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PREFACE

The interrelationship of different branches of biology is part of our evolutionary heritage, but it still frequently comes as a surprise to see how significant this is for scientific progress. Plants and bacteria do not have an immune system, at least in the sense that we know it in higher organisms, but they have a wide assortment of binding proteins that show many similarities to antibodies. They are proving of increasing utility in immunology as well as other branches of biology as is evident from the final chapter in this volume. In a very different direction, autoantibodies to nuclear constituents found in the serum of patients with systemic lupus erythematosus, and described in the chapter by Tan, are proving of great utility in basic experimental work on RNA processing after transcription. The autoantibodies are directed against important RNA–protein complexes that had only become apparent through their disease relationship in the human. These are two examples from a group of such interrelationships described in this volume.

The first chapter by Irwin Scher discusses the very interesting immune-deficient CBA/N mouse strain which has been shown to have an X-linked gene defect. A number of X-linked human immune deficiency diseases are known but the exact defect remains obscure. This mouse model promises to be of great utility in solving this question. At present the mouse strain has proven uniquely useful for studies of B cell differentiation since the defect appears to involve a subtype of B cells not recognizable in other ways. Some controversy remains about the relative position of the multiple alterations that have been described, but the significant aspects are well delineated by Dr. Scher.

The second chapter on T cell lymphokines by Altman and Katz summarizes in a very helpful fashion the vast literature on this subject. The advent of methodologies for obtaining cloned T cell lines has aided greatly in the distinction of different species of molecules. T cell hybridomas and cloned T cell lines stimulated by T cell growth factor which secrete single factors have proven particularly useful. T cell replacing factors (TRF) and antigen specific and nonspecific helper factors are discussed in special detail and their characterization as a number of specific proteins appears close at hand. In addition the work on immune interferons, suppressor factors, growth factors, and macrophage factors and their interrelationships is discussed in detail.

The potential usefulness of at least some of these isolated factors in clinical situations makes this an increasingly exciting field.

The third chapter is by Eng Tan and discusses the wide assortment of antibodies to nuclear constituents found in the serum of patients with systemic lupus erythematosus (SLE) and related conditions. He has been a pioneer in this field and much of the work stems from the efforts of his laboratory. The review is very timely because of the many new types of antibodies described recently and their potential clinical significance. It is remarkable how many interesting and unusual antibodies can be delineated. Some of them have direct significance especially with regard to the development of immune complexes which are involved in the severe renal complications of SLE. Perhaps the most interesting development is that many of these antibodies are quite specific for identifiable clinical syndromes even though it is difficult to see how they might be involved in the specific clinical manifestations. Further studies are required to understand these associations; at the moment they are useful for diagnosis. The antibodies to RNA–protein constituents appear of special interest at the present time as mentioned above.

The chapter by Cochrane and Griffin concerns an important host defense mechanism that has received insufficient emphasis in the past, the Hageman factor or contact system of plasma. In considerable part through the work of these authors, the component parts of this system have been dissected through the use of highly purified materials. Four primary proteins are involved, Hageman factor, prekallikrein, high-molecular-weight kininogen, and coagulation factor XI. This system clearly overlaps with both the coagulation and complement systems in various aspects of the inflammatory process. Components of the contact system are clearly involved in various lesions and diseases although their exact role requires further clarification. Vascular permeability, allergic reactions, joint inflammation, and bacteremic shock represent a few of the many processes implicated.

The final chapter of this volume, by Teodorescu and Mayer, concerns the interaction of bacteria with lymphocytes. This field is just beginning to develop and shows considerable promise for answering basic questions regarding cell–cell interactions as well as useful detection of lymphocyte populations. In some ways this work relates directly to the plant lectins which have proven of such value in lymphocyte studies and it is probable that bacterial lectins are responsible for many of the interactions described. A number of bacteria have been found which bind specifically to B lymphocytes or subpopulations of B cells; T lymphocyte subpopulations also bind specific bacteria. Ex-

periments to define the lymphocyte components reacting with specific bacteria are just beginning, but it is likely that at least in some instances known components reacting with monoclonal antibodies are involved. Definition of these lymphocyte components which should not be difficult may help us to understand the many cell–cell interactions involved in the immune system.

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The CBA/N Mouse Strain: An Experimental Model Illustrating the Influence of the X-Chromosome on Immunity

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I. Introduction

The influence of the X-chromosome on immune function has been evident for some years, based principally upon findings of X-linked immune deficiency diseases in man (Ayoub *et al.*, 1968; Rosen *et al.*, 1968). It was therefore of considerable interest when an inbred mouse strain was discovered which had an X-linked deficiency in its ability to respond to polysaccharide antigens (Amsbaugh *et al.*, 1972; Scher *et al.*, 1973a). Studies of this mouse strain have identified the gene, or closely linked group of genes, responsible for the immune defect and it has been named *xid* (Berning *et al.*, 1980). In this review I will attempt to summarize the known functional immune defects associated with *xid* and discuss the cellular basis for these defects. I further hope to demonstrate that the CBA/N strain has provided us with a powerful experimental model in which to study B-cell development, function, and heterogeneity.

II. Influence of the X-Chromosome on Immunity

A. Serum IgM Levels

The X-chromosomes of mice and men influence the immunological function of these species in a number of interesting ways. In addition to the well-known and profound effects illustrated by the various X-linked immunodeficiency diseases, the number of X-chromosomes of an individual directly influences the level of circulating IgM (Rhoades *et al.*, 1969; Wood *et al.*, 1970; Price *et al.*, 1974). These experiments, which were stimulated by observations that women have higher serum levels than men of IgM but not IgG or IgA (Butterworth *et al.*, 1967; Lichtman *et al.*, 1967), indicate that the mean serum IgM increases with an individual's X-chromosome complement, with $XY = XO < XX = XXY < XXX$. This phenomenon may help explain the higher titers of antibody directed against *Listeria monocytogenes* and *Escherichia coli* (presumably of the IgM class) (Gardner and Adinolfi, 1968) in normal females (XX), as compared to females with Turner's syndrome (XO) or normal males (Wood *et al.*, 1970), and the higher incidence of bacterial and viral infections in males (Washburn *et al.*, 1965; Thompson *et al.*, 1966; Asmar *et al.*, 1978; Goodman *et al.*, 1971). In studies of the influence of the murine X-chromosome on serum IgM levels, it was shown that the mean values of serum IgM were higher in female mice than in males of two different mouse

strains (Adinolfi *et al.*, 1978). However, in contrast to humans with Turner's syndrome (XO) who had lower mean serum IgM values than did normal XX females, the mean serum concentrations of IgM in XO mice were similar to that found in normal female mice.

B. Immunodeficiency Diseases in Man

Bruton's X-linked agammaglobulinemia is a malady characterized by susceptibility to recurrent bacterial infections which appear in affected boys after 6 months of age. Children with this disease have very low serum IgA, G, and M levels and either lack or have very few surface Ig-bearing lymphocytes in their circulation or tissues (Good, 1973; Cooper *et al.*, 1975). It has been shown that children with this disease have cytoplasmic Ig-bearing pre-B cells in their bone marrow (Vogler *et al.*, 1976; Pearl *et al.*, 1978). This suggests that Bruton's X-linked agammaglobulinemia is the result of a developmental arrest of B lymphocytes at the level of pre-B cells. A few patients with X-linked inheritance of panhypogammaglobulinemia appear to have normal numbers of surface Ig-bearing B lymphocytes (Siegal *et al.*, 1971; Geha *et al.*, 1973; Litwin *et al.*, 1973). These groups, along with a family with two brothers and two maternal uncles, all of whom had an associated growth hormone deficiency (Fleisher *et al.*, 1980), may represent distinctive forms of X-linked immunodeficiencies with arrests of B-lymphocyte development at different stages (Cooper and Seligmann, 1977).

Patients with X-linked immunodeficiency with increased levels of serum IgM also have increased serum IgD and low or absent serum IgG or IgA (Rosen *et al.*, 1968). These patients have normal numbers of B lymphocytes bearing surface IgM, G, or A (Cooper and Seligmann, 1977) and are apparently deficient in their ability to switch from IgM to IgG or IgA production during an immune response. This deficiency may involve the mechanism for T-lymphocyte-dependent B-lymphocyte terminal differentiation.

The Wiskott–Aldrich syndrome is a complex X-linked immunodeficiency disease with low serum IgM, normal serum IgG, and elevated serum IgA and IgE levels. Although T-lymphocyte function decreases in children with this disease as they age, delayed hypersensitivity reactions are normal early in life. The characteristic functional immune defect in these patients is their inability to mount antibody responses to polysaccharide antigens, whereas antibody responses to protein antigens are intact (Cooper *et al.*, 1968; Blaese *et al.*, 1968; Ayoub *et al.*, 1968). The primary immune defect in this condition is

unknown, as is the etiology for the associated thrombocytopenia and eczematoid dermatitis. However, the functional immune abnormalities in these patients suggest that they may have a defect in the development of a B-lymphocyte subline (Cooper and Seligmann, 1977).

An X-linked form of severe combined immunodeficiency disease has been described (Gitlin and Craig, 1963; Hoyer *et al.*, 1968). Children with this disease have variable degrees of lymphopenia, with small foci of lymphocytes in their lymph nodes and spleens. It is unclear if these patients have a primary B- or T-lymphocyte or stem cell defect, although it has been noted that three boys with this disease have normal numbers of circulating B-lymphocytes (Cooper and Seligmann, 1977).

A most interesting X-linked lymphoproliferative syndrome has been described (Purtilo *et al.*, 1975). Males with this syndrome appear well until they develop an Epstein–Barr virus infection. Thereafter, they may manifest a number of distinctive proliferative or nonproliferative clinical syndromes (Purtilo *et al.*, 1977), with different patients within the same kindred expressing different phenotypic forms of the disease. American Burkitt lymphoma, immunoblastic sarcoma of B cells, fatal infectious mononucleosis, or plasmacytoma are different proliferative forms of the syndrome, whereas acquired agammaglobulinemia, aplastic pancytopenia, or neutropenia are nonproliferative forms of the disease. The etiology of this syndrome is unclear, although it has been suggested that the variable phenotypic expression could have resulted from individual differences in the Epstein–Barr viral dose, duration of exposure, and/or age at the time of infection with the virus (Purtilo *et al.*, 1977). Recently, it has been shown that males with this disease have immune-deficient responses to the Epstein–Barr virus (Sakamoto *et al.*, 1980). The mechanism(s) responsible for this defect are unknown, but could involve defective T-cell killing of virus-infected B cells or an intrinsic defect in B-cell target cells.

C. Immunodeficiency in Mice

Studies of X-linked immunodeficiency diseases in inbred mice began in the early 1970s, when it was shown that a mouse strain carried at the National Institutes of Health failed to make antibody responses to type III pneumococcal polysaccharide (S-III) (Amsbaugh *et al.*, 1972) or polyriboinosinic–polyribocytidilic acid [poly(I-C)] (Scher *et al.*, 1973a). CBA/N male and female mice, as well as F_1 males derived from matings of CBA/N females to males of immunologically

normal strains, were unresponsive to these antigens, whereas F_1 females from these crosses made excellent responses.

The CBA/N subline (originally referred to as CBA/HN) was derived from a CBA/Harwell line, beginning in 1966. All went well with the inbreeding until the seventh generation, when a breeding crisis occurred. However, the last pregnant female from this generation had a litter which included a single male and a number of females from which the CBA/N line was derived. At the time of the original studies of these mice, 15–25 generations of brother–sister matings had occurred (Amsbaugh *et al.*, 1972). The immunological defect of this strain was not apparent from their reproductive vigor or ability to exist in a routine laboratory environment, as these animals are normal in both regards.

The gene or group of closely linked genes responsible for the immune defect of CBA/N mice has been mapped on the X-chromosome and named X-linked immune deficiency (gene symbol *xid*) (Berning *et al.*, 1980). The two mutant genes used to map *xid* were tabby (*Ta*) and hypophosphatemia (*Hyp*), with the gene order and map distances in centimorgans being *Ta*—6.6 ± 1.8—*xid*—12.2 ± 2.3—*Hyp*.

In order to study the influence of the *xid* gene of CBA/N mice on the lipopolysaccharide (LPS) unresponsiveness of C3H/J mice (Sultzer, 1968; McGhee *et al.*, 1979; Watson and Riblet, 1975), CBA/N females were crossed to C3H/HeJ males and the resultant F_1 hybrid females were mated to C3H/HeJ males (Bona *et al.*, 1979, 1980). Approximately one-half of the backcross males derived from this cross expressed a more profound immunological defect than either of the parental strains. Thus, spleen cells from these mice were unresponsive to the proliferative actions of B-cell mitogens and they failed to give antibody responses to thymus-independent type 1 (TI-1) or TI-2 antigens. The synergistic defect in B-cell function of these backcross male mice was dependent upon the presence of the *xid* gene. However, the critical gene(s) from the C3H/HeJ strain was not the defective LPS^d gene.

An interesting X-linked abnormality in the capacity of DBA/2Ha mice to respond to T cell-replacing factor (TRF) has been described (Tominaga *et al.*, 1980). B cells from (DBA/2Ha × BALB/c)F_1 male mice were incapable of responding to TRF, while their F_1 littermates gave excellent responses. This X-linked defect was associated with the inability of B cells from DBA/2Ha mice to absorb TRF activity, an action that B cells of other mice had. Thus, this defect appeared to be due to the absence of a TRF receptor on the B cells of DBA/2Ha mice.

III. Immunological Defects in CBA/N Mice

A. Thymic-Independent Immune Responses

1. Characteristics of Types 1 and 2 Thymic-Independent Antigens

Thymic-independent antigens consist of a group of substances sharing certain immunologic and physical properties, the most important being than they require little or no T-cell help in inducing antibody responses. Different authors have different criteria for including an antigen in this group; however, if an antigen is able to stimulate specific responses in *nu/nu* mice, in neonatally thymectomized, adult irradiated, and bone marrow-reconstituted mice, or in T cell-depleted cultures, it is usually considered TI. For example, the IgM responses to S-III (Howard *et al.*, 1971), LPS, polyvinylpyrrolidone (PVP) (Andersson and Blomgren, 1971), and polymerized flagellin (Feldmann and Basten, 1971) appear not to require T-cell help in certain mouse strains. Most TI antigens are large polymeric molecules that have a high density of repeating determinants or epitopes (Feldmann and Basten, 1971), are poor direct stimulators of T cells (Kruger and Gershon, 1972), are slowly degraded by biological system, and persist for long periods *in vivo* (Felton, 1949; Sela *et al.*, 1972). Continued interest in the properties of these antigens has led to the discovery of other TI antigens, such as haptenated derivatives of Ficoll (Sharon *et al.*, 1975; Mosier *et al.*, 1974), and these in turn have allowed investigators to divide TI antigens into two classes.

The distinction between TI-1 and TI-2 antigens was based upon their differential ability to induce responses in adult normal or adult CBA/N and immature normal mice (Table I) (Mosier *et al.*, 1977a,b). Earlier studies using S-III, poly(I-C), or dinitrophenyl (DNP) conjugates of Ficoll have suggested that the *xid* gene resulted in a global defect in the ability of mice to respond to TI antigens (Amsbaugh *et al.*, 1972; Scher *et al.*, 1973a, 1975a). However, when trinitrophenyl (TNP)-conjugated LPS was used to test the immune responsiveness of immune-defective mice, it was shown that they made responses which were only somewhat reduced when compared to those of normal mice at optimum concentrations of antigen (Cohen *et al.*, 1976; Mosier *et al.*, 1976). Furthermore, CBA/N mice also gave excellent responses to TNP–*Brucella abortus* (TNP-BA), an antigen, which like TNP-LPS, was shown to be TI in immune defectives as well as normals (Mond *et al.*, 1978). Additional studies demonstrated that antigens such as TNP-Ficoll, that were unable to induce responses in immune defec-

TABLE I
CHARACTERISTICS OF THYMUS-INDEPENDENT TYPES I AND II ANTIGENS[a]

	Thymus-independent types	
	I	II
Induces antibody in CBA/N mice	+	−
Induces antibody in neonatal normal mice	+	−
Accessory cell dependent	−	+
Requirement for T cells	−	±
Predominant class of antibody produced	IgM, IgG_2, IgG_3	IgM, IgG_3

[a] Data compiled from Mosier *et al.* (1977a,b), Morrissey *et al.* (1981), Mond *et al.* (1980), and Slack *et al.* (1980).

tives, also failed to induce responses from immature normal mice (Mosier *et al.*, 1977a,b). By contrast, TNP-LPS and TNP-BA induced excellent responses in both immature normal or adult immune defectives. On the basis of these findings, TNP-Ficoll was designated a TI-2 antigen, whereas TNP-LPS and TNP-BA were designated TI-1 antigens (Table I).

Early studies of different TI antigens, including polymerized flagellin (Diener *et al.*, 1970; Feldmann and Palmer, 1971), LPS (Lemke *et al.*, 1975; Yoshimaga *et al.*, 1972), TNP-Ficoll (Mosier *et al.*, 1974), and TNP-BA (Mond *et al.*, 1979b; Boswell *et al.*, 1980a), suggested that they were both thymus and macrophage independent. However, more recent studies of TNP-Ficoll have shown this antigen to be macrophage dependent (Chused *et al.*, 1976; Boswell *et al.*, 1980c). Indeed, removal of adherent cells on a Sephadex G-10 column resulted in a marked reduction or elimination in the *in vitro* responses of spleen cells to the TI-2 antigens TNP-Ficoll, TNP-dextran, and TNP-levan (Table II) (Morrissey *et al.*, 1981). Reconstitution of the responses to these antigens was achieved by the addition of irradiated Ia^+ splenic adherent cells. By contrast, control or G-10 passed cells responded equivalently to TNP-BA. Thus, although TI-1 and TI-2 antigens were not originally defined on the basis of their macrophage dependence, it now appears that this property may distinguish these two classes of TI antigens (Morrissey *et al.*, 1981).

As noted above, TNP-Ficoll was originally classified as a TI antigen on the basis of its ability to induce responses in *nu/nu* mice and in lethally irradiated mice reconstituted with anti-theta antibody (anti-

TABLE II
THE ROLE OF SPLENIC ADHERENT CELLS (SAC) IN THE STIMULATION OF TI-2 RESPONSES[a]

Responding population	SAC	PFC/10^6 cultured cells				
		TNP-BA	TNP-Ficoll	TNP-dextran	TNP-levan	No antigen
Whole spleen	–	308	120	206	308	22
SAC depleted	–	442	14	60	0	28
SAC depleted	+	N.D.	382	300	438	24

[a] Spleen cells from C57BL/10 mice were used as responding cells and were depleted of accessory cells by passage over a G-10 column. SAC were isolated, irradiated, and added back to G-10 passed spleen cells as noted. Optimum concentrations of TI-1 or TI-2 antigens were cultured with the whole spleen or SAC-depleted populations. The data are presented as the geometric mean of triplicate cultures (Morrissey *et al.*, 1981).

Thy) plus complement (C)-treated spleen cells (Sharon *et al.*, 1975). These findings were confirmed by other laboratories (Mosier *et al.*, 1974; Boswell *et al.*, 1980c). However, after extensive T-cell depletion by repeated anti-Thy + C treatment, the *in vitro* responses to TNP-Ficoll were significantly reduced or eliminated (Mond *et al.*, 1980). These responses could be restored by the addition of T cells to the cultures. By contrast, the *in vitro* responses to TNP-BA were occasionally diminished as a result of T-cell depletion, but never as drastically as the response to TNP-Ficoll. It was suggested, therefore, that TI-2 antigens required small numbers of T cells for optimal responses, whereas TI-1 antigens had either no or an even smaller requirement. Indeed, polyacrylamide beads which have been coupled to a low epitope density with TNP (Mond *et al.*, 1979b) and TNP-Dextran, which are considered to be TI-2 antigens, also require small numbers of T cells for optimal responses (Mond, personal communication). These findings, as well as those in which the response of adherent cell-depleted populations were analyzed, suggest that macrophages and small numbers of T cells are required for TI-2 but not TI-1 responses (Table I).

