minutes (Christensen and Oxelius, 1974, 1975) reduced but did not eliminate binding activity, whereas the purified IgG Fc receptor from *Streptococcus azgazardah* lost all activity after heating at 95°C for 10 minutes at pH 2.0 (Christensen and Holm, 1976).

Trypsin also destroyed activity of the purified receptor (Christensen and Holm, 1976) and both trypsin (Christensen and Oxelius, 1974, 1975; Christensen *et al.*, 1977a,b; Myhre and Kronvall, 1980b,c) and pepsin (Myhre and Kronvall, 1980b,c) reduced the binding capacity of several streptococci for IgG and IgA, although there were differences in the sensitivity of individual strains to the enzymes (Myhre and Kronvall, 1980c). Protease from *Streptomyces griseus* also reduced the agglutinating ability of streptococci against sensitized sheep erythrocytes, but the effects of trypsin or pepsin were minimal (Christensen and Kronvall, 1974).

Although the role of the Fc receptors in pathogenicity has not been investigated to any degree, an extracellular protein with a molecular weight of 46,000 reportedly is produced almost exclusively by nephritogenic group A streptococci and has been shown to be associated with immune complexes containing IgG and deposited in glomeruli (Villarreal *et al.*, 1979).

IX. Results with Pneumonococci

Conflicting results have been obtained with pneumonococci. Stephens *et al.* (1974) reported that 30 of 76 strains of *Diplococcus pneumoniae* agglutinated IgG-sensitized sheep erythrocytes. The reaction was inhibited by Fc fragments and H chains but not by Fab fragments or L chains. IgG_1 was the only highly reactive IgG subclass, and IgM and IgA were not reactive in inhibition experiments. In contrast Myhre and Kronvall (1977) found there was no detectable binding of ^{125}I-labeled human polyclonal IgG to any of 40 strains of *Streptococcus pneumoniae*. Since this test is a better measure of reactivity,

and if these results are truly indicative of all pneumonococci, then the presence of a receptor is still doubtful.

X. Concluding Remarks

In this review I have discussed the occurrence, properties, applications, biological activity, and potential significance of Ig receptors produced mainly by staphylococci and streptococci. Although they act primarily as Fc receptors, for this discussion they have been classified more broadly as Ig receptors. This is because SpA displays secondary binding activity localized in the Fab region of certain species and classes of Ig, and this has been reported to be the sole type of activity associated with polyclonal human IgE.

SpA is well characterized, and its principal reaction with IgG has been shown to account for several effector functions, expressed *in vitro* and *in vivo*, that are commonly associated with antigen–antibody complexes. The major difference aside from the site of antigen or SpA binding is the nonspecific nature of the SpA–Ig reaction in the sense that nonimmune IgG as well as antibodies appear to react with SpA in the same way. A major goal for the future is to clarify the role of these receptors *in vivo*.

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Regulation of Immunity to the Azobenzenearsonate Hapten

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I. Introduction

Regulatory events relevant to the immune response are determined by genes located on distinct chromosomes in the mouse, which influence the type and constitution of certain immunological reactions. It is the intent of this chapter to review in depth our structural, genetic, and cellular data evaluating the immunological responses to the hapten, azobenzenearsonate, coupled to proteins or cells. In so doing we will compare our data with those of our colleagues in the field. Out of necessity, only certain examples will be drawn from the literature and we wish to point out that many uncited scientific reports have helped to clarify the studies described here on the role of idiotypy in regulation.

Idiotypes associated with antibodies of a given specificity from mice of an inbred strain (Weigert and Potter, 1977) have proven useful for investigations in a number of areas. These include the nature and degree of diversity of antibodies of a single specificity; genetic variations among strains of mice with respect to their antibody repertoire; the closeness of linkage of *V* and *C* genes; the development of the repertoire of antibodies during ontogeny; immunosuppression of idiotypes and regulation of the immune response via idiotypic determinants; properties of the idiotypic network; the nature of T-cell receptors and T-cell helper and suppressor factors, and their relationship to immunoglobulins (Ig).[1]

The idiotype utilized in the studies reviewed here is that associated with antibodies to the azobenzenearsonate (ABA) hapten in strain A mice (Kuettner *et al.*, 1972; Nisonoff *et al.*, 1977). The system is useful because all mice of the A or AL/N strain respond to immunization with KLH-ABA by producing antibodies bearing a major cross-reactive idiotype (CRI); in general between 20 and 70% of the anti-ABA antibody population carries the idiotype. More recently a second idiotypic population, which is represented in the same immune sera, has been described (Gill-Pazaris *et al.*, 1979, 1980). This comprises 5–10% of the anti-ABA antibodies produced in most A/J mice and is serologically distinct from the major CRI. It is of interest that this "minor" idiotype in the A/J strain corresponds to a major idiotype associated with anti-ABA antibodies of BALB/c (Brown and Nisonoff

[1] Abbreviations: ABA, azobenzenearsonate; Ab, antibodies; anti-Id, antiidiotype; B cells, bursa equivalent or bone marrow derived; C, constant region; CRI, cross-reactive idiotype; D, diversity region; Id, idiotype; Ig, immunoglobulin; J, joining region; KLH, keyhole limpet hemocyanin; SAC, splenic adherent cells; T cells, thymus-derived cells; MA, monoclonal antibody; V, variable region; V_H, variable region of the heavy chain; V_L, variable region of the light chain.

1981; Brown *et al.*, 1981b). Thus, anti-Id prepared against A/J anti-ABA Ig reacts with an average of 40%, and as much as 60%, of the anti-ABA antibodies from BALB/c mice, although the converse is not true.

In addition to KLH, several protein carriers, when conjugated to ABA and inoculated into strain A mice, induce anti-ABA antibodies with a high content of the CRI. These include ovalbumin, edestin, and β-lactoglobulin. When bovine IgG was used as the carrier the average content of CRI in the anti-ABA Ig was considerably reduced, and an occasional A/J mouse was CRI-negative (Wang and Nisonoff, 1973). The structure of the protein, at the site to which the ABA is conjugated, nevertheless appears to influence the induction of the idiotype (Makela *et al.*, 1977). When the ABA was first conjugated to *p*-hydroxyphenylacetate (HOP), which in turn was conjugated through an acetyl linkage to lysine side chains of the carrier, the anti-ABA antibodies elicited in A/J mice contained only about one-twentieth as much of the CRI as antibodies induced by KLH-ABA. Thus the additional aceytl spacer in HOP greatly interferes with the induction of the CRI. As shown by Makela *et al.* (1976), ABA-HOP induces an intrastrain cross-reaction idiotype in A/J mice that is unrelated to that induced by KLH-ABA.

II. Strain Distribution of the CRI

The only strains identified so far that produce the CRI are the A strains (A, A/He, A/WySn), which express the Igh-1^e allotype, and AL/N (Igh-1^d); Igh-1^d and Igh-1^e are closely related serologically. It should be noted that AKR (Igh-1^d) and NZB (Igh-1^e) are CRI-negative.

III. Heterogeneity of A/J Anti-ABA Antibodies

The repertoire of anti-ABA antibodies in the A/J strain appears to be extremely large. This conclusion is derived in part from a study of the idiotypes of anti-ABA antibodies arising in A/J mice that had been immunologically suppressed with respect to the expression of major CRI (Ju *et al.*, 1977). [Suppressed mice also fail to express the aforementioned minor CRI (Gill-Pazaris *et al.*, 1980).] Anti-ABA antibodies were isolated from the sera of three mice that had been suppressed for idiotype and then hyperimmunized. The mice produced normal amounts of anti-ABA antibodies (Pawlak *et al.*, 1973). Rabbit anti-Id was prepared against affinity-purified anti-ABA Ig from each of the mice and thereafter thoroughly absorbed with normal mouse globulins. To prepare ligands for radioimmunoassays, each affinity-purified

anti-ABA antibody was radiolabeled with ^{125}I and subjected to preparative isoelectric focusing. The contents of one or two tubes constituting a single major peak were used in each case as ligand; the homogeneity of each preparation was demonstrated by refocusing. The four ligands tested were single major peaks from two of the suppressed, hyperimmunized mice and two separate peaks from the focusing pattern of antibodies of the third mouse.

One set of data is shown in Table I. Whereas 8 ng of unlabeled autologous antibodies sufficed to cause 50% inhibition in a conventional (double antibody) radioimmunoassay for idiotype, anti-ABA antibodies from 181 other A/J mice, some suppressed and some nonsuppressed, were not inhibitory, failing to cause 50% inhibition at the highest level of unlabeled antibody tested (up to 75,000 ng). Very similar results were obtained with two of the other three ligands, using the same panel of antibodies from 181 A/J mice as inhibitors. With the fourth ligand, 28% of the panel did cause 50% inhibition, but very large amounts of antibody, 800–120,000 ng, were required. These data are indicative of an extremely large repertoire of idiotypes in the A/J strain. Because of the extensive nature of the repertoire it was proposed that somatic variation contributed to the diversity of hypervariable regions (Ju *et al.*, 1977).

IV. Heterogeneity of Molecules Expressing the CRI

Amino acid sequence analyses were carried out in the laboratory of J. D. Capra on purified anti-ABA antibodies that were highly enriched

TABLE I
DISPLACEMENT FROM ANTIIDIOTYPIC ANTIBODY OF THE MAJOR PEAK (p*I* 6.7) OF THE ANTI-ABA ANTIBODY OF THE SUPPRESSED A/J MOUSE HIS-7

Inhibitor (source of anti-ABA antibody)	Number of mice	Nanograms anti-ABA antibody required for 50% inhibition[a]
Autologous (HIS-7, specifically purified)		8
	5	>10,000
Nonsuppressed A/J mice	71	>25,000
	68	>75,000
	14	>10,000
A/J mice suppressed for CRI	13	>25,000
	10	>75,000
Total number of mice tested	181	

[a] 1.5 ng of labeled ligand was used in the assay (Ju *et al.*, 1977).

for the CRI. Anti-ABA antibodies were first obtained in substantial quantity from ascites of hyperimmunized mice (Tung *et al.*, 1976). Ascitic fluids whose antibodies contained a high content of idiotype were selected and further purification was carried out by isoelectric focusing. A large proportion of the CRI^+ population in hyperimmunized mice has a p*I* of 6.7–6.9 and belongs to the IgG_1 subclass. Molecules with p*I* 6.7 or 6.9 refocus with the same p*I* value, indicating microheterogeneity with respect to this property. Antibodies that were over 90% CRI^+ were obtained by this procedure (Tung and Nisonoff, 1975).

Amino acid sequence analysis of the V_H region revealed a single major sequence (Fig. 1) (Capra *et al.*, 1978; Capra and Nisonoff, 1979). The V_L region (Fig. 2) exhibited heterogeneity at a few positions in the framework regions but appeared homogeneous in hypervariable segments (Capra *et al.*, 1977).

When monoclonal CRI^+ antibodies became available a substantial degree of microheterogeneity was revealed, both by serological and amino acid sequence analysis (Estess *et al.*, 1979, 1980; Lamoyi *et al.*, 1980a; Siegelman *et al.*, 1981; Marshak-Rothstein *et al.*, 1980; Alkan *et al.*, 1980). At a number of positions in the sequence a residue which appears with regularity in monoclonal antibodies differs from that found in the conventional induced antibody sequence. The explanation for this is uncertain, but the possibility exists that the monoclonal antibodies are not truly representative of the induced population, which was obtained from ascites after a different schedule of immunization. Arguing against this possibility is the observation that most "private" serological determinants present in monoclonal antibodies (see below) are found, albeit at a low concentration, in induced antibodies. The microheterogeneity was assessed by experiments utilizing monoclonal antibodies. Amino acid sequence data will be discussed after considering the serological data.

V. Serological Studies of Monoclonal CRI^+ Antibodies

Using the technique of Köhler and Milstein (1976) we prepared hybridomas with spleen cells of A/J mice immunized with KLH-ABA (Lamoyi *et al.*, 1980a). The tumor line used was Sp2/0-Ag14 (Shulman *et al.*, 1978), which does not secrete H or L chains before or after fusion. Affinity-purified antibodies, obtained from cloned cells, were monoclonal by the criteria of isoelectric focusing and electrophoresis in polyacrylamide gels. Monoclonal CRI^+ antibodies of each IgG subclass were obtained in pure form. Such antibodies are designated as

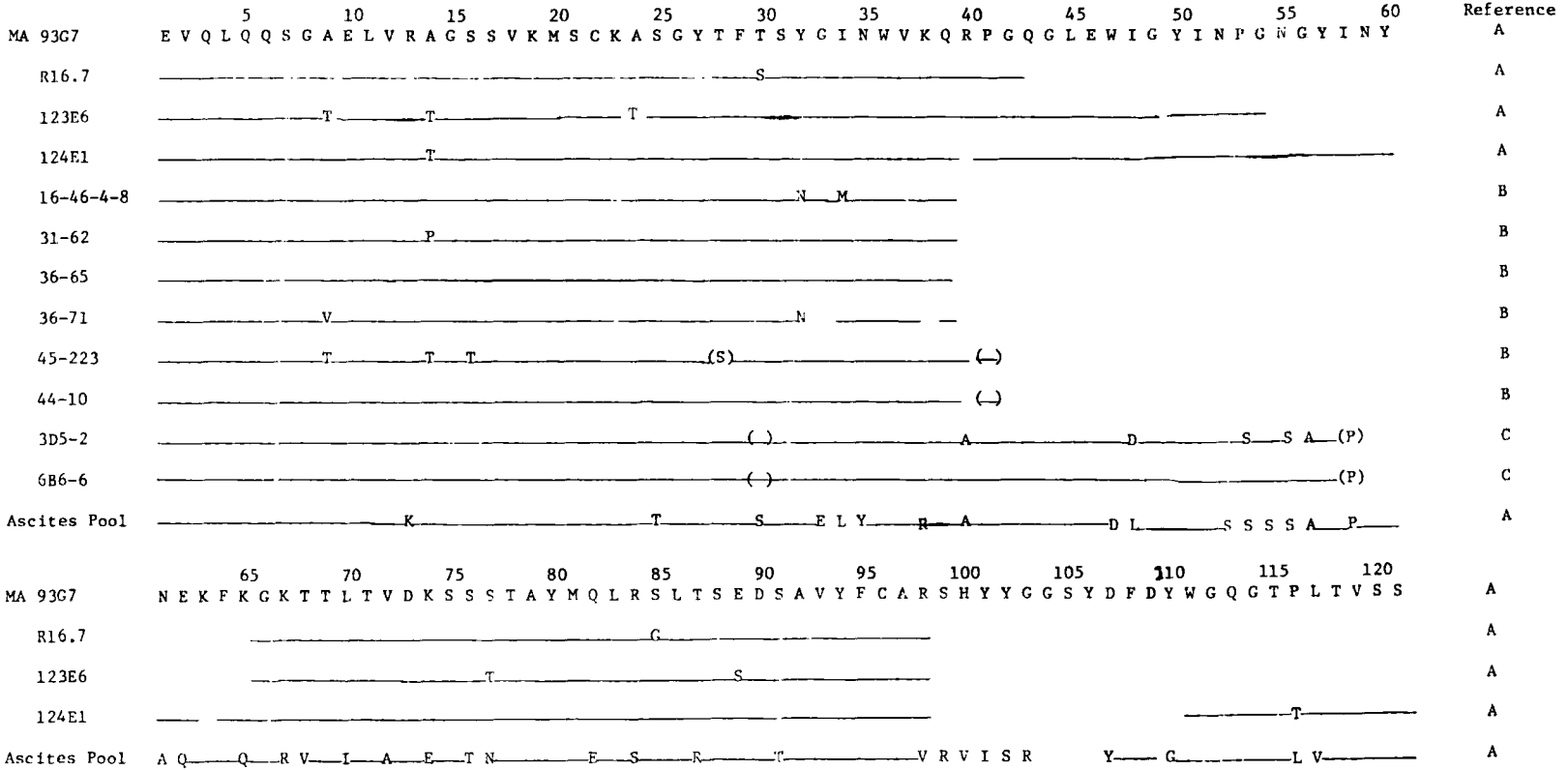

FIG. 1. Amino acid sequences of V_H regions of CRI$^+$ monoclonal antibodies and antibodies isolated from A/J immune ascites. A, Siegelman *et al.* (1981); B, Margolies *et al.* (1981); C. Alkan *et al.* (1980).

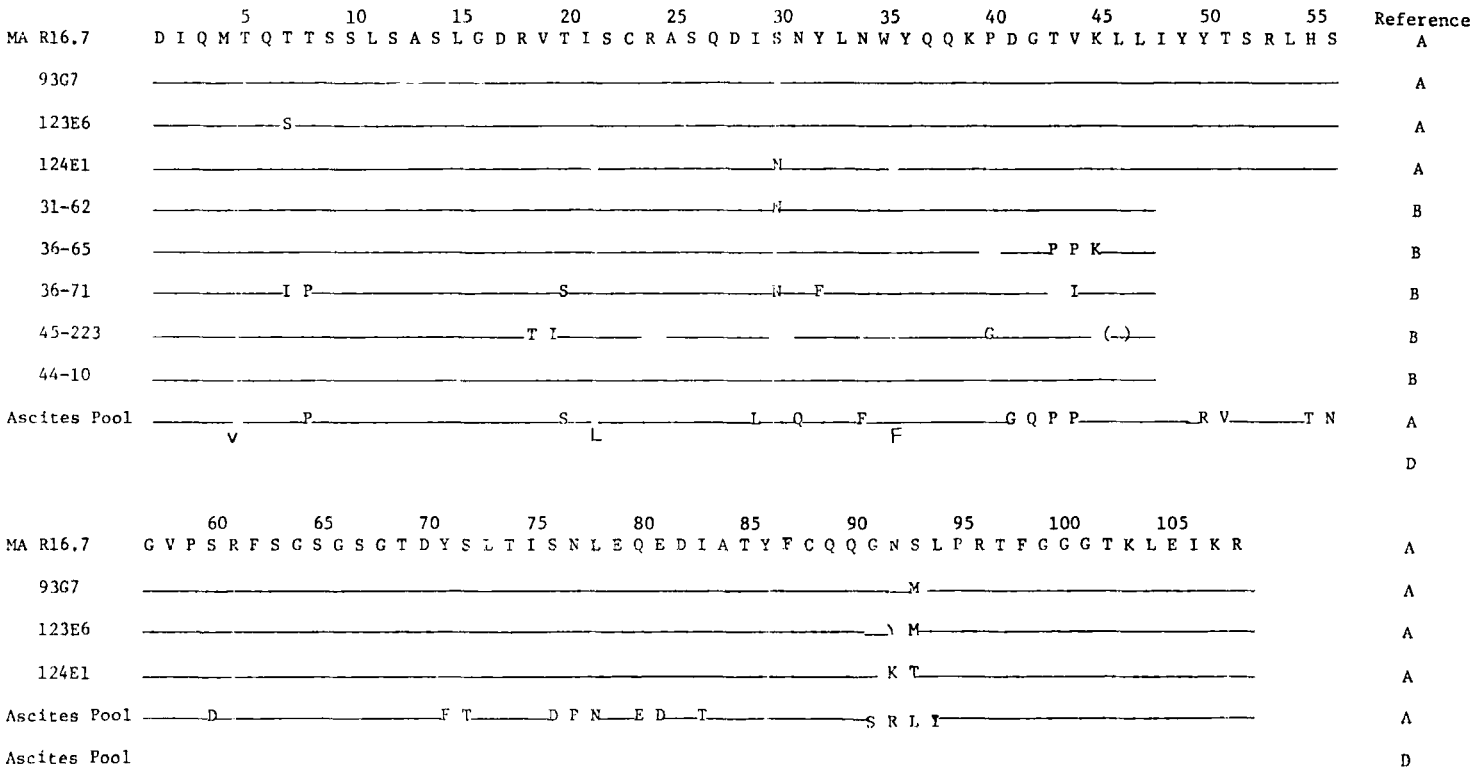

FIG. 2. Amino acid sequences of V_L regions of CRI$^+$ monoclonal antibodies and antibodies isolated from A/J immune ascites. A, Siegelman *et al.* (1981); B, Margolies *et al.* (1981); C, Alkan *et al.*(1980); D, Capra *et al.* (1977). The letters underneath the amino acid sequence of ascites pool correspond to those positions (4, 22, 36) at which two different amino acids were detected.

CRI^+ only if they cause more than 50% inhibition in the standard radioimmunoassay for the idiotype, with purified ^{125}I-labeled A/J anti-ABA antibody as the ligand.

The data on inhibition are summarized in Table II (Lamoyi *et al.*, 1980a,b). Of particular interest is the fact that individual, unlabeled monoclonal antibodies (MA), R16.7 or 93G7, can almost completely displace labeled serum anti-ABA antibody from rabbit anti-Id. On a weight basis these monoclonal antibodies are about as effective as inhibitors as unlabeled serum antibody. This indicates that a single MA possesses virtually all of the idiotopes recognized by the rabbit anti-Id. Data of this type present a problem in interpretation since, as will become evident, individual MA possess unique or "private" idio-

TABLE II

INHIBITION BY UNLABELED MONOCLONAL ANTI-ABA ANTIBODIES OF BINDING OF ANTI-Id ANTIBODIES TO LABELED ANTI-ABA LIGANDS[a]

		Nanograms required for 50% inhibition[b]		
Unlabeled inhibitor	Anti-Id against: ^{125}I-labeled ligand:	Anti-ABA Anti-ABA	R10.8 R10.8	R16.7 R16.7
Serum anti-ABA (purified)		11 (97)[c]	410 (66)	42 (94)
R16.7 (G1)		9 (94)	>2000 (5)	10 (100)
93G7 (G1)		12 (90)	>2000 (3)	13 (73)
R20.4 (G2b)		14 (86)	110 (75)	130 (55)
R26.5 (G3)		17 (85)	>2000 (3)	>2000 (47)
R13.4 (G3)		21 (87)	>2000 (4)	190 (63)
R10.8 (G2a)		180 (60)	12 (100)	>2000 (47)
R23.2 (G2b)		200 (66)	12 (100)	>2000 (48)
R9.3 (G2b)		300 (65)	14 (100)	1100 (51)
121D7 (G1)		300 (71)	>2000 (0)	>2000 (38)
R17.5 (G2b)		460 (63)	12 (100)	>2000 (48)
R24.6 (G2a)		1800 (52)	44 (86)	>2000 (43)
123E6 (G1)		1900 (51)	>2000 (15)	>2000 (12)
124E1 (G1)		2900 (47)	>2000 (0)	>2000 (17)
R22.4 (G2a)		3200 (49)	43 (86)	>2000 (45)
R18.11[d] (G3)		>2000 (20)	>2000 (2)	>2000 (0)
R19.9[d] (G2b)		>2000 (15)	>2000 (5)	>2000 (1)
R21.10[d] (G1)		>2000 (6)	>2000 (0)	>2000 (0)
R8.2[d] (G2b)		>2000 (9)	>2000 (11)	>2000 (28)

[a] Each test utilized 10 ng of ^{125}I-labeled ligand and slightly less than an equivalent amount of rabbit anti-Id. Immune complexes were precipitated by goat anti-rabbit Fc.

[b] Data are from Lamoyi *et al.* (1980a,b).

[c] The percentage inhibition by 2000 ng is in parentheses.

[d] Anti-ABA monoclonal antibodies which lack the CRI.

typic determinants not present on most other MA, and most of these determinants are found (at low concentration) in serum anti-ABA. The fact that individual MA can cause virtually complete displacement of labeled serum antibodies indicates that conventional anti-Id reacts almost exclusively with "public" determinants shared by members of the CRI^+ family. A probable explanation for this is that the "private" determinants are so diverse, and present at such low concentration in pooled serum antibody, that they are poorly immunogenic.

Further evidence for heterogeneity within the family relates to the differences in inhibitory capacity of various CRI^+ MA in the standard radioimmunoassay for idiotype (Table II) (Lamoyi *et al.*, 1980a,b). The 14 CRI^+ MA tested fell into three or four groups, based on the weight of antibody required to cause 50% inhibition, or on the degree of displacement of labeled ligand by a large excess of unlabeled MA. Those MA of which only small amounts are needed to cause 50% inhibition also inhibited the binding reaction to the extent of over 85% when 2000 ng was tested. For the weaker inhibitors much larger amounts were required for 50% inhibition and the maximal inhibition, obtained with 2000 ng, was considerably lower. The four CRI^- MA tested caused a maximum of 20% inhibition when 2000 ng was tested.

Thus the major CRI comprises a family of idiotypically related molecules. It is comparable in this respect to several other major, strain-specific idiotypes that have been investigated (Vrana *et al.*, 1980; Rao *et al.*, 1979; Reth *et al.*,1979; Ju *et al.*, 1979, 1980; Wolfe and Claflin, 1980; Clevinger *et al.*, 1980; Schilling *et al.*, 1980; Gearhart *et al.*, 1981).

VI. Private Idiotypic Determinants on CRI^+ MA

Additional evidence for microheterogeneity within the family of molecules was obtained by using anti-Id antisera prepared against individual CRI^+ MA (Lamoyi, *et al.*, 1980a,b). The antisera were thoroughly adsorbed with normal A/J immunoglobulins and BALB/c myeloma proteins. When an individual MA used as immunogen was tested as the labeled ligand, other MA were frequently incapable of displacing the ligand completely from its autologous anti-Id. Examples of such data are shown in the last two columns of Table II. When R16.7 was tested as labeled ligand, in an assay using anti-Id directed against R16.7, only a few other unlabeled MA were capable of causing 50% displacement and none of these caused complete displacement of the ligand, including 93G7, which strongly inhibits binding of serum anti-ABA to its anti-Id. Unlabeled serum anti-ABA, which con-

tained about 40% CRI^+ antibodies, was about one-fourth as effective, on a weight basis, as R16.7 and caused almost complete displacement when a large excess was tested.

The results obtained with MA R10.8 were somewhat different, in that three other CRI^+ MA were able to cause complete displacement of labeled R10.8 from its autologous anti-Id. However, a number of other CRI^+ MA were very poor inhibitors. Also, unlabeled serum anti-ABA caused only 66% inhibition when 2000 ng was tested, indicating that at least one idiotypic determinant on R10.8 is poorly represented in serum antibody.

In a number of similar systems that we have tested, pooled serum anti-ABA was generally, but not invariably, able to cause complete displacement of the labeled MA from its autologous anti-Id. In some cases, very large amounts of serum antibody were required, whereas in others (e.g., the R16.7 system discussed above) serum antibody is a strong inhibitor. Also, individual mouse antisera vary markedly in their inhibitory capacities. Even when complete displacement by serum antibody occurs one cannot conclude that the serum antibody possesses all of the determinants of the MA in identical form, since the antibody might possess one or more cross-reactive, nonidentical determinants. It is therefore difficult to decide whether all "private" determinants appear with regularity in serum antibody or whether an occasional private determinant may be present at negligible frequency in pooled serum. The latter would be possible if random somatic mutation contributes to diversity (see below). In any event, the very low frequency of appearance of certain "private" determinants in pooled serum suggests that the diversity of the CRI^+ family of molecules is very large. The presence of unique determinants on CRI^+ anti-ABA monoclonal antibodies has also been reported by Marshak-Rothstein *et al.* (1980a).

VII. Conserved Determinants in the CRI^+ Family

The fact that certain CRI^+ MA can cause virtually complete displacement of serum anti-ABA from anti-Id indicates the presence of one or more conserved idiotypic determinants. Additional evidence for this was obtained from binding experiments in which anti-Id directed against one CRI^+ MA was allowed to interact with a different CRI^+ MA, labeled with ^{125}I. Various unlabeled CRI^+ MA were then tested as inhibitors of the binding reaction. The purpose of this procedure was to rule out interactions involving "private" determinants (Lamoyi *et al.*, 1980a,b).

Some of the results are shown in Table III; the labeled ligand was MA R10.8 interacting with anti-Id directed against R16.7. It is evident that 12 of the 14 CRI$^+$ MA tested are very strong inhibitors of binding in this system and that the 12 MA are virtually equivalent in their inhibitory capacities. Very similar results were obtained with another heterologous binding system (labeled MA 121D7 reacting with anti-Id directed against 93G7); the same 12 MA were strong and virtually equivalent inhibitors. These data contrast with the results obtained with labeled serum anti-ABA as ligand, where great variation in inhibitory capacity among the same group of MA is seen (Table II). The results are consistent with the presence of one or more conserved, or public determinants in most members of the CRI$^+$ family of molecules. The fact that two monoclonal CRI$^+$ antibodies were relatively

TABLE III

INHIBITION BY UNLABELED MONOCLONAL ANTI-ABA ANTIBODIES OF BINDING OF ANTI-Id ANTIBODIES TO LABELED ANTI-ABA LIGANDS[a]

		Nanograms required for 50% inhibition[b]	
Unlabeled inhibitor	Anti-Id against: ^{125}I-labeled ligand:	R16.7 R10.8	93G7 121D7
Serum anti-ABA (purified)		15 (100)[c]	11 (100)
R16.7 (G1)		12 (100)	8 (97)
93G7 (G1)		7 (100)	9 (99)
R20.4 (G2b)		10 (92)	8 (92)
R26.5 (G3)		12 (88)	10 (99)
R13.4 (G3)		13 (100)	14 (97)
R10.8 (G2a)		8 (100)	7 (90)
R23.2 (G2b)		5 (100)	7 (91)
R9.3 (G2b)		9 (100)	10 (90)
121D7 (G1)		17 (88)	16 (100)
R17.5 (G2b)		7 (100)	7 (91)
R24.6 (G2a)		6 (100)	7 (93)
123E6 (G1)		>2000 (35)	>2000 (19)
124E1 (G1)		1900 (51)	>2000 (44)
R22.4 (G2a)		6 (100)	7 (92)
R18.11[d] (G3)		>2000 (0)	>2000 (12)
R19.9[d] (G2b)		>2000 (4)	>2000 (10)
R21.10[d] (G1)		>2000 (0)	>2000 (7)

[a] Each test utilized 10 ng of ^{125}I-labeled ligand and slightly less than an equivalent amount of rabbit anti-Id. Immune complexes were precipitated by goat anti-rabbit Fc.

[b] In the standard radioimmunoassay for idiotype. Data are from Lamoyi *et al.* (1980b).

[c] The percentage inhibition by 2000 ng is in parentheses.

[d] Anti-ABA monoclonal antibodies which lack the CRI.

poor inhibitors of the "criss-cross" binding reaction suggests that the public determinant can also exhibit microheterogeneity.

In each of the heterologous binding systems investigated phenylarsonate derivatives were found to inhibit the binding of labeled monoclonal CRI$^+$ antibodies to anti-Id prepared against a heterologous MA. The same haptens had no effect on the binding of myeloma protein T15 to its anti-Id antibodies. Conversely, phosphorylcholine inhibited the binding of T15 to is anti-Id but was without effect in the ABA system. The ability of free haptens (phenylarsonate derivatives) to inhibit binding reactions of the monoclonal antibodies is consistent with the possibility that the public determinant is in, or critically near, the hapten-binding site of anti-ABA antibody (Lamoyi *et al.*, 1980a,b).

Perhaps the strongest evidence for conserved determinant(s) on CRI$^+$ serum antibody and CRI$^+$ MA has been obtained by using monoclonal antiidiotypic reagents (Nelles *et al.*, 1981). BALB/c mice were hyperimmunized with purified A/J anti-ABA antibodies that had been polymerized with glutaraldehyde. Spleen cells from a mouse making anti-CRI antibodies were fused with the nonsecreting tumor line, SP2/0-Ag14. After cloning of fused cells, three different homogeneous anti-CRI MA were isolated. One of these was reactive with two-thirds of the major CRI present in serum. Also, each anti-CRI MA reacted with R16.7, a CRI$^+$ anti-ABA monoclonal antibody which appears to possess all of the idiotopes recognized by conventional rabbit anti-CRI antibodies. R16.7 was previously shown to cause virtually complete displacement of labeled serum CRI$^+$ anti-ABA in the conventional radioimmunoassay (Table II). The monoclonal anti-CRI antibodies failed to react with CRI$^-$ anti-ABA monoclonal or serum antibodies. That the monoclonal anti-Id reacts with a public determinant was shown by the equivalent inhibitory capacities of various unlabeled CRI$^+$ MA. Finally, two of the three monoclonal anti-CRI antibodies completely suppressed the major idiotypic component of the anti-ABA response when inoculated prior to immunization with KLH-ABA.

VIII. Amino Acid Sequences of CRI$^+$ MA

One complete and several partial amino acid sequences of V_H regions of CRI$^+$ monoclonal antibodies are shown in Fig. 1, together with the major sequence of the V_H region of CRI$^+$ serum antibody. References for the data are indicated in the legend to the figure. Hybridoma protein 93G7, whose complete V_H sequence was reported by Siegelman *et al.* (1981), is taken as the prototype. With the exception

of 123E6 the other CRI$^+$ monoclonal antibodies show over 90%, and in several instances over 95% homology with 93G7. Substitutions occur in framework and hypervariable regions, but the data are limited and detailed comparative results are not available for the third hypervariable region. Conclusions as to relative frequency of occurrence of substitutions in various segments of the V_H region cannot as yet be drawn. The sequence of antibody from A/J ascites fluid differs markedly from those of the CRI$^+$ hybridoma proteins; one-third of the positions in the V_H sequence of induced antibody differ from that of protein 93G7.

Figure 2 shows the complete amino acid sequences of V_L regions of four CRI$^+$ monoclonal antibodies, reported by Siegelman and Capra (1981). With R16.7 taken as the prototype, hybridoma proteins 93G7, 123E6, and 124E1 exhibit 1, 3, and 3 differences, respectively, in the V_L sequence (to position 108). Six of the total of seven substitutions are in hypervariable regions. For the five partial sequences (to position 47) the numbers of substitutions are 0, 1, 2, 3, and 6. Thus, with one exception, there is well over 90% homology among the L chains of the CRI$^+$ monoclonal antibodies. By contrast, the major sequence of the V_L segments of pooled serum antibody differs from that of R16.7 at 26 of 108 positions. In the monoclonal antibodies most of the substitutions, as compared to the prototype V_H or V_L sequence, can be accounted for on the basis of a single nucleotide substitution (22 of a total of 28 substitutions in the V_H sequences; 14 of 19 substitutions in V_L). In the V_L sequences of monoclonal antibodies substitutions occur most frequently although not exclusively in hypervariable regions. As already indicated, the data on V_H sequences are too limited to allow a similar conclusion.

The close relatedness of sequences of monoclonal antibodies, and the apparent randomness of the amino acid substitutions, appear consistent with the presence of a very limited number of germ line V_L and V_H genes, with somatic processes generating diversity in framework as well as hypervariable regions. Additional data are needed on sequences of D and J regions of H chains before conclusions can be drawn concerning variations in those segments of the molecule. That the major CRI is associated with a very small number of germ line genes was proposed earlier on the basis of the contrast between the regularity of appearance of the CRI and the extremely low frequency of other ("private") idiotypes associated with anti-ABA antibodies in strain A mice (Ju *et al.*, 1977). Direct evidence indicating that major intrastrain cross-reactive idiotypes are encoded by a small number of germ line genes, which are subject to somatic variations, has been re-

ported for idiotypes associated with antibodies to the 4-hydroxy-3-nitrophenylacetyl (NP) group in C57BL mice (Bothwell *et al.*, 1981) and to phosphorylcholine in BALB/c mice (Crews *et al.*, 1981).

IX. Linkage of Expression of CRI to Loci Controlling H and Kappa Chains

Linkage of genes controlling the CRI to the H chain (*Igh-C*) locus was shown by the presence of the idiotype in the anti-ABA antibodies of C.AL-20 congenic mice, which express the allotype of the CRI^+ AL/N strain ($Igh\text{-}1^d$) on a CRI^- BALB/c background (Pawlak *et al.*, 1973a,b). $Igh\text{-}1^d$ is closely related to the $Igh\text{-}1^e$ allotype of the A strain.) Such linkage was also demonstrated by conventional backcross studies, using CBA, C57BL, and BALB/c mice as mating partners with A/J (Laskin *et al.*, 1977). Linkage to the *Igh-C* locus is a commonly observed property of intrastrain cross-reactive idiotypes (Eichmann, 1975; Weigert and Potter, 1977).

The apparent absence of linkage of genes controlling the various idiotypes studied to genes encoding L chains presented a paradox, since the participation of L chains is essential for the expression of many idiotypes. We considered the possibility that, in the early genetic studies, both strains used in each backcross experiment, or contributing to the congenic strains tested, were capable of providing L chains required for the expression of the idiotype under investigation; in other words, that most strains have similar L chain repertoires. We obtained support for this view (Laskin *et al.*, 1977) by backcross studies using, as mating partners with A/J, CRI^- strains that had been shown by Edelman and Gottlieb (1970) to express a V_K polymorphism. They observed that in four strains—PL, C57, RF, and AKR—the V_K region has a cysteine-containing peptide (designated I_B) that is not found in any of the other strains tested. Subsequently, Gottlieb (1974) demonstrated that the locus controlling the expression of this peptide, designated *VK-1*, is closely linked to a locus controlling the expression of the Lyt-2,3 antigens on T cells. In backcross studies, it is therefore possible to follow kappa chain inheritance by typing for these surface antigens, a procedure that is much more rapid than the isolation and peptide mapping of L chains. Polymorphism involving the same four strains, and a similar pattern of inheritance, is exhibited if one examines the isoelectric focusing patterns of L chains of normal IgG (Gibson, 1976; Gibson *et al.*, 1978) or of anti-phosphorylcholine antibodies (Claflin, 1976; Claflin *et al.*, 1978).

When backcross studies were done with A/J mice and strains PL or C58, which exhibit the kappa chain polymorphism, the results on in-

heritance of the CRI clearly differed from those obtained with the strains previously tested. Expression of the CRI now required the presence of genes controlling both H and L chains of A/J origin (Laskin *et al.*, 1977; Gottlieb *et al.*, 1979). A subsequent study extended these findings to strain AKR and to a congenic strain having L chains of RF origin (Brown *et al.*, 1981). Thus each of the four strains exhibiting the V_K polymorphism fails to produce L chains that complement H chains of A/J origin for the expression of the CRI; i.e., when strains expressing the kappa chain polymorphism are included in genetic studies of idiotype expression, the segregation of loci governing the expression of kappa chains contributes to the inheritance of the idiotype. The results support the view that the V_K repertoire of the four strains exhibiting this polymorphism may differ considerably from that of most other inbred strains.

Linkage of an idiotype associated with the M460 (anti-DNP) myeloma protein to the *VK-1* locus has also been recently demonstrated by Dzierzak *et al.* (1980). Another cross-reactive idiotype to which genes controlling light chains contribute is that associated with antibodies to the hapten, 4-hydroxy-3-nitrophenylacetyl (NP). Imanishi-Kari *et al.* (1979) and Mäkelä *et al.* (1977) showed that the SJL strain is idiotype-negative despite the fact that it can synthesize the heavy chains required for the expression of the cross-reactive idiotype. This is attributable to the fact that these mice produce very low amounts of the required λ chains. F_1 hybrid (SJL × BALB/c) mice were idiotype-positive despite the fact that both parental strains are negative. In this hybrid, the BALB/c genes can contribute to the synthesis of the requisite λ chains.

Linkage of the expression of the anti-ABA CRI to the *VK-1* locus has been of particular interest in studies of idiotypic determinants on T-cell-derived suppressor factors. These studies will be discussed later.

Quantitative studies of the expression of the idiotype showed that mice which express A/J H chains and L chains of C57BL/6 or of BALB/c origin produce anti-ABA antibodies with a content of CRI that is only slightly lower than that observed in A/J mice (Brown *et al.*, 1981a). Thus, the three strains are comparable in their capacity to provide the requisite L chains.

Our rabbit anti-Id antisera generally contain at least two distinguishable components. Most of the anti-Id is hapten-inhibitable and interacts with one or more determinants that require the participation of the appropriate L as well as H chains. A small proportion of the anti-Id is directed to determinants present on H chains, which can be detected even when the H chains are recombined with nonspecific L

chains. In order for a given anti-ABA preparation to cause 50% inhibition in the standard radioimmunoassay for idiotype it must possess determinants to which both H and L chains contribute, since most of the anti-Id has such specificity; however, a direct binding assay, with the putative idiotype as labeled ligand, can detect idiotypic determinants associated with the H chains alone. (We have not yet identified antibodies specific for isolated L chains in our anti-Id antisera.)

An assay was therefore set up to determine whether mice that are homozygous for Igh-1^e and for Lyt-2^a,3^a (i.e., are homozygous for H chains of A/J origin and L chains of PL origin) produce anti-ABA antibodies which contain CRI^+ H chains (Brown *et al.*, 1981a). The assay made use of anti-Id prepared against the *in vitro* recombinant molecule, $H_{Id}L_N$, where the H chains are derived from CRI^+ molecules and the L chains from nonspecific IgG. It was found that the doubly homozygous mice, lacking appropriate L chains, nevertheless made use of CRI^+ H chains. However, the percentage of molecules that contained such H chains was only about one-tenth as great as that in A/J mice. Thus, in the absence of the appropriate L chains the CRI^+ H chains were still utilized, but to a considerably lesser extent. Similarly, BALB/c mice, whose anti-ABA antibodies are CRI^-, make use of L chains in their anti-ABA antibodies which are appropriate for the production of the CRI; however, only a small proportion (5–10%) of their anti-ABA antibody contains such L chains. These findings indicate that the degree of expression of a given H or L chain is somehow dependent on the complementary chains present in the repertoire of the mouse. The role of regulatory genes in the expression of a given polypeptide chain or idiotype, which might influence these observations, is unknown.

X. Regulation of the Humoral Anti-ABA Antibody Response to Idiotypic Determinants

Administration of rabbit anti-Id antibodies to A/J mice, prior to immunization with KLH-ABA, has little effect on the total anti-ABA response, but can completely suppress the appearance of the CRI (Pawlak *et al.*, 1973; Bangasser and Nisonoff, 1975). If immunization is started immediately after the administration of anti-Id, say within 2 weeks, we have never observed the reemergence of the CRI upon subsequent immunization. However, if the time interval between suppression and immunization is allowed to increase, the state of suppression gradually decays. When 5 months were permitted to elapse before immunization about half of the mice tested had regained the capacity to produce the CRI.

Fab or $F(ab')_2$ fragments of anti-Id were ineffective as suppressive reagents. The reason is uncertain. Possible explanations include the rapid clearance of such fragments, their inability to fix complement, or a requirement for the Fc fragment in the induction of regulatory cells. Indeed a similar failure to induce suppressor T cells was observed when $F(ab')_2$ of anti-Id was used in ABA-specific immunity, as gauged by delayed type hypersensitivity responses (see Section XX). For optimal suppression the anti-Id should be administered at least 2 weeks before the antigen. It was less effective when given 7 days prior to antigen and had very little suppressive effect when administered on the same day as, or 3 days after the injection of antigen (Pawlak *et al.*, 1973a).

XI. Suppression of CRI-Bearing Ab Mediated by B Cells

As indicated above, the capacity to produce the CRI eventually recovers after administration of anti-Id, provided antigen is withheld. We have never observed recovery of the capacity to produce the CRI after administration of the antigen (KLH-ABA). One possible explanation is that antigen enhances the production of a set of suppressor cells. Another is that the suppressed state is maintained in part by a mechanism of B cell clonal dominance. Once established, CRI^- anti-ABA clones may dominate the immune response by virtue of the large numbers of secondary CRI^- B cells present, as compared to the very small number of precursor CRI^+ B cells, and the greater ease of triggering secondary, as compared to primary B cells.

Experiments were carried out to determine the validity of the mechanism of clonal dominance as a means of suppression of the CRI (Eig *et al.*, 1977). BALB/c mice, which are CRI^-, were immunized with KLH-ABA and their lymphoid cells were transferred into mildly irradiated (200 rad) C.AL-20 mice that were subsequently immunized with KLH-ABA. C.AL-20 mice carry the Igh-1^d allotype of the AL/N strain on a BALB/c background, are H-2 compatible with BALB/c, but are CRI^+. It was found that the transfer of spleen cells, or spleen cells treated with anti-Thy-1.2 + C, from the immunized BALB/c mice into nonimmune C.AL-20 recipients completely prevented the appearance of idiotype upon subsequent immunization of the C.AL-20 mice, although high titers of anti-ABA were produced. The results suggest that B-cell dominance, mediated by secondary cells, can completely prevent the expression of unprimed cells having ligand-binding receptors of the same specificity. The experiments do not establish whether the phenomenon is entirely due to selective capture of anti-

gen by the scondary cells or whether some type of active suppression by B cells may also be involved. That the suppression was not due to T cells is indicated by the failure of the anti-Thy-1.2 treatment to influence the resultant suppression and by the improbability that BALB/c mice, which are CRI^-, would have CRI-specific suppressor T cells after immunization with KLH-ABA.

A subsequent study extended these findings to suppression of an entire allotype by a mechanism of B cell dominance (Brown *et al.*, 1980). In this case the antigen was KLH-TNP, the donor mice were BALB/c, and the recipients were C.B-17, a congenic strain having the Igh-1^b allotype of C57BL on a BALB/c background. A state of marked suppression with respect to the recipients' allotype was maintained for about 2 months after the adoptive transfer.

XII. Suppression of the CRI Mediated by T Cells with Antiidiotypic Receptors

Eichman (1974) showed that A/J mice, suppressed with respect to the formation of a cross-reactive idiotype associated with antibodies to Group A streptococci, have suppressor T cells, which upon adoptive transfer, selectively suppress the major idiotypic component of an antistreptococcal response in syngeneic recipients. Idiotype-specific suppressor cells were subsequently identified in the ABA system (Owen *et al.*, 1977a,b). The experiments were carried out with mice that had been suppressed with anti-Id, hyperimmunized, and rested before the adoptive transfer. The suppressor cells were found to possess antiidiotypic receptors. This was demonstrated by rosette formation, using A/J erythrocytes coated with Fab fragments possessing the idiotype. Depletion of rosette-forming splenic lymphocytes by centrifugation removed virtually all of the suppressor activity, and the rosettes themselves were found to be much more suppressive, on a per-cell basis, than the unfractionated lymphocyte population. In the hyperimmunized suppressed mice 5–10% of the splenic T cells formed rosettes. Over a long period of time we have found that the average is approximately 4–5%.

The idiotype-binding specificity of the rosette-forming T cells was shown by the absence of rosette formation when nonspecific Fab was used to coat the red cells; by the capacity of A/J anti-ABA serum to inhibit rosette formation; and by the absence of inhibition when normal A/J serum, or serum from hyperimmunized, suppressed A/J mice were tested. Similarly, rosette formation was inhibited by $F(ab')_2$ fragments of rabbit anti-Id but not by $F(ab')_2$ fragments of normal rabbit IgG. Rosette formation was also strongly inhibited by 0.2 m*M* hapten,

p-(azobenzenearsonic acid)-*N*-acetyl-L-tyrosine, which binds to anti-ABA antibodies with high affinity.

A rest period of 8–12 weeks after immunization was found necessary for the induction of high percentages of rosette-forming cells. Another requirement for the induction of rosette-forming cells was challenge by antigen. Treatment with anti-Id, without subsequent immunization, yielded a much smaller number of idiotype-specific lymphocytes. It was further shown that the receptors on the antiidiotypic cells were endogenously synthesized. Exposure to trypsin destroyed the rosette-forming capacity which, however, was largely restored after 24 hours in tissue culture. Treatment with anti-Thy-1.2 in the presence of complement eliminated the rosette-forming cells.

It was subsequently shown that a secondary response is highly resistant to the effects of suppressor T cells (Owen and Nisonoff, 1978). When recipient mice were primed with ABA, from 6 days to 4 months before the adoptive transfer of suppressor cells, normal concentrations of CRI were produced on subsequent immunization. This effect was independent of the carrier used for priming or subsequent immunization. In addition, the effect of suppressor T cells could be overcome by adding to them, before the adoptive transfer, purified secondary B cells which presumably expressed receptors for ABA. The B cells for these experiments were purified by the method of Wofsy *et al.* (1971), using hapten-conjugated Sephadex G-200 in place of polyacrylamide beads.

The absence of a specific carrier effect on CRI expression appears to rule out a carrier-specific helper T cell as the target of suppression. This, and the effect of adding secondary B cells, suggests that the primary B cell may be a direct target of suppression and that secondary B cells are resistant to suppression. Alternatively, it is conceivable that an idiotype-specific, rather than a carrier-specific helper T cell is the actual target of suppression and that the secondary response is resistant because (a) secondary B cells need much less help than primary cells, and (b) the target of suppression is a T cell but suppression is incomplete, i.e., the degree of suppression is sufficient to prevent a primary but not a secondary response. A possible objection to this hypothesis is that most of the suppressor cells, as well as the putative idiotype-specific helper cells (Woodland and Cantor, 1978; Bottomly and Mosier, 1979) have antiidiotypic receptors and would not be expected to interact with one another. The antiidiotypic suppressors might, however, stimulate idiotype-bearing suppressor cells *in vivo*, which could be the actual effectors. This will be considered again in Section XXVI.

The question arises as to how Ts with antiidiotypic receptors were stimulated. The reagents used were anti-Id antibody and antigen, both of which should interact with idiotypic, rather than antiidiotypic receptors. It seems likely that the T cells initially stimulated bore idiotypic receptors and that these in turn stimulated the production of second-order T cells with antiidiotypic receptors (Jerne, 1974). This could account for the requirement of an 8-week waiting period after hyperimmunization for optimal induction of antiidiotypic suppressor cells. The existence of Ts with idiotypic receptors, that are capable of leading to the suppression of the humoral response, was demonstrated by Lewis and Goodman (1978).

XIII. Suppression of Idiotype Induced by Idiotype-Conjugated Cells

Having shown the existence of idiotype-suppressor T cells with antiidiotypic receptors, we attempted to induce such cells without the use of antiidiotype or antigen (Dohi and Nisonoff, 1979). Rowley *et al.* (1976) had shown that challenge of BALB/c mice with myeloma protein T15, which has antiphosphorylcholine activity, reduces the subsequent humoral response to that hapten. Since nearly all serum anti-PC antibodies carry the idiotype of protein T15 it was difficult to ascertain whether the suppression was antigen- or idiotype-specific. Whether suppressor T cells were generated was not determined in those experiments. We injected idiotype-conjugated syngeneic thymocytes into A/J mice prior to immunization with KLH-ABA. High titers of anti-ABA antibodies were induced upon immunization but the CRI was not detectable in most of the recipients. Idiotype-conjugated syngeneic thymocytes similarly suppressed the appearance of the CRI in F_1(A/J × BALB/c) and C.AL-20 mice. Although injection of unconjugated antibody also induced a state of idiotypic suppression in some mice, conjugation to cells greatly enhanced the suppressive effect, particularly in the F_1 and C.AL-20 mice.

The injection of idiotype-conjugated cells induced the formation of suppressor T cells, as shown by adoptive transfer experiments. When idiotype-conjugated thymocytes were inoculated into mice that had recently received the antigen, no suppressive effects were observed. This is consistent with resistance of the secondary response to suppression, discussed above. The source of thymocytes used for producing the idiotype-conjugated cells was not relevant, i.e., there was no apparent H-2 restriction on the stimulation of suppressor T cells by the idiotype coupled to cell surfaces. These experiments indicate the feasibility of entering the network of idiotype suppressors without the use of anti-Id antibodies or of antigen.

It was found that T cells which suppress the CRI can be induced in a CRI^- strain (BALB/c) and that these suppressor cells can function across an allotype barrier (Hirai *et al.*, 1981). Syngeneic thymocytes conjugated with A/J CRI^+ antibodies were inoculated into normal BALB/c recipients. Subsequently, either thymocytes or splenic T cells from the BALB/c mice were transferred into CRI^+, H-2-compatible C.AL-20 mice. Eight of ten C.AL-20 recipients were completely suppressed with respect to the production of the CRI, although they produced normal amounts of anti-ABA antibodies after immunization with KLH-ABA. All of the control mice, which received normal BALB/c thymocytes or splenic T cells, produced the CRI, although one of nine mice showed partial suppression.

XIV. Regulation of Idiotype Suppression Mediated by Idiotypic Determinants on CRI^+ Monoclonal Antibodies

We have discussed evidence indicating that most or all of the molecules constituting the CRI share at least one highly conserved idiotypic determinant. Experiments were carried out to ascertain the extent to which expression of the CRI family can be regulated by idiotypic determinants present on a single CRI^+ monoclonal antibody (Hirai *et al.*, 1980). Two types of experiment were done. In one, the monoclonal antibody was conjugated to thymocytes and inoculated prior to immunization with KLH-ABA. The second approach was to prepare rabbit anti-Id antibodies against individual CRI^+ monoclonal antibodies and determine their suppressive effects on the idiotypic component of the humoral anti-ABA response. It was found that each of four different CRI^+ monoclonal antibodies caused virtually complete inhibition of the formation of CRI when conjugated to thymocytes and inoculated prior to immunization. There was no appreciable effect on the titer of anti-ABA antibodies produced. A fifth monoclonal antibody, coupled to thymocytes, caused significant suppression of CRI formation in three of six recipient mice.

Experiments with one of the CRI^+ monoclonal antibodies (R16.7) conjugated to syngeneic thymocytes showed that suppressor T cells were produced upon inoculation of idiotype-conjugated thymocytes. An enriched T-cell fraction, but not a B-cell fraction, was capable of adoptively transferring the suppressed state to mildly irradiated A/J recipients.

Anti-Id antibodies were prepared in rabbits against three of these five monoclonal antibodies and inoculated into A/J mice prior to immunization with KLH-ABA. The total amount injected corresponded to 30 μg of idiotype-binding capacity. Virtually complete suppression

of the CRI was observed in all of the recipient mice. Again substantial amounts of anti-ABA antibodies were produced by all of the recipients (Hirai *et al.*, 1980).

The data were interpreted as indicating that the formation of any CRI^+ antibody in this family, or the suppression of its synthesis, can trigger a network of interactions which affects most or all of the idiotypic response. The results provide further evidence for the close relatedness of members of the CRI^+ family of molecules. Furthermore, these results indicate that a discrete signal, such as an indiotype or monoclonal antiidiotype, can engender suppression.

XV. Idiotypic and Antiidiotypic T Cell Factors (TsF) That Regulate the Humoral Response

Each of the factors to be described suppresses the CRI component of the humoral response with little effect on total anti-ABA synthesis (Hirai and Nisonoff, 1980). The factors were obtained by culturing spleen cells or splenic T cells from suppressed mice for 24 hours at 37°C, removing the supernatants, dialyzing, and then culturing the supernatants with normal A/J spleen cells for 4 hours at 37°C. This culture step was absolutely necessary for suppression. The lymphoid cells were then washed and transferred into irradiated syngeneic recipients (560 rad; 5×10^7 cells per mouse), which were then immunized with KLH-ABA. The resulting antisera were analyzed quantitatively for their content of anti-ABA antibodies and CRI. The A/J mice used as a source of TsF had been suppressed with anti-Id antibodies, hyperimmunized with KLH-ABA, and allowed to rest for 3 weeks. Mice used as the source of suppressor factor had produced high titers of anti-ABA antibodies with undetectable CRI (25,000 ng of anti-ABA caused less than 50% inhibition in the standard radioimmunoassay). It was found that the cell-free culture supernatants, when mixed with normal spleen cells and adoptively transferred, caused virtually complete suppression of the CRI component of the anti-ABA response. Cell supernatants obtained from nonsuppressed, KLH-ABA-immunized mice or from nonimmune A/J mice were nonsuppressive. Treatment of the cells with antibodies directed to a brain-associated T-cell antigen and complement eliminated the capacity to produce the TsF, indicating that T cells are the source of the suppressor factor(s). Further evidence was the failure of the cells providing the factor to be bound to anti-Fab on a polystyrene dish. Quantitative considerations indicated that the suppressor activity was also not due to contaminating rabbit anti-Id or A/J anti-ABA antibodies.

XVI. Properties of TsF; Existence of Two Distinct Factors

Most of our information on these properties comes from affinity chromatography, using Sepharose 4B as the solid phase immunoadsorbent and cyanogen bromide to conjugate various proteins to Sepharose (Hirai and Nisonoff 1980).

TsF was not bound by an anti-Fab absorbent, indicating that it is not a conventional immunoglobulin. The filtrate (pass-through) from a column bearing either idiotype or antiidiotype expressed suppressor activity, but all activity was removed after successive passage through the two types of immunoadsorbents. The results indicate the presence of two types of TsF, one with idiotypic and the other with antiidiotypic receptors. This idea was supported by the observation that suppressor activity could be eluted, at pH 2.8, from either the idiotypic or antiidiotypic adsorbent. The TsF obtained by acid elution from CRI-Sepharose was, as expected, retained on repassage through CRI-Sepharose, but was not retained by a Sepharose column to which anti-ABA antibodies lacking the CRI were conjugated. (Such antibodies were obtained from suppressed, hyperimmunized mice.) This finding indicates that the adherence of TsF to CRI-Sepharose is mediated through recognition of the idiotypic determinants on the adsorbent. The coexistence in solution of separate idiotypic and antiidiotypic factors, rather than complexes of the two, is probably attributable to binding affinities that are inadequate to cause interaction at very low concentration.

TsF that carries idiotypic determinants also has anti-ABA specificity. Some of the TsF adhered to a column of ABA-bovine γ-globulin-Sepharose. The acid eluate of this column had suppressor activity which was not retained by a CRI-Sepharose column but was bound by anti-Id-Sepharose. Virtually all TsF activity (both idiotypic and antiidiotypic) was also removed by passage through a column of anti-I-J^k antibody conjugated to Sepharose (Hirai and Nisonoff, unpublished data). The molecular weight of each factor, estimated by gel filtration, was 50,000–100,000. The presence of a functionally active polypeptide in both factors was shown by the loss of activity which occurred upon exposure to trypsin and the absence of effect of DNase or RNase treatment.

The considerable length of time required to obtain experimental data on humoral responses complicates the investigation of the sequence of events that occurs. It seems probable, however, that the initial cell type induced in our suppressive regimen is idiotypic, since the inducing agents are antiidiotypic antibody and antigen. The antiidiotypic suppressor cells are probably stimulated, through a network,

by idiotype-bearing cells or factor. More detailed information on such network interactions was obtained through studies of suppression of ABA-specific delayed-type hypersensitivity, discussed in the following sections.

XVII. Thymus-Derived (T) Cell Responses to Azobenzenearsonate-Coupled Cells

In 1978, we developed a model to evaluate hapten-specific T-cell responses. The basic approach was to couple haptenic groups to the cell surface of spleen cells and inoculate syngeneic recipients with such cells by the subcutaneous route (Greene *et al.*, 1978; Greene and Bach, 1979). Mice primed by hapten-coupled cells developed sets of hapten-reactive T cells capable of mounting delayed type hypersensitivity responses (T_{DH}) or proliferation (T_P) or cell-mediated cytotoxicity (T_C) when challenged with cells coupled with the same hapten. It was possible to show that trinitrophenyl-coupled spleen cells (TNP-SC) or azobenzenearsonte-coupled cells (ABA-SC) could induce specific responses only to the homologous hapten. The hapten-coupled cells capable of priming mice *in vivo* were next characterized. The spleen cells were initially fractionated according to their adherence to glass surfaces. Only splenic adherent cells when coupled with azobenzenearsonate were capable of priming for ABA-specific T-cell responses. More recently the adherent cell has been further characterized according to its buoyant density, cell surface phenotype, and susceptibility to ultraviolet or gamma irradiation. The relevant cell is contained within a low density set of adherent cells (Tominaga and Greene, unpublished). The cell is I-A^+ and can be effectively lysed by monoclonal anti-IA antibody and complement. Interestingly, the relevant antigen-presenting cell is exquisitely sensitive to a 10-second exposure of 270–300 nm ultraviolet radiation (1.2–1.4 J/m^2/second at 20 cm) yet resistant to 1500 R of X-irradiation (Greene *et al.*, 1980). This UV sensitivity appears to be related to the inability of UV-treated ABA-coupled antigen-presenting cells (APC) to provide second signals to ABA-reactive T cells. Preliminary experiments in this and other systems, (Weinberger *et al.*, 1981; Leforte, Tominaga, and Greene, in preparation) have suggested that highly purified interleukin-1 can correct UV-induced APC defects *in vivo* and *in vitro*.

Aside from such physical characteristics of the APC necessary for hapten-specific T-cell responses, we also studied the genetic restriction of antigen priming *in vivo* using APC derivatized with hapten. By using ABA-coupled splenic adherent cells (SAC) from a variety of congenic strains and cross-immunizing, it was clearly apparent that when

ABA-SAC and recipient strains were I-A syngeneic maximal immunization resulted. Interestingly, although most allogeneic or xenogeneic splenic adherent cells were ineffective in priming for hapten-specific responses, certain allogeneic SAC could induce significant responses, suggesting that shared public I region domains were really the decisive element in presenting haptens. These features therefore establish that a small subset of I-A^+, low-density cells is capable of stimulating T-cell reactions to azobenzenearsonate (Bach *et al.*, 1979).