In addition to the apparent differences in the cellular requirements necessary for antibody responses to TI-1 and 2 antigens, recent data indicate that these antigens also induce different classes of antibodies in mice. Earlier studies had shown that the class of antibodies induced by groups A and C streptococcal carbohydrate, dextran, or phosphocholine were IgM and the rare IgG_3 subclass (Perlmutter

et al., 1978). These findings prompted a more thorough evaluation of the class of antibody stimulated by TI-1 and TI-2 antigens (Slack *et al.*, 1980). The TI-1 antigens LPS and BA stimulated little IgG_1, but large and equivalent amounts of IgG_2 and IgG_3, in addition to IgM. On the other hand, the TI-2 antigens TNP-Ficoll, Dextran-135, phosphocholine vaccine, and group A streptococcal vaccine stimulated predominantly IgG_3 in addition to IgM antibodies (Table I).

2. *Classification of Thymic-Independent Antigens as Type 1 or 2 Using CBA/N Mice*

The ability of an antigen to stimulate immune-defective CBA/N mice has provided a useful system to classify the TI antigens, dextran (Fernandez and Moller, 1977; Mond *et al.*, 1979b), levan (Morrissey *et al.*, 1981), PVP (Lindsten and Andersson, 1979), TNP-polyacrylamide beads (Mond *et al.*, 1979b), as well as different preparations of LPS (Scher *et al.*, 1977c; Mosier *et al.*, 1976; Quintáns, 1979; Fidler *et al.*, 1980) (Table III). Initially, it was shown that immune-defective F_1 male mice made equivalent or slightly lower responses than normal

TABLE III
RESPONSES OF CBA/N IMMUNE-DEFECTIVE MICE TO THYMUS-INDEPENDENT (TI) ANTIGENS[a]

		Antibody formation	
		Immune-defective	Normal
TI-2	Type III pneumococcal polysaccharide	−	+
	Polyriboinosinic–polyribocytidilic acid	−	+
	DNP-Ficoll	−	+
	Levan	−	+
	Dextran	−	+
	Polyvinylpyrrolidone	−	+
	TNP-Streptococcal pneumoniae	−	+
	Lipopolysaccharide	−	+
	Low epitope density polyacrylamide bead	−	+
TI-1	High epitope density polyacrylamide bead	+	+
	Lipopolysaccharide-associated lipoprotein	+	+
	TNP-*Brucella abortus*	+	+
	TNP-Sephadex	+	+

[a] Data compiled from Amsbaugh *et al.* (1972, 1974), Scher *et al.* (1973a, 1975a,b, 1977c), Fernandez and Möller (1977), Mond *et al.* (1979a), Morrissey *et al.* (1981), Mosier *et al.* (1976), Quintáns (1979), Fidler *et al.* (1980), Zaldivar and Scher (1979), and Lindsten and Andersson (1979).

mice to optimal concentrations of LPS extracted via the Boivin procedure and conjugated with TNP (TNP-Boivin-LPS) (Mosier *et al.*, 1976). However, at lower, suboptimal concentrations of TNP-Boivin-LPS, the responses of F_1 male mice were considerably less than those of normals. Since TNP is preferentially bound to the lipid-associated protein present in these Boivin-LPS preparations, it was of interest to study the responses of immune defectives to LPS preparations that were free of lipoprotein. Phenol-extracted LPS, which has little of the lipoprotein associated with Boivin-LPS (Melchers *et al.*, 1975), failed to induce specific anti-LPS antibody in immune-defective F_1 male mice, whereas Boivin preparations induced low responses (Fig. 1) (Zaldivar and Scher, 1979; Fidler *et al.*, 1980). Thus, CBA/N mice appear to be unresponsive to purified, lipoprotein-free preparations of LPS, and it must therefore be considered to be a TI-2 antigen,

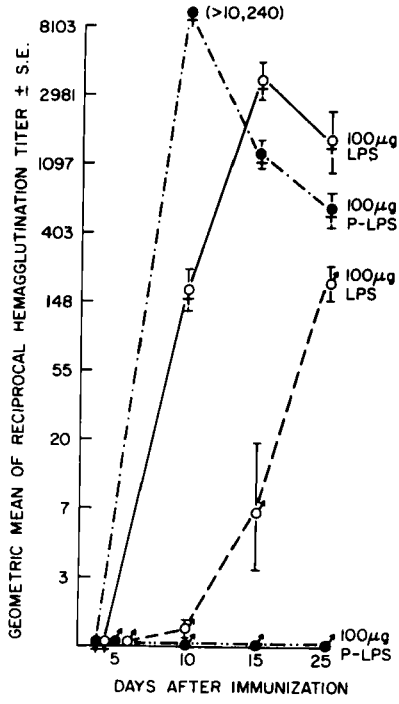

FIG. 1. Comparison of the specific antibody responses of F_1 male and female mice to different preparations of LPS. Mean reciprocal-enhanced hemagglutination titers of sera from (CBA/N × DBA/2)F_1 male (♂, ♂) and female (♀, ♀) mice after immunization with Boivin (○) preparations of *E. coli* 0111.B4 LPS or hot phenol-extracted LPS (●) (P-LPS) were measured at different times after immunization (Scher *et al.*, 1977c).

whereas the lipid-associated protein or lipoprotein found in Boivin-LPS preparations must be considered to be a TI-1 antigen. In addition to lipoprotein-free preparations of LPS, TNP-*Streptococcus pneumoniae*, levan (Morrissey *et al.*, 1981), and dextran (Fernandez and Moller, 1977; Mond *et al.*, 1979b) behave as TI-2 antigens, since they fail to induce responses in CBA/N mice. By contrast, TNP-Sephadex, which is an insoluble TI analog of TNP-dextran, is immunogenic in CBA/N immune-defective mice (Stein *et al.*, 1980b).

Poly(I-C), a putative TI-2 antigen, fails to induce specific immune responses in immune-defective CBA/N mice, whereas excellent responses were seen in normal mice (Scher *et al.*, 1973a, 1975b). This must not be due to an inability of immune-defective mice to recognize the antigenic determinants on poly(I-C) since specific anti-poly(I-C) responses are observed when the immunogen is a complex of the negatively charged poly(I-C) with positively charged methylated bovine serum albumin (Scher *et al.*, 1973a). Furthermore, after 4–5 months of repeated injections of unmodified poly(I-C) (CBA/N × NZB)F_1 male mice were capable of making specific IgG antibodies (Reeves *et al.*, 1981). It is possible that the responsiveness to poly(I-C) in immune-defective mice after repeated challenge is related to its ability to form ionic complexes with positively charged proteins *in vivo*. Alternatively, CBA/N mice may be able to respond to all TI-2 antigens under these long-term immunization procedures. Studies in which immune-defective mice have been repeatedly immunized with other TI-2 antigens over such a long interval have not been reported.

PVP acts as a typical TI-2 antigen, since it is able to induce antibody with little or no T-cell help in adult normal mice (Andersson and Blomgren, 1971), but does not stimulate immune-defective mice to form antibody (Lindsten and Andersson, 1979). However, PVP is unusual in that bone marrow cells from adult normal (Andersson and Blomgren, 1975) or immune-defective CBA/N mice (Lindsten and Andersson, 1979) were unable to reconstitute PVP responses in lethally irradiated mice, unless a source of T cells was added. Thus, bone marrow from immune-defective mice and normals behaved similarly in their requirement for T cells to support a PVP response. These findings raised the possibility that the inability of adult immune-defective mice to respond to this antigen *in vivo* was secondary to a defect of helper T-cell function. However, in adoptive transfer experiments, primed T cells, which supported a PVP response from bone marrow cells, did not influence the unresponsiveness of intact CBA/N mice to PVP.

In an interesting study of the nature of TI-1 and 2 antigens, Mond *et*

al. (1979b) coupled polyacrylamide beads with TNP-sulfonic acid to different TNP epitope densities. High epitope TNP-conjugated beads induced excellent responses in B cells derived from both immune-defective and immature-normal mice. By contrast, low epitope-conjugated beads failed to stimulate responses in B cells derived from these sources. Since both the high- and low-density TNP-conjugated beads elicited responses from *nu/nu* mice, it was reasoned that the low epitope TNP beads were an example of a TI-2 antigen, while the high epitope beads were TI-1. These data suggest that the epitope density of an antigenic determinant on a polysaccharide carrier can alter the immunogenic characteristics of that hapten–carrier complex, converting a TI-2 to a TI-1 antigen. Surprisingly, however, both the high and low epitope beads required T cells for optimal responses, a characteristic which had been previously associated with TI-2 antigens only (Mond *et al.*, 1979b; J. J. Mond, personal communication).

The persistance of the unresponsiveness of CBA/N mice to TI-2 antigens has recently been questioned (Fidler *et al.*, 1980). These workers have shown that whereas spleen cells from young adult CBA/N mice were unresponsive to both TNP-Ficoll and phenol-extracted preparations of LPS, cells from aged (>12 months) CBA/N mice responded to both antigens. Furthermore, spleen cells from aged but not young CBA/N mice supported a TNP-Ficoll response when transferred to lethally irradiated CBA/CaJ recipients. Studies by our laboratory have failed to confirm these findings, since TNP-Ficoll responses were not observed in analyses of (CBA/N × DBA/2)F_1 male mice of greater than 11 months of age (Skelly *et al.*, in preparation). The question of reconstitution of responsiveness with age in CBA/N mice remains an important and controversial issue. A number of explanations could account for the discrepant findings, among which is the possibility that the environment in the Scripps colony (where the studies of Fidler were carried out) may have altered the immune responses of aged CBA/N mice. Alternatively, genetic drift, back mutation, or contamination of the colony could have occurred (see Sections III,A,4, IV,C,1, and VI,B).

3. *Autoimmune Responses*

Polyclonal B-cell stimulators, such as LPS or poly(I-C), induce autoantibodies when administered to immunologically normal mice (Cunningham, 1976; Jacobs *et al.*, 1972). When challenged with LPS or poly(I-C), immune-defective F_1 male mice made little or no anti-DNA antibodies or autoantibodies to altered mouse erythrocytes, whereas F_1 female mice made large amounts of these autoantibodies

(Izui *et al.*, 1977; Rosenberg, 1979; Marshall-Clarke *et al.*, 1979; Kemp *et al.*, 1980; Reeves *et al.*, 1981). The absence of an anti-DNA response in immune-defective mice was apparently unrelated to the induction of circulating DNA by the injection of LPS, since these mice had DNA levels equivalent to those of normal mice after the administration of LPS (Izui *et al.*, 1977).

Crosses between CBA/N mice and strains that demonstrate autoimmune phenomena were carried out to determine if *xid* would influence the autoimmune status of NZB or MRL/1 mice. The F_1 male mice derived from crosses of CBA/N females and NZB males expressed the *xid* defect, since these mice failed to respond to TI-2 antigens (Taurog *et al.*, 1979; Romain *et al.*, 1980). However, these F_1 male mice spontaneously produced both antierythrocyte and anti-DNA antibodies, although to a much lower degree than F_1 female mice from these crosses (Romain *et al.*, 1980). On the other hand, when CBA/N mice were crossed to NZB mice to produce an NZB strain that was congenic for *xid* (NZ · CBA/N), this strain did not develop the excessive IgM production, autoantibody production, splenomegaly, splenic aneuploidy, or reduced survival characteristic of the NZB strain (Taurog *et al.*, 1981). By contrast, crosses of CBA/N with MRL/1 mice resulted in backcross male mice that were genetically greater than 99% MRL/1 and that bore *xid*, in which spontaneous autoantibody formation and lymphadenopathy were not suppressed (Cowdery *et al.*, 1980; Steinberg *et al.*, 1981). These studies provide powerful models in which to explore the role of different genes and their products on the development of autoimmunity.

B. Thymic-Dependent Immune Responses

1. Primary Thymic-Dependent Responses

The earliest studies on the responsiveness of CBA/N mice to thymic-dependent (TD) antigens were performed to contrast their responsiveness to these antigens with their inability to respond to TI-2 antigens (Scher *et al.*, 1975a; Cohen *et al.*, 1976). These *in vivo* or *in vitro* studies demonstrated that immune-defective mice developed measurable IgM antibody levels with optimal concentrations of the particulate TD antigen sheep red blood cells (SRBC). These were approximately 50% less than those of control mice, but similar to these mice when corrected for the total numbers of B cells in the spleens of the immune defects.

In more recent studies, the IgM and IgG responses of immune-defective mice to different concentrations of TD antigens were com-

pared to those of normal mice (Scher *et al.*, 1979). When optimal concentrations of the TD antigen SRBC were utilized in *in vivo* experiments, immune-defective F_1 male mice made from 10 to 50% of the IgM responses and from 1 to 10% of the IgG anti-SRBC responses of control mice (Table IV) (Scher *et al.*, 1979). Suboptimal but normally immunogenic doses of this antigen failed to induce responses in immune defectives. Similar findings were obtained in studies of the *in vivo* responses of immune-defective and normal F_1 mice to the TD antigen TNP keyhole limpet hemocyanin (TNP-KLH) (Boswell *et al.*, 1980b). Thus, when 1 or 10 μg of TNP-KLH were given to normal F_1 mice, low but easily measurable IgM anti-TNP antibody responses were observed (Fig. 2). By contrast, immune-defective F_1 mice made no antibody when challenged with these concentrations of antigen. Indeed, spleen cells from defective mice failed to make responses to this antigen at any concentration *in vitro*, but made excellent anti-TNP antibody to the TI-1 antigen TNP-BA (Boswell *et al.*, 1980b) (see Section IV,F).

2. *Secondary Thymic-Dependent Responses*

Although profound differences are seen in the primary anti-SRBC responses of immune-defective F_1 male mice when compared to female mice, these differences are reduced or eliminated when secondary responses are measured. Thus, while the $\log_2$ of the mean reciprocal direct (IgM) and enhanced (IgG) anti-SRBC hemagglutination

TABLE IV
DIRECT AND ENHANCED ANTI-SRBC HEMAGGLUTINATION RESPONSES OF (CBA/N × DBA/2)F_1 MALE AND FEMALE MICE[a]

Experiment	Sex	Day after immunization	$\log_2$ of the mean reciprocal hemagglutination titer	
			Direct (IgM)	Enhanced (IgG)
1	Female	7	7.89 ± 0.30	13.82
	Male	7	6.32 ± 0	5.82
2	Female	30 + 9	7.32 ± 0.37	14.72 ± 0.24
	Male	30 + 9	7.32 ± 0.44	12.56 ± 0.60

[a] Mice were immunized with optimum amounts of SRBC on day 0 and day 30. Seven or 9 days after immunization, sera were obtained and the hemagglutination titer of individual sera determined (Scher *et al.*, 1979).

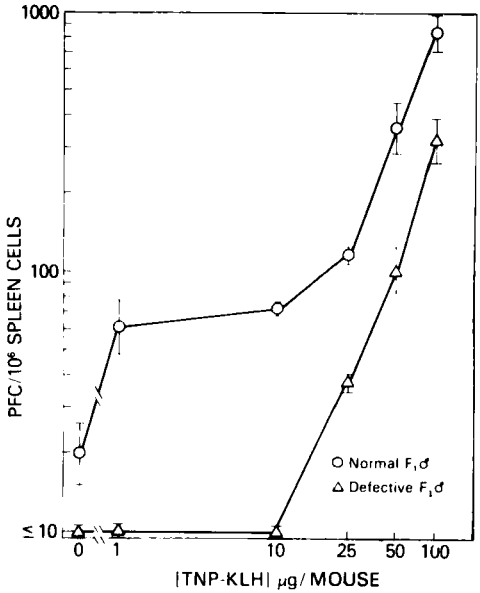

FIG. 2. Comparison of the primary *in vivo* IgM anti-TNP 4-day antibody responses of abnormal male (CBA/N × DBA/2)F_1 (Δ) and normal male (DBA/2 × CBA/N)F_1 mice (O) to graded doses of TNP-KLH (Boswell *et al.*, 1980).

titers of sera from defective and normal F_1 male and female mice were 7.9 vs 6.3 and 13.8 vs 5.8, respectively, 7 days after primary immunization with SRBC, they were 7.3 vs 7.3 and 14.7 vs 12.6, respectively, 9 days following secondary immunization (Table IV) (Scher *et al.*, 1979). Similarly, whereas the DNP antigen-binding capacity of the sera of normal F_1 female mice was considerably greater than sera of immune-defective F_1 male mice 14 days after primary challenge with DNP-KLH, the antigen-binding capacity of sera obtained from these mice 14 days after secondary challenge was equivalent (Stein *et al.*, 1980a). These findings are supported by data obtained with the splenic focus assay (E. Metcalf *et al.*, 1980). In this system the spleens of irradiated-primed immunologically normal recipient mice serve as a microenvironment in which T-cell help is maximized. Under these circumstances there were no differences in the ability of transferred immune-defective F_1 male and normal female spleen cells to form IgM or IgG anti-TNP responses to TNP-KLH.

In a recent paper by Press (1981), the Ig classes of secondary immune responses to TD antigens were analyzed. Immune-defective F_1 male mice immunized repeatedly with DNP-KLH developed signifi-

cantly reduced levels of IgG_3 anti-DNP antibody, whereas their IgG_1 and IgM anti-DNP responses were only 2-fold less than those of normal mice. However, when four synthetic polypeptide antigens under immune response (Ir) gene control, (Phe,G)-A--L, (Phe,G)-Pro--L, (T,G)-A--L, or DNP (T,G)-A--L, were used to immunize these putatively "high responder," H-$2^{k/k}$ mice, different results were observed. Thus, these antigens induced little or no IgM or IgG_3 antibodies in these mice, and the normally predominant IgG_1 isotype responses also were severely impaired. The differences in the responses to DNP-KLH and the synthetic polypeptide antigens were carrier dependent, since the F_1 male mice had significantly reduced IgG_1 anti-DNP responses to DNP-(T,G)-A--L, but not to DNP-KLH. It was noted that the responses of immune-defective mice to the heterogeneous multideterminant TD antigen DNP-KLH were comparable to the responses of these mice to TI-1 antigens, since the IgG_3 isotype was preferentially reduced. On the other hand, TD antigens, like (T,G)-A--L, which have repeating antigenic determinants and are under Ir gene control, were said to be similar to TI-2 antigens, since all isotypes of antibody were reduced/absent in CBA/N mice. However, in another report in which the isotypes of the anti-DNP responses of immune-defective mice to DNP-KLH were studied, there were severe impairment of the primary IgG_{2a} response and a total absence of a secondary IgG_{2a} response (Huber *et al.*, 1981b). Thus, the differences in the antibody responses of immune-defective mice to KLH vs TD antigens under Ir gene control are not restricted to the IgG_3 isotype, since the IgG_{2a} isotype also is reduced in their response to this TD multideterminant antigen.

When immune-defective F_1 male and normal F_1 female mice were repeatedly injected with rat erythrocytes, the nonresistant (IgM) and mercaptoethanol-resistant (non-IgM) agglutination titers were equivalent (Marshall-Clarke *et al.*, 1979). However, when the cross-reactivity of these antisera were tested with mouse erythrocytes, only the antiserum from normal females induced agglutination of these cells at a high titer. Similarly, after repeated injection with SRBC, normal F_1 female mice made antibodies which cross-reacted with horse erythrocytes, whereas immune-defective F_1 male mice did not (Gershon and Kondo, 1976).

3. *Immune Responses to Infectious Agents*

In addition to antigens which are readily classified as TI or TD, a number of investigators have studied the ability of CBA/N mice to respond to natural and experimentally induced infections. The first evidence that these mice had altered immunity to a naturally occurring

agent(s) came from studies of the serum levels of tumor-reactive antibodies in immune-defective F_1 male mice (Martin and Martin, 1975). These antibodies are readily detected in the sera of normal mice, but are present in very low amounts, or are undetectable in the sera of immune-defective mice.