The T_{DH} cell has been studied in great detail (Bach *et al.*, 1979; Greene and Benacerraf, 1980). The T_{DH} cell arises in the draining lymph nodes 4 days after priming. The cell belongs to the IA^- subset of T cells and is genetically restricted in its functions (Vadas and Greene, 1980). By transfer experiments ABA-specific T_{DH} cells manifest activity only in syngeneic or semisyngeneic recipients. Mapping of restrictions for hapten-specific immunity has suggested that K-IC region identity was necessary for successful transfer, there being no evidence for successful transfer solely with D region matching. Identity at other major genetic loci such as the *Igh-C*-linked genetic regions was not apparent in terms of restricting transfer of immunity. In addition, T_{DH} induced by ABA-coupled cells do not express cross-reactive idiotypic elements as determined by antiidiotype and complement treatment (Bach and Greene 1979, unpublished; Sy *et al.*, 1980). The T_p cell arises in the draining lymph nodes within the same time period as the T_{DH} cells (Redner and Greene, unpublished). The T_p is likely a member of the Lyt-1^+23^- family, and manifests activity in a hapten-specific manner, when restimulated *in vitro* with ABA derivatized syngeneic stimulator cells.

After priming *in vivo* with ABA-coupled cells, splenocytes contain cells which, when restimulated *in vitro* with appropriately coupled stimulators, develop cytotoxic activity (Sherman *et al.*, 1979). Evidence that priming *in vivo* primarily augments T helper (T_h) activity for cytolytic precursor cells has also been recently obtained (A. Tominaga and M. I. Greene, unpublished).

In conclusion it seems that preferential genetically restricted activation of distinct ABA specific Lyt-1^+ members (T_{DH}, T_P, T_h) occurs after *in vivo* priming with I-A^+, UV-sensitive, 1500-R resistant, low-density, ABA-coupled APC.

XVIII. Induction of Immunity with Antiidiotype Antibody

Having demonstrated that ligand-coupled cells were effective immunogens, we proceeded to determine if other immunization regi-

mens such as those using antiidiotypic reagents might be efficacious. The use of anti-id reagents had been well defined in a variety of other laboratories (Binz and Wigzell, 1976; Yamomoto *et al.*, 1979; Sy *et al.*, 1979). Priming A/J, C.AL-20, or BALB/c mice with various doses of antiidiotypic antibody subcutaneously, followed by challenge in the footpad 5 days later with ligand, resulted in immunity as judged by DTH responses in A/J and C.AL-20 but not BALB/c mice. These experiments suggested that a population of preimmune cells could react with antiidiotypic antibody. These cells should be expected to express idiotypic receptors for ligand. Indeed, treatment of A/J immune cells, induced by antiidiotype, with antiidiotype and complement abolished their capacity to transfer immunity in sharp contrast to similar treatment of immune cells induced by ligand, which were insensitive to such treatment. Thus antiidiotypic antibodies can be used to activate T_{DH} immune responses in an allotype-restricted manner. Recent experiments by Thomas *et al.* (1981) have shown that even certain monoclonal antiidiotypic antibodies can stimulate ABA-specific DTH reactions in a genetically-restricted manner. Immune cells activated by monoclonal reagents functioned only in I-A region-identical recipients. We have also found that certain batches of antiidiotypic reagents can also induce or prime for T_c activity, (Finberg *et al.*, 1981) suggesting that idiotypic T_h or pre-T_c also exist and can be activated under certain circumstances. A more interesting question relates to the presence of CRI-bearing cells in strains of the inappropriate allotype. Experiments in progress to examine this question involve priming BALB/c or C.AL-20 mice, which are allotype congenic, with rabbit antiidiotypic reagents and challenging each strain with either ligand or mouse monoclonal antiidiotypic reagents. The possible presence of CRI elements in BALB/c is an important issue since we have clearly shown that BALB/c mice can potentially develop antiidiotypic (anti-CRI) T cells (Sy *et al.*, 1981). It might be envisioned that CRI V_H genes are present in many mouse strains, but may be utilized to build receptors with different ligand specificities or regulated in some other fashion. Alternatively, BALB/c mice may lack such V_H genes but nevertheless have T cells with antiidiotypic receptors.

XIX. First-Order Suppressor Cells Activated by ABA-SC

The now classic studies of Battisto and Bloom (1966) established that hapten-coupled lymphoid cells administered intravenously induced a state of unresponsiveness determined by diminished responses upon subsequent exposure to the hapten. Studies by Henry Claman and his colleagues (Claman *et al.*, 1980) clarified that a funda-

mental characteristic of the unresponsiveness related to the stimulation of hapten-specific suppressor cells. We became interested in evaluating this issue in the azobenzenearsonate system. We have previously established in detail the general conditions, including hapten density and route of administration, necessary for the induction of suppression. In the ABA system, we found that the intravenous administration of 30 to 100 million ABA-coupled splenocytes optimally induced very potent suppressor cells demonstrable in the thymus, spleen, and lymph nodes 6 to 7 days later (Bach *et al.*, 1979; Wetzig, Foster, and Greene, unpublished). The cells capable of mediating suppression of sets of T_{DH}, T_p, or T_c cells were themselves sensitive to anti-Thy-1 and complement treatment and were functional only when given on the first and second days of the immunization of syngeneic mice (Bach and Greene, 1979; Dietz *et al.*, 1980). This feature traditionally identifies the cells as afferent-acting Ts. Such suppressor T cells (Ts) were termed first-order Ts_1 cells to distinguish them from other suppressor cell subsets which are functionally and phenotypically different and which will be described in later sections. The Ts_1 cell was later characterized not only in terms of its distinct function, but also in terms of its cell surface phenotype. The Ts_1 is Lyt-1^+23^-, and in A/J mice can by lysed by anti-CRI and complement treatment, evidence that it bears idiotypic elements (Sy *et al.*, 1981; Bromberg *et al.*, 1981). Further definitive evidence that the cell is idiotypic relates to its ability to be bound to ligand-coated plates, and subsequently lysed by antiidiotypic reagents (Deitz *et al.*, 1981). Although Ts_1 can be induced in any strain by ligand-coupled cells, it is only in those strains which have the appropriate allotype, i.e., A/J or C.AL-20, that CRI structures are expressed. In order to further assess the genetics of function of Ts_1, we generated such cells in C.AL 20 or BALB/c mice and transferred them into either C.AL 20 or BALB/c, which were then immunized with ABA-coupled cells subcutaneously. We observed that Ts_1 from C.AL-20 limited ABA-specific responses only in C.AL-20, and not in BALB/c and vice versa. We can conclude that afferent Ts_1 cells function in an allotype-restricted manner. The immediate implication of this observation is that either idiotype-antiidiotype interactions are important in suppressor pathways, or that allotype-linked genes control cellular interaction elements for suppressor cells (Yamauchi and Gershon, 1981; Rolink *et al.*, 1978).

XX. Induction of Ts_1 Cells with Antiidiotype Antibody

We were also able to induce Ts_1 cells in A/J or C.AL-20 but not BALB/c by the intravenous administration of defined amounts of anti-

idiotypic antibody (Sy *et al.*, 1979; Bach and Greene, 1979). The Ts so generated were capable of limiting responses in a hapten-specific manner, and were ligand-specific, allotype-restricted, and similar in all identifiable characteristics to ligand-induced Ts_1 cells (Sy *et al.*, 1980). It should be stressed that $F(ab')_2$ of antiidiotypic antibodies were ineffective in generating Ts. Nevertheless it has recently been possible to bypass the role of the Fc fragment by providing a specific second signal at the time of administration of antiidiotypic antibody (Bromberg *et al.*, 1981).

XXI. The Production of ABA-Specific Suppressor Molecules

Most suppressor systems studied in detail, and in particular those described in the elegant series of experiments of Takemori and Tada (1977), Tada *et al.* (1977), and Taniguichi and Tokuhisa (1980) seem to function via suppressor cell-derived factors. The azobenzenearsonate system proved no different in this regard. Ts_1 cells were obtained from the thymus, spleen, and lymph nodes of ABA-SC tolerized A/J, C.AL-20, B10.A, and BALB/c mice. Certain aliquots of cells were subjected to mechanical disruption by either sonication or repeated cycles of freezing and thawing. Another aliquot of Ts_1 was obtained from A/J mice that had been tolerized with ABA-SC and had been challenged *in vivo* with ABA-SC, 1 day prior to removal of their spleens. These splenocytes were placed in culture at 37°C for 24 and 48 hours in appropriate media and the supernatants recovered (Greene *et al.*, 1979). Cell extracts, sonicates, and supernates were all subjected to negative pressure dialysis against buffered saline, and finally adjusted to 10^8 cell equivalents per ml. Twenty million cell equivalents of factor were administered intravenously to syngeneic mice which were simultaneously immunized subcutaneously with ABA-SC. Administration of factor each day for at least the first 3 days after priming was essential to demonstrate suppressive activity. When given in this manner suppressor cell-derived factors were capable of completely abolishing the priming for T_c and T_{DH} activity in an antigen-specific manner. Having demonstrated such potent suppressive activity, we proceeded to immunochemically characterize the suppressor molecules (Greene *et al.*, 1979; Bach *et al.*, 1979). The factor was found to be a protein labile to temperatures greater than 56°C and prolonged exposures to low pH, indicating that certain tertiary structural features were necessary for factor function. Molecular sizing on Sephadex G-200 columns indicated that the proteins(s)' molecular weight was between 50,000 and 70,000. Passage over a variety of immunosorbents established that the suppressor factor obtained from Ts_1 cells was ad-

sorbed to ABA-BSA-coupled Sepharose beads and could be recovered by rapid elution with glycine–HCl (pH 2.8) or high salt. Since the factor from Ts_1 cells (TsF_1) was ligand specific, we also established that conventional immunoglobulin determinants were *not* present on the factor by its inability to be retained by columns composed of anti-mouse immunoglobulin reagents. Finally we began to analyze the genetically determined structural elements that might be present on the TsF_1. We first passed A/J TsF_1 through immunosorbents of anti-CRI Ig fixed to Sepharose, and observed that no suppressive activity was apparent in the filtrate whereas all the inhibitory activity was recovered in the acid eluate. The anti-idiotype was subjected to further purification by absorbing it to idiotype-depleted immunoglobulin, or idiotype-enriched immunoglobin and, in later experiments, to ABA-binding hybridoma-derived immunoglobulin which expressed most CRI structures found in serum anti-CRI antibody. We found that anti-CRI antibody which was purified on B-cell-derived CRI was capable of binding T-cell-derived Ts_1. Thus A/J TsF_1 has ligand binding activity mediated by its antigen binding site, which also presumably expresses CRI elements. The CRI elements, it can be considered, are similar to certain structures also present on B-cell-derived CRI-bearing immunoglobulins. However, similar fractionation of A/J, B10.A, C.AL-20, or BALB/c Ts_1-derived suppressor activity revealed that only strains with the appropriate heavy chain allotype locus, i.e., A/J and C.AL-20 make TsF which expresses CRI structures. Recently we have analyzed the precise roles of heavy chain variable region genes located on chromosome XII (Meo *et al.*, 1980), as well as variable region kappa light chain genes located on chromosome VI (D'Eustachio *et al.*, 1981). In order to accomplish this analysis we developed two approaches. In the first approach, we bred a series of mice which had the following genotypes: *Igh-1*^{e},*Lyt-3.1*; *Igh-l*^{e},*Lyt-3.2*; *Igh-l*^{a},*Lyt-3.1*; and *Igh-l*^{a},*Lyt-3.2*. (A/J mice are Igh-1^e, Lyt-3.2.) The *Lyt-3* locus governs expression of the *Lyt-3* thymocyte surface alloantigen and is closely linked to a light chain variable region genetic locus *Igk-Trp*. It has been observed (Gottlieb *et al.*, 1979; Laskin *et al.*, 1977) that mice which are homozygous for Lyt-3.1 do not express the CRI in their humoral anti-ABA antibodies despite possessing the appropriate *Igh-1*^{e} gene. A gene, *IgK-ID ABA*$^+$, linked to *IgK-Trp*^{b} and *Lyt-3.2*, is required for the expression of the CRI associated with anti-ABA antibodies from A/J mice. Consequently TsF_1 was generated in all of the strains mentioned and passed over immunosorbent columns composed of rabbit antiidiotype antibody insolubilized on Sepharose beads. We observed that regardless of whether strains possessed the genes for the appropriate light chains, all were capable of developing

idiotype-bearing T cell factors. Yet, we simultaneously verified that only mice with *Igh-1*^{e} and *Lyt*-3.2 genes could make CRI$^+$ anti-ABA Ig. At first sight, these data suggested that T-cell-derived antigen-binding material, in contrast to B-cell-derived Ig, did not require a specific light chain to express CRI determinants. However, the data cannot exclude the possibility that another light chain or light chain analog fulfills this role. In order to fully substantiate these studies, we developed a series of antiidiotypic reagents which interacted with determinants associated with the antigen binding site of CRI$^+$ anti-ABA Ig, to structures not associated with the antigen binding site, and to determinants associated directly with the heavy chain variable region. TsF_1 was generated in A/J mice and passed over affinity columns comprised of these reagents. The eluates and filtrates were collected, concentrated, and functionally evaluated. It was clearly shown that immunoadsorbents specific for the heavy chain and immunosorbents that were non-site-specific retained the TsF_1 whereas site-specific antibody did not. These results provided direct structural evidence that the CRI$^+$ elements present on TsF were contributed primarily by non-antigen-combining site, heavy chain related regions. Taken together with the aforementioned genetic experiments indicating a lack of specific V_L region gene products in TsF_1, these studies provide a compelling argument that TsF utilize V_H gene products to bind ligand. Specific V_L gene products controlled by chromosome VI, which are needed for CRI$^+$ Ig expression, seem not to be relevant.

In addition to genes located in chromosome XII, certain suppressor molecules also constitutively express gene products from chromosome XVII and in particular from the H-2 histocompatibility complex (MHC) set of genes. The contribution of H-2 MHC gene products was best analyzed by the studies of Tada (1977) and Taniguchi *et al.* (1981) who established that I-J subregion encoded determinants were present on their carrier-specific suppressor molecules. In a similar manner passage of ABA binding TsF_1 through a series of anti-H-2, anti-I region and anti-I-J region directed antibody-coupled immunosorbents showed that ABA TsF_1 also expressed I-J subregion-encoded determinants (Greene *et al.*, 1980; Dietz *et al.*, 1980). In order to determine if I-J gene products and V_H gene products were on the same molecular complex or were on two easily separable polypeptides, we passaged A/J-derived TsF first through an antiidiotypic column, after which the eluate and filtrate were tested functionally. Also, the filtrate and eluate were next passed through an anti-H-2 column. The filtrate and eluate from the second column were then evaluated for suppression of ABA-specific DTH. What we observed was that the eluate from the anti-CRI column was suppressive and was also retained on anti-H-2

columns. The filtrate from the anti-CRI columns had no discernible activity. These experiments established for the first time that XIIth chromosome V_H gene products and XVIIth chromosome I-J gene products were in the same complex or perhaps more alarmingly on the same polypeptide (Bach *et al.*, 1979). This latter idea is quite radical, since there is no presently known eukaryotic genetic mechanism that permits unlinked chromosomes to encode for peptide fragments which are later assembled as a single polypeptide. However, it is possible in this regard that the I-J region determinant is carbohydrate in nature (for example), and is a product of a regulatory rather than structural I-J genes. This suggestion has been made frequently because of the extraordinary difficulty in identifying precipitable I-J products. Recently M. Taniguchi (personal communication) and C. Waltenbaugh (personal communication) have prepared anti-I-J monoclonal antibodies. These antibodies will be of use in further definition of I-J factors.

As a consequence of these studies we can collectively summarize the pertinent biological, functional, and structural properties of first-order suppressor molecules. Ligand-activated Ts_1 cells produce TsF, which can be obtained from supernatants, or from mechanically disrupted cell lysates. TsF_1 is apparently active only when given close to the time of immunization. The reason for this time-related effect relates to the ability of TsF_1 to stimulate the activation of a second-order suppressor cell with antiidiotypic receptors and this idea will be expanded upon below. Structurally TsF_1 is a protein, does not bear conventional Ig determinants and possesses two distinct types of structures; a definable V_H gene product in certain strains of mice with the appropriate allotype, and, second, an I-J region related product. These two structures are either tightly linked or may be part of a single molecule. A variety of different effector cell types, including cells responsible for mediating cytolytic responses (T_C) (Ratnofsky *et al.*, 1981), those capable of responding in proliferation (Redner and Greene, unpublished), and those capable of responding by making inflammatory responses and typified by the delayed type hypersensitivity T cell (Greene *et al.*, 1979) are all inhibited to some degree by suppression initiated by Ts_1 cells or TsF_1.

XXII. Characterization and Mode of Action of Ts_1—the Induction of Ts_2 Cells

Waltenbaugh and colleagues (1978) first observed that an antigen-specific suppressive factor could, when administered 1 week before immunization with the appropriate antigen, lead to the complete abro-

gation of the subsequent antibody response to that antigen. Furthermore the injection of suppressive molecules prior to antigen immunization required significantly less factor to induce suppression than that needed if given at the time of antigen immunization. A detailed analysis of this phenomenon revealed that the injection of suppressive factors appeared to induce a set of suppressor T cells in the naive recipient. Study of the induction of these induced suppressor cells by suppressive factors has been made by Germain *et al.* (1979). These investigators, by an *in vitro* technique, demonstrated that the factor capable of inducing suppressor cells absolutely required a small amount of antigen be associated with it. At the time of these studies there was little known of the induced set's phenotype. At one point, it was considered to be a cell similar in nature to the cell induced by ligand in terms of its receptor specificity. In the azobenzenearsonate system the first-order suppressor factors induced in A/J mice express cross-reactive idiotypic determinants and I-J region-encoded or regulated elements. If the cell induced by suppressor factors were identical to it (TsF_1) in terms of receptor specificity, that cell would be idiotypic, and if the cell were complementary to the first, it might be antiidiotypic. CRI-positive TsF_1 obtained from ligand-specific Ts_1 cells was injected into normal syngeneic animals (Greene *et al.*, 1979; Sy *et al.*, 1980). Spleen cells were obtained from these injected animals after a period of 7 days and were found upon adoptive transfer to suppress immune responses to azobenzenearsonate-coupled cells. Cells capable of mediating this suppression are Thy-1 positive and were considered to be second-order suppressor cells (Ts_2) since they were induced by first-order suppressor cells. In order to analyze the receptor on Ts_2 cells we first attempted to lyse the Ts_2 cells with anti-CRI antibody and complement. It was not possible, however, to eliminate the function of Ts_2 cells by such treatment, suggesting that they were distinct from the first-order ligand-generated suppressor cell. Their receptor specificity, however, was elucidated when the Ts_2 cells were subjected to binding to either ligand-coupled polystyrene plates or CRI-coupled polystyrene plates. It was found that suppressor cells induced by TsF_1 could be bound to and recovered from idiotype-coated plates but not ligand-coated plates (Sy *et al.*, 1980). Therefore Ts_2 cells are antiidiotypic. Moreover, whereas Ts_1 cells are only active in the afferent mode, that is when given early in the immunization regimen, Ts_2 cells were active not only in the afferent mode but were also active when given late in the immune response (efferent mode). It therefore appears that Ts_2 cells, in contrast to the TsF_1 cells, bear antiidiotypic receptors and also act in a different manner, being func-

tionally capable of suppressing ongoing immune responses. In further contrast to the previous observation by Waltenbaugh *et al.* and Germain *et al.*, the TsF_1 is distinct with respect to the requirement for ligand. The induction of Ts_2 in the azobenzenearsonate system does not appear to require antigen at all. We have been successful in inducing Ts_2 cells, using antigen-free conventional TsF_1 or, alternatively, TsF_1 obtained from a long-term T-cell hybridoma (see Section XI). In the latter case the TsF_1 clearly cannot possibly contain ligand. In conclusion, the second cell induced by others, and that induced in the azobenzenearsonate system are probably distinct and may represent different participants in the suppressor pathway.

XXIII. The Characterization of a Soluble Product Elaborated by Second-Order Suppressor Cells

In order to determine whether a suppressor molecule could be obtained from the second-order cells Ts_2 were generated in A/J mice and were subjected to mechanical disruption. The molecular products obtained after high-speed centrifugation were then analyzed for their ability to mediate suppression of the DTH response to azobenzenearsonate-coupled cells. It was found that Ts_2-derived suppressive factor (TsF_2) was also capable of inhibiting immune responses in an antigen-specific manner and was similarly capable of inhibiting immunity in an efferent or afferent mode. An analysis of the TsF_2 molecule was next undertaken (Dietz *et al.*, 1981). TsF_2 lacked conventional immunoglobulin determinants as determined after passage through affinity reverse immunosorbents. The TsF_2 was retained by immunosorbents directed to H-2 products. In contradistinction to TsF_1, which binds to azobenzenearsonate–protein conjugates coupled to Sepharose, TsF_2 binds to idiotype-coupled Sepharose beads and not at all to ligand-coupled beads. Thus, TsF_2 is antiidiotypic in nature. In marked contrast to the function of TsF_1, which can act in strains that differ with respect to their H-2 and Igh genes, TsF_2 demonstrated both H-2 and Igh-1 linked genetic restriction. This characteristic was determined by the ability of TsF_2 induced in A/J mice to function in A/J ($H\text{-}2^a$) mice but not A.BY ($H\text{-}2^b$) mice. Furthermore C.AL-20-derived TsF_2 could only function in C.AL-20 but not BALB/c mice. These results collectively suggest that the Ts_2 activity is both H-2 and idiotype-, or at least allotype-restricted. It is apparent that the H-2 restriction of the second-order regulatory cell or product is related to the way in which the TsF_1 is presented to precursor Ts_2 cells. Thus, when A/J TsF_1 is administered by A.BY, the subsequent Ts_2 and TsF_2 are restricted to

H-2^b rather than the H-2^a gene products present on the TsF_1 itself. The Ts_2 so generated caused suppression only in H-2^b mice (Takaoki and Greene, unpublished). Evidence that the TsF_2 factor is Igh restricted at the same time that it is H-2 restricted might suggest that TsF_2 may be a different type of molecule than TsF_1. Detailed analyses of the TsF_2 in terms of whether this is a single factor or multiple factors has not as yet been completed. If one views the family of suppressor molecules that have been described in the literature (Tada *et al.*, 1976; Fresno, 1981; Kontianen *et al.*, 1980), it is apparent that in cases in which no H-2 restriction was observed the factor in question is of the TsF_1 type whereas in cases in which H-2 restriction was observed the factors may in fact represent the products of Ts_2 cells. The identification of the Ts_2 set and its derived molecule, TsF_2, can help unify several models of suppressor cell activity that have been described in the literature (Germain and Benacerraf, 1980; Eardley *et al.*, 1978; Greene and Sy, 1980; Tada *et al.*, 1980). The TsF_2 molecule may be of use in determining what is meant by H-2 restriction in suppressor systems. Does the factor have anti-H-2-like activity, or does it interact with certain acceptor sites in a genetically restricted manner (Tada *et al.*, 1980; Schrader, 1980)? Studies in progress will hopefully resolve this issue. Another intriguing possibility is that the TsF_2 molecules' H-2 determinant is itself recognized by the third subset of suppressor cells. If this is the case then H-2 restriction is related to the Ts_3 cell being restricted to interact with certain I-J structures on the TsF_2 molecule and not vice versa.

XXIV. The Induction of Antiidiotypic Suppressor Cells by the Use of Idiotype-Coupled Syngeneic Spleen Cells

Another series of experiments which have been performed by our laboratories to probe hapten-specific regulation is the use of idiotype coupled directly to the syngeneic spleen cell surface by the use of various coupling reagents (Dohi *et al.*, 1980; Sy *et al.*, 1980). We have already discussed such experiments, using the humoral antibody response as a probe. The following discussion relates to the DTH response. The use of idiotype-coupled cells to induce antiidiotypic suppressive cells was in fact first employed in the azobenzenearsonate system. Purified CRI^+ antibody, or anti-ABA antibody obtained from hyperimmune suppressed (HIS) mice, or normal A/J immunoglobulin were independently coupled to A/J splenocytes and used to induce suppressor cells. It was found that only idiotype-coupled cells were capable of inducing suppressor cells demonstrably active in in-

hibiting the immune response to azobenzenearsonate-coupled cells. Cells coupled with HIS immunoglobulin, which lacks CRI^+ molecules, or normal A/J immunoglobulin-coupled cells were ineffective. These experiments suggest that certain structures present on the idiotypic immunoglobulin are decisive in the activation of suppressor cells and rule out allotypic or isotypic effects. The suppressor cells which were induced by such treatment were determined to be antiidiotypic by their ability to be retained by CRI^+ antibody coupled to polystyrene-coated plates (Sy *et al.*, 1980; Dietz *et al.*, 1980; Greene and Sy, 1981). Phenotyping of such cells (Sy *et al.*, 1981) has revealed these suppressor cells to be $Lyt\text{-}1^+2^+$ as opposed to suppressor cells induced by ligand which, as mentioned, are $Lyt\text{-}1^+23^-$. Hence suppressor cells induced by ligand are distinct from those induced by idiotype coupled to cell surfaces. This is a somewhat of an anomaly since idiotype coupled to the cell surface should be equivalent to antigen coupled to a cell surface. An explanation for this question, however, has come from recent studies done in collaboration with Abul Abbas (Abbas and Greene, in preparation). These studies have shown that myeloma idiotype coupled to the cell surface can induce antiidiotypic Ts_1 cells. Thus, it might be envisioned that idiotype on cells does in fact behave as ligand and is capable of inducing more than one type of suppressor subset. However, in the case of ABA-specific DTH, only antiidiotypic Ts_2 can be evaluated. Antiidiotypic Ts_1 may be irrelevant for the suppression of DTH but are probably of significance in the B-cell response.

XXV. The Demonstration of Allotype and/or H-2 Restrictions between Ts_1 and Ts_2 and the Target of the Ts_2 Cell

During our course of study on idiotype-coupled cells as an inductive signal for the generation of antiidiotypic suppressor cells we made the following observations. It was possible to use idiotype-coupled cells to induce suppression of DTH to the ABA hapten in C.AL-20 mice but not in BALB/c mice. It is known of course, as mentioned above, that C.AL-20 mice are capable of responding to azobenzenearsonate conjugates by producing CRI^+ antibody whereas BALB/c mice cannot. It was considered that the observed failure to induce suppression of DTH in BALB/c might be explained in two ways. The first explanation was that the BALB/c mouse could not recognize idiotype and consequently could not generate an antiidiotypic suppressor cell response. This would imply that the absence of idiotype-encoding genes in the genome precluded its ability to recognize idiotypic ele-

ments. An alternative explanation is that the BALB/c mice in fact generated antiidiotypic suppressor cells but failed to generate, upon immunization with azobenzenearsonate-coupled spleen cells, an appropriate target for such antiidiotypic suppressor cells. In order to test this notion antiidiotypic suppressor cells were generated in BALB/c mice by the injection of idiotype-coupled spleen cells intravenously and the suppressor cells so generated transferred to either C.AL-20 mice or to BALB/c mice. The C.AL-20 and BALB/c were immunized subcutaneously with syngeneic azobenzenearsonate-coupled cells. It was found that antiidiotypic suppressor cells generated in BALB/c, although incapable of adoptively transferring suppression to BALB/c mice, could transfer suppression into C.AL-20 mice (Sy *et al.*, 1981). These results suggest that the failure of idiotype-coupled cells to generate unresponsiveness in BALB/c mice was due to the inability of such mice to generate the apppropriate idiotype-bearing target. This notion therefore suggests that genetic restrictions must be analyzed in terms of the inductive signal and the target of the intermediate cell in the suppressor pathway. That is, if the inductive signal represents idiotype-bearing factor or idiotype-coupled cells then the suppressor cell so activated will only be able to manifest its suppression if it encounters in the adoptive host a similar idiotype-bearing target (Greene *et al.*, 1981; Greene and Sy, 1981; Sy *et al.*, 1981). Therefore it is necessary to define the target of the antiidiotypic suppressor Ts_2 cell in greater detail.

XXVI. The Demonstration of an Idiotype-Bearing Ts_3 Subset

The injection of azobenzenearsonate-coupled spleen cells subcutaneously leads to the induction of a variety of effector cells. The DTH effector cell was shown to lack the appropriate idiotype; it is insensitive to antiidiotype and complement treatment. Thus it is unlikely that the immune effector cell itself represents the target of the antiidiotypic suppressor cells. An idiotype-bearing target, however, is apparently necessary, as evidenced by the transfer of suppression from the BALB/c strain, in which antiidiotypic suppressor cells were induced, into a C.AL-20 recipient. We considered the possiblity that the idiotype, although not present on the mature T_{DH} cell, was expressed on a precursor of these same cells. This consideration was excluded since Ts_2 are capable of manifesting suppression at the time of challenge, that is when the mature effector T_{DH} cell is already active. Another consideration was that the T_{DH} cell required an idiotype-specific helper cell for its maximal activation. In this regard attempts to dem-

onstrate a helper cell in the DTH response to ABA have been inconsistent and hence were considered to be inconclusive. It was also possible that in the ABA suppressor pathway another suppressor participant exists which expresses the idiotype and which occurs concomitant with the immune cell after antigen administration. Such an idiotype-bearing, ABA binding suppressor cell has been found in the innovative studies of Thomas *et al.* (1981). An auxiliary cell described by Sy (Sy *et al.*, 1978) in the immune animal primed to dinitroflourobenzene (DNFB) was first shown to be necessary for function of effector suppressor cell subsets. It has been found in that system that DNP-specific effector phase suppressor T cells required a cyclophosphamide-sensitive T cell present in the immune population. The interaction of effector suppressor cells with the auxiliary, cyclophosphamide-sensitive suppressor cell was required subsequently to inhibit the immune effector cell itself. It is clear from what we have described above that the data in the ABA system may be explained by the presence of a T auxiliary-like cell coexisting with the immune population. The Ts_3 set is our terminology for the Ts auxiliary and appears to be a cell similar to that described by J. F. A. P. Miller and colleagues (Thomas *et al.*, 1981). In a series of experiments we have observed that treatment of immune lymph node cells from A/J mice with antiidiotype antibody and complement did not abolish immune function, but rendered these cells insusceptible to suppression by antiidiotypic Ts_2. The generation of antiidiotypic suppressor cells in BALB/c mice and the cotransfer of such Ts_2 BALB/c cells with immune lymphocytes generated in C.AL-20 suppresses the DTH response of the cell. Pretreatment of C.AL-20 immune cells with antiidiotype antibody and complement prevented BALB/c Ts_2 cells from interacting with them and consequently such C.AL-20 immune cells now manifested significant amounts of immunity. Thus it is apparent that an idiotype-bearing suppressor cell, induced by ligand, coexists with effector cells and interacts with the Ts_2 antiidiotypic cell to manifest suppression. The Ts_3 has also been found to be Thy-1^+ and Lyt-1-2^+. This Ts_3 subset apparently requires two distinct signals. The first signal is ligand, and the second is Ts_2 mediated. In experiments in which the specificity of Ts_3 has been evaluated it was apparent that once having been activated the Ts_3 cell is capable of manifesting suppression in an idiotype nonspecific way (Sy *et al.*, 1981). Idiotype-bearing C.AL-20 Ts_3 cells and antiidiotypic Ts_2 cells were cotransferred into BALB/c mice which had been preimmunized with azobenzenearsonate-coupled BALB/c cells. It was observed that the antiidiotypic Ts_2 cells appeared to interact with the idiotypic Ts_3

C.AL-20 immune cell population and suppressed the BALB/c immune cells activated by ligand-coupled cells. These results clearly suggest that, once activated Ts_2 plus Ts_3 function in an idiotype nonspecific manner. In more recent experiments, we have demonstrated that once Ts_2 and Ts_3 have interacted in the appropriate allotype and H-2 restricted manner suppression is not only idiotype nonspecific but may also be antigen nonspecific.

Thus we have defined three suppressor cell subsets present and operative in the suppressor cell pathway. The first cell is activated by ligand and, in the azobenzenearsonate system and the appropriate mouse strain, is idiotype positive. The first cell (Ts_1) mediates its effect by the induction and stimulation of a complementary set of cells which can be shown to be antiidiotypic. These second-order cells are capable of elaborating an antiidiotypic product much like the idiotypic Ts_1. In this case, however, the product is both H-2 and Igh restricted in its activity. Alternatively, two types of Ts_2 cells or TsF_2 are needed, one mediating H-2 restriction, the other necessary for idiotype recognition. Still another more intriguing possibility, as mentioned, is that H-2 restriction is determined by Ts_3 recognizing certain H-2 determinants on the TsF_2. Whatever the case, the Ts_2 interacts with an idiotype-bearing Ts_3 coexisting with the immune effector cell population. The Ts_3 itself apparently requires two separate signals for its activation. Once activated the Ts_3 is capable of mediating its effect in a nonspecific mode. The regulation of immunity to azobenzenearsonate can be thus understood to represent a series of steps, dominated by idiotype and antiidiotype events. In the case of the Ts_2–Ts_3 interaction an additional complexity relates to the H-2 restriction observed. The nature of genetic restriction, however, between the Ts_2 and its target might conceivably represent a necessary recognition of the Ts_2 cell by the Ts_3 cell. Additionally, we suggested that idiotype-coupled cells under certain circumstances might also be able not only to induce antiidiotypic Ts_2 cells but also antiidiotypic Ts_1 cells. If this were the case and if each set of cells had a prescribed function, as apparently is the case, then the antiidiotypic Ts_1 cell must interact with an idiotypic Ts_2 population and the idiotypic Ts_2 population would have to be able to interact with an antiidiotypic Ts_3 population. The antiidiotypic Ts_3 population would consequently have to interact with an idiotype-bearing effector cell. Since in the T cell responses to azobenzenearsonate idiotype-bearing T effector cells have not been defined, we would not be able to detect an antiidiotypic Ts_1. However, as mentioned, the experiments in collaboration with Abbas have already demonstrated that antiidiotypic Ts_1 cells can be induced by

idiotype-coupled cells. Therefore it is apparent that the suppressor pathway conceivably could exist in two levels: at the first level there is a set of cells dominated by and activated by ligand; in this case the Ts_1 is idiotypic, the Ts_2 is antiidiotypic, and the Ts_3 is idiotypic. A second set of cells would also exist: in this case Ts_1 would be antiidiotypic, Ts_2 would be idiotypic, and Ts_3 would be antiidiotypic. Indeed, these two sets of cells might exchange members. We can therefore entertain the notion that the events described previously in terms of the humoral response might occur through the aegis of an antiidiotypic antibody Ts_1 triggering an idiotypic Ts_2 which thereafter interacts with an antiidiotypic Ts_3 and leads to suppression of the idiotypic component of the antibody response (Table IV, mechanism 5). In this case Ts_3 would specifically suppress the CRI complement of humoral response.

In Table IV group 1 is exemplified by the studies of regulation of ABA-induced DTH (Greene and Benacerraf 1980). Most of the response (hapten) would be suppressed after idiotypic Ts_3 are activated. Group 2 is an example of regulation triggered by CRI^+ antibody on cell surfaces (Dohi and Nisonoff, 1980) or myeloma idiotype-coupled cell-induced regulation (Abbas and Greene, in preparation). In this case the inductive signal primarily activates Ts_1 cells which are antiidiotypic. In this situation the Ts_3 subset is primed by B-cell idiotypic structures and triggered by idiotypic Ts_2 factors. In this example regulation is primarily idiotype-specific. Hapten-specific immunity would only be partially affected. In contrast (group 3) idiotype on cells may also directly activate antiidiotypic Ts_2 cells which trigger hapten-primed idiotypic Ts_3 cells. It is this phenomenon which has been apparently observed and demonstrated in the ABA DTH system

TABLE IV

Signal	Ts_1 stimulate	Ts_2 trigger	Ts_3
1. Inductive signal is hapten	Idiotypic	Antiidiotypic	Idiotypic
2. Inductive signal is idiotype on cells	Antiidiotypic	Idiotypic	Antiidiotypic
3. Inductive signal is idiotype on cells	—	Antiidiotypic	Idiotypic
4. Inductive signal is antiidiotypic	Idiotypic	Antiidiotypic	Idiotypic
5. Inductive signal is antiidiotypic	—	Idiotypic	Antiidiotype

(Greene and Sy, 1981). Once again the Ts_3 cell would be idiotypic and capable of suppressing most of the hapten-specific reaction. Finally (groups 4 and 5) antiidiotypic signals could conceivably activate idiotypic Ts_1 or idiotypic Ts_2 cells to induce regulation which is either hapten or idiotype-specific, respectively (Greene *et al.*, 1980; Sy *et al.*, 1980; Nisonoff and Greene, 1980). It should be stressed that this concept implies that the receptor repertoire of Ts_1 and Ts_2 cells are identical and triggering of one subset rather than the other by idiotype may depend on a variety of other biologic considerations such as the type of cell membrane H-2 structures that are associated with the idiotypic element. At any rate, this idea permits a greater latitude in prediction of the outcome of regulatory signals and accommodates more of the published data than previous pathway theories (Germain and Benacerraf, 1981).

XXVII. T-Cell Hybridomas Which Secrete TsF_1 Polypeptides

Our interest in the molecular basis of regulation necessitated a more detailed examination of suppressor molecules. It had already been established that azobenzenearsonate-induced suppressor factors obtained from Ts_1 cells were proteins of mass 50–70 kd, and could be shown to have I-J-encoded determinants as well as idiotypic structures. We took advantage of the well-established technique of Köhler and Milstein (1975) as modified by others (Taniguchi *et al.*, 1979) to fuse the BW5147 thymoma with ABA-induced Ts_1 cells (Whitaker *et al.*, 1981; Takaoki *et al.*, 1981). One such resultant clone termed F12 was initially screened for its ability to secrete a product identifiable by a radioimmunoassay developed by Pacifico and Capra (1980). Briefly this assay is a competitive radioimmunoassay in which T-cell hybrid supernates are evaluated for their ability to inhibit the binding of monoclonal anti-ABA antibody to ABA-bovine serum albumin linked to polystyrene plates. By this semiquantitative assay we were able to examine a variety of potential T-cell clones. Interesting clones were next functionally evaluated in terms of their ability to produce unresponsiveness as assayed by cell-mediated cytotoxic responses, or delayed-type hypersensitivity reactions *in vivo*.

In this manner F12 was found and shown to be effective in inhibiting DTH and ABA-specific cytotoxicity. F12 supernates were administered *in vivo* to A/J mice which had been primed with ABA-coupled cells subcutaneously. Dilutions of up to 1/100 of the original supernates were still markedly suppressive. Moreover the factor could be obtained from subclones of the F12 and this material was as active as

from the parental line. Finally, it should be noted that the clones were remarkably stable, the lines have been in culture for about 1 year with no evidence that any major cellular function has changed. We also characterized the cell lines phenotypically, analyzing the hybrid lines using the cytofluorograph, and observed that the F12 line was both Thy-1.1 and Thy-1.2 positive and Lyt-1.2 positive but Lyt-2.2 and Ig negative. The presence of both theta markers is in accord with the fact that the parental line BW5147 is Thy-1.1 and the A/J Ts_1 cell is Thy-1.2 positive. Attempts to determine if the *in vitro* maintained line bore idiotypic markers or I-J-encoded elements were consistently negative.

We evaluated the F12 product in terms of its immunochemical properties as determined by a panel of immunosorbents. As described previously for conventional TsF_1 (Greene *et al.*, 1979) and substantiated by testing the hybridoma, the F12 product was shown to bear I-J-encoded elements as well as cross-reactive idiotypic structures. These results suggest to us that F12 is in fact a functioning long-term T-cell hybridoma.

We also observed in the course of these studies another T-cell hybridoma termed F3 capable of producing molecules detectable by the same radioimmunoassay. F3 however produced factors which lacked suppressive effects in our functional assays. The F3 cell product could not inhibit the generation of cytotoxicity or suppress the generation of DTH responses to azobenzenearsonate when administered into A/J mice. However, it was possible to resolve the defect of the F3 molecule by a series of manipulations. Our studies of the activity of presuppressor cells (Bromberg *et al.*, 1981) had indicated that molecular signals achieved under the influence of an allogeneic effect could stimulate the generation of suppressor T cells from a presuppressor (pre-Ts) pool. These studies have shown that appropriately administered allogeneic cells or factors could trigger pre-Ts that had been minimally activated by ligand. It should be noted that the doses of ligand-coupled cells had been titrated so as to determine the precise amount needed to generate Ts and pre-Ts. Using this principle of providing the appropriate second signal we have recently been able to use an allogeneic effect to bypass the F_3 detect. At first sight, the F3 product appeared to be a defective F12-like molecule. Recently we have found suggestive evidence that F3 does not have conventional I-J determinants. Thus it is likely that careful scrutiny of such hybridoma products may reveal "mutants" which are interesting in terms of their disordered construction and consequently may provide significant insight into the mechanism of subcellular assembly of T-cell sup-

pressor molecules subcellularly. In conclusion, whereas F12 is idiotypic and I-J bearing, F3 appears to be idiotypic but lacks the same I-J markers. Consequently F3 seems to be a defective product that requires further study.

XXVIII. Chemical Studies of the F12 Polypeptide

Because of the availability of large amounts of hybridoma products we undertook to determine the fine molecular characterization of the F12 molecule. These studies (Nepom *et al.*, in preparation) have revealed that F12 can be precipitated most efficiently by 40% saturated ammonium sulfate. Passage of these molecules over Sephacryl G-200 revealed that most functional activity was contained within the 50–70 kd region. Other chemical characteristics such as p*I* and chain structure are being studied.

In summary a detailed molecular analysis of T-cell hybridoma products has been undertaken, and will be discussed elsewhere. The factor is extremely potent and capable of engendering suppression of a variety of T-cell functions in an antigen-specific manner. Finally the molecules appear to be the product of the Ts_1 subset of cells. This latter point deserves particular emphasis since published analyses of other suppressor products have not established the nature of the Ts (Fresno *et al.*, 1981; Taussig *et al.*, 1979; Taniguchi *et al.*, 1981; Yamauchi *et al.*, 1981; Tada *et al.*, 1981).

XXIX. A Synthesis of Information on Suppressor Pathways

It is clear from what has been described above that the essential features of a suppressor pathway as initially outlined by Gershon and colleagues (1978) also relates to the azobenzenearsonate system. Work by Dorf and colleagues in the 4-hydroxy-3-nitrophenyl acetyl (NP) system has revealed many features similar to those described herein as well as certain fundamental differences (Weinberger *et al.*, 1980; Okuda *et al.*, 1981). One critical difference in the NP system relates to the ability of a relatively uncharacterized Ts_1 factor derived from a T-cell hybridoma to suppress selective idiotypic antibody responses as well as hapten-specific DTH responses. At the present time administration of TsF from the F12 has not been shown to be capable of inhibiting the production of CRI-bearing anti-ABA antibody *in vivo*. This difference remains to be clarified. The detailed studies of Fresno *et al.* (1981) have revealed that a cloned T cell line makes a 70,000-dalton product which is capable of suppressing the immune response to

sheep erythrocytes. The 70,000-dalton H-2 negative moiety spontaneously disintegrates into a 45 and a 24-kd piece. It was suggested that the 24-kd piece binds antigen but does not manifest suppression, whereas the 45-kd piece manifests nonspecific suppression and does not bind antigen. Fresno further speculated that the antigen binding piece in some manner can trigger the larger piece, after binding antigen. These results, it should be stressed, relate to products elaborated from an Lyt-23-bearing clone of cells. Taniguchi and Tada (1980) have also generated antigen-specific hybridomas and these have been described as bearing I-J and also having a V_H binding site for antigen. More recently Taniguchi has shown that treatment of a KLH-specific hybridoma product with dithiothreitol disrupted the KLH-specific T-cell hybridoma product into two separate polypeptides. Alkylation prevented the two chains from reassociating indicating the presence of interchain disulfide bonds. Previously Taniguchi and Tada (1979) had observed that extracts of hybridomas seemed to spontaneously dissociate into two separable polypeptides. The study of reduction and alkylation was performed on secreted molecules from a similar KLH-specific hybridoma. In accord with the previous study (Taniguchi and Tada, 1979) the antigen binding piece was incapable of mediating suppression unless combined with the I-J determinant containing polypeptide.

More recently Yamauchi and Gershon (1981) have shown that certain Lyt-1^+-derived suppressor supernatants contain two already dissociated entities, one I-J bearing and the other mediating antigen binding. By a variety of experiments the authors concluded that the I-J-bearing entity was decisive in determining V_H restriction. This idea is quite provocative and exciting. However, the latter studies were not performed with hybridoma products and consequently it cannot be absolutely excluded that the I-J-restricting polypeptide was in fact a suppressor factor with antiidiotypic activity. Consequently it might be argued that the reassociation of the antigen binding element with the I-J antiidiotypic factor in the presence of antigen may have unknowingly created the situation where a complex in fact determined the allotype restriction. At any rate it can be said that these studies provide suggestive evidence that the I-J-bearing peptide determines allotypic restrictions but it is necessary that more rigorous experiments be performed to substantiate the idea.

Another functional T-cell hybridoma has been reported by Kapp *et al.* (1980). These workers have found a 20–60 kd molecule capable of suppressing the plaque response of DBA/1 mice to the synthetic copolymer L-glutamic acid–L-alanine–L-tyrosine. The suppressor mole-

cule was also shown to express I-J subregion-encoded determinants. The study did not resolve the suppressor cell type responsible for the factor production, nor did the experiments establish whether the factor was a single or complex molecule. Additionally Kontiainen *et al.* (1981) have recently observed that the parental BW5147 line might contribute I-J determinants to suppressor products elaborated by hybridomas. If this is the case, then certain public I-J-encoded domains also may be determinants of TsF activity. Recently another suppressor T-cell hybridoma has been produced (Sorenson and Pierce, 1981) in which I-A determinants are present in lieu of I-J region-encoded structures. These TsF also are restricted to inhibit I-A syngeneic primary plaque-forming splenocytes. In this regard at least two other types of TsF bear determinants other than I-J, and in both cases appear to be I-C region related (Rich and Rich, 1976; Moorehead, 1979; Claman *et al.*, 1980).

It is obvious that a great deal of information will be generated shortly by various groups on the nature and structure of these biologically active molecules. Because of the distinct types of antigen assay systems and purification techniques employed it is not possible to gain any complete understanding at present. The opportunity to speculate on the complex regulatory events, however, allows us to predict that through the use of long-term T-cell hybridomas or T-cell clones it may be possible, with time, to isolate pure factors and T-cell hormones and assay these molecules in standard *in vivo* and *in vitro* systems. Once this occurs we will be able to precisely manipulate this pathway.

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Immunologic Regulation of Lymphoid Tumor Cells: Model Systems for Lymphocyte Function

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I. Introduction

The past decade has witnessed remarkable progress in immunology. The phenomena of immune induction, suppression and unresponsiveness, the regulatory interactions between lymphoid cells and accessory cells, and the genetic controls of such interactions are now firmly established on the basis of a large body of experimental evidence. The next phase in the analysis of the immune system has to be devoted to redefining these biologic phenomena in more quantitative and, ultimately, mechanistic terms. Several recent advances have made it feasible to design experimental systems for elucidating the basic mechanisms underlying immune responses and their regulation. Some of these developments include techniques for long-term culture

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of functional lymphoid cells, the characterization of growth factors and biologically active mediators that can be purified to homogeneity, the availability of monoclonal antibodies as highly specific probes for lymphocyte populations, and, perhaps most significantly, the advent of molecular biological techniques for defining the genetic basis of cellular differentiation. One of the major problems in applying these techniques in immunology is the vast heterogeneity of the mammalian immune system. Thus, the immune system is known to consist of many functionally and phenotypically distinct but interacting clones of cells that are exquisitely specific, so that only very few cells in an organism are responsive to any one antigenic determinant. Moreover, since lymphoid cells constantly arise, undergo a normal process of differentiation and are subsequently regulated by positive amplification and negative feedback loops, it is difficult to isolate desired functional clones at any given time for studying the mechanisms that control their growth and function. One approach for defining the biochemical and molecular basis of lymphocyte function, which has been actively investigated only during the last 5 years or so, is based on exploring the possibility that tumors of immunocompetent cells are susceptible to regulatory stimuli much like their nonneoplastic counterparts. The goal of this article is to detail the experimental systems in which tumor cell lines have been employed as models for normal lymphocytes, with particular emphasis on their physiologic validity, the current state of the analyses, and their future potential. Although lymphoid neoplasms are widely used to study oncogenesis and tumor immunity, these aspects will not be addressed in the present article. Moreover the biochemical analysis of tumor cell products such as antibodies and immunologic mediators derived from tumors of T lymphocytes and macrophages will not be considered in any detail, since our focus is on the use of tumor cells themselves as model systems. Throughout we will point out the resemblance between results obtained with tumor cells and normal immunocompetent cells. Finally, the major caveats and problems in the use of tumors as model systems will be highlighted, so that optimal experimental conditions can be established to extend the phenomenology to mechanistic analyses.

II. Potential Applications of Lymphoid Tumors: General Considerations

Ever since immunologists recognized the existence of clones of lymphoid cells committed to interact with distinct antigenic determinants and the vast size of the immune repertoire, it has been apparent

that issues such as lymphocyte differentiation, triggering and inactivation by antigens, and regulation of one clone by other cells cannot be analyzed in detail with conventional systems of immune responses. Several experimental approaches have been attempted to circumvent the problems inherent in the complexity and heterogeneity of the immune system.

Antigen-specific lymphocyte purification. In spite of many different attempts to enrich or isolate lymphocytes reactive with defined antigens by affinity purification (Haas and von Boehmer, 1978), the technology has remained cumbersome and frequently unreliable, and the yields of cells are extremely small. The studies of Nossal and associates along these lines have been perhaps the most fruitful. Thus, hapten-specific B lymphocytes can be enriched on hapten-gelatin coated plates and used to define the requirements for activation as well as tolerogenesis (Nossal and Pike, 1979). Such approaches have led to concepts such as clonal abortion and clonal anergy in the induction of B-cell tolerance (Nossal and Pike, 1980), which state that an immature B-cell clone, upon interacting with an antigen, loses its ability to express surface receptors or be subsequently triggered and is thus rendered tolerant to the antigen. Whether these studies can be extended to further molecular analysis of the mechanisms of triggering or inactivation is an open question. Affinity purification of different subsets of T lymphocytes by binding to antigens (Taniguchi and Miller, 1978) or antigens in association with cell surface histocompatibility determinants (e.g., Kees *et al.*, 1978) is feasible, but generally results in functional depletion or recovery of enriched rather than homogeneous functional populations.

Polyclonal activators. The use of stimuli that affect many clones of lymphocytes irrespective of antigenic specificity is another experimental approach for defining the basis of cellular activation. Many different polyconal activators are available; among these are bacterial lipopolysaccharides (LPS) and antiimmunoglobulin (Ig) antibodies for B cells, and plant lectins, e.g., phytohemagglutinin (PHA) and concanavalin A (Con A) for T lymphocytes. Important advances in defining the requirements for inducing B-cell proliferation and differentiation, such as the role of helper T-cell-derived factors and macrophages, have been made using LPS and anti-Ig as inductive signals (Moller, 1980). Moreover, the use of polyclonal activators permits analysis of the early cytoskeletal, ionic, and biochemical events associated with lymphocyte activation (Oppenheim and Rosenstreich, 1976). However, it is unclear whether or not the effects of polyclonal stimuli are always fundamentally similar to the more physiologically relevant an-

tigen-specific triggering of a clone, and with such activators it may not be possible to analyze negative effects, i.e., functional suppression.

Lymphocyte clones and long-term culture. Recent progress in our understanding of the growth requirements for lymphocytes has led to the successful maintenance of cloned, nontransformed, antigen-specific lymphocytes in long-term culture. The most dramatic results are with T cell lines, and clones of cytolytic, helper, suppressor, and proliferating T cells and the cells that mediate delayed hypersensitivity have been established in many laboratories (Moller, 1981; Nabel *et al.*, 1981). Such clones are being utilized for defining the recognition patterns of T cells, for studying functional T-cell-derived soluble mediators, and, more recently, for analyzing the interactions between different T-cell subsets such as suppressor and helper cells (Fresno *et al.*, 1981). Using essentially similar approaches, colonies and cloned lines of B lymphocytes can also be derived from nonimmune or specifically immunized cell populations (Pillai and Scott, 1981; Howard *et al.*, 1981). Difficulties in maintaining clones for prolonged periods and limitations on the number of functional cells that can be recovered are some of the major technical problems with lymphocyte cloning at present. Moreover, it is conceivable that long-term clones represent either a subset of normal cells, or cells that have become abnormal during growth *in vitro*. Such potential artifacts have not been critically evaluated for a large number of clones, to date.

The final experimental approach to analyzing lymphocyte biology at the level of single cells is the theme of this article, namely the use of *lymphoid tumors as model systems.* The term "lymphoid tumor" is used in this article to refer to all tumors of immunocompetent cells, including myelomas and tumors derived by somatic cell hybridization. Tumor cells have two important properties that are particularly significant in this context. First, they are homogeneous and easily obtained in bulk quantities. Therefore, they are convenient sources of differentiation markers and of target determinants recognized by other cells (e.g., regulatory T cells) or factors (e.g., growth and differentiating inducing substances). In addition, if tumor growth or function can be regulated, the morphologic and molecular basis of such regulation can be analyzed. Second, a tumor cell is, by definition, autonomous in its growth and function and, unlike normal lymphocytes, can be studied in isolation. Putative inductive, suppressive, and regulatory stimuli such as antigens, polyclonal activators, macrophages, T lymphocytes, or soluble mediators can be added at will and in any desired sequence to define their mechanisms of action. It is obvious that tumors are physiologically valid models only to

the extent that they resemble their nonneoplastic cells of origin. In fact, a large number of experimental and clinical observations have established that neoplasms frequently respond to physiologic stimuli much like their normal counterparts. For instance, the long-recognized association between hormonal status and tumors of hormone-responsive tissues (such as breast, prostate, and endometrium) provide situations for biological experimentation as well as rational therapy. Cell biologists and endocrinologists have used a variety of nonlymphoid tumors as targets for growth and differentiation-inducing stimuli. Among the many elegant examples are the requirement of estrogens for the growth of cultured mammary tumor cells (Lippman *et al.*, 1979), nerve-growth factor induced differentiation of pheochromocytoma cells *in vitro* (Goodman *et al.*, 1979), and the induction of enzymes involved in melanin biosynthesis in cultured melanoma cells (Pawelek, 1976). Such *in vitro* systems are being actively investigated to define the biochemical and molecular biologic basis of cellular growth and differentiation and its control by extrinsic agents.

Immunologists have appreciated the value of tumor products for over two decades. The concept that immunoglobulins secreted by myeloma cells reflect the properties of normal antibodies was established by the pioneering studies of Kunkel and associates using human myelomas (Kunkel, 1965). This soon led to the widespread use of monoclonal myeloma proteins for studying antibody structure (Edelman, 1973), and for identifying allotypic and idiotypic markers (Natvig and Kunkel, 1973). In addition, the discovery that antibodies secreted by some spontaneous human and chemically induced murine myelomas are fortuitously specific for defined, often simple antigenic determinants such as haptens and polysaccharides (Natvig and Kunkel, 1973; Potter, 1977) has provided systems for analyzing the thermodynamics of antigen–antibody interactions and the structure of the antigen combining sites of Ig molecules. More recently, myelomas have proved to be vital sources of genes and mRNA coding for Ig molecules, which are being used to redefine the genetic basis of antibody synthesis and the generation of antibody diversity (Moller, 1981b). Moreover, immunization with myeloma proteins is a widely used protocol for inducing antiidiotypic antibodies and T lymphocytes, and this approach has proved invaluable for dissecting the role of idiotype-specific immunity in regulating immune responses (Eichmann, 1978). The advent of hybridoma technology has led to a situation where one is no longer restricted by the limited availability of antigen-binding myelomas, since homogeneous antibodies can be readily produced against any antigen. Monoclonal (hybridoma) anti-

bodies are not only powerful affinity probes, labeling reagents, and potential therapeutic or diagnostic tools, but can also be used as models for normal antibody molecules, much like myeloma proteins. Finally, the ability to prepare T-cell hybridomas that secrete biologic regulators of lymphocyte growth or antigen-specific suppressor factors (Kapp *et al.*, 1980; Taniguchi *et al.*, 1979; Harwell *et al.*, 1980), and the discovery that some spontaneous macrophage and T cell tumors liberate factors like interleukin 1 and 2 that are secreted by normal cells (Gillis *et al.*, 1980; Mizel *et al.*, 1978) make structural and biologic analyses of these immunoregulatory molecules a feasible goal.

Despite these long recognized uses of tumor products in immunologic research, the concept that tumors of immunocompetent cells can be used as models for analyzing lymphocyte function is a relatively novel one. During the last 5 years, this possibility has received increasing attention, and experimental support for this notion is already compelling. The physiologic validity of the tumors as models can only be established by demonstrating similarities between the tumor and nonneoplastic cells. Such evidence is necessarily circumstantial and based on phenomenology rather then mechanistic similarities, so that all studies with tumors must be considered with the caveat. Moreover, this approach implies that negative or aberrant results with tumors are difficult to interpret with regards to their physiologic relevance, although it is conceivable that such unexpected results or aberrations of the neoplastic state might provide valuable insights into the mechanisms operative in the usual or "normal" physiologic phenomena. Finally, perhaps the most elegant demonstration of the importance of tumors as model systems will come if they suggest phenomena that have not been appreciated in physiologic immune responses but, upon subsequent investigation, prove to exist. In fact, situations where this might be true are already being discovered.

In subsequent sections we will focus on the application of tumors of immunocompetent cells to the study of normal lymphocyte differentiation and function. Initially we will review the evidence that such tumors represent neoplastic transformations of well-characterized differentiative stages or subsets of lymphocytes. The remainder of this article will deal with experimental systems for specific immunologic activation and suppression of tumor growth and function. It should be pointed out that the majority of the reported studies have been done with B-cell tumors, since T lymphocyte neoplasms and the regulation of physiologic T cells are relatively poorly characterized, to date. Moreover, it is clear that although answers to many of the major unresolved issues in cellular immunology have not yet been definitively

established, the stage is set for immunologists, biochemists, and molecular biologists to pool the technological advances, and tumor systems may well be a valuable approach for elucidating the mechanisms underlying immune phenomena.

III. Relationship of Lymphoid Tumors to Differentiative Stages or Subsets of Normal Lymphocytes

If tumors of immunocompetent cells are to be used as models for physiologic immune responses, it is important to first establish which stage of normal lymphocyte differentiation or subset of lymphoid cells is represented by a particular tumor. The reason for this is that B and T lymphocytes at different stages of their ontogenetic pathway show fundamentally different responses to antigens and immunoregulatory stimuli. In theory, it may also be possible to use tumors as models of development, i.e., to study the ontogeny of normal immune cells and its regulation by analyzing tumors, as has been done for other systems such as myeloid cell differentiation (Sachs, 1978). However, our current knowledge of lymphocyte development, especially in mice, is largely based on studies of normal cells. Thus, the principle of identifying the differentiative stage or subset of a lymphoid tumor is to detect markers that are known to characterize normal B and T lymphocytes in terms of their lineage and ontogeny. A large number of serologically and immunochemically defined differentiation markers are known; among these, the most widely used are the pattern of biosynthesis and expression of Ig for B cells, and cell surface alloantigens as well as production of functional mediators for T lymphocytes. Studies of tumor cells by these techniques need to be considered with several points of caution. First, it is clear that not all the stages in B and T lymphocyte differentiation, which must form the basis for comparisons with neoplasms, are defined to date. For instance, during the last 2 years, previously unsuspected stages in the antigen-induced maturation of suppressor T lymphocytes (Germain and Benacerraf, 1981) and subsets of B cells with strikingly different requirements for activation (Singer *et al.*, 1981) have been identified, and it is reasonable to predict that similar complex subpopulations will be discovered in other functional lymphoid classes. Monoclonal antibodies against cellular differentiation antigens may be an invaluable tool for such analyses, as has already been elegantly demonstrated for human T lymphocytes (Reinherz and Schlossman, 1980). In fact, the application of hybridoma technology to the study of lymphocyte development is likely to provide results that might not be predicted by studies with conven-

tional reagents such as heterologous antibodies or alloantisera (Springer, 1981). Studies with monoclonal antibodies should, however, be interpreted with caution, since the restricted specificities of such antibodies might result in cross-reactivity with antigens that are largely dissimilar but share minor structural domains that are serologically indistinguishable (Milstein and Lennox, 1980). Second, although many or most lymphoid tumors phenotypically resemble a particular subpopulation of normal lymphocytes, at least some tumor cells express combinations of surface markers that are not found together on normal cells. Whether such tumors represent instances of aberrant gene expression or of dedifferentiation associated with the neoplastic state is unknown. Third, it is conceivable that apparently "inappropriate" surface antigens on tumor cells may not be artifacts of the neoplastic state but may reflect a technical inability to identify the specific marker on normal cells. For instance, Fc receptors and Ia antigens were detected on murine T-cell tumors at a time when they were considered specific for B lymphocytes (and certain nonlymphoid cells); only later were these molecules clearly identified on a small subpopulation of normal T cells (Warner, 1978). Finally the presence of unsuspected reactivities (e.g., antiviral antibodies) in typing reagents such as alloantisera may lead to findings with tumor cells that cannot be related to the normal sequence of lymphocyte differentiation.