After infection with malaria, polyclonal (Greenwood and Vick, 1975) as well as specific antiplasmodial (Cohen and McGregor, 1963) antibody formation is observed. In studies of the susceptibility of immune-defective mice to malaria infection, these responses were measured and differences from normal mice were noted. Thus, the polyclonal IgM and IgG responses of infected immune-defective F_1 male mice were delayed and lower in absolute magnitude than those of normal F_1 mice (Table V) (Hunter *et al.*, 1979; Jayawardena *et al.*, 1979). The specific IgM antiplasmodial antibodies of immune-defective mice were also depressed during the infection, while the IgG antiplasmodial antibodies, although initially depressed, approached those of normal F_1 mice on day 19 of infection (Table V) (Hunter *et al.*, 1979). By contrast, when immune-defective mice were immunized with killed preparations of *S. typhimurium*, their IgM anti-salmonella responses were equivalent to those of F_1 female mice, whereas their IgG responses were from 16- to 65-fold less than immunologically normal mice (O'Brien *et al.*, 1980). These immunological abnormalities were associated with a markedly reduced resistance of immune-defective mice to malaria and salmonella infections. Thus, defective mice had more severe and longer lasting malarial infections when compared to controls (Hunter *et al.*, 1979; Jayawardena *et al.*, 1979). These infections were often fatal in defectives, but rarely were

TABLE V
RADIOIMMUNOASSAY FOR IgG AND IgM ANTI-PLASMODIAL ANTIBODY IN CBA/N MICE INFECTED WITH *P. yoelii*[a]

	Reciprocal titer			
	IgM		IgG	
(CBA/N × DBA/2)	Day 12	Day 19	Day 12	Day 19
Male	2.5	6.6	2.1	14.0
Female	28.2	35.5	30.0	41.0

[a] Mice were infected with *P. yoelii* and their sera obtained at day 12 and 19. Specific titers of IgG or IgM antiplasmodial antibody was determined using a solid phase radioimmunoassay (Hunter *et al.*, 1979).

in normals. The levels of parasitized erythrocytes were higher in immune-defective than normal mice, and these erythrocytes persisted for longer periods in the defectives. Inoculation of small numbers of *S. typhimurium* into *xid* mice often leads to fatal infections. Indeed, immune-defective mice were approximately 10-fold more susceptible to the letahl effects of *S. typhimurium* than were normal mice (O'Brien *et al.*, 1979).

Immune-defective mice appear to be unable to mount specific antibody responses to a number of infectious agents. Thus, after challenge with whole or disrupted A/A ichi/Z/68 influenza virus, CBA/N mice had undetectable levels of antiinfluenza antibodies, while normal strains made excellent responses (Lucas *et al.*, 1978).Similarly, antiphosphocholine (PC) antibody was not observed in the sera of immune-defective mice which had been challenged with *Ascaris suum* (Brown *et al.*, 1977) or the C_5 variant of *S. pneumoniae* (Quintáns, 1977) (see section dealing with PC responses in CBA/N mice for more thorough discussion of this topic). The abnormal responses of immune-defective mice to PC determinants present in the capsules of these bacteria may explain their virulence in these mice, which had an LD_{50} of 10 vs 100,000 for normal mice (Yother *et al.*, 1981). Thus, strains of this organism, which showed differential killing of immune defectives and normal mice, were all encapsulated and their capsules all bound anti-PC antibodies.

In a paper recently reported by Lee and Ihle (1981), CBA/N mice were inoculated as newborns with the Moloney leukemia virus. These mice, like normals, develop an acute viremia after infection. However, unlike normal newborn mice, they fail to develop leukemia later in life and do not have detectable proliferative T-cell responses against the virus. The appearance of viral proteins in the sera of infected CBA/N mice indicated that they provided an environment in which the virus would replicate, although specific antibody responses to the virus were not measured. These data supported the views of the authors of this report, who have suggested that chronic immune stimulation is essential for the development of leukemia.

4. Immune Responses to Phosphocholine

The abnormal immune reactivity of CBA/N mice to TI-2 and TD antigens does not entirely define the defect of these mice, as has been demonstrated in studies of their reactivity to the PC hapten. When normal mice are immunized with PC coupled to a TD carrier, their antibody responses are characterized by (a) a restriction in the PC clonotype repertoire (Sher and Cohn, 1972; Cosenza and Kohler,

1972a,b; Gearhart *et al.*, 1975), with idiotype predominance in most inbred strains (Lieberman *et al.*, 1974; Claflin and Davie, 1974); (b) anti-PC responses in neonatal normal mice which do not develop until 7 to 10 days of life (Siegal *et al.*, 1976, 1977); and (c) early-appearing (1 to 2 weeks of life) PC-reactive clones which are easily tolerized (E. Metcalf *et al.*, 1977, 1979). Early studies on this subject with CBA/N mice suggested that the expression of the *xid* defect resulted in an absolute unresponsiveness to the PC hapten (Mond *et al.*, 1977; Quintáns and Kaplan, 1978). Thus, anti-PC responses were not observed even when PC-conjugated TI-1 antigens, such as LPS or BA, were used as carriers. The failure of immune-defective mice to make conventional anti-PC antibodies was supported by the data of Kishimoto *et al.* (1979) who, however, demonstrated that these mice were able to mount an IgE anti-PC response. More recent reports on this topic indicate that immune-defective CBA/N mice can make conventional anti-PC responses (Table VI) (Kenny *et al.*, 1980, 1981; Clough *et al.*, 1981; Kohler *et al.*, 1981). Although the immunization procedures and PC carriers used in these studies were different, their results are similar. Thus, PC-KLH induced primary responses in immune defectives that were either 10-fold less than in normals (Kenny *et al.*, 1980, 1981),

TABLE VI
ANTIPHOSPHOCHOLINE ANTIBODIES IN NORMAL AND IMMUNE-DEFECTIVE MICE[a]

Group and sex	Experiment number	Number of animals per group	mg anti-PC antibody per ml serum			
			V_HPC idiotype		T-15 idiotype	
			1	2	1	2
(CBA/N × BALB/c)F_1 female	1	5	N.T.	1131	N.T.	729
	2	5	682	1132	899	478
	3	5	614	2333	948	1757
(CBA/N × BALB/c)F_1 male	1	5	N.T.	243	N.T.	<6
	2	5	19	984	<3	<6
	3	15	39	305	<3	<1

[a] Mice were immunized with 200 mg PC-KLH in CFA. Serum anti-PC antibody was analyzed by an inhibition RIA using 125I-H8 myeloma protein for both the anti-T-15 and anti-VHPC assay. Standard curves were generated using known concentrations of cold H8. The values shown represent the geometric mean for the sample populations, and all standard errors were < or = 1.69 (Kenny *et al.*, 1981).

or were not detectable (Clough *et al.*, 1981). By contrast, the secondary anti-PC responses of immune-defective mice were excellent and in some instances appraoched those of control mice (Table VI, V_H PC idiotype).

The discrepant results in these studies may result from the different assays used to detect anti-PC antibody. The reports in which anti-PC antibody was not detected utilized a plaque-forming cell (PFC) assay (Mond *et al.*, 1977; Quintáns, 1977; Quintáns and Kaplan, 1978; Kishimoto *et al.*, 1979). The PFC assay, although sensitive in detecting IgM anti-PC responses, detects IgG anti-PC PFC with poor sensitivity (Cosenza and Kohler, 1972a; Claflin *et al.*, 1974; Lee *et al.*, 1974). Thus, in the presence of low or no IgM, but high IgG anti-PC antibody, the PFC assay could register no response, whereas the radioimmunoassay would be positive. By contrast, in the later studies demonstrating responses in immune defectives, anti-PC antibodies were detected by direct binding to PC in a solid-phase radioimmunoassay, or by their interaction with anti-V_H PC antisera (Kenny *et al.*, 1980, 1981; Clough *et al.*, 1981; Kohler *et al.*, 1981). The anti-V_H PC antisera are particularly useful in these studies, since they react with an antigenic determinant(s) common to all murine PC-binding antibodies (Claflin and Davie, 1975).

The quantitative differences in the primary anti-PC responses of immune-defective mice were associated with aberrations in the idiotype and class of anti-PC antibodies produced by these mice. Thus, whereas T-15 was the predominant anti-PC idiotype produced by (CBA/N × BALB/c)F_1 female mice in response to PC-KLH, the majority of immune-defective F_1 male mice from these crosses failed to produce this idiotype (Table VI). Furthermore, after challenge with PC-KLH, immune-defective mice failed to make IgM or IgG_3 anti-PC antibodies in either a primary or secondary response (Kenny *et al.*, 1981; Clough *et al.*, 1981). The absence of anti-PC antibodies of the IgG_3 class or of the T-15 idiotype also was observed after immunization of immune-defective F_1 male mice with PC-LPS (Kohler *et al.*, 1981). In contrast to the results observed with PC-KLH, IgM anti-PC antibodies were stimulated by PC-LPS in immune-defective CBA/N mice. Thus, immune-defective mice are capable of responding to PC when it is present on a TD or TI-1 carrier, but these responses are unusual in the idiotype, class, and amount of antibody made.

The preimmune sera of normal mice contain low levels of antibody with the T-15 idiotype, whereas the sera of immune-defective mice contain no detectable antibody of this idiotype (Kenny and Guelde, 1981). However, a small percentage (3 to 10%) of CBA/N and F_1 male

mice did have low levels of T-15 idiotype in their sera. When immunized with PC-KLH, these mice made anti-PC responses which were equivalent in every way to the responses made by F_1 female mice. Indeed, F_1 male mice, which had circulating T-15 idiotype antibodies, were also capable of responding to TNP-Ficoll. Additional studies will be required to establish the basis of these observations. Conceivably, these mice could carry *xid* in either the homozygous or hemizygous state and not express the CBA/N immune defect, or they could be the product of a back mutation or a contamination in the original CBA/N strain with the loss of *xid*. Preliminary studies using young CBA/N mice that had no circulating T-15 idiotype antibodies when initially tested indicate that over a period of approximately 10 months this phenotype did not change (Kenny *et al.*, unpublished observations). These data suggest that, since the appearance of T-15 idiotype-responsive CBA/N mice is not age related, this capacity is inherited rather than acquired (see Sections III,A,2, III,A,4, IV,C,1, and VI,B).

5. *Antibody Affinity*

Analysis of the affinity of antibodies produced by CBA/N mice has yielded variable results, apparently depending upon the type of antigen utilized for immunization. In the first report of this topic, Gershon and Kondo (1976) reported that CBA/N mice failed to make cross-reacting antibody to horse erythrocytes after hyperimmunization with SRBC. Such antibody is found in high titer in the hyperimmune sera of control mice and is of high avidity. These findings suggested that CBA/N mice might have a general inability to produce high-affinity antibody to TD antigens. This was supported by data showing that mice with the CBA/N immune defect have a lower percentage of high-avidity anti-TNP PFC than normal mice after either *in vitro* (Mosier *et al.*, 1976) or *in vivo* (Quintáns, 1979) immunization with TNP-LPS. In both of these studies, the frequency of high-avidity anti-TNP PFC was measured by determining the number of PFC inhibited by low concentrations of the free TNP-hapten.

A detailed analysis of the affinity of antihapten responses of immune-defective F_1 male and normal F_1 female mice to three different haptens, DNP, 4-hydroxy-5-bromo-3-nitrophenol (NBrP), and azobenzene-arsonate-5-hydroxyphenylacetyl (ABA-HOP), was reported recently (Stein *et al.*, 1980a). When hyperimmunized with these haptens conjugated with KLH, or chicken γ-globulin, the relative affinity of the anti-DNP antibodies (measured by an ammonium sulfate precipitation assay with [^{3}H]DNP-lysine) and the NBrP or ABA-HOP anti-

bodies (measured by hapten-phage inactivation assay) was equivalent in immune-defective and normal F_1 mice. The appearance of high-affinity antibody directed against these haptens occurred over the same time course in the defective and normal mice. Furthermore, in a different study, the inhibition profiles of the IgG_1 anti-DNP antibodies produced in immune-defective and normal mice after immunization with DNP-KLH, were similar when ε-DNP-caproic acid was used as the inhibitor (Press, 1981). However, different results were obtained when the affinity maturation pattern of the IgG_{2a} anti-DNP response to DNP-KLH response in normal and defective mice was studied (Huber *et al.*, 1981b). This study employed a highly sensitive radioimmunoassay that measures both the magnitude and affinity of the anti-DNP antibody of each isotype in individual serum samples (Tsu and Herzenberg, 1980). Immune-defective mice made smaller but relatively normal affinity IgG_1 responses compared to normal mice. On the other hand, their primary IgG_{2a} response was found to be severely impaired and their secondary response was completely absent after challenge with DNP-KLH. The affinity of the IgG_{2a} anti-DNP response of the defective F_1 male mice was 10 times lower after primary immunization and 100 times lower after boosting with antigen than was the affinity of the IgG_{2a} responses in normal mice (Huber *et al.*, 1981b).

These findings differ from those in studies on the IgG_1 anti-(T,G)-A--L antibody response in immune-defective mice. As noted previously, this antigen elicits little or no IgM or IgG_3 and very little IgG_1 antibody in these mice (Press, 1981). However, the little IgG_1 antibody that was made had different binding properties than that made by normal mice, when tested by competitive inhibition of binding using a 1 : 1 copolymer of L-Glu : L-Tyr. By contrast, the inhibition curves obtained were similar when (T,G)-A--L was used as the inhibitor. Thus, CBA/N-defective and normal mice appear to produce similar populations of IgG_1 antibodies when immunized to the TD antigen DNP-KLH, but produce different populations of anti-TNP, anti-DNP, or anti-(T,G)-A--L antibodies after immunization with TNP-LPS or (T,G)-A--L, respectively, or when the IgG_{2a} anti-DNP response to DNP-KLH is measured.

6. *Serum Immunoglobulin Levels*

In the first report on CBA/N mice, Amsbaugh *et al.* (1974) showed that their serum IgM levels were only approximately 20% of those of normal mice. This trait was inherited in an X-linked manner, being associated with the inability of backcross animals to respond to S-III.

Perlmutter *et al.* (1979) studied the level of serum Ig isotypes of CBA/N inbred as well as F_1 mice derived from this strain. The serum levels of IgG_1, IgG_{2a}, IgG_{2b}, and IgA were similar in immune-defective and normal F_1 mice, with the IgG_1 and IgA being slightly lower in defectives when compared to normals. By contrast, the serum IgM levels in the F_1-defective mice were approximately 25% of normals, and the serum IgG_3 levels were approximately 15% of normals. After administration of Boivin-LPS or phenol-LPS to immune-defective mice, large increases in their serum IgM levels were noted, which were less than but paralleled those of normals (Amsbaugh *et al.*, 1974; Cohen *et al.*, 1976; Scher *et al.*, 1977c). By contrast, only minor increases in the serum IgG_3 were observed in defective mice after stimulation with LPS when compared to normal controls (Perlmutter *et al.*, 1979). Indeed, while IgM-secreting cells were easily detectable, they were 10-fold less prevalent in the spleens of immune defectives than in controls, and IgG_3-secreting cells of immune defectives were only detected after stimulation with LPS.

7. *Cytotoxic Activities*

The ability of immune-defective F_1 male mice to reject allogeneic C57BL/6 skin grafts was shown to be equivalent to their F_1 female littermates (Scher *et al.*, 1975a). Equivalent *in vitro* cytotoxicity was also demonstrated when the secondary cytotoxic reactivity of spleen cells from these mice to allogeneic tumor cells was tested. Similarly, the natural killer activity of spleen cells from immune-defective mice to allogeneic tumor cells or chicken RBC in unprimed *in vitro* assays was normal (Scher *et al.*, 1975a; Nunn and Heberman, 1979; Gerson *et al.*, 1980). By contrast, when tested in an antibody-dependent cytotoxicity assay with antisera directed against the EL-4 tumor line, spleen cells from F_1 male defective mice were considerably less active in killing than those from F_1 female mice (Scher *et al.*, 1975a).

IV. Functional Properties of Lymphocytes and Accessory Cells in CBA/N Mice

A. T Cells

The T-cell proliferative responses induced by concanavalin A (Con A) or phytohemagglutinin were equivalent in immune-defective and normal F_1 mice, as was the ability of these mice to reject allogeneic skin grafts or to kill allogeneic tumor cells in *in vitro* secondary cytotoxicity assays (Scher *et al.*, 1975a). These assays are thought to

reflect T-cell function and therefore indicate that, with regard to their proliferative and cytotoxic activities, the T cells of immune-defective and normal mice are functionally indistinguishable. As noted previously, early studies using optimum concentrations of TD antigens found that immune-defective mice had IgM responses which were similar to those of normals, on a per B-cell basis (Scher *et al.*, 1975b; Cohen *et al.*, 1976). This suggested that these mice had functional T-cell helper activities which were equivalent. Indeed, in adoptive transfer studies, the primed functional helper T-cell activity of defective and normal F_1 male and female mice was similar (Janeway and Barthold, 1975).

In later studies of the TD antibody responses of immune-defective mice to SRBC or TNP-KLH, it became apparent their responses were not normal (Scher *et al.*, 1979; Boswell *et al.*, 1980b) (see Sections III,B,1 and III,B,2). In order to define the cellular basis of these abnormalities, the T-cell helper activity of primed activated T cells derived from these mice was tested in adoptive transfer experiments (Scher *et al.*, 1979). In these experiments, increasing numbers of (CBA/N × DBA/2)F_1 male- or female-activated T cells were given to lethally irradiated F_1 female mice along with T cell-depleted F_1 female spleen or bone marrow populations as a source of B cells (Fig. 3). Under these conditions no differences were seen in the activities of a wide range of different numbers of T cells from either source to support an IgG anti-SRBC response. Furthermore, in both *in vitro* and *in vivo* systems, when graded numbers of helper T cells from immune-defective or normal mice were compared, their abilities to help B cells from normal mice make IgM antihapten antibody were equivalent in the presence of hapten–protein conjugates (Boswell *et al.*, 1980b; Janeway *et al.*, 1980a).

These conclusions have been supported and extended by studies in which the function of two classes of helper cells were analyzed in defective CBA/N mice (Janeway *et al.*, 1980a). These distinct helper T-cell (Th) activities, called Th-1 and Th-2, are antigen specific. However, whereas Th-1 requires a hapten–carrier bridge, Th-2 does not (Janeway *et al.*, 1980b). Th-2 appears to require Th-1 to provide help; and Th-2, if stimulated with antigen, will augment the idiotype or allotype of the resultant antibody response. Both of these activities were present in CBA/N mice when tested in an adoptive secondary *in vivo* system.

Prior to the publications of the studies demonstrating PC responsiveness of CBA/N mice, it was reported that the T cells of immune defectives did not support a T-15 predominant response when used in

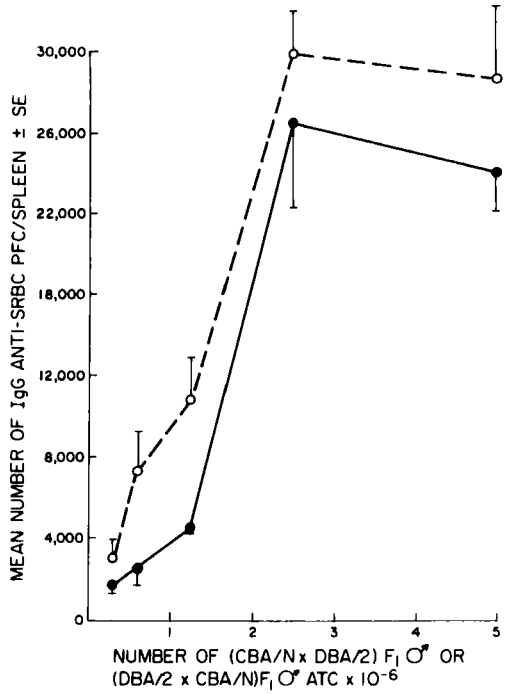

FIG. 3. A comparison of the helper activity of graded numbers of activated T cells from abnormal male (CBA/N × DBA/2)F_1 (●) or normal male (DBA/2 × CBA/N)F_1 (○) mice. The 7-day IgG anti-SRBC PFC responses of lethally irradiated (CBA/N × DBA/2)F_1 male mice were measured after immunization with SRBC and reconstituted with anti-theta + C-treated spleen cells from normal F_1 male mice, along with the activated T cells as noted (Scher *et al.*, 1979).

adoptive transfer experiments (Bottomly and Mosier, 1979). In these studies lethally irradiated (CBA/N × BALB/c)F_1 mice were used as recipients of B and/or T cells derived from immune-defective F_1 male or normal female mice. F_1 male T cells were able to support a PC response by F_1 female B cells which was quantitatively similar to that of F_1 female T cells. However, the predominance of the T-15 idiotype, which occurred with F_1 female T cells, was not seen when F_1 male T cells were used as helpers. When F_1 female T cells that were primed to a second carrier were added to male T cells and restimulated with PC conjugated to the carrier used to prime the male cells, along with the second free carrier, reconstitution of T-15 predominance was seen. Thus, under conditions where free carrier was present for the stimulation of the female cells, F_1 male cells, primed to a different PC protein, were able to support a T-15 predominant response. These findings

support the concept that at least two helper T cells are important in the generation of idiotype-specific TD responses (Janeway *et al.*, 1980b). They also imply that the T-cell helper function which is defective in CBA/N mice is the idiotype-specific subset (Bottomly and Mosier, 1979). However, as noted previously, the PC-PFC assay used in this study may not have detected IgG responses (Cosenza and Kohler, 1972a; Claflin *et al.*, 1974; Lee *et al.*, 1974). The interpretation of this report is further complicated by studies in which it was shown that the majority of (CBA/N × BALB/c)F_1 males, given neonatal BALB/c liver cells, made T-15 predominant responses when challenged with PC antigens 2–4 weeks after reconstitution (Quintáns *et al.*, 1979; Quan *et al.*, 1981). Furthermore, Kaplan and Quintáns (1979) have shown that the T cells of immmune-defective mice provide normal PC-specific helper activity with regard to their ability to support a T-15 predominant anti-PC response.

Goodman and Weigle (1979a) have shown that splenic T cells of normal mice were able to augment polyclonal B-cell responsiveness to phenol-extracted LPS. This T cell-mediated enhancement was dependent on the ability of the T cells to divide, and was manifested when T cells interacted with B cells soon after cultures were initiated. Studies using LPS-nonresponder C3H/HeJ mice indicated that the LPS had a direct action on the T cells. Using this technique, it was shown that B cells from (CBA/N × CBA/CaJ)F_1 male and female mice were receptive to the T-cell help provided by F_1 female cells (Goodman and Weigle, 1979b). However, T cells derived from F_1 male mice were unable to augment the LPS-induced polyclonal responses of either F_1 male or F_1 female B cells. Furthermore, treatment with antimouse thymocyte serum reduced the polyclonal response of F_1 female spleen cells to LPS, but did not alter the F_1 male response.

Thus, while there appears to be subtle abnormalities in the T-cell function of CBA/N immune-defective mice, these cannot explain the abnormalities in their TD antibody responses. Indeed, while it is clear that the T cells of immune defectives do not augment a B cell LPS response, the lower B-cell responses to this mitogen in these mice was shown to be at least partially intrinsic to their B cells, since the LPS proliferative studies of T cell-depleted *in vitro* cultures remained low when compared to normals (Scher *et al.*, 1975a). Furthermore, although it has been suggested that T cells play an important role in the responses of mice *in vitro* to the TI-2 antigen TNP-Ficoll, the demonstration that splenic T cell-depleted populations of normal F_1 female cells will reconstitute TNP-Ficoll responses in defective F_1 male mice (see Section VI,A) suggests that T cells do not play a primary role in

the cellular mechanism(s) underlying the CBA/N immune defect. Finally, although it is possible that CBA/N mice lack T cells which are important for the predominance of the T-15 response in normal mice, it is postulated that the development of these cells is dependent on the presence of circulating T-15, suggesting that this putative T-cell defect is not a primary but a secondary abnormality (Bottomly and Mosier, 1979).

B. MACROPHAGES

The macrophage function of immune-defective CBA/N mice has been tested in a number of ways. In studies of the facultative intracellular bacterium *S. typhimurium*, it was shown that mice with the susceptibility gene *Ity*^{s}, such as the BALB/c strain, succumb early during the course of the infection (<10 days) and to very low doses of the bacterium (Plant and Glynn, 1976). By contrast, although the expression of the *xid* trait in F_1 male or CBA/N mice resulted in an increased susceptibility to *S. typhimurium*, the LD_{50} at 10 days postinfection for F_1 male mice was only 10-fold less than for F_1 female mice, whereas there was a 1000-fold difference between the 28-day LD_{50} for these mice (Figs. 4 and 5) (O'Brien *et al.*, 1979). These data suggested that

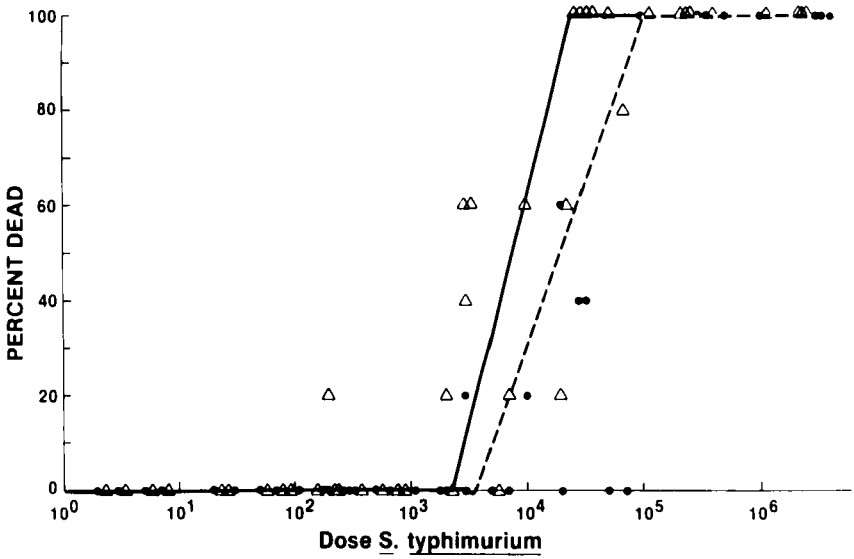

FIG. 4. Each point represents the percentage of five (CBA/N × DBA/2)F_1 female mice that were dead at 10 (●) and 28 (Δ) days postinfection with *S. typhimurium*. The LD_{50} obtained by extrapolation from the graphs at 10 and 28 days postinfection are 2×10^4 and 8×10^3, respectively (O'Brien *et al.*, 1979).

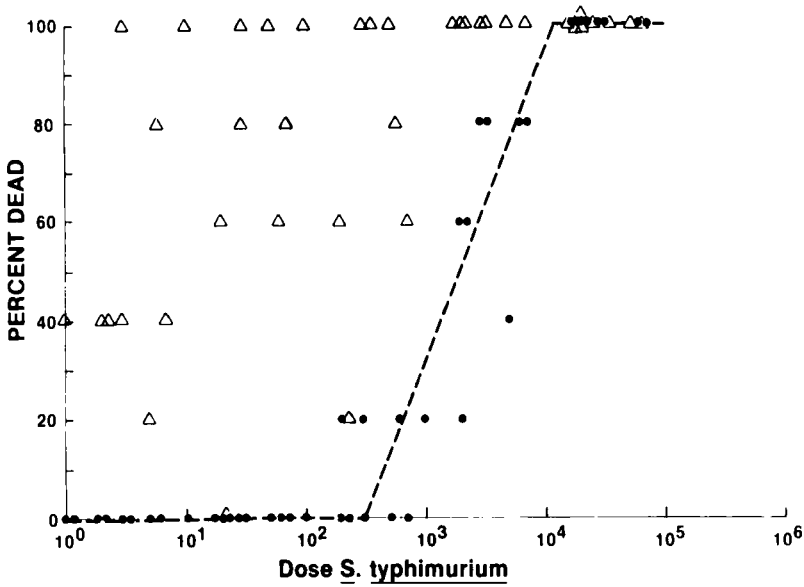

FIG. 5. Each point represents the percentage of five (CBA/N × DBA/2)F_1 male mice that were dead at 10 (●) and 28 (Δ) days postinfection with *S. typhimurium*. The LD_{50} obtained by extrapolation from the graphs at 10 days postinfection (O) is 2×10^3 (O'Brien *et al.*, 1979).

the mechanism(s) responsible for the control of the bacteria early in the course of the infection was similar in these mice, but that the F_1 male mice were defective in controlling the multiplication of the *S. typhimurium* later in the infection. This was supported by the finding that F_1 female anti-*S. typhimurium* serum, when given to F_1 males, allowed them to resist subsequent challenge with *S. typhimurium* with the same vigor as normal mice (O'Brien *et al.*, 1981). Indeed, a comparison of the early net growth of *S. typhimurium* in the spleens of *Ity*^{s} and *xid* mice indicated that, unlike the salmonella sensitivity of BALB/c *Ity*^{s} mice, the susceptibility of (CBA/N × DBA/2)F_1 male mice could not be attributed to the early failure of their reticuloendothelial cell organs to contain multiplication of the bacteria, since during the early stages of infection their net growth in the spleens of F_1 male and female mice was equivalent (O'Brien *et al.*, 1979).

In a more direct analysis of the macrophage function of immune-defective mice, Rosenstreich *et al.* (1978) tested the ability of LPS to release prostaglandins, or the monokine, lymphocyte-activating factor (LAF) from these cells. Macrophage LAF production is known to be induced by LPS through its direct effect on macrophages. When

thioglycollate-induced macrophages derived from (CBA/N × DBA/2)F_1 male and female mice were tested for their ability to produce LAF at different concentrations of LPS, they were shown to be equivalent. Furthermore, LPS-induced synthesis of prostaglandins by macrophages of F_1 male or female mice was similar, a finding which is not surprising in view of the important role played by prostaglandins in the initiation of endocytotoxicity *in vivo* (Skarnes and Harper, 1972; Rosenstreich *et al.*, 1977) and the known lethal effects of LPS in immune defectives (Zaldivar and Scher, 1979). These findings, as well as those which demonstrate that F_1 male mice are normally sensitive to the adjuvant effects of LPS *in vivo* and that their spleen cells produce normal amounts of colony-stimulating factor (Apte *et al.*, 1977), suggest that, at least with regard to these functions, their macrophages are normal.

In experiments designed to determine the cellular basis of the abnormalities in the immune responses of CBA/N mice, the antigen-presenting functions of their macrophages was studied (Boswell *et al.*, 1980c). Because of the known importance of antigen-presenting accessory cells in the induction of both TD and TI-2 responses, it was possible that abnormal macrophage function could be responsible for the low/absent responses of immune-defective mice to these antigens. Macrophage enriched, spleen adherent cells (SAC) prepared by their glass adherence properties consist of 50–80% latex-ingesting cells, 8–15% nonphagocytic surface Ig^+ cells, approximately 1% Thy 1^+ cells, and 15–25% of cells negative for these markers (Singer *et al.*, 1978). SAC from normal and immune-defective F_1 spleens were pulsed with TNP-KLH and graded numbers were added to normal spleen cells depleted of adherent cells by Sephadex G-10 passage (Fig. 6) (Boswell *et al.*, 1980c). These different preparations of SAC had equivalent capacities to present the TNP-KLH. Furthermore, SAC derived from immune-defective F_1 and normal mice presented TNP-Ficoll to normal-adherent cell-depleted spleen cells equivalently (Fig. 6). Thus, in their abilities to resist the growth of *S. typhimurium*, to act as an antigen-presenting cell for TNP-KLH or TNP-Ficoll, and to produce LAF and prostaglandins in response to LPS, the macrophages/accessory cells of immune-defective mice functioned normally.

Recently it has been shown that an antiserum detecting I-region-associated (Ia) antigen (anti-Ia.W39) could block the T-cell proliferative responses induced in $H\text{-}2^b$ mice by beef insulin (Rosenwasser and Huber, 1981). These findings, coupled with those suggesting that anti-Ia alloantiserum blockade is at the level of the antigen-presenting macrophage (Schwartz *et al.*, 1976; Thomas *et al.*, 1977;

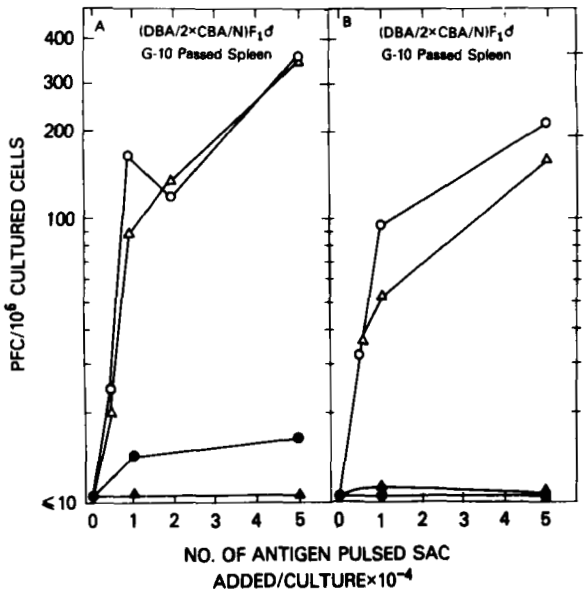

FIG. 6. *Comparison of the antigen-presenting accessory cell function of SAC from* normal male (DBA/2 × CBA/N)F_1 (○) or abnormal male (CBA/N × DBA/2)F_1 mice (Δ) pulsed with TNP-KLH (open figures, A), TNP-Ficoll (open figures, B), or medium (closed figures, A and B). Pulsed accessory cells were added to 5×10^5 G-10 passed spleen cells from normal male (DBA/2 × CBA/N)F_1 mice (Boswell *et al.*, 1980).

Shevach, 1978), suggest that Ia.W39 is expressed by these cells and is important for the presentation of beef insulin to T cells. It was of particular interest, therefore, that a T-cell proliferative response to beef insulin was not observed in (CBA/N × C57BL/6)F_1 male mice, whereas their F_1 female littermates gave excellent responses (Rosenwasser and Huber, 1981). Since Ia.W39 is not detected on T cells, it was suggested that the failure of immune-defective F_1 male mice to respond to beef insulin was the result of the inability of their macrophages to present this antigen. Indeed, although F_1 female T cells gave excellent proliferativr responses to beef insulin presented on F_1 female macrophages, F_1 male macrophages did not present. Thus, it appears that F_1 male macrophages do not express Ia.W39 and are functionally deficient in their ability to present beef insulin to T cells.

The evidence that B cells express determinants encoded for by the minor lymphocyte-stimulating locus (Mls) is quite strong, and will be discussed in the section dealing with B cells. It has been suggested that Mls-encoded determinants are also present on mouse macrophages (Schirrmacher *et al.*, 1975). Thus, peritoneal cavity cells,

purified by adherence to polystyrene tubes, were able to induce Mls-determined mixed lymphocyte reactions (MLR). In order to reduce the possibility that contaminating lymphocytes were not the stimulators in these experiments, the authors treated their adherent cells with anti-B and anti-T cell serum + C, pretreated the adherent monolayers with trypsin, or precultured the cells for 10 days, manipulations that did not reduce the capacity of the adherent cells to stimulate an Mls-determined MLR. Since spleen cells of immune-defective mice do not stimulate an Mls determined MLR, their macrophages may be defective in expressing Mls determinants. However, in the studies noted above, the frequency of residual surface Ig-bearing cells in the populations treated to enrich for adherent cells was not measured, and the Mls-determined MLR observed may have been due to B cells contaminating these populations.

C. B Cells

Proliferative and Polyclonal Responses to Mitogens or Anti-Ig Antibodies

Mitogens have proven to be useful tools to selectively probe for the presence of functional B cells in lymphocyte populations. For example, LPS and poly(I-C) directly stimulate proliferation in B but not T cells (Greaves and Janossy, 1972; Scher *et al.*, 1973b). Analyses of the responsiveness of CBA/N immune-defective mice to these mitogens revealed proliferative responses which were between 4- and 10-fold lower, and LPS induced polyclonal antibody responses which were as great as 100-fold lower in these mice than in controls (Scher *et al.*, 1975a; Cohen *et al.*, 1976). These unusually low responses were not due to differences in the dose of mitogen used to stimulate, or the kinetics of responses in defective mice, since they were noted with different concentrations of mitogens and after 24–96 hours of exposure. The influence of suppressor cells and/or the lower frequency of B cells found in the spleens of immune-defective mice (Scher *et al.*, 1975a) was ruled out, since populations of these cells that were depleted of T cells by anti-Thy antibody + C treatment continued to have reduced responses to LPS (Fig. 7). Subsequent studies have demonstrated an even greater reduction in the proliferative responses of B cells from immune-defective mice when cultured in the absence of fetal calf serum (FCS) (Glode and Rosenstreich, 1976; Kincade, 1977; Rosenstreich *et al.*, 1978).

In order to study the cellular basis of the reduced responses of CBA/N mice to B-cell mitogens, the mice were stimulated with TNP-

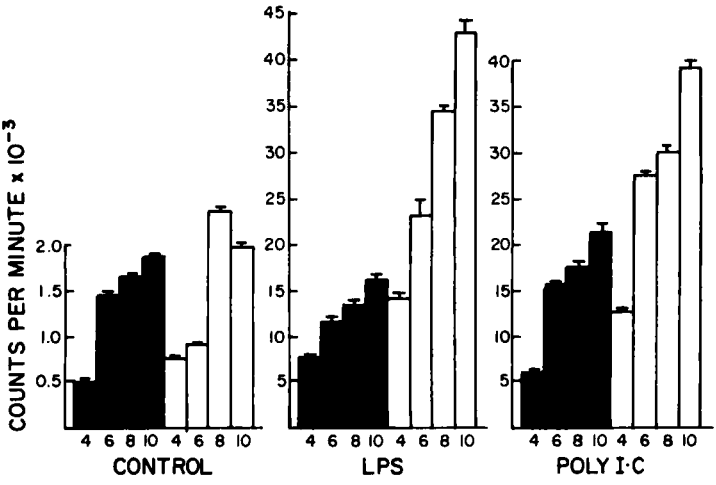

FIG. 7. Comparison of the proliferative uptake of [^{3}H]thymidine by increasing numbers of (CBA/N × DBA/2)F_1 male (closed bars) or female (open bars) spleen cells, depleted of adherent cells and T cells, in response to the B-cell mitogens LPS and poly(I-C) (Scher *et al.*, 1975a).

LPS and the responder cell frequency and magnitude of the PFC response was measured (Quintáns, 1979). By limiting dilution analysis, the frequency of TNP-LPS responding cells, in cultures of spleen cells from immune-defective mice, was approximately 3- to 4-fold less than the frequency of responding cells of normal spleen cells. By contrast, no differences were seen in the size of the responding anti-TNP clones derived from immune-defective or normal mice (i.e., PFC generated from individual precursor cells). Thus, the differences in the TNP-LPS-induced anti-TNP antibody responses of immune-defective and normal mice were due to a lower frequency of TNP-LPS responsive precursor cells in the defective mice rather than to the size of their clonal response. In another study of this topic, young adult immune-defective F_1 male mice produced 50- to 100-fold fewer IgM-secreting B cells after stimulation with phenol-LPS than their normal F_1 female littermates (Huber and Melchers, 1979). Similar differences were observed in the frequency of responding B cells in immune-defective vs normal mice after stimulation with the lipoprotein present in Boivin-LPS preparations. It also was noted in this study that the frequency of B cells responding to B-cell mitogens present in FCS was "at least 10-fold" less in immune defectives than in normals.

The LPS or poly(I-C)-induced proliferative responses of B cells derived from young adult vs aged immune-defective mice have been

studied. Responses of B cells from aged (>11 months) CBA/N mice have been reported to reach levels similar to those of normals (Whitlock and Watson, 1979; Fidler *et al.*, 1980). In contrast, however, similar studies have revealed no influence of age on the responsiveness of spleen cells from immune-defective mice to a number of different preparations of LPS (Nariuchi and Kakiuchi, 1981; Skelly *et al.*, in preparation) (see Sections III,A,2, III,A,4, and VI,6).

The responsiveness of B cells from immune-defective mice to other B-cell mitogens including dextran sulfate (Fernandez and Moller, 1977; Nariuchi and Kakiuchi, 1981), β-mercaptoethanol, purified protein derivative (PPD), Fc fragments of human immunoglobulin (FcHIg) (Fidler *et al.*, 1980; Nariuchi and Kakiuchi, 1981), and human B cell-activating factors (Cameron and Wood, 1978) has also been studied. PPD functioned like LPS and poly(I-C) in that it induced lower proliferative responses in B cells from immune-defective as compared to normal mice. By contrast, β-mercaptoethanol did not induce B-cell proliferation in immune defectives, while human B cell-activating factor induced equivalent responses in normals and immune defectives.

Fc fragments derived from human Ig induced strong proliferative responses in CBA/CaJ mice, whereas young adult CBA/N mice were unresponsive (Morgan *et al.*, 1980). However, the responsiveness of 12-month-old CBA/N mice was not significantly different from the response of 3-month-old or 12-month-old CBA/CaJ mice. Similarly, the low proliferative responses observed in young adult CBA/N spleen cells after stimulation with PPD were markedly improved and approached those of normal mice in 12-month-old CBA/N mice, whereas the spleen cells of aged CBA/N mice remained unresponsive to dextran sulfate (Nariuchi and Kakiuchi, 1981) (see Section VI,3).