Despite these caveats, it is important to highlight the relationships of tumors to normal cells in the immune system. In the following sections the major results with murine and human tumors of immunocompetent cells will be described. Rather than listing all the tumors that have been studied, an attempt will be made to define the approaches that are used, their validity and their role in establishing that different tumors are known that do, in fact, represent neoplasms of virtually all the identified stages or subsets of normal lymphocytes (Seligmann *et al.*, 1978).

A. B Lymphocyte-Derived Tumors

The current view of the differentiation of normal B lymphocytes in man as well as experimental animals is based largely on the expression of Ig as well as other surface markers such as receptors for Fc and C3 and Ia alloantigens (Table I). The earliest cell type that can be shown to belong to the B lymphocyte lineage by Ig expression is the pre-B cell, which contains cytoplasmic μ chains, does not synthesize light chains, and expresses little or no surface Ig (Kincade, 1981). In fact, Epstein–Barr virus (EBV) transformed lymphoblastoid cell lines

TABLE I

DIFFERENTIATIVE STAGES OF NORMAL B LYMPHOCYTES AND REPRESENTATIVE TUMORS

Stage of differentiation	Immunoglobulin expression	Representative tumors	
		Spontaneous	Experimentally induced
Pre-B cell	Cytoplasmic μ^+, surface Ig^- (due to synthesis of μ but not light chains)	Human pre-B leukemias[a]	Abelson virus-transformed lines[b]; 70 Z/3[c]
Immature B lymphocyte	Surface IgM^+ (transcription and translation of $C\mu$ membrane gene)	Human B-CLL, B-cell lymphomas	Virus, chemical carcinogen and radiation induced[d,e] (e.g., WEH1 231, 38C-13)
Mature B lymphocyte	Surface IgM^+ IgD^+ (alternative processing of primary RNA transcript containing V_H, $C\mu$ and $C\delta$ segments)	Human B-CLL, B-cell lymphomas; BCL_1, other BALB/c lymphomas[f,g]	Virus, chemical carcinogen and radiation induced[h]
Activated B lymphocyte	Expression of other Ig isotypes ("heavy chain class switching," possibly due to deletion of preceding C region genes); low rate Ig secretion	Human B-CLL, B cell lymphomas[i]	EBV-transformed lymphoblastoid lines
Antibody-secreting cell	High rate Ig secretion, reduced density of surface Ig (failure to transcribe and/or translate C region membrane segment genes)	Myelomas	Myelomas, hybridomas

[a] Vogler *et al.* (1978); Hurwitz *et al.* (1979); Smith *et al.* (1981).

[b] Baltimore *et al.* (1979).

[c] Paige *et al.* (1978).

[d] Warner *et al.* (1979).

[e] Bergman and Haimovich (1977).

[f] Strober *et al.* (1979).

[g] Laskov *et al.* (1981).

[h] The majority of experimentally induced murine B lymphomas have not been studied for IgD expression; evidence for maturational stage comes from the presence of other Ig isotypes (e.g., IgG) and/or additional cell surface markers (Fc and C3 receptors).

[i] Fu *et al.* (1979).

that do not synthesize any Ig but bear surface Fc and/or C3 receptors may represent even more immature B lymphocyte precursors (Fu and Hurley, 1979; Fu *et al.*, 1980). Toward the end of gestation in the mouse, B lymphocytes begin to express IgM; at this stage the usual result of encounter with antigen is inactivation (tolerance) rather than triggering. Soon after birth, receptors for Fc and C3 are expressed, and during the first week of life B cells also express IgD and Ia antigens, and become immunocompetent (Paige *et al.*, 1978). Further stimulation of these mature B cells with antigens or polyclonal activators leads to the appearance of cells with other surface Ig isotypes (i.e., IgG, IgA, IgE), this phenomenon being called "heavy chain class switching." In addition, the pattern of Ig expression changes such that the cells begin to actively secrete Ig and show a gradual reduction in the density of surface Ig. Thus, the terminally differentiated antibody-forming cell (AFC), which secretes antibody at a high rate, may express little or no surface Ig (Melchers and Andersson, 1973; Sidman, 1981). Although this general scheme of B cell differentiation is supported by a large body of experimental evidence, recent studies employing heteroantisera and monoclonal antibodies in man and mice clearly demonstrate that even surface Ig-positive B lyphocytes may be composed of multiple distinct subpopulations (Wang *et al.*, 1979; Stashenko *et al.*, 1980; Nadler *et al.*, 1981; Brooks *et al.*, 1981; Lanier *et al.*, 1981). The existence of B cell heterogeneity is further supported by differential reactivities of B lymphocyte subsets to various polyclonal activators (Gronowicz and Coutinho, 1974) and the acquisition of cell surface markers associated with maturation, such as the Lyb5 alloantigen (Ahmed *et al.*, 1977). In any event, analysis of a large number of spontaneous tumors in mice and man, as well as experimentally induced tumors, clearly demonstrates that neoplasms representing distinct differentiative stages of B lymphocytes have been characterized (Table I).

1. Tumors of Pre-B Cells

Among the best studied tumors of pre-B cells are those induced in mice or by *in vitro* transformation by Abelson murine leukemia virus (Baltimore *et al.*, 1979; Boss *et al.*, 1979). Although early studies suggested that these lymphomas were mostly membrane IgM-bearing tumors of mature B cells (Premkumar *et al.*, 1975), more recent work has clearly established that the majority of these tumors are cytoplasmic Ig-positive and membrane Ig-negative (Siden *et al.*, 1979). Most Abelson virus transformed lymphoma cells do not express surface Ig apparently because they do not synthesize light chains, similar to nor-

mal pre-B cells in mouse fetal liver (Levitt and Cooper, 1980; Siden *et al.*, 1981). However, the μ only phenotype is not a universal finding in all normal or neoplastic cells that are surface Ig-negative, presumed pre-B cells (Seligmann, 1979). A nitrosourea-induced murine leukemia, 70 Z/3 (Paige *et al.*, 1978), as well as several human lymphocytic leukemias (Vogler *et al.*, 1978; Hurwitz *et al.*, 1979; Smith *et al.*, 1981) and established cell lines (Preud'homme *et al.*, 1978) contain cytoplasmic but no surface Ig and thus appear to represent neoplasms of pre-B cells. Biosynthetic labeling studies suggest that some human chronic lymphocytic leukemia (CLL) cells that bear little or no surface Ig synthesize an excess of light chains over heavy chains (Hannam-Harris *et al.*, 1980). This is clearly contrary to the results obtained with normal and neoplastic pre-B cells; however, these CLL cells also contained no detectable cytoplasmic Ig, so that their relationship to the normal pathway of B lymphocyte differentiation remains uncertain. Finally, Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines obtained from patients with X-linked agammaglobulinemia frequently show patterns of Ig biosynthesis and expression that are characteristic of pre-B cells (Fu *et al.*, 1980). This is in marked contrast to EBV-transformed lines obtained from normal individuals, which usually represent tumors of activated B lymphocytes (vide infra). It is likely that the preferential induction of pre-B cell tumors by transformation of cells from patients with agammaglobulinemia reflects the absence of mature B lymphocytes which are generally transformed by EBV and overgrow cultures if the transformation is attempted with normal lymphocytes.

Recent analysis of μ gene expression in pre-B cell-derived tumors suggests that at least some Abelson lines contain mature messenger RNA (mRNA) for μ chains but not light chains (Kemp *et al.*, 1980). Such observations have led to the concept that during B-cell ontogeny, the first immunoglobulin gene to be rearranged, i.e., to undergo joining of the variable (V), diversity (D), joining (J), and constant (C) region segments, is the μ gene. Similarly, pre-B-cell hybridomas derived by fusion of fetal liver cells with a myeloma contain rearranged genes for μ chains but not light chains (Maki *et al.*, 1980). Fusion of a cytoplasmic μ-positive, light chain-negative Abelson tumor with a myeloma that does not express light chains of Ig molecules leads to the synthesis of light chains (κ or λ) from the Abelson line (Riley *et al.*, 1981). This suggests that the embryonic light chain gene of the Abelson tumor can be rearranged and expressed in its mature form. In contrast to these observations, similar analyses of the Ig genes of 70 Z/3 pre-B leukemia cells show the presence of rearranged

κ chain DNA (Maki *et al.*, 1980) but no light chain synthesis (Paige *et al.*, 1981). This implies independent transcriptional control of the expression of heavy and light chains of Ig molecules, although it is presently unclear whether or not the DNA rearrangement observed in 70 Z/3 cells is present at any stage of normal B cell ontogeny. Other Abelson virus-transformed lines may express light chains but no heavy chain and, therefore, no surface Ig (Siden *et al.*, 1979). Such cells may contain a μ gene that is aberrantly or abortively rearranged, but this possibility has not yet been formally established by gene cloning experiments. Walker and Harris (1981) have recently demonstrated a Cμ RNA species in 3 or 4 Abelson virus transformed lines examined that is clearly different from the μ mRNA and is not translated into a detectable polypeptide. The same Cμ RNA is present in some T-cell-derived tumors, and the possibility exists that the Cμ gene may be transcribed in B and T cells at a stage before the acquisition of translational competence. The function of this Cμ RNA, which clearly lacks the properties of mature messenger RNA, is presently unknown. Finally, it is important to note that the pattern of Ig expression by tumors of presumed pre-B cell origin can be strikingly altered by culture conditions (Siden *et al.*, 1979) and by exposure to polyclonal activators that are often present in fetal calf serum (Paige *et al.*, 1978). Thus, variations in Ig biosynthesis or expression of heavy and light chain genes among different tumors may reflect artifacts of *in vitro* propagation rather than variations that are to be expected in normal B lymphocyte development.

2. *Tumors of Surface Ig-Positive B Cells*

The majority of *human non-Hodgkin's lymphomas* are derived from B lymphocytes, as judged by the expression of surface Ig, Fc, and C3 receptors and B-cell alloantigens (D/DR antigens, the equivalent of murine Ia antigens) (Berard *et al.*, 1978; Mann *et al.*, 1979; Aisenberg, 1981). Double labeling of surface Ig in tissues of patients with CLL and B-cell-derived lymphomas has established the monoclonality of the majority of these tumors (Levy *et al.*, 1977). Analysis of surface markers and Ig biosynthesis in such neoplasms clearly demonstrates that individual cases of CLL reflect the entire spectrum of B cells, corresponding to immature, mature, and activated normal B lymphocytes. Thus, some tumors show unbalanced synthesis of light and heavy chains with little or no surface Ig and may be tumors of immature cells (Hannam-Harris *et al.*, 1980). A large number of cases express both IgM and IgD on the surfaces of leukemic cells, and both isotypes have serologically identical idiotypic determinants (Fu *et al.*,

1974). Other CLL cells phenotypically resemble normal B lymphocytes that are stimulated by antigen or polyclonal activators, in that (1) the cells spontaneously secrete IgM that is reflected as a monoclonal band in the patients' sera (Nies *et al.*, 1973; Fu *et al.*, 1979), and (2) individual cells exhibit heavy chain class switches, i.e., they express IgM and IgG, or IgG to IgA (Rudders and Howard, 1977; Rudders and Ross, 1975). The idiotypic identity of the serum IgM and cell surface Ig, or of the different Ig isotypes detected on these cells, shows that such differentition to Ig secretion (Fu *et al.*, 1979) or heavy chain switching (Rudders and Ross, 1975) takes place within the single neoplastic clone in each case. It is, however, unclear whether these differentiative events are spontaneous, or induced by immunological or other host factors. In any event, it is clear that CLL cells are not "frozen" in differentiation, since nonsecreting tumor cells can be induced to secrete Ig by several different experimental manipulations (vide infra). It should also be mentioned that the association of EBV antigens, particularly the EB nuclear antigen (EBNA) with a variety of human B-cell tumors, led to the concept that this virus selectively transforms B lymphocytes. Evidence supporting this view is the ability of EBV to activate normal B cells and to induce lymphoblastoid lines from normal human and murine B lymphocytes *in vitro* (Rosen *et al.*, 1977; Steinitz *et al.*, 1977).

Further phenotypic analysis of human B-cell-derived tumors is difficult because of several reasons. First, many cell surface markers such as Fc receptors and D/DR alloantigens are present on a variety of normal non-B lymphocytes, so that their expression on cells from patients with clinically diagnosed CLL may be difficult to interpret. This problem is compounded by the fact that many of the studies to date have been done with peripheral blood lymphocytes and only a few investigators (Hurley *et al.*, 1978) have cloned the leukemic cells and established cell lines derived from the leukemic lymphocytes. Second, a number of investigators have reported the presence of dual T- and B-cell markers on Ig-producing lymphoma and leukemia cells (Boumsell *et al.*, 1978; Foon *et al.*, 1980; Aisenberg and Wilkes, 1980). In these cases the possibility of normal T cells or a second neoplastic clone contaminating the leukemic B-cell population can generally be readily excluded. Thus, the expression of dual markers may be an example of aberrant gene expression. An alternative possibility that has been raised recently is that some of these apparently T-cell-specific surface antigens that are present on leukemic B cells may be related to viral antigens such as the p69/71 oncornavirus glycoprotein of the G_{IX} antigen system of mice (Wang *et al.*, 1980).

In contrast to the clinical situation, *spontaneous animal lymphomas of the B lymphocyte lineage* are relatively uncommon. The most thoroughly analyzed of these is the BCL_1 tumor, which was discovered as a spontaneous tumor in a 2-year-old BALB/c mouse (Slavin and Strober, 1978). The BCL_1 leukemia has been subsequently characterized as an IgM+, IgD+, λ+, Fc receptor+, nonsecreting tumor presumably derived from a virgin (nonactivated) B lymphocyte (Strober *et al.*, 1979; Knapp *et al.*, 1979; Krolick *et al.*, 1979). Other spontaneous B cell lymphomas have been observed in old (>15 months) BALB/c mice and adapted to *in vitro* growth (Kim *et al.*, 1979; Kanellopoulos-Langevin *et al.*, 1979). Analysis of cell surface Ig in four of these tumors by lactoperoxidase-catalyzed iodination and specific immunoprecipitation has shown that three express IgM and IgD in association with λ light chains (Laskov *et al.*, 1981). It is somewhat surprising that all four spontaneous B cell lymphomas that express surface IgM and IgD (i.e., BCL_1 and the three studied by Laskov *et al.*, 1981) do so in association with λ light chains, since in mice both cell surface Ig and serum Ig show a 10- to 20-fold predominance of κ over λ. There are several possible explanations for this finding. For instance, a selective process might favor survival of λ bearing transformed B cells, or an oncogenic virus might selectively transform a particular B-cell subpopulation. In fact, it has been reported that EBV "preferentially transforms" human B lymphocytes bearing μ, κ Ig (Steel *et al.*, 1977). An intriguing possibility is that the λ chain participates in the formation of a receptor for an oncogenic virus, and transformation results from specific, repeated virus–receptor interactions, as has been suggested in T-cell leukemogenesis (McGrath *et al.*, 1980). Other spontaneous murine tumors that have been claimed to be derived from B lymphocytes include the reticulum cell sarcoma of SJL mice (Scheid *et al.*, 1981); more recent studies, however, indicate that this neoplasm arises from macrophages (Ford *et al.*, 1981).

A variety of protocols have been employed to *induce lymphomas experimentally* in mice or with human lymphocytes. The studies of Klein and associates, among others, with EBV transformation of normal lymphocytes have been mentioned above; the lymphoblastoid lines that are derived by this approach appear to represent tumors of activated B cells. Other murine leukemia viruses that have been reported to induce surface Ig-bearing B cell lymphomas in mice include Friend virus (Cheseboro *et al.*, 1976) and Gross virus (Numata *et al.*, 1981). In addition, as discussed in Section III,A,1, Abelson murine leukemia virus induces pre-B-cell tumors preferentially, although some of these tumors may resemble more mature B cells phenotypi-

cally. Chemical carcinogens that produce B-cell tumors are nitrosourea derivatives (Kim *et al.*, 1979), benzanthracenes (Bergman and Haimovich, 1977; Bergman *et al.*, 1977), and mineral oil (Warner *et al.*, 1975). Two aspects of these studies are noteworthy. First, intraperitoneal administration of mineral oil generally produces antibody-secreting plasmacytomas rather than nonsecreting B cell lymphomas (vide infra). Second, Bergman and Haimovich (1977) induced a tumor with the characteristics of mature B lymphocytes by injecting a thymectomized mouse with a chemical carcinogen. The general applicability of such a protocol for producing B cell lymphomas, albeit unlikely, needs to be explored more thoroughly. Occasional B lymphomas have been observed in X-irradiated mice (Warner, 1978). A surface IgM, λ bearing tumor, CH1, has been induced in double congenic B10.A $H\text{-}2^a$ $H\text{-}4^b$ mice following adoptive transfer of lymphocytes from donors that were hyperimmunized with sheep erythrocytes, but the tumor Ig is not specific for the immunizing antigen (Lynes *et al.*, 1978; Lanier *et al.*, 1978). In fact, so far it has not been possible to experimentally produce nonsecreting B cell tumors with defined antigenic specificities. Finally, mice undergoing graft-versus-host reactions, or F_1 hybrid mice injected with parental lymphoid cells frequently develop lymphoid malignancies that are most commonly of host origin and often represent tumors of Ig-producing B cells (Gleichmann *et al.*, 1978). Lymphomagenesis could be a result of the activation of previously latent murine leukemia viruses which might cause the chronic and repeated stimulation of lymphocytes bearing receptors for the viral envelope glycoproteins (McGrath *et al.*, 1980; McGrath and Weissman, 1979). Consistent with such an hypothesis is the observation that clones of strain A alloreactive T cells which recognize a hybrid determinant(s) present on $(C57BL/6 \times A)F_1$ stimulator cells, when injected into F_1 mice, develop into alloreactive T cell lymphomas, presumably because of persistent stimulation of the injected clones by host antigens (Fathman and Weissman, 1980). An alternative or additional factor in the induction of lymphomas during persistent graft-versus-host reactions may be the production of T-cell-derived soluble factors that stimulate the growth and/or differentiation of other lymphocytes (Delovitch *et al.*, 1981). In the final analysis, it is clear that in the majority of experimental protocols for inducing tumors of B lymphocytes, neither the mechanism of tumorigenesis nor the precise target of the oncogenic event is clearly established. However, biosynthetic and morphologic analyses have corroborated the basic notion that these tumors reflect the normal pathway of B-cell differentiation. In fact, Andersson *et al.* (1974) have shown that five IgM

producing BALB/c tumors can be categorized as representatives of activated B lymphocytes at different times following stimulation by lipopolysaccharide (LPS) *in vitro*. Such studies are not only necessary preludes to the use of tumors as model systems, but serve to strengthen the evidence supporting a well-defined pattern of B lymphocyte ontogeny and differentiation.

3. *Tumors of Antibody-Secreting Cells: Myelomas and Hybridomas*

The most widely studied animal tumors of the B lymphocyte lineage are antibody-secreting myelomas (plasmacytomas). The monoclonal Igs produced by these tumors and the discovery that some myeloma proteins are specific for defined, often simple, antigenic determinants have been of enormous value for immunochemists and cellular immunologists (Potter, 1977). More recently, the development of somatic cell hybridization techniques has made it feasible to produce monoclonal cell lines whose products react with virtually any desired determinant (Kohler and Milstein, 1975). Hybridization of fetal liver cells with myelomas results in the production of tumors that represent earlier stages in B-cell differentiation, i.e., pre-B cells (Burrows *et al.*, 1979). It is of interest to determine if somatic cell hybridization techniques can be used to produce tumors of nonsecreting B cells reactive with defined antigens, which can then be used as models for antigen- or idiotype-specific B-cell triggering and regulation. In fact, fusion of nonsecreting cells with a drug-marked myeloma generally results in hybrids that secrete the Ig produced by the nonsecreting normal parent (Laskov *et al.*, 1979; Levy and Dilley, 1978). The reason why pre-B cell hybridomas can be produced by a similar hybridization procedure is that pre-B cells, lacking rearranged light chain genes, are incapable of secreting Ig molecules. The possibility that fusion of B cells with relatively immature drug-marked tumors may give rise to nonsecreting, antigen-specific hybrids is currently being investigated in a number of laboratories. Finally it should be added that EBV transformation of immune lymphocytes is another procedure for producing cell lines secreting antibodies of desired specificities (Steinitz *et al.*, 1977). This technique, however, may not have significant advantages over somatic cell hybridization, and experience with it is limited, to date.

4. *Immunoglobulin Gene Expression in B Lymphocyte-Derived Tumors*

The application of recombinant DNA technology to studies of Ig genes in tumors of Ig-producing cells has led to the evolution of con-

cepts that are dramatically changing the way immunologists view the immune system, its development and its functional regulation. To date the bulk of these studies have been done with tumor cells, for technical reasons, and the physiologic relevance of these results is entirely dependent on the validity of tumors as models for normal immunocompetent cells. Hence, the molecular biology of the antibody gene system is not only of great topical interest but is obviously relevant to the theme of this article. However, our main focus in this article is on the use of tumor cells as targets for functional regulation, and the rapidly developing views of the genetic basis for antibody production have been the subject of several recent reviews (e.g., Johnson *et al.*, 1981; Leder *et al.*, 1981). Therefore, in the following section only some of the results that bear most directly upon issues of lymphocyte regulation will be outlined, without any attempt to discuss experimental details. The studies of Tonegawa and associates using murine myeloma and embryonic cells first showed that during ontogeny, the genes coding for Ig light chains became rearranged such that the introns separating variable (V), joining (J), and constant (C) region exons were deleted and the transcribed mRNA was composed of contiguous, joined V–J–C segments (Brack and Tonegawa, 1977; Bernard *et al.*, 1978). This basic phenomenon has been confirmed for all murine and human Ig heavy and light chain genes analyzed so far (Johnson *et al.*, 1981; Leder *et al.*, 1981). The possible combinatorial associations of different V_L and J_L regions for light chains or V_H, D (diversity), and J_H regions for heavy chains with the appropriate C regions provide an elegant mechanism for generating the highly diverse antibody repertoire. Moreover, recent studies employing hybridomas that secrete phosphorylcholine (PC)-specific IgM and IgG antibodies suggest that somatic point mutations in certain segments of the relevant genes may also contribute to antibody diversity (Gearhart *et al.*, 1981). With respect to the differentiative events that occur in cells of the B lymphocyte lineage, three types of gene rearrangements are particularly important.

1. Expression of membrane vs secreted IgM: The initial observation that the $C\mu$ mRNA in B-cell tumors had a size of 2.4 or 2.7 kb depending on whether the predominant form of IgM was secreted or membrane, respectively, soon led to the identification of the gene segment encoding the hydrophobic membrane anchor portion of the IgM molecule (Alt *et al.*, 1980; Rogers *et al.*, 1980). Subsequently it was shown that membrane or secreted μ could be expressed by alternative RNA splicing of a primary transcript contain-

ing the V_H, Cμ secreted, and Cμ membrane segments (Early *et al.*, 1980). The intriguing question of how antigens or polyclonal activators influence the splicing mechanism, leading to IgM secretion by previously membrane IgM bearing B cells, remains unresolved.

2. Simultaneous expression of μ and δ heavy chains on B lymphocytes: Using BALB/c lymphomas that express surface IgM and IgD, Moore *et al.* (1980) have cloned the Cμ and Cδ genes and the intervening segment. The results suggest that simultaneous expression of Cμ and Cδ with the same V_H region is mediated by two forms of RNA processing of a primary transcript that contains the V_H, Cμ, and Cδ segments. In contrast, secretion of IgD by a rat myeloma is apparently associated with deletion of the Cμ gene and a translocation of the V_H to the Cδ gene.

3. Heavy chain class switching: Analysis of the heavy chain encoding genes of myelomas that secrete different Ig isotypes suggests that during class switching (i.e., switching from expression of IgM to IgG, IgE, IgA), a V_H gene is joined to a C_H gene by deleting the intervening DNA, and the gene order is 5′-V_H-Cμ-Cδ-Cγ_3-Cγ_1-Cγ_{2b}-Cγ_{2a}-Cϵ-Cα-3′ (Honjo *et al.*, 1981; Davis *et al.*, 1980). It is likely that precise nucleotides ("switch segments") located 5′ to each C gene are involved in class switching, but the mechanisms regulating the switching process, and the effects of different types of antigens, regulatory T lymphocytes, and other environmental factors on the Ig isotype that is predominantly expressed in a particular immune response are unknown. Moreover, it is unclear whether such a linear deletion model is true for nonneoplastic B cells. In fact, myeloma mutants have been found to secrete Ig isotypes in a pattern that is inconsistent with a simple linear deletion model (Radbruch *et al.*, 1980).

This outline of an evolving area of immunologic research has necessarily omitted a large number of experimental studies. However, it does point out that if the function and differentiative stage of neoplastic cells can be altered by experimental manipulations, the technology is now available for analyzing the molecular basis of these phenomena in detail.

B. T Lymphocyte-Derived Tumors

The differentiation pathway of T lymphocytes is more difficult to define in functional as well as phenotypic terms compared to B lymphocytes. There are two major reasons for this. First, the antigen receptor on T cells and the effector molecules produced by these

lymphocytes are incompletely defined. As a result, the stage of differentiation of a particular T cell has to be judged by the expression of phenotypic markers, such as the Lyt alloantigens in mice or antigens recognized by monoclonal antibodies in man, and these surface markers may or may not be involved in cellular functions. Second, T lymphocytes consist of a large number of subpopulations that are distinct in terms of (1) function (helper, suppressor, and cytotoxic T cells and the cells that mediate delayed hypersensitivity), (2) specificity [T cells that recognize antigens or Ig determinants in association with major histocompatibility complex (MHC)-encoded structures or in an MHC-unrestricted manner], and (3) surface phenotype. This heterogeneity greatly complicates the analysis of T-cell differentiation. In any event, the most immature cell in the T lymphocyte lineage is an unresponsive cell that expresses the Lyt 1,2,3 antigens in mice and reacts with specific monoclonal antibodies in man (Table II) (Cantor and Boyse, 1977; Reinherz and Schlossman, 1980; Ledbetter *et al.*, 1981). During intrathymic maturation at least some helper and cytolytic lymphocyte precursors acquire the property of MHC restriction, i.e., they "learn" to recognize foreign antigens in association with MHC-encoded determinants present on nonlymphoid thymic cells (Zinkernagel and Doherty, 1979; Bevan and Fink, 1978). Further differentiation of T lymphocytes may be influenced by "hormones" secreted by thymic epithelial cells (Scheid *et al.*, 1973), and is accompanied not only by the acquisition of immune responsiveness but also by changes in cell surface antigens in mice and man (Cantor and Boyse, 1977; Reinherz and Schlossman, 1980). Such changes in antigenic profiles may not only be markers for maturation but may also have important functional consequences, since at least some of the antigens of the Lyt series are involved in T-cell functions (Ledbetter *et al.*, 1981b). Many aspects of this scheme remain unresolved. For instance, it is unclear if all T-cell subsets arise from a common progenitor, and whether MHC restriction is acquired in the thymus alone or other sites (Katz, 1980). The basic T-cell populations listed above (see Table II) can probably be further subdivided. For instance, different subsets of helper T cells recognize antigens in an MHC-restricted manner (Paul and Benacerraf, 1977), Ig idiotypes without MHC restriction (Bottomly and Mosier, 1981), and Ig isotypes with presently undefined restrictions (Mongini *et al.*, 1981). Suppressor T lymphocytes consist of antigen- and idiotype-specific subsets that are sequentially activated by complimentary interactions (Germain and Benacerraf, 1981). Moreover, other components of immunoregulatory circuits exist that can be identified by functional and phenotypic properties

TABLE II

DIFFERENTIATIVE STAGES AND SUBPOPULATIONS OF NORMAL T LYMPHOCYTES AND REPRESENTATIVE TUMORS

Differentiative stage or subset	Surface markers		Soluble mediator(s)	Representative tumors[b]	
	Murine	Human[a]		Spontaneous	Experimentally produced
Immature T cell, thymocyte	Lyt 1^+23^+ TL^+	OKT9, OKT10		Human, murine thymomas; T-ALL[d]	Carcinogen, radiation induced[e]
Helper T lymphocyte	Lyt 1^+23^-	OKT4	IL-2, TRF; antigen-specific factors	Sezary syndrome; [f] spontaneous IL-2 producing lymphomas[g]	Hybridomas[h]
Suppressor T[c] lymphocyte	Lyt 1^-23^+ $I\text{-}J^+$	OKT5 OKT8	Antigen-specific factors; Con A-induced polyclonal factors	Some T-ALL[i]	Hybridomas[j]
Cytotoxic T lymphocyte	Lyt 1^-23^+	OKT5 OKT8		Rare murine lymphomas[k]	Hybridomas[l]
T_{DH} (T lymphocyte-mediating delayed hypersensitivity)	Lyt 1^+23^-		Numerous lymphokines (e.g., MIF, MAF)	None known	Hybridoma[m]

[a] Only markers defined by the OKT series of monoclonal antibodies (Reinherz and Schlossman, 1980) are listed; similar subsets have been identified by other investigators using different antibodies (e.g., Haynes *et al.*, 1981).

[b] Many more tumors have surface markers that are characteristic of known T-cell subsets but their functional properties are undefined and, therefore, these are not listed.

[c] The possibility of multiple stages in the suppressor pathway with different specificities and surface marker profiles is discussed in the text. The table outlines properties most characteristic of effector suppressors.

[d] Reinherz *et al.* (1979); Aisenberg (1981). [e] Mathieson *et al.* (1978). [f] Broder *et al.* (1976). [g] Gillis and Watson (1980); Gillis *et al.* (1980). [h] Harwell *et al.* (1980); Stull and Gillis (1981); Takatsu *et al.* (1980). [i] Broder *et al.* (1981); Kai *et al.* (1980). [j] Taniguchi *et al.* (1979); Kapp *et al.* (1980); Esshar *et al.* (1980); Okuda *et al.* (1981b); others listed in text. [k] Chang and Eisen (1979). [l] Kaufman *et al.* (1981). [m] Jones *et al.* (1981).