CBA/N mice also differ from normal mice in their capacity to be stimulated by anti-Ig antibodies. Splenic B-cell proliferation was induced in all immunologically normal strains tested when cultured with soluble anti-κ, anti-μ (Sieckmann *et al.*, 1978a,b; Mongini *et al.*, 1978), or anti-δ antibodies (Sieckmann *et al.*, 1980; Sieckmann, 1980; Pure and Vitetta, 1980), as well as insolubilized anti-Ig antibodies (Parker, 1975; Puré and Vitetta, 1980). The T cell-independent nature of this stimulation has been established (Sieckmann *et al.*, 1978a,b), while the role of macrophages remains unresolved (Sieckmann *et al.*, 1978a,b; Mongini *et al.*, 1978). In contrast to the excellent proliferation observed in cultures of lymphoid cells of normal mice, cultures of B cells from adult immune-defective mice were refractory to anti-Ig stimulation (Fig. 8) (Sieckmann *et al.*, 1978a,b; 1980; Sieckmann, 1980).

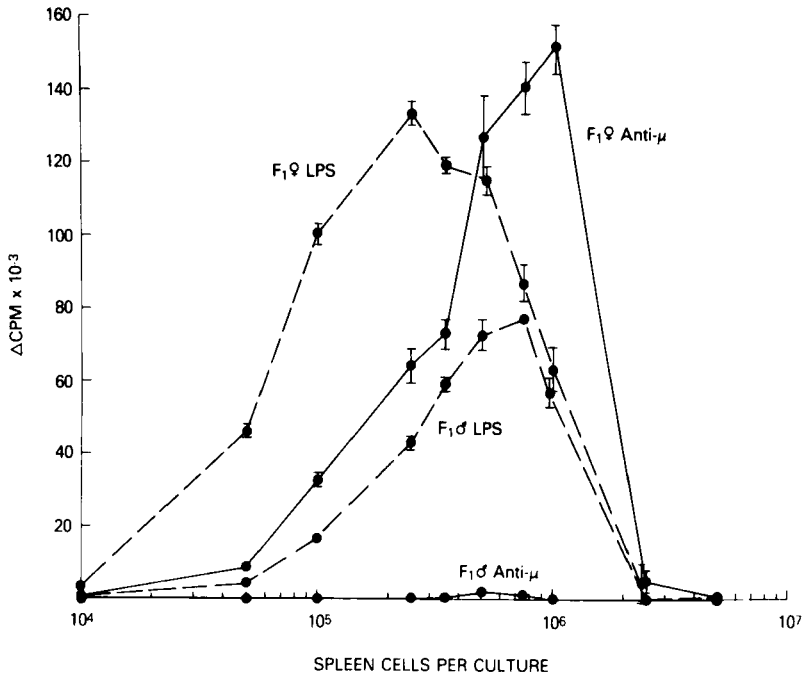

FIG. 8. Comparison of the proliferative responses (Δ counts per minute of [^{3}H]thymidine) of increasing numbers of (CBA/N × DBA/2)F_1 male or female spleen cells cultured in the presence of a goat anti-μ antiserum or LPS (Sieckmann *et al.*, 1978b).

D. Susceptibility to Tolerance

The susceptibility of CBA/N B cells to tolerance induction was of particular importance in view of the functional defects demonstrated in these cells when analyzed with TI-2 antigens or B-cell mitogens. A number of approaches have been employed in studies on this subject (E. Metcalf *et al.*, 1980; McKearn and Quintáns, 1980; Merchant *et al.*, 1978a,b). In early studies it had been demonstrated that a larger proportion of neonatal splenic B cells and a smaller proportion of adult bone marrow B cells were sensitive to tolerance induction when exposed to a tolerogen *in vitro* (E. Metcalf and Klinman, 1976). The tolerance induced in this system was independent of suppressor T cells, since treatment of the spleen cells of 3-week-old mice with anti-Thy + C did not alter the susceptibility of these cells to the tolerogen. When adult immune-defective F_1 male mice were tested in the splenic focus system, greater than 50% of the DNP-specific splenic

B cells were shown to be susceptible to tolerance induction, as opposed to less than 10% in normal mice (Table VII) (E. Metcalf *et al.*, 1980).

The tolerance induced in adult bone marrow is distinct from that of neonatal bone marrow or spleen cells, since while the frequency of IgM-producing adult bone marrow B cells is reduced by this treatment, both IgM- and IgG-producing neonatal B cells are reduced (E. Metcalf and Klinman, 1977). Exposure of adult F_1 male spleen cells to tolerogen in this system resulted in a reduction in both the IgM and the IgG responses (E. Metcalf *et al.*, 1980). Thus, the splenic B cells of adult immune-defective mice resemble the B cells of normal neonatal mice, but not bone marrow B cells of adult normal mice with regard to their susceptibility to tolerance induction.

A different approach to the analysis of the tolerance susceptibility of immune-defective mice was taken by McKearn and Quintáns (1980), who preincubated the tolerogen TNP-fowl γ-globulin (TNP-FGG) with B cells of immune-defective or normal mice prior to challenge with the TI-1 antigens TNP-BA or TNP-LPS. TNP-FGG affected the subsequent responses of normal neonatal B cells to TNP-BA and

TABLE VII

EFFECT OF *in Vitro* TOLERANCE INDUCTION ON THE ISOTYPE OF MONOCLONAL ANTI-DNP ANTIBODY RESPONSES IN (CBA/N × DBA/2)F_1 FEMALES AND MALES

Donor F_1 spleen cells	Tolerogen (10^{-6} *M* DNP-MγG)	Number of clones per 10^6 cells transferred[a]	Heavy-chain class: IgM — Number of clones per 10^6 cells transferred[b]	Heavy-chain class: IgG_1 — Number of clones per 10^6 cells transferred
Females	−	1.98 ± 0.21	0.83 ± 0.17	0.58 ± 0.08
	+	1.94 ± 0.26	0.74 ± 0.37	0.56 ± 0.18
Males	−	1.44 ± 0.26	0.56 ± 0.06	0.43 ± 0.06
	+	0.52 ± 0.15	0.04 ± 0.03	0.16 ± 0.08

[a] Donor spleen cells (4–6 × 10^6) were transferred to each recipient mouse. Recipient fragment cultures were incubated in the presence or absence of 10^{-6} *M* DNP-MγG for 24 hours, washed, and stimulated with DNP-Hy at 10^{-6} *M* DNP. DNP-specific clones were detected by radioimmunoassay of culture fluids with ^{125}I-labeled anti-mouse Fab, IgM, or IgG_1.

[b] The frequency of DNP-specific clones that produced both IgM and IgG_1 anti-DNP antibody in the absence or presence of tolerogen is: F_1 female, 0.39 and 0.31; F_1 male, 0.25 and 0.25, respectively (Metcalf *et al.*, 1980).

TNP-LPS differently. The responses to TNP-LPS were easily reduced, whereas those to TNP-BA were not. Thus, while 10^{-4} μg of FGG reduced the TNP-LPS responses of B cells of normal neonatal (4-day-old) mice by greater than 50%, it had no effect on the response of these cells to TNP-BA. When B cells of CBA/N adult mice were tested in this system, they were susceptible to tolerance induction to TNP-LPS but not TNP-BA at 10^{-4} mg/ml of tolerogen, while the B cells of adult normal mice required approximately 100 μg/ml of tolerogen to reduce the responses to either of these antigens.

The influence of DNP-Ficoll on subsequent responses of immune-defective F_1 male mice to the TD antigen DNP-KLH was studied in order to determine if the immune-defective cells would be influenced by this TI-2 antigen (Merchant *et al.*, 1978a,b). Exposure of immune-defective mice to low doses of DNP-Ficoll, 4 days to 2 hours prior to challenge with DNP-KLH, resulted in the complete suppression of the response to DNP-KLH. This suppression was most effective with highly substituted DNP-Ficoll conjugates and was also observed with TNP-S-III. Treatment of normal mice with DNP-Ficoll prior to immunization with DNP-KLH did not appear to alter their subsequent responses to DNP-KLH. It is important to note that in contrast to the profound effects that the DNP-Ficoll had on the DNP-KLH-induced anti-DNP responses in the primary response of immune defectives, DNP-Ficoll had no effect on the secondary anti-DNP-KLH responses of these mice. Thus, immune defectives were highly susceptible to DNP-Ficoll-induced suppression in primary but not secondary anti-DNP-KLH responses. Although it is likely that these effects of DNP-Ficoll on DNP-KLH response were the result of B-cell receptor blockade, the known influence of T cells on both TNP-Ficoll and S-III responses (Braley-Mullen, 1974; Baker, 1975; Mond *et al.*, 1980) makes it possible that these effects were mediated via T cells.

E. B-Cell Colony Formation

Clusters and colonies of up to 3000 cells develop in semisolid agar when murine spleen, lymph node, peritoneal cavity, thoracic duct, or blood cells are plated under appropriate conditions (D. Metcalf *et al.*, 1975). Important for the growth of these cells are mitogens, including LPS or other stimulatory factors, which can be supplied by SRBC, β-mercaptoethanol (D. Metcalf, 1976), macrophages (Kurland *et al.*, 1977), or the agar itself (Kincade *et al.*, 1976). The cells formed in the clusters or colonies bear Fc receptors; up to approximately 70% react with anti-μ antibodies, and their morphology is that of immature lymphoid and plasma cells (D. Metcalf *et al.*, 1975). The numbers of col-

onies formed under these conditions are proportional to the number of B cells placed in culture (D. Metcalf *et al.*, 1975; D. Metcalf, 1976; Kurland *et al.*, 1977). Thus, these semisolid agar cultures provide an assay for measuring the *in vitro* development of B-cell colonies.

The nature of the cell or cells involved for the development of these colonies was studied by analyzing the inhibitory effects of antisera directed against different lymphoid and/or macrophage cell surface constituents (Kincade *et al.*, 1978b). Thus, when adult spleen or lymph node cells were used as a source of the B-cell colonies, inhibition occurred when anti-Ia or anti-δ antiserum was incorporated into the soft agar medium. Colonies were also inhibited if the adult spleen or lymph node cells were first treated with anti-Ia antiserum + C. By contrast, when fetal or neonatal mouse tissue or adult bone marrow was used to generate colonies, anti-δ antiserum had little or no effect on colony formation. Differences in the inhibitory capacity of anti-Ig antiserum were also noted when neonatal and adult cells were compared for their ability to generate B-cell colonies. Thus, although it was clear that the semisolid agar cultures provide a method to functionally assess the development of B-cell colonies *in vitro*, the nature of the critical progenitor cell(s) in this system was elusive. What was apparent was that the colony-forming B cells derived from different lymphoid organs and at different stages of B-cell development represented a certain proportion of the B cells from these sites, that the maturity of these progenitors was variable and dependent on the different antisera used to inhibit, and that they were not representative of B cells as a whole (Kincade *et al.*, 1978b).

When cells of immune-defective mice were assayed for their ability to form B-cell colonies *in vitro*, it was shown that these mice lacked this function (Kincade, 1977). By contrast, the frequency of granulocyte–macrophage progenitors, as well as multipotential stem cells in the bone marrow of immune-defective mice, was normal. Cell-mixing experiments indicated that the inability of immune-defective mice to develop colonies was due to an intrinsic defect or absence of the progenitor of the colonies in these mice. It was concluded from these studies that CBA/N immune-defective mice lack a lineage of functionally specialized B cells (Kincade, 1977; Kincade *et al.*, 1978b).

F. Interactions of B Cells with T Cells and T Cell-Derived Helper Factors

As noted previously, when immune-defective mice were immunized with small doses of TD antigens, their primary IgG or IgG

responses were noted to be either absent or very low when compared to those of normal mice (Scher *et al.*, 1979; Boswell *et al.*, 1980b). These differences were not due to abnormalities in the T helper or accessory cell presentation function of immune-defective mice, since these functions were equivalent in these and normal mice when tested in adoptive transfer or in *in vitro* experiments (Janeway and Barthold, 1975; Scher *et al.*, 1979; Boswell *et al.*, 1980b). However, when lethally irradiated F_1 mice were reconstituted with primed F_1 T cells, along with B cells (anti-Thy treated spleen or bone marrow) from F_1 male mice, they made approximately 10-fold lower IgM or IgG anti-SRBC responses than mice reconstituted with F_1 female B cells (Fig. 9) (Scher *et al.*, 1979). Increasing the number of F_1 male B cells or primed T cells in these adoptive transfer experiments did not alter the low responses. These findings demonstrate that F_1 male B cells have a defect in their ability to respond to the TD antigen SRBC in the presence of T cells which provide sufficient help to F_1 female B cells.

The capacity of the F_1 male B cells to respond to SRBC was considerably improved if the B cells were derived from mice that had been primed to SRBC. However, the IgG anti-SRBC responses of these cells remained approximately 7-fold less than those of primed F_1 female B cells (Scher *et al.*, 1979). Different results were obtained by E. Metcalf *et al.* (1980) who demonstrated that the IgM and IgG anti-TNP responses of these mice to TNP-KLH were equivalent to normals. These studies utilized KLH-primed, lethally irradiated F_1 female mice as recipients for defective F_1 male or female spleen cells. Splenic fragments, containing accessory and helper T cells of the primed recipient as well as the B cells from the donor, were then cultured in the presence of TNP-KLH to study the products of individual B cells. Under these conditions T-cell help is said to be maximized.

The TD B-cell function of immune-defective mice was also tested in an *in vitro* Mishell–Dutton assay (Boswell *et al.*, 1980b). Under these conditions, defective F_1 male splenic B cells failed to make anti-TNP antibody when cultured with TNP-KLH in the presence of accessory and T cells from normals or defective F_1 mice. This failure was not the result of death of the F_1 male B cells in these cultures, since these cells made excellent responses to the TI-1 antigen TNP-BA. Thus, it is apparent that the B cells of immune-defective mice are unable to support a TD antibody response equivalent to that of normal mice under certain *in vitro* or *in vivo* conditions. This is presumably a reflection of the abnormal TD responses observed in intact CBA/N mice when suboptimal doses of antigen are used to challenge. However, priming of the B cells from defective mice either eliminates or lowers

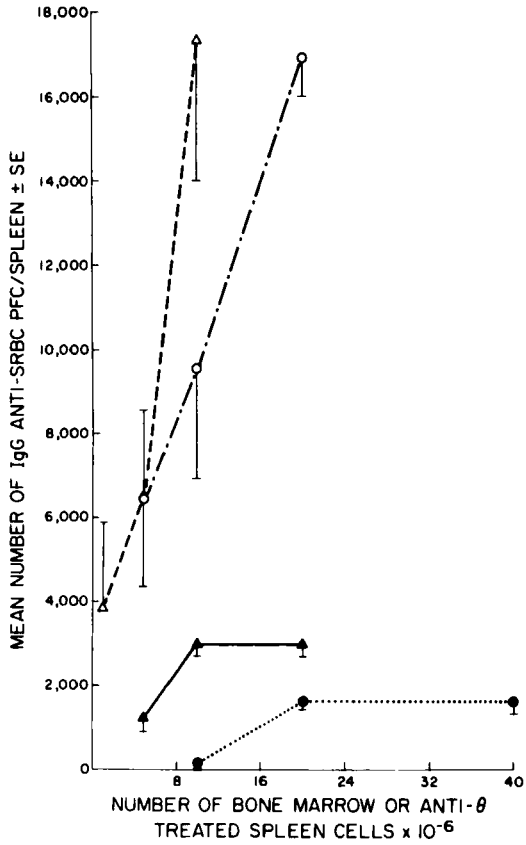

FIG. 9. Comparison of the ability of graded numbers of bone marrow (O) or splenic B cells (Δ) from abnormal male (CBA/N × DBA/2)F_1 (closed symbols) or normal male (DBA/2 × CBA/N)F_1 mice (open symbols) to make IgG anti-SRBC responses in adoptive transfer experiments. Lethally irradiated (CBA/N × DBA/2)F_1 male mice were immunized with SRBC and reconstituted with bone marrow or anti-theta treated spleen cells, as noted, plus 2 × 10^6 (DBA/2 × CBA/N)F_1 male-activated T cells (Scher *et al.*, 1979).

the differences in the TD responses of these cells when compared to those in normals. Furthermore, in the splenic focus assay, where accessory and T-cell help are maximized, the TD IgM and IgG antibody responses of B cells from immune-defective mice are equivalent to those of normals.

The mechanism(s) responsible for the abnormal TD B-cell responses of immune-defective mice has not been fully elucidated (see Section VI,A). However, the failure of B cells from these mice to respond to

certain T cell-derived factors may help to explain this phenomenon. The two factors studied in this system are allogeneic effect factor (AEF) and TRF. AEF is an antigen-nonspecific helper factor which reconstitutes the *in vitro* antibody responses of murine T cell-depleted B cells to TD antigens (Armerding and Katz, 1974). In order to determine if the B cells of immune-defective mice were responsive to AEF, T cell-depleted spleen cell cultures of (CBA/N × DBA/2)F_1 male and female mice were stimulated with TI-1, TI-2, or TD antigens in the presence of AEF (Yaffe *et al.*, 1980). AEF was able to reconstitute the responses of B cells from F_1 male or female mice to the TD antigen SRBC in these experiments and also augmented the TNP-BA responses from these cells, as well as the TNP-Ficoll responses from the F_1 female cells. However, AEF had no effect on the inability of F_1 male B cells to respond to TNP-Ficoll.

A cloned helper T-cell line, L2, was derived by stimulating leukocyte culture cells with H-2 and Mls-disparate spleen cells (Glasebrook *et al.*, 1981). This line, when activated by Mls-bearing spleen cells, produced a polyclonal stimulation of B cells which was mediated by soluble factors. When L2 cells were stimulated by F_1 female, Mls^+ spleen cells, they produced polyclonal activation of both normal F_1 female and immune-defective F_1 male B cells. The activation induced in male and female cells was quantitatively and qualitatively distinct, since no IgM anti-TNP PFC were observed when male cells were plaqued against SRBC which were conjugated with 20 TNP groups/SRBC (TNP_{20}SRBC), while female cells gave 550 PFC/10^6, whereas when plaqued against TNP_{80} SRBC, the response of the male cells was 50% that of the female cells.

These experiments gave quite distinct results from those obtained using TRF, a factor produced by Con A-stimulated T cells which is also able to reconstitute TD responses from T-cell-depleted spleen cell populations (Schimpl and Wecker, 1975). TRF reconstituted an SRBC response and augmented the responses of F_1 female T-depleted spleen cell populations to both TNP-BA and TNP-Ficoll (Yaffe and Scher, 1981). By contrast, TRF had no effect on the T-depleted spleen cells of immune-defective F_1 male mice, so that no reconstitution of the SRBC or augmentation of the TNP-BA responses was seen.

An interesting correlation exists between these findings with TRF, the functional properties of anti-Lyb 3 (see Sections V,F, VI,A, and VI,B) and the abnormal responses of defective mice to TD antigens. When normal mice are challenged with low doses of SRBC, their responses can be markedly augmented by the administration of small amounts of anti-Lyb 3 antiserum (Huber *et al.*, 1979). This antiserum

detects specificities on approximately 50% of normal adult splenic B cells, but does not react with CBA/N B cells (see Section V,F). Anti-Lyb 3 serum has no effect on the responses of immune-defective mice to these or any doses of SRBC. It has been postulated that the Lyb 3 receptor of normal B cells is in fact a receptor for T-cell helper factors (Huber *et al.*, 1979) and in a more recent paper it was noted that molecules binding to TRF are contained in this receptor (Huber and Borel, 1981). Evidence that the Lyb 3 receptor plays a role in the activation of B cells has also been obtained in studies of B-cell tolerance (Huber and Borel, 1981; Sherr *et al.*, 1981), the induction of autoantibodies (Kemp *et al.*, 1980), and in alteration of the IgG isotype during a TD response (Huber *et al.*, 1981b).