(Gershon *et al.*, 1981). Finally, during the last 5 years it has become apparent that many T-cell functions can be experimentally reproduced with antigen-specific and nonspecific factors (Tada and Okumura, 1979; Moller, 1981).

In principle, the assignment of a lymphoid neoplasm to a particular T-cell subset is based on surface phenotype, biologic function, and the liberation of an identifiable mediator or factor. It is, however, apparent that given the incomplete definition of normal T-cell populations, the classification of many tumors is incomplete or unreliable.

1. Tumors of Helper and Suppressor T Lymphocytes

About 15–20% of human acute lymphoblastic leukemia (ALL) cases and small fractions of chronic lymphocytic leukemias and non-Hodgkin's lymphomas appear to represent malignant proliferations of T lymphocytes as determined by the absence of Ig production, rosette formation with sheep erythrocytes, and reactivity with heterologous anti-T-cell antisera (Aisenberg, 1981). The use of other surface markers such as Fc receptors and D/DR alloantigens is generally less helpful as these are not uniquely present on normal or neoplastic T lymphocytes. Further analysis of ALL cases with monoclonal antibodies reveals considerable heterogeneity, with different cases showing predominant reactivity with antibodies that are presumed to be specific for helper/inducer T cells (e.g., OKT4) or suppressor cytotoxic cells (e.g., OKT8) (Reinherz *et al.*, 1979; Nadler *et al.*, 1980; Haynes *et al.*, 1981). In the absence of functional assays, it is unwise to definitively characterize these tumors as helper or suppressor T cell derived, particularly since recent studies indicate that suppressor function can be experimentally induced in OKT4-positive normal T lymphocytes that were originally believed to represent helper/ inducer cells exclusively (Thomas *et al.*, 1981). In some cases of ALL, the neoplastic T cells secrete factor(s) that suppress the pokeweed mitogen (PWM)-induced Ig secretory response of normal B lymphocytes (Broder *et al.*, 1981). Interestingly, cells from the patient reported by Broder *et al.* (1981) needed to interact with normal T cells to develop full suppressor activity and surface markers, suggesting the presence of suppressor inducer and effector suppressor cells as in murine systems (Germain and Benacerraf, 1981). Other cases of T-ALL and T-CLL also appear to be examples of neoplasms of cells that are suppressive for B lymphocytes *in vitro* (Broder and Waldmann, 1978; Uchiyama *et al.*, 1978; Kai *et al.*, 1980). Incubation of a human T-cell leukemic line, CEM, with Con A induces suppressor cells that inhibit the DNA synthetic response of an established B-cell line to anti-Ig

(Kishimoto *et al.*, 1977). This is obviously similar to the induction of suppressor cells by culturing normal human and murine T lymphocytes with Con A (Rich and Pierce, 1974). However, correlative studies of surface phenotype, functional effect, and clinical manifestations of immunosuppression have not yet been systematically attempted in the majority of patients with T-cell lymphomas and leukemias. The converse, i.e., T lymphocyte tumors apparently derived from the helper T-cell subset, has been clearly documented in cases of cutaneous T-cell lymphoma, which range from mycosis fungoides to the frankly leukemic form, Sezary's syndrome. In this disorder, the neoplastic T cells generally react with monoclonal antibodies specific for human helper T lymphocytes, and are capable of providing helper function for polyconal stimulation of normal B lymphocytes (Haynes *et al.*, 1981; Broder *et al.*, 1976; Berger *et al.*, 1979). Cell lines derived from Sezary cells have also been typed as belonging to the helper cell population (Haynes *et al.*, 1981). In contrast, recently a suppressor cell line has been obtained by long-term propagation of lymphocytes from a case of cutaneous T-cell lymphoma in the presence of interleukin-2 (IL-2, T cell growth factor); it is, however, unclear whether this line originated from normal or malignant cells (Fleisher *et al.*, 1981). Finally, a human T-cell tumor line has been found to actively secrete IL-2 *in vitro* following lectin stimulation (Gillis and Watson, 1980); this presumably represents a neoplasm of helper/inducer T lymphocytes. It is an intriguing possibility that some tumor cells might secrete growth-inducing factors and also express receptors for these factors, so that neoplastic proliferation is a result of continuous autostimulation.

Although murine tumors have been shown to possess cell surface markers characteristic of normal helper/inducer or suppressor/cytotoxic T lymphocytes, to date functional activity has only been identified in lines derived by somatic cell hybridization (see below). Rare T lymphomas that are cytotoxic for ^{51}Cr-labeled target cells in the presence of the lectin, Con A, have been described (Chang and Eisen, 1979). A radiation-induced splenic lymphoma in a B10.BR mouse that secretes IL-2 following lectin stimulation is now known, and widely used as a monoclonal source of this factor (Gillis *et al.*, 1980).

2. *T-Cell Tumors with Unknown Function*

It is clear that despite the occurrence of T-cell neoplasms with demonstrable biologic function (see above), the majority of human and animal T-cell-derived lymphomas and leukemias are not known to produce any mediators. The surface phenotype of human T lym-

phomas and leukemias has been outlined above. Old mice (>1 year of age) of many strains, in particular AKR and C58, frequently develop thymic lymphomas which are believed to be the result of activation of murine leukemia viruses. In addition, BALB/c and other mouse strains develop T-cell leukemias following X-irradiation or exposure to chemical carcinogens such as ethylnitrosourea. Different tumors express alloantigens that are markers of particular normal T-cell subsets, i.e., they are phenotypically Lyt 1^+2^+, Lyt 1^+2^-, or Lyt 1^-2^+, and the majority are positive for the thymus leukemia (TL) antigen (Krammer *et al.*, 1976; Mathieson *et al.*, 1978; Kim *et al.*, 1978).

3. T Lymphocyte Hybridomas

One of the most impressive advances that has stemmed from the somatic cell hybridization technique is the development of T-cell hybrids that mediate specific immunologic functions, usually by liberating mediators. Such tumor lines are invaluable as monoclonal sources of mediators that can be purified to homogeneity and analyzed biochemically. In addition, recent experiments suggest that T-cell hybrids can also be used for studying MHC-restricted receptors for antigen, and for delineating the mechanisms by which different T-cell subsets communicate with one another. In general, these hybridomas can be divided into two broad groups on the basis of the antigenic specificity of the functional mediators they produce.

1. T-cell hybrids that secrete antigen-specific mediators: By fusion of specifically immune T lymphocytes with the drug marked AKR thymoma, BW5147, hybrid lines have been established that secrete antigen-specific helper and suppressor molecules (Taniguchi and Miller, 1978; Kontiainen *et al.*, 1978; Taussig *et al.*, 1978; Taniguchi *et al.*, 1979; Hewitt and Liew, 1979; Esshar *et al.*, 1980; Kapp *et al.*, 1980; Lonai *et al.*, 1981; Whitaker *et al.*, 1981; Okuda *et al.*, 1981). The expression of idiotypic and MHC-encoded determinants on these factors, and their complete structural analysis, are already well under way. Interestingly, Okuda *et al.* (1981b) have produced hybridomas that represent three distinct antigen- and idiotype-specific stages in the suppressor cell pathway. The analysis of the specificities and structures of the elaborated factors should be useful in delineating the processes that occur in normal suppressor T-cell induction (Germain and Benacerraf, 1981). Moreover, molecular biologic analysis of the antigen-specific proteins and the genes coding for these are likely to be valuable for solving one of the major enigmas of modern immunology, the nature of the T-cell receptor for an-

tigen. Such studies are currently in progress in numerous laboratories, but to date no definitive results have been obtained.

2. T-cell hybrids that secrete antigen nonspecific mediators: T-cell hybrids have now been produced that secrete the following biologic mediators either constitutively or subsequent to stimulation with Con A or PHA: IL-2 (Stull and Gillis, 1981; Harwell *et al.*, 1980); thymus replacing factor (TRF, which promotes the differentiation of B lymphocytes to AFC) (Takatsu *et al.*, 1980); allogeneic effect factor (Katz *et al.*, 1980); and macrophage-activating and inhibiting factors (Jones *et al.*, 1981). Such hybridomas should provide some definitive answers to presently unresolved issues about the nature of factors, their target specificity (e.g., whether IL-2 promotes the growth of B and T cells), and the number of diverse biologic activities that can be expressed by a single molecular species. Perhaps the most exciting possibilities lie in the ability to use monoclonal factors and cloned target cells to analyze the molecular mechanisms whereby surface-generated immunoregulatory stimuli lead to specific changes in gene expression. The elegant study of Kappler *et al.* (1981) showing that receptors for antigen can be introduced into functional T-cell hybridomas makes it feasible to analyze the T-cell receptor and its reactivity pattern in quantitative and molecular terms (see Section VII). Similar analyses of T-cell receptors should be possible using hybrids of MHC-restricted, tumor-specific cytotoxic T cells that have been constructed recently (Kaufman *et al.*, 1981). Finally, Finn *et al.* (1979) have transformed antigen-specific murine helper T cells with radiation leukemia virus to produce a helper cell line. This technique, however, has largely been superseded by the development of T-cell hybrids that have different functions.

It should also be mentioned that murine macrophage tumor lines, notably the P388D1, have been shown to secrete interleukin-1 (IL-1), which may play a central role in lymphocyte growth and differentiation (Mizel and Mizel, 1981), and to present antigen to specifically immune T lymphocytes much like normal antigen-presenting cells (D. I. Beller, personal communication).

From the preceding discussion it is apparent that a large number of spontaneous, experimentally induced and fusion-derived tumors are now available that represent different maturational stages and functional subsets of B and T lymphocytes. Prior to their use as models for normal immunocompetent cells, it is important to consider potential problems with their classification and whether such neoplasms are

truly homogeneous or not. These issues are particularly relevant in view of the fact that the pattern of response of a cell to a given stimulus is determined to a large extent by the differentiative stage of the cell; moreover, intratumor heterogeneity can complicate the interpretation of results obtained using tumors as model systems.

C. Limitations of Tumor Classification and the Problem of Intratumor Heterogeneity

Some of the major problems in classifying lymphoid tumors in terms of differentiative stage or phenotypic/functional subpopulation are listed in Table III. Contaminations in typing reagents (e.g., antiviral antibodies) and the nonspecificity of phenotypic markers (e.g., Fc and C3 receptors and Ia or Ia-like alloantigens, which are present on many cell types) have been mentioned previously. The presence of dual markers believed to be specific for different cell types on an individual tumor cell may reflect aberrant gene expression, or the presence of virus-related antigens (Wang *et al.*, 1980). In some instances, apparent expression of such dual markers can be due to the presence of two or more sublines within a given tumor (Zatz *et al.*, 1981), although this possibility can generally be excluded by rigorous cloning of lines adapted to growth *in vitro*. Abnormal patterns of Ig biosynthesis in B-cell tumors or alloantigen expression in T-cell tumors may be intrinsically associated with the neoplastic phenotype, and may render a particular tumor not useful as an appropriate model system. It should be pointed out that monoclonal antibodies can be valuable for establishing the phenotypic identity of a tumor but in some instances can also

TABLE III
Limitations in Classification of Lymphoid Tumors

1. Nonspecificity of reagents (e.g., antiviral antibodies contaminating heterologous and alloantisera)
2. Nonspecificity of markers [e.g., Fc receptors, C3 receptors, Ia (D/DR) alloantigens]
3. Expression of dual (i.e., T and B cell) markers, which may be due to (1) aberrant gene expression, and (2) presence of two or more lines or differentiative stages within a given tumor
4. Abnormal patterns of differentiation or marker expression (e.g., excess free Ig light chain synthesis in some human B-CLL)
5. "Contamination" of tumor cells with normal lymphocytes (in human tumors that are not adapted to culture and cloned)
6. Intratumor microheterogeneity, which may be due to (1) cell cycle-related variations, (2) appearance of mutants or variants, (3) "spontaneous" differentiation, leading to simultaneous presence of multiple stages of maturation within a given tumor line

produce unexpected cross-reactivity patterns that are difficult to interpret on the basis of current knowledge (Milstein and Lennox, 1980).

Heterogeneity within monoclonal neoplasms is manifested in many different ways, such as variations in expression of surface antigens or differentiated functions among individual cells. For instance, individual cells in a cloned BALB/c myeloma MOPC315 adapted to culture exhibit marked variations in (1) expression of membrane Ig, (2) rate of Ig secretion, and (3) susceptibility to immunoregulatory stimuli such as antigens and suppressor T cells (Abbas, 1979). The presence of such heterogeneity raises major problems in the use of tumors as targets for immune regulation. Thus, if only a fraction of a myeloma population secretes Ig, and if only some of these can be specifically suppressed, biochemical analysis of the mechanisms of suppression using the entire population may be unreliable or not feasible. Clearly the ideal situation is one in which all cells in a tumor population are phenotypically identical and react to positive or negative stimuli in a consistent and predictable manner. Since the optimization of experimental systems may have to include approaches for obtaining more homogeneous cloned tumor cells, it is important to consider the causes for intratumor heterogeneity. To date, three main factors can be identified, although in many instances the mechanism(s) contributing to a major portion of the heterogeneity are unknown.

1. Cell cycle-related variations: Studies using tumor lines that are synchronized by metabolic inhibition or from which individual cells in different phases of the cell cycle are isolated by velocity sedimentation or flow cytometry have clearly shown that the expression of many surface antigens, e.g., Ig, MHC-encoded determinants, viral antigens, blood group glycolipids, and other differentiation antigens, varies according to the stage of the cell cycle (e.g., Buell and Fahey, 1969; Takahashi *et al.*, 1969; Cikes, 1970; Killander *et al.*, 1974; Williams *et al.*, 1979; Lanier and Warner, 1981). In most instances, cells in the G_1 and S phases of the cell cycle express more differentiated or specialized functions than cells in G_2. There are, however, numerous reports indicating that variations in surface antigen expression among cells of cloned tumor lines cannot be accounted for by cell cycle related changes (e.g. Leibson *et al.*, 1980).
2. Presence of distinct subpopulations: Some tumor lines, such as culture-adapted murine myelomas, show rates of mutation as high as 1 in 10^3 cells per generation (Coffino and Scharff, 1979). Mutants of myeloma cells can lose their ability to produce Ig, or begin pro-

duction of variant Igs that differ in heavy chain isotype or allotypic and idiotypic markers or antigen-binding affinity (Hausman and Bosma, 1979; Francus *et al.*, 1978; Radbruch *et al.*, 1980; Cook and Scharff, 1977). These findings clearly emphasize the importance of either frequently recloning tumor lines that are used in experimental studies, or restarting the lines from frozen stocks. The presence of distinct subpopulations within a tumor can reflect ongoing clonal evolution rather than mutations. For instance, cloned, culture-adapted S107 and MOPC315 BALB/c myeloma cells show marked variations in surface receptor expression as measured by flow cytometry or hapten-binding assays (Leibson *et al.*, 1979; Abbas, unpublished observations). Sublines isolated by cloning in soft agar represent populations that express high or low densities of membrane Ig, and this expression correlates strongly with the rate of antibody secretion. Although the sublines remain phenotypically stable, even within each line the cells are heterogeneous. Moreover, we have never observed evolution of a high receptor line to a low receptor phenotype or vice versa, so that it is not possible to account for the heterogeneity on the basis of ongoing differentiation in the tumor population.

3. Evidence for "spontaneous" intratumor differentiation: The possibility that many or all tumor lines consist of stem cells that are continuously undergoing differentiation to various extents in the absence of obvious external stimuli has been raised in many systems, and may be the major cause of cell-to-cell variations observed in cloned tumor lines. Studies with nonlymphoid tumors such as neoplasms arising from embryonic tissues (Martin, 1975) and myelogeneous and erythroid leukemias (Sachs, 1978) have firmly established the concept that such apparently spontaneous differentiation does occur in tumors. Several lines of evidence suggest that a similar phenomenon exists in tumors of immunocompetent cells as well.

a. Rohrer *et al.* (1977) have shown that MOPC315 cells growing in diffusion chambers implanted in the abdomens of BALB/c mice consist of two distinguishable populations—a small cell, clonogenic population which upon transfer, develops into the larger antibody-secreting plasmacytoid cells. Although the relationship between these two populations has not been formally established at the single cell level, the available evidence suggests that the smaller cells are stem cells and the larger ones their differentiated progeny. A nonsecreting lymphoma-like variant of the MOPC315 tumor has been isolated from the parent myeloma (Gebel *et al.*,

1979). This variant presumably represents nondifferentiating, clonogenic stem cells, but so far it has not been possible to induce it to differentiate into secreting sells.

b. B-cell lymphomas are not "frozen" in their state of differentiation, as implied by the frequent occurrence of secreting tumor cells or cells showing patterns of heavy chain class switching in patients in whom the majority of the tumor cells are nonsecreting, typical CLL B lymphocytes (Fu *et al.*, 1979; Rudders and Ross, 1975). Moreover, fusion of nonsecreting CLL cells with a myeloma leads to hybrids that secrete Ig bearing the idiotypic markers of the CLL (Laskov *et al.*, 1979; Levy and Dilley, 1978).

c. The oncogenic event in tumors of immunocompetent cells may occur at a stage of differentiation much earlier than the phenotypic manifestation of the tumor. For instance, patients with myelomas contain, in their bone marrow, greatly increased numbers of pre-B cells that contain cytoplasmic idiotypic determinants typical of the myeloma (Kubagawa *et al.*, 1979). This observation implies that differentiative stages from pre-B cells to high rate AFC are all capable of being present within a given B-cell derived tumor. It should, however, be pointed out that some studies showing idiotype-bearing small lymphocytes in patients or mice with myelomas were originally interpreted as evidence for intratumor differentiation but are now believed to be due to the presence of T lymphocytes bearing Fc receptors for the myeloma Ig heavy chain (Hoover *et al.*, 1981).

From these kinds of data, the main point that emerges is that it is clearly conceivable that lymphoid neoplasms in which the constituent cells express differentiated functions are actually composed of transformed stem cells that differentiate during tumor growth. In fact, by analogy with many tumors of nonlymphoid tissue, this possibility is a likely one. Nevertheless, there is little direct evidence definitively establishing a continuing process of intratumor maturation in neoplasms of immunocompetent cells. This is obviously an important issue, since any detectable effect of an exogenous stimulus on tumor growth or function may be due to an effect on the stem cells or on their differentiated products, and the two may be biologically and mechanistically quite distinct. Moreover, the presence of cells at different stages of maturation within a cloned tumor line may give rise to partial effects of immunoregulatory stimuli, even though the line is apparently "homogeneous."

The availability of tumors arising from cells of the immune system, and the ability to mark these tumors phenotypically and functionally,

means that it is possible to use them as models for normal lymphocyte function. In the following sections we will review experimental systems designed to examine the effects of physiologic immunoregulatory stimuli on tumor growth and function. Wherever possible, an attempt will be made to relate these studies to normal lymphocyte biology, and to highlight situations in which recently developed techniques can be applied to provide clues to the mechanisms of immune regulation.

IV. Regulation of B Lymphocyte-Derived Tumors by Polyclonal Stimuli

Agents that stimulate or inactivate normal lymphocytes by interacting with surface receptors other than the combining sites for antigens are powerful tools for dissecting the maturational stages and ontogenetic pathways of lymphoid cells (Gronowicz and Coutinho, 1975). In addition, such polyclonal stimuli can be used to analyze the biochemical basis of immune induction or unresponsiveness (Oppenheim and Rosenstreich, 1976), and ultimately for defining the mechanisms by which surface-generated signals are transduced into alterations in functional gene expression. A large number of polyclonal activators of B and T lymphocytes have been described, including plant lectins, microbial products, antibodies directed against membrane macromolecules such as Ig and β_2-microglobulin, and products of macrophages and lymphocytes themselves, particularly of T lymphocytes. The availability of these agents made it feasible to test their effects on neoplastic lymphoid cells. In fact, since the antigenic specificity of the majority of human and animal lymphomas and myelomas remains undefined, attempts to regulate their growth and function can only be made with polyclonal stimuli. In the following section we will describe the studies dealing with activation and inhibition of B-cell-derived tumors by agents that are not antigen- or idiotype-specific.

In the last decade, experimental systems of polyclonal regulation of normal B lymphocytes have led to the establishment of several important concepts. It is known that the response of lymphocytes to different polyclonal B-cell activators (PBAs) depends on the maturational stage of the cell (Gronowicz and Coutinho, 1975; Coutinho and Moller, 1975). This response is usually manifested by enhanced proliferation and/or differentiation. PBA-induced B-cell differentiation results in IgM secretion, reduced density of membrane Ig, and heavy chain class switching, and thus mimics the antigen-driven sequence of differentiative events. Some PBAs (such as dextran sulfate and LPS)

trigger B lymphocytes without an absolute requirement for T cell help. In contrast, others can stimulate growth but not differentiation without overt cooperative influences from T cells and yet others require the participation of T cells for inducing both growth and differentiation. The prototype of the latter category is anti-Ig antibody, which stimulates (1) optimal DNA synthesis in mature murine B lymphocytes only in the presence of a T-cell factor that has not yet been separated from IL-2 preparations that are required for T lymphocyte growth, and (2) differentiation to Ig secretion in the presence of a distinct T-cell-derived mediator termed thymus-replacing factor (TRF) (Parker, 1980; Sieckmann, 1980). Such studies have been interpreted to strongly support the Bretscher–Cohn two-signal model for lymphocyte activation, which stated that triggering of B lymphocytes requires one signal delivered via the membrane Ig receptor (i.e., by antigen or anti-Ig antibody), and another signal from helper T cells that is now known to be mediated by factors interacting with appropriate receptors on target B lymphocytes (Bretscher and Cohn, 1970). The current view of the two-signal model is that antigen (or anti-Ig antibody) renders resting B cells responsive to T-cell-derived factors which then drive the target B lymphocytes to proliferate and/or differentiate (Parker, 1980; Vitetta *et al.*, 1980). Although the use of factors emphasizes the role of non-MHC-restricted T lymphocytes in B-cell activation, MHC-restricted helper T cells may also be important in polyclonal responses, as has been clearly demonstrated for antigen-specific immune induction (Katz, 1977). In addition, macrophages participate in polyclonal triggering by presenting the PBA to B lymphocytes and by liberating growth and differentiation-inducing factors such as IL-1 (Unanue, 1981). Recent studies of Singer *et al.* (1981) elegantly demonstrate the different triggering requirements of antigen-specific B cells at different stages of maturation. Thus, cells lacking the Lyb 5 surface marker secrete antibody in response to an antigen only in the presence of MHC-restricted helper cells, whereas Lyb 5-positive B lymphocytes can be activated by antigen together with TRF. Whether a similar difference applies to PBA-responsive B lymphocytes is an open question.

A number of murine and human B lymphocyte-derived tumors have been used to analyze the phenomenon of PBA-induced activation. The obvious advantages of murine systems are the ease of experimental manipulations and the large body of knowledge that now exists regarding the cellular phenomenology as well as the molecular and genetic basis of lymphocyte activation and differentiation. Human tumors offer a vast resource of potential models for polyclonal regula-

tion but, as detailed in Section IV,B, most of the studies suffer from a lack of clonal systems with precisely defined properties and controlled variables.

A. Studies with Murine Tumors

1. Differentiation of Tumors of Pre-B Cells

Abelson virus-induced tumors, which are believed to be neoplasms of pre-B cells, are susceptible to a variety of regulatory stimuli. Some lines that synthesize μ chains but not light chains and do not express surface Ig can be induced to synthesize light chains, assemble intact 8 S IgM molecules, and express these on the membrane following brief culture with LPS or pokeweed mitogen (Rosenberg *et al.*, 1979). LPS, butyric acid, and conditioned media from PWM-stimulated spleen cells have been shown to increase the proportion of Abelson-transformed cells containing cytoplasmic μ chains, but in these studies no membrane Ig was expressed and production of light chains was not measured (Boss *et al.*, 1979). Similarly, dimethyl sulfoxide (DMSO) induces surface IgM and Fc receptors in Abelson lines (Weimann, 1976), and LPS as well as agents that increase cyclic AMP stimulate modest increases in Fc receptor expression in some lines (Burchiel and Warner, 1980). Many of these inducing agents are known to have a similar differentiative effect on nonlymphoid tumors such as friend virus-induced erythroleukemia (Friend *et al.*, 1971) and myelogenous leukemia cells (Sachs, 1980). Surface Ig expression has also been induced in >90% of 70 Z/3 pre-B cell leukemia cells (from an initial level of <5% surface Ig-positive) by LPS and dextran sulfate (Paige *et al.*, 1978). In a study of synchronized 70 Z/3 cells, Sakaguchi *et al.* (1980) have shown that LPS added in the G_1 or S phase of the cell cycle induces κ mRNA and increased κ chain synthesis and surface Ig expression within 12 hours (in the G2 phase). It is likely that the LPS-stimulated increase in κ mRNA is at the level of transcription itself (Perry and Kelley, 1980), providing a simple system for studying a change in gene expression following a membrane signal. It should be pointed out that the 70 Z/3 line, unlike many Abelson-derived tumors, does express a κ chain gene in the adult, rearranged configuration but the majority of cells fail to synthesize light chains due to unknown reasons (Section III,A). Thus, although the phenomena of surface Ig induction in these two types of pre-B cell tumors appear basically similar, the operative mechanisms could be fundamentally different. In any event, these experiments demon-

strate that neoplastic pre-B cells, upon appropriate stimulation, acquire at least some of the phenotypic characteristics of B lymphocytes. However, several issues remain to be clarified. First, lines that apparently represent neoplastic pre-B cells may be composed of subsets at various differentiative stages, particularly with regards to Ig gene rearrangements and transcriptional or translational control mechanisms. Second and more importantly, culture conditions alone can markedly influence the Ig phenotype in these tumors. For instance, the biosynthesis of Ig shows an inverse relationship to growth rate (Siden *et al.*, 1979), and some batches of fetal calf sera may themselves contain agents other than LPS (endotoxin) that induce surface Ig expression (Paige *et al.*, 1978). Finally, sublines of a cloned tumor such as 70 Z/3 often show considerable differences in Ig expression (Ralph, 1979). Nevertheless, in the final analysis, studies aimed at using Abelson virus-transformed and chemically induced pre-B cell neoplasms as models for normal B lymphocyte differentiation hold considerable promise. More precise definition of the initial tumor phenotype and the effects of inducing agents in molecular terms is likely to provide valuable information about the mechanisms that regulate the ontogenetic development of the early cell types in the B lymphocyte lineage.

2. *Activation of Surface Ig Expressing Tumors by PBAs*

Relatively few murine neoplasms of mature B lymphocytes have been successfully used as targets for immunoregulatory stimuli, to date. A notable exception that has emerged recently is the spontaneous BALB/c tumor BCL_1 which possesses the phenotypic characteristics of a virgin (unactivated) mature B lymphocyte (Krolick *et al.*, 1979; Strober *et al.*, 1979). Polyclonal activators such as LPS, PPD, and dextran sulfate stimulate cell growth and induce IgM secretion of BCL_1 cells isolated from the spleens of tumor-bearing mice (Knapp *et al.*, 1979). LPS or lipid A stimulation of a culture-adapted line induces secretion of pentameric IgM in 20–40% of BCL_1 cells in the absence of added macrophages or T lymphocytes; moreover, high concentrations of LPS (>100 μg/ml) suppress tumor cell growth *in vitro* (Gronowicz *et al.*, 1980). Soluble $F(ab')_2$ fragments of anti-μ and anti-δ antibodies suppress DNA synthesis by BCL_1 cells harvested from the spleens or peripheral blood of tumor-bearing mice, and block LPS-induced stimulation of growth (Isaakson *et al.*, 1980). However, if these *in vivo* passaged tumor cells are cultured for 4 or more days, they respond to insolubilized anti-Ig or $F(ab')_2$ anti-μ with a marked proliferative response (Isaakson *et al.*,

1981). It appears likely that brief culture results in the maturation of the tumor cells to a stage of responsiveness, since similar opposing effects of anti-Ig antibodies are known to occur with mature and immature normal B lymphocytes (Isaakson *et al.*, 1980; Vitetta *et al.*, 1980). In addition, pre-cultured BCL_1 cells exposed to insolubilized anti-Ig together with T cell helper factors obtained by Con A stimulation or from mixed lymphocyte reactions differentiate to Ig-secreting cells; neither anti-Ig nor T cell factors alone is a sufficient differentiative signal (Isaakson *et al.*, 1981). Two aspects of these studies are significant. First, the secreted IgM that is induced by LPS or a combination of anti-Ig +T cell factors is idiotypically identical to the membrane Ig expressed by unstimulated BCL_1 cells or carries only the λ light chain establishing the monoclonality of the elicited response (Gronowicz *et al.*, 1980; Isaakson *et al.*, 1981). Second, hemolytic plaque assays show that only a small proportion of the tumor cells are induced to secrete Ig. For instance, in the studies of Isaakson *et al.* (1981), 2×10^5 precultured peripheral blood lymphocytes from BCL_1-bearing mice gave approximately 800–1000 Ig-secreting cells following incubation with Sepharose-coupled anti-Ig and T-cell factors. Although in these experiments, the precise numbers of tumor cells at the initiation and end of culture were not specified, the results of Gronowicz *et al.* (1980) with LPS stimulation of culture adapted BCL_1 cells also show that only a minority of the cells can be induced to secrete Ig. Whether this reflects technical limitations of the detection methods or culture conditions, or the presence of subpopulations or maturationally distinct stages within the tumor line, is not known. Finally, the induction of secretion is accompanied by a decline in the density of membrane IgM (Strober *et al.*, 1979). Recently, Northern blot analysis of μ mRNA in unstimulated and LPS-stimulated BCL_1 cells has shown that LPS induces a parallel increase in the membrane and secreted forms of mRNA, suggesting that the effects of LPS on this tumor cannot be accounted for entirely by a change in transcription (S. Strober, personal communication). Similar studies are in progress in other laboratories (E. S. Vitetta, personal communication).

Employing an essentially similar strategy, Schrader and colleagues have shown that LPS induces increased secretion and reduced surface expression of IgM in the B cell tumor, WEHI 231 (Boyd *et al.*, 1981). In contrast, anti-Ig antibodies markedly inhibit growth of the tumor *in vitro*, an effect that is independent of Fc receptors (Boyd and Schrader, 1981). Thus, this cloned tumor line, like BCL_1, is responsive to signals delivered via surface Ig as well

as receptors for polyclonal activators. Preliminary studies indicate that induction of Ig secretion in WEHI 231 cells by LPS is not accompanied by a commensurate increase in the levels of secretory type μ mRNA (John Schrader, personal communication). This suggests that control of membrane expression vs secretion of Ig in WEHI 231 lymphoma cells (as in BCL_1) might be translational, although definitive evidence in support of this hypothesis is lacking, to date.

Finally, anti-Ig antibodies have been shown to inhibit proliferation of MOPC104E myeloma cells *in vitro* (Barnhill *et al.*, 1976) and reduce tumor growth *in vivo* (Manning and Jutila, 1973). However, these experiments were done with tumor cells passaged in ascites form and, therefore, contaminated with host lymphocytes and macrophages, so that the effect may be due to antibody-dependent cellular cytotoxicity rather than a surface Ig-mediated growth suppressive signal.

B. Studies with Human Tumors

The effects of polyclonal mitogens on human lymphoid neoplasms, particularly CLL cells, has been studied for many years, not only to develop clonal systems for lymphocyte triggering or inactivation but also in a search for possible prognostic and therapeutic clues. Early attempts to stimulate CLL cells with lectins and PBAs were generally unsuccessful, and it was widely believed that this reflected functional immaturity of the neoplastic cells. More recently, however, with the increased awareness of culture conditions, growth factors, and collaborative interactions of B cells with helper T lymphocytes and macrophages, several groups have shown that CLL cells are, in fact, responsive to polyclonal stimuli. In general, the responses consist of regulation of proliferation or differentiation (Ig secretion, heavy chain class switching). Although most of the experimental systems to date leave many issues unanswered, a large number of experiments demonstrate the potential value of such an approach.

1. Growth Regulation of CLL Cells

In a series of studies, Robert and co-workers (Robert, 1979) have analyzed the response of whole and T-cell-depleted peripheral blood lymphocytes from patients with CLL to a battery of PBAs that are known to stimulate normal B lymphocytes at different stages of maturation. CLL cells show definite but variable DNA synthetic responses to LPS, dextran sulfate, anti-β_2-microglobulin antibody, PPD, and PHA. With some mitogens (dextran sulfate, LPS), the stimulation is

essentially independent of T cells, whereas with others (e.g., PHA) the response of CLL cells may actually be indirect, i.e., as a result of PHA-induced production of soluble mediators from T lymphocytes present in the peripheral blood (Robert and Nilsson, 1979). That the proliferating cells are the CLL cells themselves is shown by the monoclonality of the intracellular Ig in the blasts that develop in PBA-stimulated cultures (Robert, 1979). These analyses suggested that the responsiveness of CLL cells depends on two major factors: the degree of maturation of the neoplastic B cell clones and the presence of accessory cells, in particular T lymphocytes, which presumably serve a helper function. Mitogen responsiveness may also be a prognostic indicator since it reflects the degree of differentiation of the neoplastic cells, with cells responding to LPS and dextran sulfate being relatively immature and reflecting a poor prognosis (Robert *et al.*, 1980). Other CLL cells show enhanced DNA synthesis and proliferation only when stimulated with anti-Ig antibodies (Kishimoto *et al.*, 1977, 1981) or polyclonal mitogens together with allogeneic T lymphocytes (Saiki *et al.*, 1980; Nowell *et al.*, 1981). In contrast to these stimulatory effects, a number of studies show depressed growth of CLL cells in the presence of agents such as LPS, dextran sulfate, and anti-Ig (Smith *et al.*, 1972; Ralph and Nakoinz, 1974; Ralph *et al.*, 1974; Kishimoto *et al.*, 1977; reviewed by Ralph, 1979). The reason for the apparently aberant response pattern is unclear; nevertheless, it does indicate that leukemic lymphocytes express receptors for polyclonal ligands similar to normal B lymphocytes. Finally, CLL lymphocytes are also susceptible to suppressor T cell influences, as evidenced by the ability of a Con A-stimulated T cell line to inhibit the proliferative response of CLL cells to anti-Ig antibodies (Kishimoto *et al.*, 1977).

2. *PBA-Induced Differentiation of CLL Cells*

In addition to growth stimulation PBA can induce enhanced synthesis and secretion of Ig in CLL cells *in vitro*. Fu *et al.* (1978, 1979) have found that CLL cells can be stimulated to differentiate into cytoplasmic Ig-rich plasma cells by culture with PWM together with allogeneic T lymphocytes or culture supernatants from mixed lymphocyte reactions. The cytoplasmic Ig bears the same idiotypic determinants as the surface Ig expressed on the leukemic clone. Enhanced Ig biosynthesis is generally seen in only 10–25% of the peripheral blood lymphocytes from leukemic individuals, and is accompanied by a decline in the density of membrane Ig. Moreover, PWM by itself, and other PBAs even with allogeneic T lymphocytes, are ineffective, and this type of differentiation is seen only with leukemic cells from pa-

tients with a serum monoclonal Ig band, implying that the cells that can be triggered are neoplastic counterparts of activated B lymphocytes (Fu *et al.*, 1979). More recent studies of Fu, Kunkel, and associates (H. G. Kunkel, personal communication) indicate that similar differentiation can be induced in some cases of CLL without serum monoclonal Ig bands (vide infra). Anti-Ig and anti-idiotypic antibodies block the differentiative effect of PWM and allogeneic T cells and do not stimulate the CLL cells by themselves (Fu *et al.*, 1979). The role of allogeneic T cells may be to secrete helper factors which are known to include IL-2 and MHC-restricted differentiative agents (Delovitch *et al.*, 1981). Saiki *et al.* (1980) have reported essentially similar findings with CLL cells which can be stimulated to secrete IgM by culture with PWM and allogeneic T cells. A low level of IgG secretion was also induced, suggesting the occurrence of heavy chain class switching. However, the leukemic cells expressed membrane IgG in addition to IgM (although no cytoplasmic IgG was detected), so that the occurrence of class switching at the clonal level is not established in this case. Anti-Ig and antiidiotypic antibodies, together with supernatants from PHA-stimulated normal T lymphocytes, also induce IgM secretion in CLL cells (Kishimoto *et al.*, 1981). Mitogen and alloantigen-activated T cells liberate factors such as TRF that can induce Ig secretion in established human B lymphoblastoid lines in the absence of any overt PBA (other than substances possibly present in fetal calf sera) (Kishimoto *et al.*, 1978; Muragachi *et al.*, 1981). The studies of Muragachi *et al.* (1981) are particularly interesting since they showed that the induction of Ig secretion in a B-cell line is independent of cell proliferation, and the cells from this line absorb TRF activity but not IL-2 from supernatants of PHA-stimulated normal T cells. In addition to T cell-derived influences, supernatants from a macrophage cell line, J774, obtained after LPS stimulation have been shown to enhance the T-cell-dependent Ig secretion observed with B lymphoblastoid lines (Kishimoto *et al.*, 1978). This presumably reflects the known effect of IL-1 on antibody responses, either by stimulating B lymphocytes directly or by enhancing helper T-cell function (e.g., Calderon *et al.*, 1975). Finally, some CLL cells may have a basic biosynthetic defect that results in the secretion of free light chains even though intact Ig molecules can be detected in the cytoplasm following stimulation by polyclonal activators (Maino *et al.*, 1977).

In contrast to the T cell dependence of the differentiation of neoplastic B lymphocytes demonstrated by most investigators, Robert *et al.* (1979) have shown that CLL cells can be induced to secrete Ig by PBAs in the absence of added T lymphocytes or T-cell-derived factors. It is possible that the patients' own T lymphocytes provided the

necessary helper signal(s), although T lymphocytes from most patients with CLL are known to be markedly deficient in helper function (Chiorazzi *et al.*, 1979).

In summary, experimental conditions have been developed to induce many of the proliferative and differentiative events in neoplastic B lymphocytes *in vitro* that are seen following polyclonal activation of normal B cells (Table IV). The studies with human CLL cells need to be evaluated with some important points of caution. First, in experiments where the targets of PBAs are peripheral blood lymphocytes from CLL patients and not cloned cell lines, it is obviously important to demonstrate that the responding cells are from the leukemic clone rather than "contaminating" normal lymphocytes. Approaches that have been used to successfully establish this point include karyotyping of responding cells (Hurley *et al.*, 1980; Nowell *et al.*, 1981), the expression of idiotypic markers on the induced synthesized or secreted Ig (Fu *et al.*, 1978; Kishimoto *et al.*, 1981), and the monoclonality of the Ig light chain (Robert, 1979). Second, many of these studies are poorly controlled with respect to the clinical and therapeutic stage of the disease, the functional competence of the patients' own nonneoplastic lymphocytes and the possible presence of suppressor

TABLE IV
REGULATION OF B LYMPHOCYTE-DERIVED TUMOR CELLS BY POLYCLONAL STIMULI: SUMMARY[a]

Tumor cell type	Polyclonal stimulus	Induced event(s)
Pre-B cell tumors (Abelson virus-transformed lines, 70 Z/3)	LPS, other PBA, DMSO	Expression of surface IgM (induction of light chain mRNA and synthesis of Ig light chains)[b]
Surface Ig-positive B-cell tumors (murine: BCL_1, WEHI 231; human: B-CLL, lymphoblastoid lines)	1. LPS, other PBA[c] 2. Anti-Ig antibody + T cell derived factors[d] 3. T cell factors alone[e]	1. Enhanced proliferation 2. Induction of IgM secretion 3. Secretion of IgG (possibly representing IgM to IgG class switching)
Surface Ig-positive B-cell tumors (murine: WEHI 231; human: B-CLL)	LPS, dextran sulfate anti-Ig antibody, other PBA	Inhibition of DNA synthesis and cellular proliferation[f]

[a] Experimental details are described in the text.
[b] Paige *et al.* (1978); Rosenberg *et al.* (1979); Sakaguchi *et al.* (1980).
[c] Robert (1979); Strober *et al.* (1979); Boyd *et al.* (1981).
[d] Isaakson *et al.* (1981); Kishimoto *et al.* (1981).
[e] Muragachi *et al.* (1981).
[f] Ralph (1979).

cells. The current paucity of markers for B-cell subpopulations also makes it difficult to define the maturational stage of the neoplastic B cell clone in each patient. Third, in all reported studies, differentiation (in terms of Ig secretion) has been induced in a minor fraction of the leukemic B cells, ranging from about 10 to 25%, and the reasons for this are unclear. The possible induction of heavy chain switching (IgM to IgG) is even more uncertain since only low levels of IgG secretion have been induced and it is unclear whether the production of IgG was induced in cells that originally did not synthesize γ heavy chains (Kishimoto *et al.*, 1977, 1981; Saiki *et al.*, 1980). Clearly many of these problems can be resolved if attempts are made to clone leukemic B cells *in vitro* and then to activate them with polyclonal stimuli under defined conditions.

The studies with murine tumors (Abelson-transformed lines, 70 Z/3, BCL_1, and WEHI 231) have already been extended to the stage of molecular analysis of differentiative events, although the definitive answers are not yet available. In addition to examining the changes in Ig gene expression induced by polyclonal activators, a number of other central issues can be tackled with these systems. For instance, anti-Ig antibodies inhibit the spontaneous and LPS-induced proliferation of BCL_1 cells that are freshly isolated from tumor-bearing mice or adapted to *in vitro* growth, but stimulate precultured, *in vivo* passaged tumor cells (Strober *et al.*, 1979; Isaakson *et al.*, 1980, 1981). It should be feasible to exploit these opposing effects to determine the functional consequences of the cytoskeletal, ionic, and other early biochemical alterations that occur after the binding of anti-Ig to the membrane Ig molecules of B cells (Braun and Unanue, 1980). Moreover, since these tumor lines clearly possess receptors for T-cell-derived helper factors, questions such as the differences between IL-2, TRF, and the B-cell maturation and replication-inducing factor (if distinct from IL-2) can be resolved (Melchers *et al.*, 1980). Ultimately, it should also be possible to determine the nature of the interactions between various T-cell factors and their receptors on target B cells as well as the biochemical consequences of such interactions, by using factors derived from monoclonal sources (i.e., T-cell hybridomas) and cloned B-cell tumors as targets.

V. Specific Antigen and Antibody-Mediated Regulation of Immunoglobulin-Producing Tumor Cells

The stimulation or inactivation of homogeneous tumor lines by polyclonal stimuli has demonstrated potential for producing significant

results. There are, however, numerous advantages in extending these studies to regulation of tumor cells by ligands specific for the antigen receptors. First, regulation of target cells by interaction of antigen or antiidiotypic antibody with the combining site of the membrane-bound receptor is the sine qua non of all specific immune responses. Thus, antigens both stimulate and inactivate ("tolerize") specific lymphocytes (Klaus and Abbas, 1978), and under different conditions antiidiotypic antibodies have been shown to trigger or inhibit both B and T lymphocytes (Eichmann, 1978; Kelsoe *et al.*, 1980). Second, and perhaps more important, much more is known about lymphocyte membrane receptors for antigen than for polyclonal activators in terms of structure, physiology, and, more recently the genetic basis of their expression (Warner, 1974; Schreiner and Unanue, 1976; Section III,A,4). The obvious disadvantage of using tumors as models for antigen- or idiotype-specific regulation is that such studies are restricted to those tumor lines whose antigenic specificity is defined or against which specific antiidiotypic antibodies can be produced. Antigen-binding murine myelomas (Potter, 1977) initially provided numerous systems for investigating antigen-specific regulation. In addition, it has been demonstrated by many investigators that antiidiotypic antibodies can be raised against the combining sites ("complimentarity determining residues") as well as framework regions of Ig molecules that are secreted or expressed on surface membranes (e.g., Odermatt *et al.*, 1978). Finally the advent of somatic cell hybridization techniques makes it feasible to construct homogeneous antibody-secreting cell lines with virtually any desired antigenic specificity or idiotypic marker. As detailed in the following section, such approaches have been used to convincingly establish the concept that tumor lines derived from cells of the immune system are susceptible to positive and negative regulation by receptor-specific ligands. It should be pointed out that so far the majority of the studies have focused on specific suppression of growth and/or function rather that differentiation or activation of tumor cells.

1. Suppression of Myeloma Function by Antigens

The concept that fully differentiated, high rate antibody-secreting cells can be suppressed by brief exposure to antigen (Schrader and Nossal, 1974; Klaus, 1976; Baker *et al.*, 1971) led to attempts to demonstrate this phenomenon with homogeneous, cloned lines of murine myeloma cells specific for defined antigenic determinants (Table V). Among the earliest studies were done with a culture adapted line of the mineral oil-induced BALB/c myeloma, MOPC315, which secretes

TABLE V
EFFECTS OF ANTIGENS ON Ig PRODUCTION BY ANTIGEN-SPECIFIC TUMOR LINES

Tumor Line	Ig produced	Antigen exposure	Effect
MOPC315 (culture line)	IgA, λ_2 specific for DNP, TNP	DNP-γ-globulins, DNP-containing immune complexes *in vitro*	Inhibition of IgA synthesis and secretion; no effect on growth or membrane receptor expression[a,b]
TEPC15 (ascites)	IgA, κ specific for PC	R36a pneumococcus *in vitro*	Inhibition of growth and Ig secretion[c]
MOPC104E (ascites)	IgM, λ specific for α1,3-dextran	Oxidized dextran B1355 *in vitro*	Inhibition of membrane receptor expression; no effect on growth or IgM secretion[d,e]
Fluorescein-specific hybridoma	IgM, specific for fluorescein (Flu)	Flu–protein conjugates *in vitro*	Inhibition of Ig synthesis and secretion; no effect on growth[f]

[a] Abbas and Klaus (1977).
[b] Abbas and Klaus (1978).
[c] Kim *et al.* (1978).
[d] Bankert *et al.* (1978).
[e] Abbas (unpublished).
[f] Boyd and Schrader (1980).

an IgA, λ2 antibody specific for 2,4-dinitrophenol (DNP) and 2,4,6-trinitrophenol (TNP). Exposure of MOPC315 cells to multivalent DNP conjugates of bovine, human, and rabbit γ-globulin *in vitro* leads to a time-dependent reduction in the biosynthesis and secretion of the myeloma Ig without affecting the expression of membrane receptors (Abbas and Klaus, 1977). Comparably substituted DNP conjugates of albumin or hemocyanin are ineffective, but immune complexes of DNP-hemocyanin and antihapten or anticarrier antibody are as suppressive as DNP-globulins (Abbas and Klaus, 1978). These studies extended the well-known tolerogenic properties of globulins and immune complexes (Klaus and Abbas, 1978) to a clonal system employing tumor cells as the targets. Several additional parameters of the antigen-specific suppression of MOPC315 cells were established: the inhibition of antibody production is not accompanied by any effect on the growth of or DNA synthesis by the myeloma cells; it is reversible following removal of the antigen; and since $F(ab')_2$ fragments of globulins or of antibodies used to prepare immune complexes are also suppressive, the suppressive signal is apparently not delivered via classical Fc receptors. In fact, the features of antigen-mediated suppression of myeloma cells are strikingly similar to previous results ob-

tained with normal B lymphocytes and AFC, suggesting that the myeloma system is indeed physiologically valid (Table VI). Subsequent experiments using antigens trace-labeled with radioactive markers suggested that their suppressive effect is related to the extent to which they are endocytosed by the myeloma cells, although the basis for differences in internalization of different DNP-proteins remains unclear (Abbas, 1979a). It is conceivable that endocytosed antigen and/or antigen–receptor complexes interfere with antibody synthesis at the level of transcription, translation, or posttranslational assembly of heavy and light chains, although to date there is no direct proof for any such mechanism.

Using similar approaches, Kim *et al.* (1978) showed that culture of phosphorylcholine (PC)-specific TEPC15 myeloma cells (IgA, κ antibody) with the PC-bearing pneumococcus, R36a, leads to inhibition of myeloma growth and antibody production. These experiments, however, were done with tumor cells that were maintained by passage as ascites, and the presence of host cells contaminating the tumor population may complicate interpretation of the results. Tumor cells depleted of host lymphocytes and macrophages showed trivial suppression of Ig secretion following culture with the specific antigen (Kim *et al.*, 1978). Bankert *et al.* (1978) have shown that oxidized dextran B1355, which is a potent tolerogen for normal α1,3-dextran-specific B lymphocytes, suppresses the expression of membrane receptors by the α1,3-dextran binding BALB/c myeloma, MOPC104E (IgM, λ). This suppression is not due to persistent surface binding and receptor occupancy by the oxidized ligand, and is not observed with the nontolerogenic, native form of dextran B1355. Bankert *et al.* suggest that this system is a model for B lymphocyte tolerance since anti-Ig antibodies and antigens block receptor expression in neonatal B cells, which are exquisitely sensitive to tolerance induction (Sidman and Unanue, 1975; Raff *et al.*, 1975; Nossal and Pike, 1975), and highly tolerogenic polymeric antigens such as polysaccharides have the same effect in adult B lymphocytes (Klaus *et al.*, 1977). Moreover, oxidized dextran does not inhibit IgM secretion by MOPC104E cells (A. K. Abbas, unpublished observations; R. B. Bankert, personal communication). This intriguing selective suppressive effect on receptor expression but not Ig secretion is the reverse of what is seen in MOPC315 cells exposed to DNP-globulins (Abbas and Klaus, 1977). Such experiments provide situations in which it should be possible to analyze the mechanisms whereby the expression of genes encoding membrane and secretory Igs is selectively altered in the same target cell. The antigen-specific regulation of myeloma cells has been re-

TABLE VI
COMPARISON OF ANTIGEN-MEDIATED SUPPRESSION OF MOPC315 CELLS AND DNP-SPECIFIC NORMAL B CELLS AND AFC

Feature	Tolerance induction in DNP-specific mature B cells	Blockade of DNP-specific AFC	Suppression of MOPC315 cells
Induced by	Polymeric DNP-proteins and DNP-polysaccharides; DNP-globulins; immune complexes[a,b,c] (reviewed in Klaus and Abbas[d])		DNP-globulins, immune complexes[e,f]
Multivalency of antigen	Highly multivalent antigens are more suppressive; for AFC, ≤3–4 moles DNP/50,000 MW of carrier are required to induce blockade[b,c]		≤9 moles DNP/50,000 MW carrier required. (Need for higher valency may be due to low affinity of M315 IgA for DNP)[e]
Kinetics of induction	Increases with increasing exposure to antigen[a,g]		Requires ≈24 hour culture; increases with increasing exposure[e]
Recovery after removal of antigen	Not established	Inversely related to initial duration of exposure and antigen concentration[g]	Inversely related to initial duration of exposure and antigen concentration[e]
Role of Fc receptors	May be involved[h]	Not known	Not involved[e,f]
Effect on membrane receptor expression	May be suppressed or not affected[i,j,k]	Not known	Not affected[e,f]
Effect on synthesis of DNA and non-Ig protein	Not known	Not known	Not affected[e,f]

[a] Abbas *et al.* (1977).
[b] Schrader and Nossal (1974).
[c] Klaus (1976).
[d] Klaus and Abbas (1978).
[e] Abbas and Klaus (1977).
[f] Abbas and Klaus (1978).
[g] Klaus (1976a).
[h] Work of Vitetta *et al.*, quoted by Howard (1981).
[i] Nossal and Pike (1975); studies done with immature B lymphocytes.
[j] Nossal and Pike (1980); studies done with immature B lymphocytes.
[k] Klaus *et al.* (1977).

cently extended to a hybridoma prepared by fusion of specifically immune spleen cells with a nonsecreting myeloma line. Boyd and Schrader (1980) showed that brief incubation of a fluorescein (Flu)-specific IgM-secreting hybridoma with multivalent Flu conjugates of gelatin, human γ-globulin, or bovine albumin results in inhibition of antibody synthesis and secretion. Similar to the results of Abbas and Klaus (1977), this suppression affects only Ig production and not the biosynthesis of other proteins, is not accompanied by an effect on hybridoma cell growth, is reversed upon removal of the antigen, and does not involve interactions with Fc receptors, which are not detectable on the hybridoma cells. Moreover, the experiments of Boyd and Schrader suggested that the suppressive effect of Flu–protein conjugates is related to their binding to and persistence on the surface of the hybridoma cells, although no attempt was made to examine the internalization of antigen.

In addition to functional inhibition, administration of large doses of the specific antigens to mice bearing antigen-binding myelomas (MOPC104E, TEPC15, or MOPC315) has been reported to retard the growth of the myeloma (Paraf *et al.*, 1975; Bhoopalam *et al.*, 1980). Such an effect may not be a direct result of antigen–receptor interactions at the tumor cell surface but could involve the formation of immune complexes and complement-mediated lysis and/or antibody-dependent cellular cytotoxicity (ADCC). Attempts to inhibit tumor cell growth *in vitro* by culture with specific antigens have generally proved unsuccessful. Although Barnhill *et al.* (1976) reported that dextran B1355 reduces DNA synthesis by MOPC104E cells *in vitro*, we have failed to confirm this finding (Abbas, unpublished observations). In the final analysis, antigens appear to inhibit Ig production by some myeloma and hybridoma cells but generally have little or no direct effect on tumor cell growth. These experimental results provide simple systems which may be useful for analyzing the mechanisms by which ligand–receptor interactions result in functional alterations, and may be relevant to the mechanisms of B-cell tolerance.

2. *Idiotype-Specific Suppression of Tumor Growth*

The observation of Lynch *et al.* (1972) that BALB/c mice immunized with the protein, M315, produced by the isologous myeloma, MOPC315, resisted challenge with viable myeloma cells generated considerable interest as a model for specific tumor immunity and suppression of tumor cell growth by idiotype–antiidiotype interactions. Subsequently the basic phenomenon of actively induced or passively transferred idiotype-specific immunity has been demonstrated with

several different tumors, including MPC11 and TEPC15 myelomas (Freedman *et al.*, 1976; Beatty *et al.*, 1976) and surface Ig-positive lymphomas (Sugai *et al.*, 1974; Krolick *et al.*, 1979; Lanier *et al.*, 1980). Transfer of resistance with serum in the isologous mouse model has been demonstrated infrequently (Beatty *et al.*, 1976). In most instances, this form of tumor immunity does not appear to be mediated by antiidiotypic antibody. For instance, BALB/c anti-M315 idiotypic antibody does not fix complement or sensitize tumor cells for antibody-dependent cellular cytotoxicity *in vitro* (Frikke *et al.*, 1977; Lynch *et al.*, 1979; Abbas, unpublished results), and essentially similar results are obtained with other tumors (Sugai *et al.*, 1979). In contrast, heterologous (rabbit) antibodies directed against idiotypic determinants or constant regions of the myeloma Ig (Schrieber and Leibson, 1978), as well as complement-fixing monoclonal antiidiotypic antibodies (Mahony *et al.*, 1981) are capable of sensitizing the appropriate target cells for complement-dependent lysis and/or ADCC, indicating that the tumors themselves are susceptible to these immune effector mechanisms. In the more extensively studied situations of isologous idiotype-specific tumor immunity, several lines of evidence suggest that immune lymphoid cells play a major role. For instance, the resistance to tumor growth can be abrogated by postimmunization thymectomy (Daley *et al.*, 1979), and some investigators have demonstrated the presence of T lymphocytes in idiotype-immune mice that are cytolytic or cytostatic for tumor cells expressing the relevant surface idiotype (Sugai *et al.*, 1974; Flood *et al.*, 1980). In contrast, BALB/c mice immunized with the M315 IgA do not contain cells that lyse ^{51}Cr-labeled MOPC315 cells or suppress myeloma cell growth *in vitro* (Abbas, unpublished observations; R. G. Lynch, personal communication). In summary, this system of idiotype-specific transplantation resistance to myeloma or lymphoma cells is a complex one in which immunoregulatory T cells may play the predominant role, and is of interest primarily as a model for tumor immunity.

Finally, prolonged culture of cloned myeloma lines, in particular MOPC315, with isologous antiidiotypic antibody alone does not alter myeloma growth or function. In fact, the only demonstrable effect of the antibody is a modulation of surface receptor molecules which are rapidly reexpressed following removal of the antibody (Milburn and Lynch, 1982; Abbas, unpublished observations). These experiments, therefore, hold little promise for providing systems to study the biologic effects of idiotype–antiidiotype interactions.

In conclusion, antigen–receptor interactions in antibody-secreting tumor cells have functional consequences that are similar in certain

respects to normal B lymphocytes and antibody forming cells (AFC). At this stage, it is appropriate to consider the implications of receptor-mediated signal delivery in myeloma cells. The generally accepted view of lymphocyte activation and suppression is that both inductive and tolerogenic signals can be delivered via antigen-binding receptors whose hydrophobic portion is embedded in the plasma membrane and whose C terminals are available in the cytoplasmic aspect, perhaps to interact with cytoskeletal elements (Braun and Unanue, 1980). It is, however, unclear whether normal cells that secrete antibody at high rate as well as their neoplastic counterparts carry true membrane receptors. Many studies have established that as B lymphocytes differentiate, the density of surface Ig gradually declines. Nevertheless, a large number of investigations have demonstrated the presence of antigen-binding Ig molecules on AFC as well as myeloma cells that are accessible to external ligands (reviewed in Klaus and Abbas, 1978; Klaus, 1979). The issue whether these are true receptor molecules with hydrophobic membrane domains or simply secretory Ig en route to secretion remains unresolved. On the one hand, experiments showing direct correlations between rate of Ig secretion and density of membrane Ig in S107 and MOPC315 myeloma variants and sublines (Loken *et al.*, 1980; Abbas, unpublished observations) suggest that the surface receptor molecules may, in fact, be secretory Ig. However, at least some myelomas such as MOPC104E possess functional genes encoding hydrophobic membrane domains of μ constant regions (Rogers *et al.*, 1981) and proteins that represent precursors of true receptor Ig (Sidman, 1981). Moreover, differences in the sensitivity of membrane versus secreted Ig to inhibition of glycosylation (Hickman and Wong-Yip, 1979) or of protein synthesis (Bankert *et al.*, 1978) also suggest that these pools of Ig are distinct. Finally, selective suppression of secreted or membrane Ig by antigens (see above) or suppressor T lymphocytes (Section VI) further support the concept of different intracellular pools. It is however, clear that such findings are not seen with all antibody-secreting tumors. For instance, some hybridoma cells do not express precursors of membrane Ig (Sidman, 1981), and the J558 tumor, unlike MOPC104E (both being specific for α1,3-dextran) does not contain pools of receptor and secretory Ig that can be readily distinguished by inhibiting protein synthesis with cycloheximide (Bankert *et al.*, 1978). In the final analysis, two basic points remain unresolved. First, it is not yet clearly established which myelomas express "true" receptor Ig molecules as defined by protein structure and identification of a gene segment encoding the membrane domain. Second, the possibility exists that sig-

nal delivery might occur via membrane Ig molecules that are not embedded in the plasma membrane by hydrophobic segments but are in the process of being secreted. Although this latter hypothesis contradicts the accepted view of signal transduction as a consequence of specific ligand receptor interactions, it can only be formally proved or excluded by experimentation. The studies detailed above amply illustrate experimental systems which make such approaches feasible.

VI. T Lymphocyte-Mediated Regulation of B-Cell-Derived Tumor Cells

The roles of helper and suppressor T lymphoctes in humoral and cell-mediated immune responses are probably the most intensively investigated phenomena in cellular immunology during the last decade. Nevertheless, studies in which the final readout is a conventional immune response do not permit definitive answers to some fundamental questions, including (1) the molecular events that occur in target cells following reception of immunoregulatory signals, (2) the relative contributions of antigen-reactive, idiotype-specific and nonspecific ("polyclonal") T lymphocytes to the immune response, (3) the possible involvement of other cell types, e.g., macrophages, which cannot be excluded from a system in which an immune response is the final assay, and (4) the target determinants that are recognized by regulatory T cells or their functional products (e.g., receptors for growth factors, or extrinsic antigens in association with self MHC-encoded membrane molecules). Such issues can be resolved if the targets for immunoregulatory stimuli are homogeneous, monoclonal cells, such as cloned lines or tumors of immunocompetent cells. In the following section we will review the experimental evidence showing that the growth and/or function of tumors of lymphocytes and AFC can be affected by both helper and suppressor T lymphocytes, and that these results are often strikingly analogous to the results obtained by studying physiologic immune responses.