The interactive properties of CBA/N B cells were recently studied using T-cell helpers generated in chimeric mice (Singer *et al.*, 1981). In these experiments T-cell helpers were obtained from chimeras of H-$2^{b/b}$ recipients that had been reconstituted with H-$2^{b/k}$ bone marrow after lethal irradiation. These T cells are tolerant to both donor and host MHC determinants and can only recognize the host MHC determinants. Thus, H-$2^{b/k}$ T-cell helpers from these chimeras can recognize H-$2^{b/b}$, but not H-$2^{k/k}$ accessory cells and/or B cells. CBA/J B cells (H-$2^{k/k}$) were able to make excellent PFC responses to SRBC when used in adoptive transfers with H-$2^{b/b}$ accessory cells and/or B cells. By contrast, CBA/N B cells (H-$2^{k/k}$) failed to make responses in this system, but made excellent responses with T-cell helpers from F_1 mice that recognized H-$2^{b/b}$ and H-$2^{k/k}$ cells. These data indicate that the T-cell helper activation of B cells from immune-defective CBA/N mice is genetically restricted, while the activation of CBA/J B cells is not.

G. Ability of B Cells to Accept Accessory Cell Antigen Presentation

TI-2 and TD antigens require antigen-presenting cells in order to induce B-cell activation (Rosenthal and Shevach, 1973; Thomas and Shevach, 1976; Singer *et al.*, 1979; Boswell *et al.*, 1980c; Kirkland *et al.*, 1980), while TI-1 antigens are able to induce activation in the absence of antigen-presenting cells (Lemke *et al.*, 1975; Yoshimaga *et al.*, 1972; Mond *et al.*, 1979a; Boswell *et al.*, 1980a,b,c). Although there is controversy as to the nature of the cell(s) which present TI-2 antigens, with some groups presenting evidence that adherent macrophages play this role (Boswell *et al.*, 1980c) while others suggest that B cells and not macrophages are important (Kirkland *et al.*, 1980), it is clear that this function is intact in immune-defective mice (Boswell *et al.*, 1980b). It was therefore possible that abnormal TI-2 and TD re-

sponses in immune-defective mice were the result of their B cells being less responsive to accessory cell activation signals than B cells from normals and defectives. This was studied by presenting TNP-BA to B cells from immune-defective or normal F_1 mice in suspension or via antigen-presenting cells (Fig. 10) (Boswell *et al.*, 1980b). As TNP-BA is a TI-1 antigen, one would expect that it would induce responses in the B cells derived from both sources, no matter how it was presented. However, while TNP-BA induced responses in cultures of spleen cells derived from immune-defective F_1 male and normal F_1 female mice when added as a suspension, only F_1 female B cells were activated when TNP-BA was presented using pulsed-adherent spleen cells. These data indicate that the B cells of immune-defective F_1 male mice are not capable of accepting accessory cell activation signals.

It is of particular interest that in studies of the association of macrophages with splenic lymphocytes, it was shown that B but not T cells bound to these cells (O'Toole and Wortis, 1980). Indeed, it also was shown that only a subpopulation of B cells was able to bind to macrophages and that this population bore the Ia.W39 alloantigen. This alloantigen had been described previously as being absent from the B

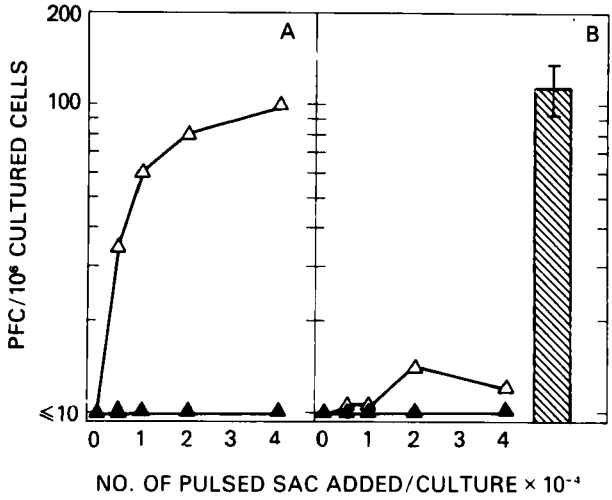

FIG. 10. Comparison of the ability of normal male (DBA/2 × CBA/N)F_1 G-10 passed (A) and abnormal male (CBA/N × DBA/2)F_1 G-10 passed (B) spleen cells to accept graded numbers of accessory cell presented TNP-BA (△). Accessory cells were pulsed with media as control (▲). The response to TNP-BA suspension by the (CBA/N × DBA/2)F_1 male G-10 passed spleen cells is also indicated in the bar graph (Boswell *et al.*, 1980).

cells of immune defectives and from Lyb 3^- B cells of normal mice (Huber, 1979). Furthermore, whereas B cells from immunologically normal F_1 female mice were able to bind to macrophages, those from immune-defective F_1 male mice were not (O'Toole and Wortis, 1980). Although the role of macrophages in the activation of B cells by anti/Ig antibodies or TI-2 antigens is controversial (Sieckmann *et al.*, 1978a,b; Bowell *et al.*, 1980c; Kirkland *et al.*, 1980; Mongini *et al.*, 1978), the roles of these cells in the activation of B cells to TD antigens and their importance in the generation of B-cell colonies in soft agar *in vitro* are not (Rosenthal and Shevach, 1973; Thomas and Shevach, 1976; Singer *et al.*, 1979; Kurland *et al.*, 1977). Thus, the findings of O'Toole and Wortis (1980), coupled with the data indicating that CBA/N B cells cannot accept the activation signals provided by antigen-presenting accessory cells (Boswell *et al.*, 1980a,b), may pinpoint the mechanism underlying at least some of the functional differences between the B cells of immune-defective and normal mice.

V. Surface Membrane Characteristics of B Cells of CBA/N Mice

A. Frequency and Numbers of B Cells

Spleens from either CBA/N or defective F_1 male mice are considerably smaller than those of normal mice, and the number of nucleated cells/spleen reflects this difference (Scher *et al.*, 1975a). Thus, whereas adult CBA/N male and female as well as immune-defective F_1 male mice had mean numbers of nucleated spleen cells of approximately 60×10^6, CBA/J male and female and immunologically normal F_1 female mice had approximately 100×10^6 nucleated cells/spleen. Similarly, the frequency of κ-bearing cells in these mice, detected by fluorescein or ^{125}I-conjugated anti-κ antibodies was approximately 50% of normals. In these studies, where conventional microscopic techniques were utilized, the mean frequency of κ-bearing B cells was approximately 24% in the immune-defective vs approximately 42% in normal mice (Scher *et al.*, 1975a). It has been suggested that with age the frequency of Ig-bearing cells in immune-defective mice increases to that of normals (Whitlock and Watson, 1979). However, although the frequency of Ig-bearing spleen cells of 60-week-old immune defectives had reached that of young adult normal mice in this study, the frequency of these cells in aged normals was not shown. In a more recent study where the frequency of IgM-bearing spleen cells was analyzed with the fluorescence-activated cell sorter (FACS), three individual 51-week-old immune-defective F_1 male mice had a mean of

32.0%, while their normal F_1 female littermates had a mean of 49.5% positive cells (Scher *et al.*, 1980).

The frequency of surface IgM-bearing cells in other lymphoid organs of immune-defective mice also has been studied (Scher *et al.*, 1980). Immune-defective F_1 male mice had approximately one-half to one-third of the surface IgM-bearing bone marrow (3.4 vs 7.2%), Peyer's patch (13.9 vs 39.3%), or lymph node cells than their normal F_1 female littermates.

B. Surface Immunoglobulin Isotypes and Light Chains

Studies of murine B cells using lactoperoxidase-catalyzed surface iodination, immunoprecipitation with polyvalent anti-Ig reagents, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), have demonstrated two major isotypes of surface Ig on murine B cells (Vitetta *et al.*, 1975). These isotypes, μ and δ, are present on the majority of adult splenic cells (Scher *et al.*, 1980). However, cells bearing only μ predominate in the lymphoid organs of neonatal mice (Vitetta *et al.*, 1975; Scher *et al.*, 1980). When the splenic B cells of immune-defective mice were first studied using radioiodination and immunoprecipitation techniques, it was shown that adult immune-defective mice had a much higher μ to δ ratio of ^{125}I than was found in the splenic cells of normal mice (Finkelman *et al.*, 1975). Although the ratio of μ vs δ radioactivity in the defectives resembled that observed in the spleens of neonatal normal mice, it was not clear if this similarity was due to a high incidence of $\mu^+\delta^-$ cells in the immune-defective mice.

This question has been studied using the technique of rapid flow microfluorometry with a FACS (Scher *et al.*, 1976a, 1980), which can quantitatively determine the amount of fluorescence on individual lymphoid cells. In early studies using this approach it was shown that the splenic B cells of immune-defective mice had a high mean fluorescence intensity after labeling with fluorescein-conjugated anti-μ antibodies as compared to the B cells of normal mice (Scher *et al.*, 1976a). Thus, whereas approximately 24% of the surface IgM-bearing cells in normal adult mice bore relatively high densities of surface IgM, approximately 63% of surface IgM-bearing cells from adult CBA/N mice had this characteristic (Fig. 11). Indeed, when the surface IgM-bearing splenic cells of developing CBA/N mice were analyzed by this technique, the percentage of these cells with a relatively high density of μ was 59–63% (Fig. 11). By contrast, the density of μ on B cells of developing normal mice decreased as these animals matured, with the percentage of μ-bearing cells of high relative density decreasing from

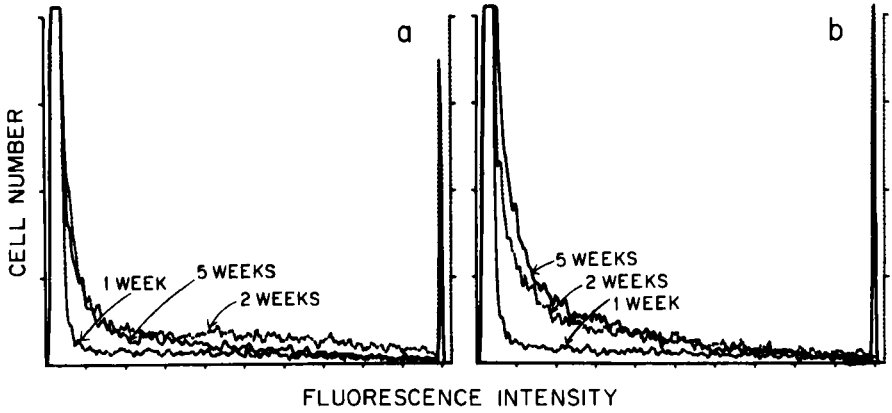

FIG. 11. The anti-μ flourescence profiles of spleen cells from developing normal BALB/c (b) and CBA/N mice (a). The fluorescence intensity is plotted on the x axis with increasing intensity toward the right.

49, 41, 33, and 27% at 7, 14, and 35 days, respectively. These data suggested that the high μ/δ ratio in adult immune-defective mice was in part related to the relatively high density of μ on their cells. However, it was still possible that these mice had a predominance of the $\mu^+\delta^-$ cells found in the spleens of neonatal normal mice.

The availability of affinity-purified heterologous anti-mouse δ antibodies made it possible to resolve this problem (Scher *et al.*, 1980). Virtually all of the μ-bearing spleen B cells of adult immune-defective F_1 male and normal F_1 female mice also bore δ, since the frequency of μ- and δ-bearing cells in the spleens of these mice was similar. The density of δ on the F_1 male cells was only slightly less than that of the normal F_1 female cells. Thus, the high ratio of μ to δ present on immune-defective adult splenic B cells is the result of the high relative density of μ on these cells, and is not due to a predominance of $\mu^+\delta^-$ cells in these mice. The cells of immune-defective mice are similar in this regard to a large fraction of B cells found in the spleens of developing immature normal mice.

Although the numbers of μ- and δ-bearing cells are equivalent in the spleens of adult immune-defective and normal mice, indicating that few if any $\mu^+\delta^-$ cells exist in this organ at this stage of development, this situation is quite different when immature mice are studied. Thus, larger numbers of μ^+ than δ^+ cells are found in the spleens and bone marrow of both normal and immune-defective mice earlier in development. However, $\mu^+\delta^-$ cells become a small minority of splenic B cells in the spleens of immune defectives at approximately 120 days,

whereas these cells are absent or in very small numbers in the spleens of normal mice at 50 days of age (Fig. 12) (Scher *et al.*, 1980).

Recently it has been suggested that there is a shift in the κ to λ light chain ratio as murine B cells develop (Haughton *et al.*, 1978; Golding *et al.*, 1980). Adult murine splenic B-cell surface Ig possesses predominantly (approximately 95%) κ light chains (Hood *et al.*, 1967). However, analyses of the κ/λ ratio on the B cells of developing mice found that there was a more balanced expression of light chains on immature B cells (Haughton *et al.*, 1978; Golding *et al.*, 1980). These studies suggested that the shift to the κ predominant phenotype occurred in normal mice at 4 to 6 weeks of age, in apparent coincidence with the development of large numbers of $\mu^+\delta^+$ B cells. When studied in CBA/N mice, the low κ/λ ratios typical of neonatal B cells persisted throughout life (Golding *et al.*, 1980). In addition, in a double-blind study of F_2 mice ($F_1 \times F_1$), the low κ/λ ratio was associated with an inability of these mice to respond to TI-2 antigens or to form B-cell colonies in soft agar.

The results of these studies are distinct from those obtained in two

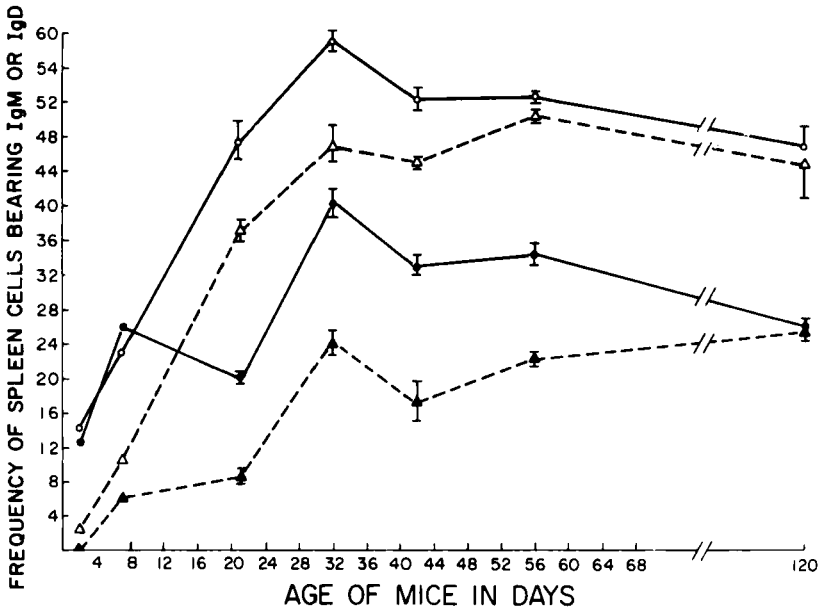

FIG. 12. The frequency of μ-bearing spleen cells in abnormal male (CBA/N × DBA/2)F_1 (●——●) and normal female F_1 mice (○——○) during development and in adults. The frequency of δ-bearing spleen cells in abnormal (▲---▲) and normal (△--△) mice is also indicated (Scher *et al.*, 1980).

more recent publications, that employed FACS analysis and affinity purified anti-λ antibodies (Kessler *et al.*, 1981; McGuire and Vitetta, 1981). These authors found only minor differences in the κ/λ ratios of immature and adult B cells of either CBA/N or normal mice, with λ-bearing cells representing only a minor fraction of the B cells. Although the findings in the studies of the F_2 mice of Golding *et al.* (1980) are most provocative, the extensive documentation of the reactivity of the anti-λ reagent used in the latter two studies rules out any significant differences in the κ/λ ratios of CBA/N and normal mice. Further studies to identify the additional specificities contained in these serum which distinguished immune-defective F_2 mice from normals would be most interesting.

C. Surface I-Region-Associated Antigens

Surface Ia antigens can be found on virtually all B cells of mature mice (Kearney *et al.*, 1977; Mond *et al.*, 1980). These antigens appear early in the differentiation of B cells, since Ia antigens are found on the surface of B cells derived from neonatal and adult bone marrow as well as neonatal spleen cells. However, μ^+Ia^- B cells have been demonstrated in the bone marrow of 4-week-old mice (Hammerling *et al.*, 1975, 1976). These μ^+Ia^- bone marrow cells expressed Ia after stimulation with LPS, a phenomenon which was suppressed if the cells were first treated with anti-Ig antibodies + C. Furthermore, Kearney *et al.* (1977) demonstrated that a large proportion of surface IgM-bearing fetal spleen and adult bone marrow cells was surface Ia^-. The similarity of the surface Ig characteristics of immune-defective CBA/N adult B cells to those of neonatal normal B cells suggested that there might be a high percentage of surface Ia^- cells among the surface IgM-bearing B cells of adult CBA/N mice. This question was studied using a mouse anti-mouse Ia antisera (ATH anti-ATL) and rapid flow microfluorimetry (Mond *et al.*, 1980). The frequencies of surface Ia- and surface IgM-bearing cells in the lymphoid organs of adult immune-defective F_1 male mice were virtually identical, demonstrating that there was no increase in the proportion of μ^+Ia^- cells in these mice.

In studies of an antiserum which was initially developed to identify possible allelic polymorphism of Lyb 3 (see Section V,F), Huber (1979) detected a cytotoxic specificity in some preparations which was distinct from the noncytotoxic anti-Lyb 3 specificity. Thus, whereas anti-Lyb 3 binds approximately 50% of B cells derived from all normal mouse strains by immunofluorescence (Huber *et al.*, 1979), these antisera were cytotoxic for 25–35% of B cells of $H\text{-}2^b$ mice only (Huber, 1979). Mapping studies demonstrated that at least one gene coding for

the marker was located in the I-A subregion of the major histocompatibility complex (MHC), and that it was possible that a second gene in the I-B region was also necessary for the expression of this marker. Because this antiserum was made by immunization of (CBA/N × C57BL/6)F_1 male mice with C57BL/6 spleen cells, the absence of this Ia antigen from B cells of immune-defective mice is indicated by definition. It is significant that although virtually all of the surface IgM-bearing B cells of adult immune-defective and normal mice bear surface Ia antigens, this I-A specificity, termed Ia.W39 (Demant, 1979), is present on approximately 50% of normal Lyb 3-bearing B cells and is not expressed on B cells of immune-defective CBA/N mice. The Ia antigen bearing this specificity has a similar two-chain structure and two-dimensional gel pattern to conventional Ia molecules. Interestingly, Ia.W39 appears to be synthesized in the cytoplasm but not expressed on the surface of B cells of either immune-defective or normal neonatal mice (Huber *et al.*, 1981a).

D. Minor Lymphocyte-Stimulating Determinants

In mice, the MLR is under the independent control of two genetic systems, the multigenic major histocompatibility complex (Dutton, 1966) and the unigenic non-H-2 Mls locus (Festenstein, 1973). A number of studies have demonstrated that Mls-determined markers are present on B but no T cells. In the first of these studies, von Boehmer and Sprent (1974) demonstrated that thymus cells were not stimulatory, whereas spleen cells and B cells isolated by electrophoresis from thoracic duct lymphocytes were stimulatory. It was also shown that while anti-Thy + C treatment enhanced the stimulatory capacity of spleen cells in an Mls-determined MLR, treatment with an antiserum known to kill B but not T cells (anti-mouse B-lymphocyte antisera) (Raff *et al.*, 1971) abolished the stimulatory capacity of these cells (Ahmed *et al.*, 1977a). Correlations between the presence of B cells and Mls-determined MLR were also demonstrated by positive selection of Ig-bearing cells via cell sorting and by the absence of stimulatory activity from spleen cells of mice depleted of B cells by anti-μ antibody treatment (Ahmed *et al.*, 1977a).

The Mls locus appears to code for four codominant alleles termed Mls^a, Mls^b, Mls^c, and Mls^d. Interestingly, lymphoid cells from mice that express the Mls^b allele, as found in the BALB/c strain, fail to stimulate an Mls distinct MLR, but are excellent responders. The inability of splenic B cells of mice to stimulate an Mls-determined MLR is not unique to Mls^b mice, since neonatal spleen cells derived from strains of mice with other Mls alleles were also nonstimulatory

(Ahmed *et al.*, 1977a). Indeed, the capacity to stimulate an Mls-determined MLR did not appear until 2 weeks of age in the DBA/2 (Mls^a) strain. With further maturation (approximately 4–5 weeks of age), these mice acquire the capacity to stimulate at the same level as adults. The inability of neonatal spleen cells to stimulate an Mls-determined MLR was shown to be an intrinsic property of these cells, since positively selected, neonatal, Mls^a, surface Ig-bearing cells were also nonstimulatory, and neonatal spleen cells from these mice did not suppress the responses of adult Mls^a splenic cells.

When CBA/N spleen cells were used as responders in Mls-determined MLR, stimulation was seen with both C3H/HeN and CBA/J spleen cells (Ahmed and Scher, 1976). By contrast, CBA/N spleen cells were unable to stimulate responses in either C3H/HeN or CBA/J responders, suggesting that CBA/N mice bore the nonstimulatory Mls^b allele (Table VIII). However, spleen cells of F_1 female mice derived from crosses of CBA/N with C3H/HeN mice were able to induce excellent responses in C3H/HeN cells, whereas F_1 male spleen cells were nonstimulatory. These data are supported by experiments using a cloned T-cell line, L2, which recognizes Mls^a but not H-2 determinants on allogeneic spleen cells (Glasebrook *et al.*, 1981). Spleen cells from immune-defective F_1 male mice derived from crosses of CBA/N and DBA/2 ($Mls^{a/a}$) mice failed to stimulate the L2 line, whereas F_1 female cells were stimulatory. Thus, the inability of CBA/N mice to induce an Mls-determined MLR appeared to be due to an X-linked failure of the B cells of these mice to express these determinants, and was not due to CBA/N mice bearing the nonstimulatory Mls^b allele.

TABLE VIII
STIMULATORY CAPACITY OF CBA/N SPLEEN CELLS IN AN Mls-DETERMINED MLR

Stimulator cells			Stimulation index of responder cells[a]		
Strain	H-2	Mls	CBA/N	CBA/J	C3H/N
CBA/N	k/k	?/?	1.0	1.0	1.2
CBA/J	k/k	d/d	4.3	1.0	4.0
C3H/N	k/k	c/c	2.3	3.7	1.0
(CBA/N × C3H/N)F_1 ♀			6.1	5.1	5.7
(CBA/N × C3H/N)F_1 ♂			1.1	1.0	1.1

[a] The stimulation index was calculated by dividing the mean uptake of [^{3}H]TdR in cpm of the experimental cultures by the mean uptake of controls (Ahmed and Scher, 1976).

E. Complement Receptors

The complement receptor (CR) can be detected on approximately 30% of adult spleen cells, or 50% of B cells from this organ (Bianco *et al.*, 1970; Bianco and Nussenzweig, 1977). This receptor is absent from the spleens of newborn mice and the appearance of cells bearing the CR receptor lags considerably behind that of surface Ig-bearing cells (Gelfand *et al.*, 1974b). Thus, the CR serves as a marker to distinguish two populations of adult splenic B cells, with the population lacking this receptor presumably representing the less mature fraction (Gelfand *et al.*, 1974a). Selection of adult splenic B cells on the basis of their surface μ density has demonstrated a negative correlation between the frequency of B cells bearing the CR and the presence of increasing amounts of surface μ on these cells (Scher *et al.*, 1977a). On the basis of these data, the two populations of adult splenic B cells already alluded to can be characterized as μ high, low-frequency CR bearing, and μ low, high-frequency CR bearing. Studies on adult immune-defective F_1 male mice indicate that approximately 10% of the B cells of adult were CR^+, whereas 50% of F_1 female B cells bore this receptor (J. Mond, A. Ahmed, and I. Scher, unpublished data). These findings are in agreement with another study which showed that the percentage of CR^+ lymphocyte was lower in the spleens of CBA/N than in normal CBA control mice (Lindsten and Andersson, 1981). Thus, when antibody and complement-coated erythrocytes (EAC) were utilized that were made with different dilutions of sera containing complement, the percentage of CR^+ lymphocytes was 38 vs 27, 36 vs 27, and 37 vs 13 at 1 : 4, 1 : 20, and 1 : 40 serum dilutions in CBA and CBA/N mice, respectively.

F. Lyb Antigens

The cumulative functional studies on CBA/N immune-defective mice led to the proposal that the expression of the *xid*-encoded defect resulted in the failure of a unique B-cell subpopulation to appear (Scher *et al.*, 1976a, 1977b; Ahmed and Scher, 1976). The putative population which was absent in adult immune defectives was presumably that which appeared late in the development of normal mice, and which would be distinguished on the basis of the high density of surface μ, the presence of Mls-encoded surface antigens, and the high frequency of CR cells. The presumed absence of these cells in immune-defective CBA/N mice provided a stratagem for the development of antisera that would detect B-cell alloantigens present on the

late-developing subpopulation in the spleens of adult immunologically normal mice.

Two approaches to the development of such antisera have proven successful. In one approach immune-defective F_1 male mice were immunized with lymphoid cells from the normal parental strain; i.e. (CBA/N × BALB/c)F_1 male anti-BALB/c which identified an antigen termed Lyb 3, and (CBA/N × C57BL/6)F_1 male anti-C57BL/6 (Huber *et al.*, 1977; Huber, 1979), which identified I-A region controlled specificities (see Section V,C), in addition to Lyb 3. A different approach was taken by Ahmed *et al.* (1977b), who utilized immune-defective (CBA/N × DBA/2)F_1 male lymphoid cells as well as DBA/2 thymus cells to absorb a C57BL/6 anti-DBA/2 antiserum. This antiserum was shown to have two specificities, a cytotoxic specificity termed Lyb 5, and a functional specificity termed Lyb 7 (Subbarao *et al.*, 1979a,b).

Anti-Lyb 3 antiserum [(CBA/N × BALB/c)F_1 male anti-BALB/c] requires no absorption for specificity and by immunofluorescence reacts with approximately 50% of B cells, derived from immunologically normal adult mice and 15–20% of B cells from 18-day-old mice (Huber *et al.*, 1977). The spleen cells of newborn normal mice, as well as those derived from the spleens of immune-defective mice, showed no reactivity with anti-Lyb 3 antiserum. Although correlations between Lyb 3 and Mls determinants, μ density, or the CR have not been made, Huber *et al.* (1979) have shown that 85–90% of surface IgG_{2^-}, IgG_{3^-}, or IgA-bearing lymph node or spleen cells bear Lyb 3. Immunoprecipitation studies with this antiserum performed on surface membrane-radioiodinated spleen cells have resulted in the identification of a B cell associated polypeptide of 68,000 molecular weight (Cone *et al.*, 1978).

After extensive absorption with DBA/2 thymus and (CBA/N × DBA/2)F_1 male spleen cells, a cytotoxic C57BL/6 anti-DBA/2 antiserum was prepared which reacted with a fraction of F_1 female but not male spleen cells (Ahmed *et al.*, 1977b). This antiserum killed approximately 25–30% of spleen cells from F_1 female or DBA/2 mice, 60% of positively selected Ig-bearing cells from these strains, and 75% of positively selected Ig^+, CR-bearing splenic B cells. The frequency of low-density μ-bearing spleen cells lysed by this antiserum from these immunologically normal adult mice was 81% compared to lysis of only 21% of high-density μ-bearing spleen cells. The cells bearing a low density of μ on their surface, which were highly susceptible to lysis with anti-Lyb 5 antisera, were shown in a different study to also

bear the CR and to be Mls stimulatory, while the high-density μ-bearing cells were not stimulatory and had few CR-bearing cells (Scher *et al.*, 1977a). These data indicate that the Lyb 5 reactivity was specific for the low-μ, Mls-stimulatory, high-frequency CR-bearing B cells found in the spleen of adult normal mice. This was supported by data demonstrating that anti-Lyb 5 antisera + C did not kill neonatal normal B cells.

In the course of analyzing the functional properties of Lyb 5^+ and Lyb 5^- B-cell subpopulations, it was shown that anti-Lyb 5 antisera had important effects on B-cell function in *in vitro* cultures (Subbarao *et al.*, 1979b). Thus, when present continuously, anti-Lyb 5 antiserum suppressed the TNP-Ficoll or TNP-dextran responses by approximately 70–90%. By contrast, anti-Lyb 5 antiserum had little or no effects on the anti-TNP *in vitro* responses of splenic cells to either TNP-LPS or TNP-BA. The finding that AL/N mice were sensitive to the cytotoxic activity, but resistant to the blocking activity of these antisera, suggested that these activities were dependent upon the recognition of different determinants on Lyb 5^+ B cells. This was confirmed in studies of progeny of (C57BL/6 × DBA/2)F_1 × C57BL/6 mice, where it was shown that the loci specifying Lyb 5 and those specifying for the blocking activity were not linked. The blocking activity has been defined as anti-Lyb 7. Antisera with monospecific anti-Lyb 7 blocking activity were prepared from anti-Lyb 5 antisera by utilizing AL/N lymphoid cells for absorption (Subbarao *et al.*, 1979a).

Since B cells of immune-defective mice fail to stimulate Mls-determined MLR (Ahmed and Scher, 1976), it was possible that anti-Lyb 5 or anti-Lyb 7 was directed against Mls-encoded antigens. In addition, since the B cells of immune-defective mice have a high ratio of surface μ to δ (Finkelman *et al.*, 1975; Scher *et al.*, 1980) and since immune defective spleen cells are used as adsorbents to make anti-Lyb 5 antisera specific (Ahmed *et al.*, 1977b), it was also possible that the blocking action of Lyb 7 reflected incompletely absorbed anti-δ antibody. However, it was shown that the genes coding for both Lyb 5 and Lyb 7 segregate independently of Mls genes and that the genes controlling Lyb 7 and the C_H region of δ heavy chain are not identical (Subbarao *et al.*, 1979a).

Although the activities of anti-Lyb 5 and anti-Lyb 7 are not due to anti-δ antibodies in these antisera, analyses of backcross mice revealed an apparent linkage of the genes controlling the expression of Lyb 7 and the IgC_H locus. Analysis of 19 recombinant inbred strains revealed seven with the phenotype for Lyb 7 and the IgG_{2a} allotype, a finding which is consistent with the linkage between these two loci. This

linkage is particularly provocative in view of the role of the products of these Lyb 7 and IgC_H loci in the activation of B cells (Subbarao *et al.*, 1979a).

VI. Cellular Basis of the CBA/N Immune Defect

A. Intrinsic Nature of B-Cell Defect

The absence of TI-2 responses in immune-defective mice and the apparent B-cell abnormality in this strain could have been related to an isolated dysfunction in otherwise normal B cells of these mice, the absence of a subpopulation of B cells which was normally responsive to TI-2 antigens, or the absence of clones of B cells capable of reacting with the determinants expressed by these antigens. The latter possibility was the easiest to rule out because, while it was clear that immune-defective mice were unresponsive to TNP when it was presented on Ficoll, these mice made excellent responses to TNP-BA, TNP-Boivin-LPS, and TNP on TD carriers (Scher *et al.*, 1975a; Mond *et al.*, 1978; E. Metcalf *et al.*, 1980; Boswell *et al.*, 1980b). Furthermore, as noted previously, when exposed to very small amounts of DNP-Ficoll or DNP-S-III, F_1 male mice were rendered incapable of responding to DNP-KLH (Merchant *et al.*, 1978a,b). Additional evidence that immune-defective CBA/N mice have B cells which express receptors for TI-2 antigens is provided by the finding that anti-poly(I-C) antibody can be induced in these mice if the poly(I-C) is first complexed to methylated-bovine serum albumin (Scher *et al.*, 1973a). Furthermore, the fusion product of NS-1 plasmacytoma cells and B cells of immune-defective mice produced anti-S-III antibody (Schroer *et al.*, 1979). Finally, while F_1 male immune-defective mice made no responses to dextran, they made substantial responses when the polysaccharide determinants present on dextran were coupled to KLH and the mice repeatedly challenged with this antigen (Stein *et al.*, 1980a).

Although it was possible that the initially described defects of CBA/N mice could have been associated with an isolated dysfunction in otherwise normal B cells, the demonstration that the B cells of these mice had wide-ranging defects made this less likely. Thus, while an inability to respond to polysaccharide antigens, such as poly(I-C) or S-III, could have been the result of a single defect in a particular activation pathway, it would be difficult to explain all the abnormalities in B-cell function, which include the ease with which they are tolerized and their low/unusual/absent responses to TD and PC antigens, mitogens, anti-Ig antibodies, and T-cell helper factors on this

basis. Furthermore, the unusual surface membrane phenotype of B cells of CBA/N mice suggests that they do not represent a "normal" B cell with a restricted defect.

The inability of immune-defective CBA/N mice to respond to the TI-2 antigens strongly implied that the B cells of these mice were intrinsically defective. This was studied with experiments in which spleen cells from either (CBA/N × DBA/2)F_1 male or female mice were used to reconstitute lethally irradiated F_1 mice of both sexes (Table IX) (Scher *et al.*, 1975b). In every instance, the subsequent responsiveness of the reconstituted mice to poly(I-C) was associated with the sex of the donor strain, with F_1 female cells inducing responses in both recipients, while F_1 males cells were ineffective. Similarly, whereas F_1 male bone marrow cells were unable to reconstitute the TNP-Ficoll responses of lethally irradiated male or female mice 8 weeks after reconstitution, mice of either sex given female bone marrow equivalent responses. Thus, F_1 female cells were able to reconstitute the TNP-Ficoll responses of lethally irradiated male or female mice in acute and chronic experiments, whereas F_1 male spleen cells were not. These experiments suggested that the defect of CBA/N mice was intrinsic to these cells and not related to a microenvironmental abnormality, since the F_1 male microenvironment neither suppressed the TNP-Ficoll responsiveness of F_1 female spleen cells acutely, nor did it suppress the development of TNP-Ficoll responsive cells from F_1 female bone marrow over 8 weeks.

This hypothesis was supported by the ability of anti-Thy 1.2 + C treated F_1 female spleen cells to reconstitute the TNP-Ficoll responses in defective F_1 male mice (Scher *et al.*, 1975b) and the reconstitution of PVP responses by CR^+ lymphocytes from normal CBA mice in

TABLE IX
RECONSTITUTION OF RESPONSES TO TI-2 ANTIGENS IN LETHALLY IRRADIATED F_1 MICE

Sex of (CBA/N × DBA/2)F_1 recipient	Responsiveness to Poly(I-C)[a]		Responsiveness to TNP-Ficoll[a]	
	Donor source		Donor source	
	♀ Spleen	♂ Spleen	♀ Bone marrow	♂ Bone marrow
♀	4/4	0/4	3/3	0/3
♂	6/7	0/7	3/3	0/3

[a] Number of mice responding/number of mice reconstituted (Scher *et al.*, 1975b).

CBA/N recipients (Lindsten and Andersson, 1981). These studies did not rule out the possibility that macrophages were playing an important role in this reconstitution. However, this was unlikely, since F_1 female lymph node cells were also effective in reconstituting F_1 male mice and very few macrophages exist in these populations. Furthermore, as noted in the section dealing with macrophage function in CBA/N mice, subsequent studies have shown that immune-defective CBA/N macrophages present TNP-Ficoll to B cells as well as normal macrophages (Boswell *et al.*, 1980b).

The ability of the F_1 female splenic B cells to reconstitute responsiveness in the absence of recipient F_1 male irradiation suggested that these cells took up residence in these mice, although it was possible that the female cells were able to transfer the ability to respond to TI-2 antigens to the resident male B cells without establishing residency. This question was directly studied using CBA/CaN-T6, CBA/CaN, and immune-defective CBA/N mice, all of which accept reciprocal skin grafts (Volf *et al.*, 1978). Nonirradiated CBA/N mice given normal CBA/CaN-T6 cells became lymphoid cell chimeras, exhibiting donor type T6 mitoses. By contrast, normal CBA/CaN recipients did not exhibit significant numbers of donor type mitoses after receiving equivalent numbers of CBA/CaN-T6 cells. The lymphoid cell chimerism in the CBA/N host appeared in spleen, lymph node, and Peyer's patches, but not in bone marrow or thymus, suggesting that a subpopulation of lymphocytes was responsible for the chimerism. Indeed, stimulation of recipient CBA/N spleen cells *in vitro* indicated that the chimeric donor T6 cells were responsive to LPS, but not phytohemagglutinin. Furthermore, in similar studies, the genotype of cells derived from colonies of CBA/CaN-T6 reconstituted CBA/N mice was T6 and not that of the recipient CBA/N strain (Paige *et al.*, 1979). Thus, nonirradiated immune-defective but not normal CBA/CaN mice are able to accept B cells from CBA/CaN-T6 mice in their peripheral lymphoid organs to form a stable chimera. These data, coupled with those summarized in the section dealing with the B-cell surface membrane characteristics of CBA/N mice, give evidence for a missing B-cell subpopulation in these mice.

B. Changes in the Functional Properties of CBA/N B Cells with Age

As noted in previous sections, a number of recent reports have indicated that with advanced age (>12 months) certain of the immune defects of CBA/N mice improve or in some cases disappear (Rosenberg, 1979; Whitlock and Watson, 1979; Fidler *et al.*, 1980; Morgan *et*

al., 1980; Nariuchi and Kakiuchi, 1981). With one exception, these studies did not attempt to analyze the surface membrane characteristics of B cells of aged CBA/N mice, and in that instance it was noted that the frequency of B cells in the spleens of aged immune-defective F_1 male mice had increased to normal levels. These findings were not confirmed in later studies (Scher *et al.*, 1980; Skelly *et al.*, in preparation). There are also conflicts in the literature with regard to the improvement in the LPS proliferative and polyclonal antibody responses induced in F_1 male or CBA/N mice as they aged, with two groups reporting increased responses (Whitlock and Watson, 1979; Fidler *et al.*, 1980) and two groups reporting no improvement (Nariuchi and Kakiuchi, 1981; Skelly *et al.*, in preparation). However, increased proliferative responses were noted in aged CBA/N mice to PPD by two groups (Fidler *et al.*, 1980; Nariuchi and Kakiuchi, 1981), and Rosenberg (1979) has shown that an autoantibody response induced by injection of LPS or malaria parasites increased as a function of age. These data may be explained by the finding that there appears to be a substantially delayed maturation of B cells in immune defectives as compared to normal mice (Scher *et al.*, 1980). If the acquisition of δ by all μ-bearing spleen cells is used as a marker for maturation, immunologically normal mice have equivalent splenic μ- and δ-bearing cells by 7 weeks of age, whereas this does not occur in immune-defective mice until 14–15 weeks of age. Thus, the delayed appearance of IgD or a different and unknown B cell receptor/marker on the surface of IgM^+, $Lyb\ 5^-$ B cells of CBA/N mice could explain their improved responses to these mitogens with age. Alternatively, other factors, such as macrophage and/or T-cell maturation, could play a critical role in the improvement in certain of the immunological functions of CBA/N mice with age.

It is much more difficult to explain the appearance of a B-cell function in aged CBA/N mice which was totally absent in young adult CBA/N mice on this basis. This is particularly true of responsiveness to TNP-Ficoll, which has been shown to be correlated with the presence of $Lyb\ 5^+$ B cells. Unfortunately, the literature is also contradictory on this point, with one group noting the appearance of responsiveness to DNP-Ficoll with age (Fidler *et al.*, 1980) and a second not seeing such responsiveness (Skelly *et al.*, in preparation). The situation is further complicated by a report indicating that in some colonies, a low percentage of young adult, putatively defective F_1 male or CBA/N mice were immunologically normal (Kenny and Guelde, 1981). However, when the surface membrane characteristics of B cells derived from aged F_1 male mice that were unresponsive to DNP-Ficoll were studied,

they maintained their immature phenotype, since they had a high density of surface μ, did not express Lyb 5, and stimulated a very poor Mls-defined MLR (Skelly *et al.*, in preparation). The critical issue in this area is the nature of the B cells in the aged CBA/N mice that respond to DNP-Ficoll; i.e., are these mice responding because they have acquired low-μ density, Lyb 3^+, Lyb 5^+, Mls^+ B cells, or have their Lyb 5^- B cells acquired the capacity to respond to TI-2 antigens by virtue of the age of the animal. The answer to the question is not yet known. Indeed, it is essential for those laboratories that have CBA/N mice that respond to TI-2 antigens to determine if this trait is transmitted to their young adult offspring and if they show any evidence of other disparities (i.e., MLR reactivity, allotype, H-2 type) when compared to a CBA/N line that does not respond to DNP-Ficoll as it ages.