1. Effects of Helper T Lymphocytes on Lymphomas and Myelomas

The experiments described in Section IV demonstrated that T lymphocytes that are activated by alloantigens or polyclonal mitogens such as PHA or Con A can markedly influence B-cell-derived lymphomas by (1) enhancing growth, (2) inducing secretion of Ig in tumor cells that normally express only membrane Ig, (3) enhancing Ig secretion by lymphomas that secrete antibody at low rates, and (4) possibly inducing a switch from IgM to IgG production. In different tumors, these changes are induced by the T cells alone or in the presence of

polyclonal B-cell activators such as LPS, PWM, or anti-Ig antibodies. The effects clearly mimic the differentiative events induced in non-neoplastic B lymphocytes by helper T cells, and are presumably mediated by non-antigen-specific helper factors such as B-cell replication and maturation-inducing factors (which may or may not be identical to IL-2), TRF, and allogeneic effect factors (Melchers *et al.*, 1980; Altman and Katz, 1980). Essentially similar results are obtained using monoclonal factors liberated by spontaneous T-cell tumors or T-cell hydridomas. The ability of monoclonal B-cell-derived neoplasms to respond to inductive signals from helper T lymphocytes makes it feasible to analyze the biochemical basis and functional consequences of this interaction. It is clearly important to determine if different response patterns of B-cell tumors is related to differences in the maturational stages of the tumors, as assessed by the expression of markers such as the Lyb 5 surface antigen (Signer *et al.*, 1981). Moreover, if methods can be developed for producing antigen-specific B-cell tumors or hybridomas that secrete little or no Ig, it should be possible to study differentiation induced by signals delivered via membrane Ig receptors for the specific antigens, and the collaboration between MHC-restricted antigen-reactive helper cells and the appropriate receptor-bearing neoplastic B-cell targets.

Extensive studies of the regulation of B-cell-derived tumors by antigen-specific helper T lymphocytes have been done by Lynch and coworkers using the DNP-specific BALB/c myeloma, MOPC315 (reviewed by Lynch *et al.*, 1979). The basic protocol of these experiments was to implant MOPC315 cells together with TNP-coupled sheep erythrocytes (SRBC) within diffusion chambers in the abdomens of syngeneic mice that were previously immunized with SRBC at doses known to preferentially induce helper or suppressor T cells. Thus, immunoregulatory molecules liberated by the carrier-specific T lymphocytes would be focused on to the TNP-specific receptors on the myeloma targets by the hapten–SRBC complex acting as a bridge. Such experiments showed that in mice containing SRBC-specific helper T cells there is a significant enhancement of myeloma growth as well as function in terms of the numbers of antibody-secreting tumor cells recovered from the diffusion chambers (Rohrer and Lynch, 1977, 1978). Conversely, in the presence of SRBC-specific suppressor cells, MOPC315 growth and function are inhibited (see below). This phenomenon is carrier-specific, i.e., TNP-SRBC but not TNP-rabbit erythrocytes could focus SRBC-specific T-cell signals on to the myeloma; it can be transferred to naive recipients with T cells from appropriately immunized mice; and the same results are ob-

tained with localized (subcutaneous) or disseminated MOPC315 myeloma (Rohrer and Lynch, 1979). The enhancing effect of helper T cells on a tumor that is apparently derived from terminally differentiated, antibody-producing cells is best explained by the presence of less differentiated stem cells in the tumor population (Rohrer *et al.*, 1977). Helper T cells might serve to enhance the differentiation of clonogenic stem cells; however, it has not yet been possible to induce differentiation of nonsecretor variants of MOPC315, which may be tumors of the stem cells that do not differentiate spontaneously like the parent myeloma (R. G. Lynch, personal communication). Moreover, attempts to enhance the growth or function of culture-adapted MOPC315 cells *in vitro* using carrier-specific helper T lymphocytes or T-cell lines and appropriate TNP-carrier conjugates have so far been unsuccessful (Abbas, unpublished observations). One possible explanation for this is that the culture-adapted tumor line no longer shows the relatively clear distinction between nonsecreting stem cells and differentiated cells that is apparent in the line maintained by passage *in vivo*. Consistent with such a view are recent preliminary observations of Langman and Cohn (R. Langman, personal communication) with the α1,3-dextran binding myeloma, MOPC104E. Brief (24–48 hour) incubation of ascitic but not culture-adapted MOPC104E cells with dextran-coupled SRBC, antigen-presenting cells, and a cloned line of SRBC-specific helper T lymphocytes results in a marked increase in the proportion of antibody-secreting myeloma cells. Thus, these experiments indicate that antigen-specific tumors are responsive to specific T-cell helper influences. It is, however, clear that further analysis of the operative mechanisms will be greatly simplified if these studies can be done *in vitro* rather than the cumbersome *in vivo* experiments that are described above.

2. *Effects of Antigen-Specific and Nonspecific Suppressor T Cells on Ig-Producing Tumors*

In a series of experiments done in parallel with those demonstrating enhancement of MOPC315 growth and differentiation by helper T cells, Rohrer and Lynch (1978, 1979) have shown that SRBC-specific suppressor T cells can suppress the growth and function of MOPC315 targets in the presence of TNP-SRBC. Similar to the phenomenon of carrier-specific T cell help, this suppression can be transferred to naive recipients with immune T lymphocytes and can be mediated by a diffusible factor. Moreover, both help and suppression by carrier-specific T lymphocytes require the presence of the relevant TNP-carrier conjugate as well as macrophages in contact with myeloma cells

(Rohrer and Lynch, 1977, 1978; Lynch *et al.*, 1979). The involvement of macrophages in the effector phase of positive and negative T-cell-mediated immune regulation has been suggested by many different experiments (e.g., Basten *et al.*, 1975; Unanue, 1981). Whether the macrophages serve to "focus" the appropriate regulatory mediators on to the target cells, or are themselves activated to produce the final effector molecules (Tadakuma and Pierce, 1978; Aune and Pierce, 1981) remains to be established.

Attempts to demonstrate antigen-specific or nonspecific suppression of myeloma cells *in vitro* have not proved successful, to date. Suppressor T cells generated in BALB/c mice by immunization with aqueous hemocyanin or the synthetic polypeptide, L-glutamic acid L-alanine L-tyrosine (GAT), do not affect culture-adapted MOPC315 cells in the presence of the relevant DNP–carrier conjugates (Abbas, unpublished observations). Hapten-specific suppressor cells or their secreted factor(s) that inhibit the effector phase of TNP-specific delayed hypersensitivity (Zembala and Asherson, 1974) do not have any effect on MOPC315 cells that are lightly haptenated or cultured in the presence of TNP-proteins. Similarly, suppressor T lymphocytes induced by polyclonal stimulation of normal splenocytes with Con A (Rich and Pierce, 1974) also have no effect on the growth or function of MOPC315 or MOPC104E cells *in vitro*. Such negative results could merely reflect the lack of susceptibility of certain neoplasms to regulatory T cells. Alternatively, carrier-specific and Con A-induced suppressors or their liberated factors might act on helper T lymphocytes and the early (inductive) phase of immune response (Tada and Okumura, 1979; Tadakuma and Pierce, 1978), so that their failure to inhibit an autonomous tumor of differentiated AFC is not surprising. Recent studies of hapten-specific suppression of humoral and cell-mediated immune responses suggest that effector suppressors can be generated even after a single immunization with an antigen, but that the specificity of these suppressors is for idiotype rather than hapten (Sherr and Dorf, 1981; Sy *et al.*, 1981). The regulation of myeloma cells for antiidiotypic suppressors is discussed below.

3. *Regulation of Myeloma Cells by Idiotype-Reactive T Lymphocytes*

Investigations of idiotype-specific resistance to myelomas (Section V,3) suggested the possibility that idiotype-reactive T lymphocytes may be important in regulating the growth of target cells. These observations provided the initial stimulus for examining the effects of anti-idiotypic T cells on tumor function. In fact, such systems have now

emerged as valuable models for studying the mechanisms of action of regulatory T lymphocytes.

The initial studies of Rohrer *et al.* (1978, 1979) used the protocol first employed for demonstrating the regulation of MOPC315 cells by carrier-specific helper and suppressor T lymphocytes. In these experiments, ascitic MOPC315 cells were enclosed within diffusion chambers and implanted in the abdomens of BALB/c mice previously immunized with purified M315 IgA. The myeloma cells show a striking block in antibody secretion which can be reversed by treatment with proteolytic enzymes and can be transferred to naive recipients with M315-immune T lymphocytes. Thus, it appeared that idiotype-specific T cells release a soluble mediator(s) that blocks Ig secretion by the relevant idiotype-bearing myeloma target. Moreover, a block in secretion is not accompanied by any effect on membrane receptor expression. Subsequently this same phenomenon was demonstrated *in vitro,* by culturing MOPC315 cells and idiotype-specific T lymphocytes either together or separated by cell-impermeable membrane (Milburn and Lynch, 1981). The suppressor cells inhibit the biosynthesis of IgA but not non-Ig proteins, they do not affect myeloma cell growth, their effect is reversible, they can be specifically bound to M315-coupled immunoabsorbents, and suppression is competitively inhibited by a large excess of myeloma proteins. In these experiments, Ig synthesis and secretion by MOPC315 cells is inhibited up to 80–90% within 24 hours after exposure to the antiidiotypic suppressor cells. Removal of the suppressors reverses the inhibition within 12–24 hours, implying a rapid but reversible effect of idiotype-reactive suppressor factors on the myeloma.

Suppressor T cells reactive with myeloma idiotypic determinants have also been induced by immunizing BALB/c mice intravenously with syngeneic idiotype-coupled splenocytes (Abbas *et al.*, 1980a). This protocol was based on the induction of hapten- or protein antigen-specific suppressors by iv immunization with lymphoid cells to which the hapten or antigen is coupled covalently (Battisto and Bloom, 1966; Miller *et al.*, 1979). In the MOPC315 system, suppressor cells generated in this manner and cocultured with the myeloma targets suppress IgA synthesis and secretion by 40–60% without inhibiting the growth of or receptor expression by the myeloma cells. Moreover, their idiotypic specificity was established by their ability to bind to dishes coated with the appropriate myeloma protein (Abbas *et al.*, 1980a). In a subsequent series of experiments it was shown that suppressors could be induced that were specific for determinants (presumably idiotypic) of either M315 (IgA, λ_2) or MPC11 (IgG2b, κ) mye-

loma proteins. When cocultured with a somatic cell hybrid derived by fusion of MOPC315 and MPC11 cells and selected on the basis of its ability to secrete both Igs, each suppressor inhibits secretion of only the idiotype that is recognized on the hybrid cell surface (Abbas *et al.*, 1980b, 1981). This effect cannot be accounted for by the presence of variants secreting only one Ig in the hybrid myeloma population. However, the degree of suppression observed is only 40–50%, and this does not permit more detailed biochemical studies of the operative mechanisms. Such a selective effect suggests a compartmentalization of signal delivery that may have several parallels in the immune system. For instance, neonatal B lymphocytes exposed to anti-Ig antibodies show a prolonged inhibition of the expression of surface Ig but not other membrane macromolecules (Sidman and Unanue, 1975). Recent experiments of Fresno *et al.* (M. Fresno and H. Cantor, personal communication) indicate that a cloned line of suppressor T cells inhibits the production of only a limited number of peptides by cloned helper cells of the same antigen specificity, and the consequence of this is abrogation of helper function. Clearly the molecular basis of such selective functional effects is an issue of great interest.

More recent studies by Abbas *et al.* (1981b) suggest that the suppressors induced by iv immunization with idiotype-coupled cells correspond to an early stage in the suppressor T cell circuit which has been referred to as "suppressor inducer" (Eardley *et al.*, 1978) or "T_{s1} cell" (Germain and Benacerraf, 1981). Administration of a soluble extract of T_{s1} cells, when accompanied by priming with antigen (or myeloma idiotype, in this case), generates a population of effector suppressors (effector Ts). In the MOPC315 system, such effector Ts inhibit antibody secretion by MOPC315 targets more rapidly and completely than T_{s1}, are capable of mediating suppression across a cell-impermeable membrane provided macrophages are present with the myeloma targets, and have a surface Ly phenotype (Lyt 1^-2^+) that is different from T_{s1} (which are either Lyt 1^+2^+ or require an Lyt 1^+2^+ cell for their final effect) (Abbas *et al.*, 1981b). Moreover, effector Ts specific for M315, like the T_{s1}, suppress IgA but not IgG secretion by the MOPC315-MPC11 somatic cell hybrid. Rohrer *et al.* (1981) have also reported preliminary evidence suggesting that immunization with myeloma protein can induce Lyt 1^+2^- suppressor inducers in addition to Lyt 1^-2^+ effector suppressor cells. Similar observations of the circuits generated by antigen- and idiotype-specific T cells and their effects on normal humoral and cell-mediated immune responses have recently been made by numerous investigators (Dohi and Nisonoff, 1979; Sy *et al.*, 1981; Sherr and Dorf, 1981; Greene *et al.*, 1981). Taken

together, such results provide emphatic confirmation of the physiologic importance of idiotype–antiidiotype interactions first proposed by Jerne (1974).

The effects of idiotype-reactive suppressor T cells on the MOPC315 myeloma can be accounted for by effector phase suppression of differentiated tumor cells. In contrast, Kans *et al.* (1981) have found that HOPC8 (antiphosphorylcholine-secreting) myeloma cells injected into BALB/c mice that are neonatally suppressed by administration of antiidiotype antibody show a pattern of delayed maturation from clonogenic stem cells to antibody-secreting cells. This maturational delay is idiotype-specific, but a role for T lymphocytes has not yet been formally demonstrated. It is of interest that the HOPC8 myeloma cells used in these studies are maintained by passage *in vivo* and are presumably composed of stem cells and differentiated cells, as discussed in Section III,C.

In summary, these functional effects of idiotype-reactive suppressor T lymphocytes on myeloma targets provided an excellent model system for further analyzing the molecular basis of action of at least one type of regulatory T cells. The MOPC315 systems described above have several important advantages. First, idiotype-specific suppressor T cells are readily induced and their effect is rapid and occurs *in vitro*. Second, and more important, the phenomenon shows three types of selectivity that make it particularly interesting—suppressors block the synthesis of IgA but not other proteins by MOPC315 cells (Milburn *et al.*, 1981); they inhibit Ig secretion but not receptor expression (Rohrer *et al.*, 1979; Abbas *et al.*, 1980a); and they inhibit secretion of one of two Igs produced by a hybrid myeloma cell (Abbas *et al.*, 1980b, 1981). Finally, the ability to induce suppression across a membrane and to reverse the suppression by treating myeloma targets with proteolytic enzymes implies that a soluble mediator is active. Once such a mediator can be purified, or obtained from a T-cell hybridoma, biochemical analysis of its effect on the relevant myeloma target will be greatly simplified.

4. *Interaction of Cytolytic T Lymphocytes with Syngeneic Myeloma Targets*

Many different types of tumors, including neoplasms of lymphocytes and AFC, are routinely used as target cells for studying the activity and specificity of alloreactive, virus-specific and hapten-specific cytolytic T lymphocytes (CTL). The availability of tumor cells with membrane Ig receptors for defined haptens raises the possibility that haptenic determinants noncovalently associated with the receptors

can also function as targets for MHC-restricted, hapten-specific CTL. Such a situation would provide a model for studying the interaction of antigen-specific, MHC-restricted T cells with an antigen-binding target (such as a B lymphocyte), and could be used for analyzing the structure of the target determinant(s) that are recognized by MHC-restricted T cells. Along these lines, Abbas *et al.* (1980c) found that BALB/c TNP-specific CTL interact with syngeneic MOPC315 cells to which soluble TNP-proteins are bound. The interaction is measured by functional suppression of the myeloma targets, which is a sensitive assay for cytolysis or prelytic cell injury (Abbas, 1979b). The two most important features of this phenomenon are (1) the CTL are MHC-restricted, and (2) they only recognize haptens that are bound to specific Ig receptors on the target myeloma (Abbas *et al.*, 1980c). Thus, these results indicate that the CTL recognize receptor-bound antigen in some form of association with surface MHC-encoded determinants. More recent experiments have shown that TNP-specific CTL also inhibit antibody secretion by normal anti-TNP B cells *in vitro*, and in this situation the CTL appear to recognize TNP in association with I-A antigens on the B lymphocytes (Moser *et al.*, 1981). The central role of the I region in immune regulation is well established, and a large body of evidence indicates that extrinsic antigens activate a variety of T cells only if they are associated with self Ia on the surfaces of antigen-presenting cells (Paul and Benacerraf, 1977; Benacerraf, 1978). The studies of Moser *et al.* (1981) demonstrate yet another situation where a noncovalent complex of antigen and Ia determinants provides the target for T lymphocytes. These experiments do not establish a physiologic immunoregulatory role for hapten-specific CTL. However, studies of Bosma and co-workers strongly suggest that in a system of allotype suppression, the suppressors may actually be allotype-specific, MHC-restricted CTL (Bosma and Bosma, 1977; Snodgrass *et al.*, 1981). The ability to use the antigen receptors of cloned myeloma lines as targets for MHC-restricted T lymphocytes may prove useful in defining the molecular specificity of the receptor(s) on such T cells, and could thus provide insights into a number of immune phenomena in which MHC-restricted T cells are critically involved.

VII. Functional Regulation of Tumors of T Lymphocytes and Antigen-Presenting Cells

Until recently, there has been a paucity of well-defined tumors of T lymphocytes and antigen-presenting cells whose function is characterized and can be measured. This situation has changed dramatically with the development of somatic cell hybrid lines that secrete anti-

gen-specific or nonspecific immunoregulatory molecules, and the recognition of spontaneous murine and human tumors that produce IL-1 and IL-2 (Section III,B). The biochemical analysis of the various factors is beyond the scope of this article. The production of measureable products also makes it feasible to use the tumor cells themselves as targets for immune regulation. In the following section, experimental systems using T-cell tumors and macrophage-derived cell lines as models for normal T lymphocytes and macrophages, respectively, will be described.

A. Studies with T Lymphocyte-Derived Tumors

A large body of evidence accumulated during the last 5 years supports the view that many or most T lymphocyte functions are MHC restricted, i.e., T cells recognize antigens in association with MHC-encoded surface determinants. However, detailed analysis of this phenomenon is hampered by the failure to define the structure of the T-cell receptor and difficulties in performing experiments to study the binding of antigen or antigen + MHC molecules to purified T lymphocytes. Recently, Kappler and co-workers have circumvented these problems inherent in using normal T cells by introducing antigen-binding receptors into a functional T-cell hybridoma. Initially, hybridomas that produce IL-2 following stimulation with Con A were developed, the IL-2 being assayed by its ability to support the growth of an IL-2-dependent cytolytic T-cell line (Harwell *et al.*, 1980). One of these hybridomas, termed FS6, was then fused with normal T cell blasts that proliferate in response to protein antigens (ovalbumin, hemocyanin, human γ-globulin) presented only on syngeneic antigen-presenting cells, and receptor-bearing hybrids selected by their ability to produce IL-2 in response to antigen on presenting cells (Kappler *et al.*, 1981). The elegant experiment involved a double fusion, in which two T cell blasts, each recognizing a different antigen in association with a different MHC product, were sequentially fused with the parent FS6 line. Attempts to stimulate the resultant "double hybrid" with the two antigens on the two types of presenting cells clearly show that the receptors introduced into the FS6 parent retain the specificity patterns of the T-cell blasts (Kappler *et al.*, 1981). This result demonstrates that the "antigen + MHC" specificity of T-cell recognition cannot be due to two independently segregating receptors, one for antigen and one for self MHC products. In fact, either a single T-cell receptor recognizes a complex of antigen + MHC determinants or two associated and not independent receptors act in concert for functional recognition (Matzinger, 1981). Perhaps the most

significant aspect of the approach of Kappler *et al.* is the enormous potential of using such antigen-specific hybrids for studies of antigen binding and its biochemical consequences, for identifying the chromosomal location of the gene(s) encoding T-cell receptors by classical methods of somatic cell genetics, and ultimately for defining the molecular structure of these receptors and the genetic basis of their expression. The essential reason why this experimental system is so valuable is that it has been used to produce functional T-cell tumors which liberate a detectable factor only upon MHC-restricted antigenic stimulation, whereas most other antigen-specific hybridomas prepared to date secrete helper or suppressor factors constitutively. Whether this basic approach can be modified to produce hybrids into which receptors from other T-cell subsets (e.g., suppressor cells) are introduced is a question that will undoubtedly be answered by experiments that are currently in progress in a number of laboratories.

Attempts to analyze the antigen nonspecific interaction between macrophages and T lymphocytes or specific interactions between T-cell subsets in clonal tumor systems have been made only recently. One such study employs a murine radiation-induced T-cell-derived tumor, LBRM33, which produces IL-2 upon Con A stimulation. Occasional sublines of the tumor fail to respond to mitogen activation. However, preculture of one such variant with IL-1 derived from a macrophage tumor line restores its ability to secrete IL-2 in response to Con A (Gillis and Mizel, 1981). This result suggests that at least one function of macrophage products like IL-1 is to stimulate the differentiation of T-cell precursors that are unresponsive to inductive signals. In fact, IL-1 is routinely assayed by its ability to stimulate or enhance the proliferation of immature thymocytes and different experiments indicate that it promotes the function of helper T and/or B lymphocytes, although the mechanisms underlying these effects are not yet defined. The studies of Gillis and Mizel not only suggest a probable operative mechanism but provide a system for further analyzing the interaction between IL-1 and its receptor on target cells. It is also of interest that this result is, in some aspects, similar to the two-signal activation of B lymphocytes and their neoplasms (Section IV). Thus, in the case of B cells, anti-Ig or antigen is believed to render them responsive to T-cell-derived helper factors, and in immature T lymphocytes, macrophage products may induce responsiveness to further antigenic or polyclonal stimulation.

Finally, the production of hybridomas that apparently represent different stages in the pathway of T suppressor cell induction (Okuda *et al.*, 1981b) raises the possibility of analyzing the intercommunication

between these stages in greater detail. The feasibility of such studies is suggested by some preliminary experiments of Taniguchi *et al.* (1981), who have produced a series of hemocyanin-specific suppressor T-cell hybridomas. One such hybridoma constitutively secretes a factor that suppresses hemocyanin-specific helper cells in the early phase of an immune response, and therefore this hybrid is presumably derived from suppressor inducer or T_{s1} cells. More interestingly, the factor acts on a second hybridoma and induces this to liberate product(s) that suppress the effector phase of the response. Thus, the product of one hybridoma can, by itself, stimulate the production of an immunoregulatory effector molecule from another hybridoma. With the recent development of antigen-specific helper T-cell hybridomas (Lonai *et al.*, 1981), it should be possible to analyze the biologic consequences of suppressor cell–helper cell interactions in molecular terms, as is being done with cloned lines of antigen-specific T lymphocytes (Fresno *et al.*, 1981).

B. Studies with Macrophage-Derived Tumors

To date, tumors of macrophages, such as P388D1 and J774, have been used primarily for isolation and biochemical characterization of secreted products, in particular IL-1 (Mizel and Mizel, 1981). The other well-established role of macrophages in immune responses, which was, in fact, the first to be recognized, is to present antigens to immunocompetent lymphocytes in an immunogenic form, often in association with MHC-encoded (I region) surface determinants. Recent studies demonstrate that the macrophage cell line, P388D1, is capable of presenting antigen to specifically immune syngeneic T lymphocytes and thus inducing a proliferative T-cell response (D. I. Beller and E. R. Unanue, personal communication). One important aspect of this antigen presentation phenomenon that has been demonstrated only in the last 2 years is that lymphokines liberated by antigen or mitogen-stimulated T cells induce the expression of Ia (but not other H-2 encoded) determinants on normal, previously Ia-negative, macrophages *in vitro* and elicit an influx of Ia-positive macrophages *in vivo* (Steeg *et al.*, 1980; Scher *et al.*, 1980). Thus, the macrophage–T cell interaction stimulates a series of positive feedback loops that serve to enhance the immunogenicity of antigens and the responsiveness of T cells (Unanue, 1981). The same lymphokines can also induce Ia expression in cultured P388D1 cells *in vitro* (Beller, personal communication). Attempts to use the P388D1 tumor to study the receptor for this lymphokine and to define the molecular basis for selective induction of Ia antigens are currently in progress. Along the same lines, re-

cent studies of Lanier, Chesnut, Kappler, Warner, and Grey (L. L. Lanier, personal communication) have shown that B-cell-derived lymphomas can present antigen to antigen-specific T cells and hybridomas in an Ia-restricted manner, supporting the concept that B cells can perform at least one of the immunologic functions typically attributed to macrophages (Chesnut and Grey, 1981). It is apparent that surface Ig molecules on the B-cell-derived tumors do not play a role in this phenomenon. It is, however, conceivable that the B-cell tumors will be susceptible to T lymphocyte-mediated regulation of Ia determinants, since it is known that Ia expression on normal B lymphocytes can be enhanced by interaction with anti-IgD antibodies (Mond *et al.*, 1981). Thus, the vast and readily available resource of B-cell-derived tumors (Section III,A) may be valuable for exploring issues of antigen presentation in clonal systems that are presently restricted by the paucity of functional macrophage cell lines. Finally, a human macrophage cell line, U937, can be induced by conditioned media from alloantigen- or mitogen-stimulated lymphocytes to express morphologic and biologic characteristics of activated macrophages, such as enhanced expression of Fc receptors, antibody-dependent phagocytosis, and ADCC (Larrick *et al.*, 1980). Such systems may be useful for studying the biochemical and functional properties of activated macrophages.

VIII. Conclusions

Neoplasms of immunocompetent cells are clearly remarkable in that they exhibit many of the phenomena that are well-established with normal lymphocytes. Spontaneous, carcinogen-induced, and fusion-derived lymphoid tumors are now available that represent virtually all the known differentiative stages and subsets of normal B and T lymphocytes. More importantly, experimental conditions have been developed to alter the function or state of differentiation of many of these tumors. This article has focused on clonal systems employing tumor cells as targets for immune regulation, with a view to identifying the types of issues that can be tackled with such approaches and the most promising areas of future investigation. Several topics related to lymphoid tumors that do not bear directly on the theme of this article have not been dealt with in any detail. These include the biochemical and biologic properties of antigen-specific and nonspecific regulatory factors produced by lymphoid tumors and hybridomas; the biochemistry of Ig molecules and the nature of the genes coding for them as well as the mechanisms underlying Ig expression, as revealed by studies with Ig-producing tumors; the cellular and molecular basis

of leukemogenesis; and the mechanisms of tumor immunity that have been inferred from host responses to lymphoid neoplasms.

The scope of this article has been limited to detailing the studies that best demonstrate the feasibility of using lymphoid tumors for analyzing the biochemical and molecular basis of lymphocyte differentiation and regulation and the interactions between immunoregulatory cells or soluble mediators with their specific receptors on the relevant targets. Among the many experimental systems that have been explored, a few appear to hold the greatest promise. The induction of light chain synthesis and surface Ig expression in tumors of pre-B cells by polyclonal activators reflects early events in B-cell ontogeny that can be explored by currently available techniques. Second, Ig secretion and heavy chain class switching induced in nonsecreting B-cell lymphomas by polyclonal activators with or without T-cell-mediated helper effects is a model for normal B-cell differentiation that is amenable to mechanistic analysis at the molecular level. In addition, many such tumors must bear receptors for helper T-cell-derived growth and/or differentiation inducing factors, and can, therefore, be used to define the structure and properties of the receptors as well as the nature of the specific ligand–receptor interactions. The third potentially valuable system is the antigen-mediated inhibition of Ig production by hapten-specific myelomas and hybridomas. This effect is observed rapidly *in vitro*, and is apparently only a consequence of the interaction of the antigen with specific receptors. This system may be a model for antigen-specific B-cell tolerance or "effector cell blockade," and provides a simple experimental situation for analyzing functional suppression induced by an antigen–receptor interaction. Fourth, the suppression of myeloma cell function by idiotype-reactive T lymphocytes *in vitro* lends itself to studies of the biochemical basis of T-cell-mediated suppressive effects. These experiments are particularly useful because of the highly selective effect of suppressor cells on idiotype secretion, so that internal controls for the specificity of suppression are available. Finally, the recently developed technique for introducing antigen-specific T-cell receptors into T lymphocyte hybridomas by somatic cell hybridization offers exciting possibilities for analyzing T-cell receptors and the mechanisms of receptor-mediated signal delivery in these cells.

There are, however, a number of problems that need to be solved before the concept of using tumors as model systems realizes its full potential. The relationship of many neoplasms to the ontogenetic pathway or functional subpopulation of normal cells is inadequately defined, particularly with human lymphomas and leukemias. The de-

velopment of precise markers, especially the more recently identified markers of maturation (such as the Lyb 5 antigen for B lymphocytes), and the use of monoclonal antibodies should help resolve many of the present uncertainties. Studies using impure tumor cell populations rather than cloned lines are often difficult to interpret. However, with the remarkable advances in techniques of long-term culture and our understanding of the growth requirements of various cells, it is now possible to develop cloned lines of animal and human lymphoid tumors and to use these for analyses of differentiation and regulation. The inherent microheterogeneity in tumor populations and the possibility of continuous spontaneous differentiation need to be more precisely defined, and flow cytometry and cell-sorting techniques offer means of doing this better than has been possible to date (Warner *et al.*, 1979). The lack of antigen-specific tumors of the B lymphocyte lineage other than a few murine myelomas limits our ability to study specific immune induction and regulation in biochemical terms. Somatic cell hybridization may offer a means for producing antigen-binding tumors that represent virgin or activated B cells and retain their susceptibility to immunoregulatory stimuli. In fact, hybridization techniques have so far largely been used to prepare reagents such as antibodies or T-cell-derived factors for structural and biologic studies and for clinical use. Several recent studies elegantly demonstrate that hybridomas are valuable for much more than their products. Along the same lines, T-cell-derived tumors and hybridomas that secrete immune mediators can themselves be used as targets for specific regulation; such studies have received little attention so far. Finally, all results obtained with tumor cells must be considered with the caveat that they may not precisely reflect the phenomena seen with nonneoplastic lymphocytes. To date, tumors have been shown to respond to immunoregulatory stimuli much like their normal cells of origin, but this is only circumstantial evidence for the physiologic validity of tumors as model systems. Moreover, at least part of the explanation for the many striking similarities between lymphoid neoplasms and normal lymphocytes is that all the investigations are inherently biased toward emphasizing such similarities, and apparently aberrant results using neoplastic cells are difficult to explore and, therefore, are largely ignored. Nevertheless, direct and detailed comparisons of phenomena observed with tumors and normal cells, such as the antigen-induced blockade of antibody production, strongly support the value of tumors as model systems. In addition, studies with tumor cells have suggested the existence of phenomena that have only later been demonstrated in physiologic immune responses, such as the

suppression of antibody production by antigen or allotype specific cytolytic T lymphocytes. In the final analysis, it is clear the experimental systems have already been developed, and as the conditions are optimized, it is likely that combined immunologic, biochemical, and molecular biologic studies using lymphoid tumors as model systems will provide answers to issues that are among the most central unresolved questions in cellular immunology.

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