C. The Functional Properties of B-Cell Subpopulations of Adult Normal Mice; Comparisons with CBA/N B Cells

If the absence of a subpopulation of B cells from the peripheral lymphoid organs of adult CBA/N mice was associated with the functional immune defects of these mice, one would predict that the subpopulation of B cells present in the spleens of normal mice, which resemble those of immune defectives, would behave functionally like those of adult CBA/N or neonatal normal mice. This has been studied in part using the technique of cell sorting. Spleen cells of normal adult mice were stained with fluorescein-conjugated anti-μ antibodies and the brightly stained or high-density μ-bearing B cells were separated from the dull or low-density μ-bearing cells (Scher *et al.*, 1977a). The low μ-bearing B cells were excellent stimulators in an Mls-determined MLR and had a high frequency of cells bearing the CR (Table X). By contrast, the high μ-bearing B cells were poor stimulators of an Mls-determined MLR and had a low frequency of cells bearing the CR. These cells can therefore be characterized as μ high, Mls^-, $CR^\pm$ (like CBA/N and normal neonatal B cells), and μ low, Mls^+, CR^+ B cells.

As noted previously, the B cells of adult immune-defective mice fail to proliferate in response to either anti-Ig, anti-μ, or anti-δ antibodies, whereas the B cells of adult immunologically normal mice give excellent responses (Sieckmann *et al.*, 1978b, 1981; Sieckmann, 1980). These responses were absent when spleen cells of mice less than 4 weeks of age were studied, and adult levels of responsiveness did not occur until 12 weeks (Fig. 13) (Sieckmann *et al.*, 1979). In order to determine if the high-density μ, low-density δ B cells of normal mice made different proliferative responses to these antibodies than low-density μ B cells, spleen cells were stained with fluorescein-

TABLE X

DISTRIBUTION OF Mls$^+$ AND CR$^+$ B LYMPHOCYTES AMONG POPULATIONS WITH DIFFERENT DENSITIES OF SURFACE IgM

Donor strain[a] (Mls, H-2)	Donor cell type[a]	Percentage of total cells	Frequency of CR$^+$ lymphocyte	Stimulation index observed with responder strain (Mls, H-2)[b]	
				BALB/cN (b/b, d/d)	C3H/HeJ (c/c, k/k)
DBA/2 (a/a, d/d)	Unsorted	100.0	31.4	6.2	7.4
	Ig$^-$, 8–80	42.8	3.6	0.9	3.6
	Ig$^+$, 140–1000	43.1	52.7	13.8	9.9
	Ig$^+$, 140–240	14.8	74.7	18.5	7.8
	Ig$^+$, 240–400	14.0	29.8	2.6	6.3
	Ig$^+$, 400–1000	14.7	21.4	5.1	6.7
				C3H/HeJ (c/c, k/k)	BALB/cN (b/b, d/d)
CBA/J (d/d, k/k)	Unsorted	100.0	31.6	5.8	11.9
	Ig$^-$, 0–40	42.1	4.8	3.4	4.5
	Ig$^+$, 80–1000	45.8	58.4	11.1	14.8
	Ig$^+$, 81–164	19.4	71.8	19.5	11.6
	Ig$^+$, 165–299	13.9	32.1	7.2	11.4
	Ig$^+$, 300–1000	12.4	19.8	3.1	14.0

[a] Donor cells were stained with Fl anti-Ig or anti-IgM and B lymphocytes with different densities of surface Ig were isolated using the fluorescence-activated cell sorter (FACS) (see Fig. 11).

[b] Populations of cells isolated from the FACS were irradiated (1000 R) and 1×10^5 cells were used with 4×10^5 responder cells in mixed lymphocyte cultures (Scher *et al.*, 1977a).

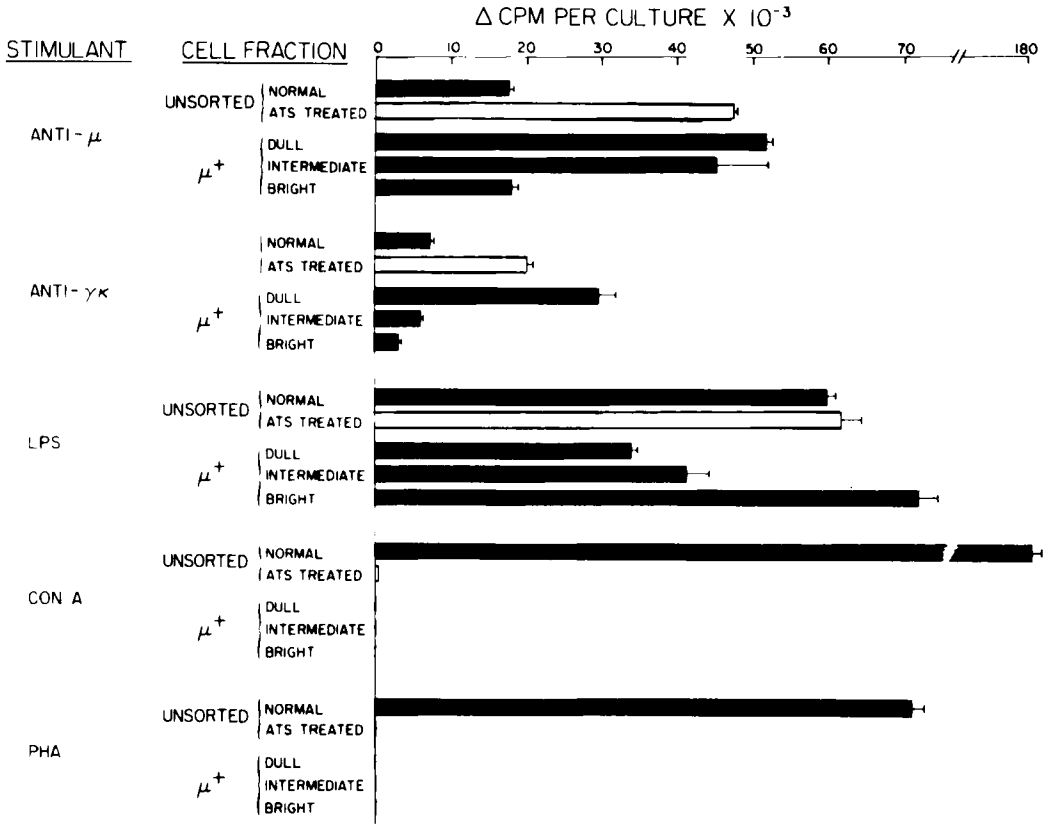

FIG. 13. Stimulation of spleen cells having various membrane IgM densities with anti-μ, anti-$\gamma\kappa$, LPS, Con A, and PHA. DBA/2 spleen cells treated with anti-thymocyte antibody + C and incubated with a fluorescence-conjugated rabbit anti-mouse μ. Cells were sorted into three fractions, each being 33% of the total IgM^+ cell population. The sorted cells were then cultured in triplicate with the above-noted stimulants and their ability to incorporate [^{3}H]thymidine was determined (Sieckmann *et al.*, 1980).

conjugated anti-μ or δ and were separated by cell sorting (Sieckmann *et al.*, 1979, 1981; Sieckmann, 1980). The low-density μ B cells isolated by this technique gave greater proliferative responses to anti-μ antibodies than the original unsorted B cells, while the responses of the high-density μ cells were considerably less than those of the unsorted B cells. These data correlated well with those obtained with low- or high-density δ cells, which had low and high proliferative responses to anti-μ antibodies, respectively. Thus, the low μ, high δ splenic B cells of normal mice gave excellent responses to anti-μ antibodies, whereas the high μ, low δ cells of these mice, which resemble

the B cells of immune-defective mice and neonatal normal mice, gave much lower responses.

A second approach to this problem is the use of anti-Lyb 5 antisera to kill Lyb 5^+ cells, so that the function of the remaining Lyb 5^- cells can be analyzed. As noted previously, in such experiments the Lyb 5^- spleen cells of normal mice had μ to δ ratios which resembled those of normal neonatal B cells and, like these cells, they were unable to stimulate Mls-encoded MLR (Table XI) (Ahmed *et al.*, 1977b; Ahmed and Scher, 1979). These Lyb 5^- cells were able to lyse antibody-coated erythrocytes in *in vitro* antibody-dependent cell-mediated cytotoxicity studies, but failed to kill antibody-coated nucleated target cells, resembling immune-defective spleen cells in this regard. Lyb 5^- B cells obtained in this manner also failed to respond to TNP-Ficoll and were not responsive to the T-cell replacing activity of TRF, but made excellent responses to TNP-BA (Ahmed and Scher, 1979; Boswell *et al.*, 1980b; Yaffe and Scher, 1981) (Table XI). With regard to these functional properties, the Lyb 5^- cells of normal adult mice resemble those of adult immune-defective and normal neonatal mice. Furthermore, the activation of these Lyb 5^- B cells by T-cell helpers from chimeric mice was genetically restricted, as was the activation of B cells of CBA/N, but not CBA/J mice (Singer *et al.*, 1981). Taken together, these findings strongly support the hypothesis that the absence of low μ, high δ, Mls^+, CR^+, Lyb 3^+, 5^+, 7^+, Ia.W39$^+$ cells in immune-defective mice is the cause of most of their functional immune defects. Thus, their inability to respond to anti-Ig antibodies or to kill antibody-coated nucleated target cells and their failure to produce antibodies to TI-2 antigens correlate with the functional attributes of normal B-cell populations that were depleted of their Lyb 5^+ cells.

Finally, the functional properties of low μ, high δ, Mls^+, CR^+, Lyb 3^+, 5^+, Ia.W39$^+$ cells of normal mice can be studied by analyzing the effects that anti-Lyb 3 antisera have on the immune function of normal mice. Anti-Lyb 3 acts to prevent tolerance (Huber and Borel, 1981; Sherr *et al.*, 1981), augments the production of autoantibodies (Kemp *et al.*, 1980), and accelerates and augments the amount and affinity of specific IgG_{2a} antibody formation (Huber *et al.*, 1981b) during an immune response. These data suggest that the susceptibility to tolerization (E. Metcalf *et al.*, 1980; McKearn and Quintáns, 1980), the low levels of autoantibody formation after challenge with polyclonal B-cell activators (Rosenberg, 1979; Marshall-Clarke *et al.*, 1979; Kemp *et al.*, 1980; Reeves *et al.*, 1981; Taurog *et al.*, 1979), and the low levels and affinity of the IgG_{2a} anti-DNP antibodies after immunization of immune-defective mice with DNP-KLH (Huber *et al.*, 1981b), as well

TABLE XI
CHARACTERISTICS OF Lyb 5⁻ ADULT SPLENIC B CELLS

Challenge		Responses of adult (CBA/N × DBA/2)F_1 ♀ spleen cells: Control[a]	Responses of adult (CBA/N × DBA/2)F_1 ♀ spleen cells: Anti-Lyb 5 + complement
TNP-BA	TNP-PFC/spleen[a]	45,854	42,680
TNP-Ficoll		26,538	1,528
Antibody-coated SRBC	Percentage specific ^{51}Cr release[a]	51.4	48.7
Antibody-coated L-1210		26.9	2.4

Responder		Stimulator (CBA/N × CBA/J)F_1 ♀ H-$2^{k/k}$, $Mls^{b/d}$ spleen cells: Control	Stimulator (CBA/N × CBA/J)F_1 ♀ H-$2^{k/k}$, $Mls^{b/d}$ spleen cells: Anti-Lyb 5 + complement
(CBA/N × C3H/HeJ)F_1 ♀ H-$2^{k/k}$, $Mls^{b/c}$	Stimulation index[a]	10.8	1.9

[a] The responses of the control (cells treated with absorbed anti-Lyb 5 + C) or experimental cells were determined by measuring TNP-PFC, in the case of TNP-BA or TNP-Ficoll responses, percentage specific ^{51}Cr release in the case of antibody-dependent cell-mediated cytotoxicity, and stimulation index in the case of Mls-determined MLR (Ahmed and Scher, 1979).

as the other abnormalities in their primary and secondary TD responses, are the result of the absence of B cells which can accept T-cell-derived helper factors. With regard to these functions, however, this view is more difficult to defend when considering the other immune defects that CBA/N mice demonstrate. Unlike responses to B-cell mitogens, anti-Ig antibodies, and TI-2 antigens, which are absolutely or relatively independent of T and accessory help, interactions between T, B, and accessory cells are critical in the functions that are influenced by anti-Lyb 3. Indeed, it has been noted that a recently described "hapten-specific" suppression mechanism can induce responses in normal mice which are similar to the TD anti-KLH responses of immune-defective mice (Herzenberg *et al.*, 1980). Thus, as has been pointed out in a recent paper, the *xid* defect could impair TD responses by the induction of hapten-specific suppression without altering the development of memory B cells (Huber *et al.*, 1981b). Stud-

ies which distinguish between these possibilities, i.e., that abnormalities in TD responses and/or tolerance are the result of the absence of a subpopulation of B cells, or the result of active suppressive mechanisms, may resolve these issues.

D. B-Cell Lineage

The studies described heretofore give evidence for the existence of at least two distinct subpopulations of B cells, the so-called Lyb 5^+ and 5^- cells. What has not been addressed is the lineage of these cells, i.e., are the Lyb 5^+ cells a distinct and separate subline of B cells, or do they appear as the result of the differentiation of Lyb 5^- B cells? The development of surface Ig^+ B cells proceeds from the earliest distinguishable progenitor of these cells, the cytoplasmic μ-bearing, surface Ig^- pre-B cell (Raff *et al.*, 1976). Pre-B cells give rise to surface IgM-bearing B cells and these surface Ig cells are then the progenitors of all subsequent B cells. This has been demonstrated in studies where anti-μ antibodies are administered chronically to newborn mice beginning at birth (Lawton *et al.*, 1972; Lawton and Cooper, 1974). Under these circumstances, surface Ig-bearing cells fail to appear. Thus, the suppression of these earliest surface IgM-bearing cells at birth precludes the development of all classes of B cells, including Lyb 5^+ or 5^- cells.

The sequential appearance of $\mu^+\delta^-$, followed by $\mu^+\delta^+$ cells early in ontogeny, suggests that these populations represent different stages in the development of a single lineage (Scher *et al.*, 1980). Indeed, the disappearance of one function attributed to an early B-cell subset, concomitant with the appearance of a second function attributed to a later appearing subset, gives evidence for this hypothesis. Thus, as B-cell tolerance susceptibility was lost at approximately 1 week of age, the ability to respond to PC-conjugated haptens was acquired (Sigal *et al.*, 1976, 1977; E. Metcalf and Klinman, 1976). These data would imply that $\mu^+\delta^-$ cells, which may be those responsible for the tolerance susceptibility that neonatal mice demonstrate (Vitetta and Uhr, 1975; Cambier *et al.*, 1977; Scott *et al.*, 1977), give rise to surface $\mu^+\delta^+$ cells that are resistant to tolerance induction and that are responsive to PC antigens.

The appearance of B cells capable of stimulating Mls-determined MLR and expressing Lyb 3, Lyb 5, Lyb 7, Ia.W39, and CR at 2 weeks of age does not coincide with the disappearance of surface $\mu^+\delta^+$ cells lacking these characteristics. Thus, the spleens of adult mice bear these two subpopulations of B cells in equivalent numbers when studies of the surface membrane or functional characteristics of these cells

are made (Ahmed *et al.*, 1977b; Huber *et al.*, 1977; Gelfand *et al.*, 1974a; Scher *et al.*, 1977a,b). These findings are compatible with the Lyb 5^+ cells being the direct progeny of Lyb 5^- cells, or their being a distinct subline.

As previously noted, transfers of adult spleen or bone marrow cells from normal mice to immune-defective mice reconstitute the responsiveness of these mice to TI-2 antigens, the PC hapten, and their capacity to form colonies in soft agar (Scher *et al.*, 1975b; Kincade *et al.*, 1978a; Paige *et al.*, 1979; Quintáns and Kaplan, 1978; Quintáns *et al.*, 1979). This reconstitution occurs because of the establishment of a stable B-cell chimera. In these chimeras, the donor B cells function to reconstitute the CBA/N immune defect directly, and do not influence the functional properties of the defective B cells (Volf *et al.*, 1978; Paige *et al.*, 1979). Interestingly, while spleen and bone marrow cells were able to reconstitute TNP-Ficoll or PC-KLH responses (detected with the IgM PC-PFC assay), transfers of neonatal liver cells did not result in reconstitution (Quintáns *et al.*, 1979). However, with low-dose irradiation of the immune-defective recipients, normal neonatal liver cells reconstituted these functions after 15 days. One explanation for these findings is that, while there is a deficiency in the late-developing subpopulation of B cells that is present in normal adult spleen or bone marrow, allowing such cells to persist in immune defectives and form a stable chimera, no such deficiency exists for more immature progenitor B cells. These cells would presumably not persist in immune defectives to form chimeras, unless the defective mice were given low-dose irradiation. If the CBA/N defect resulted in the absence of an entire lineage of B cells, one would expect that the immature progenitors of this lineage would also be absent or deficient, and that noenatal liver cells from normal mice would persist and reconstitute the defect. This interpretation must, however, be considered in light of results obtained using normal fetal liver cells in attempts to reconstitute the ability of immune-defective mice to form B-cell colonies if soft agar. In contrast with the data obtained with PC antigens or TNP-Ficoll, normal fetal liver cells were the most active source of cells able to reconstitute this function (Kincade and Paige, 1979; Kincade *et al.*, 1978a). These data support the hypothesis that multiple lineages of B cells diverge early in development to yield functionally restricted progeny. However, in studies of the characteristics of murine colony-forming B cells, it was shown that they comprise an equivalent proportion of the B cells in different sites and development (Kincade *et al.*, 1978b). Thus, the cells capable of forming these colonies could have been derived from fetal liver cells as a differ-

entiated cell which although capable of forming colonies in soft agar, could not form B cells capable of responding to TNP-Ficoll or make an IgG anti-PC response. These questions can be studied more directly with different experimental approaches. The outcome of these investigations may clarify the question of B-cell lineage.

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REFERENCES

Adinolfi, A., Haddad, S. A., and Seller, M. J. (1978). *J. Immunogenet.* **5,** 149.

Ahmed, A., and Scher, I. (1976). *J. Immunol.* **117,** 1922.

Ahmed, A., and Scher, I. (1979). *In* "B Lymphocytes in the Immune Response" (M. Cooper, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 115–124. Elsevier, Amsterdam.

Ahmed, A., Scher, I., and Sell, K. W. (1977a). *Cell. Immunol.* **30,** 122.

Ahmed, A., Scher, I., Sharrow, S. O., Smith, A. H., Paul, W. E., Sachs, D. H., and Sell, K. W. (1977b). *J. Exp. Med.* **145,** 101.

Amsbaugh, D. F., Hansen, C. T., Prescott, B., Stashak, P. W., Barthold, D. R., and Baker, P. J. (1972). *J. Exp. Med.* **136,** 931.

Amsbaugh, D. F., Hansen, C. T., Prescott, B., Stashak, P. W., Asofsky, R., and Baker, P. J. (1974). *J. Exp. Med.* **139,** 1499.

Andersson, B., and Blomgren, H. (1971). *Cell. Immunol.* **2,** 411.

Andersson, B., and Blomgren, H. (1975). *Nature (London)* **253,** 476.

Apte, R. N., Hertogs, C. F., and Pluznik, D. H. (1977). *J. Immunol.* **118,** 1435.

Armerding, D., and Katz, D. H. (1974). *J. Exp. Med.* **140,** 19.

Asmar, B. I., Slovis, T. L., Reed, J. R., and Dajani, A. S. (1978). *J. Pediatr.* **93,** 389.

Ayoub, E. M., Dudding, B. A., and Cooper, M. D. (1968). *J. Lab. Clin. Med.* **72,** 971.

Baker, P. J. (1975). *Transplant. Rev.* **26,** 3.

Berning, A., Eicher, E., Paul, W. E., and Scher, I. (1980). *J. Immunol.* **124,** 1875.

Bianco, C., and Nussenzweig, V. (1977). *Contemp. Top. Mol. Immunol.* **6,** 145.

Bianco, C., Patrick, R., and Nussenzweig, V. (1970). *J. Exp. Med.* **132,** 702.

Blaese, R. M., Strober, W., Brown, R. S., and Waldmann, T. (1968). *Lancet* **1,** 1056.
Bona, C., Mond, J., Kessler, S., Scher, I., and Paul, W. (1979). *In* "B Lymphocytes in the Immune Response" (M. Cooper, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 357–361. Elsevier, Amsterdam.
Bona, C., Mond, J. J., and Paul, W. E. (1980). *J. Exp. Med.* **151,** 224.
Boswell, H. S., Ahmed, A., Scher, I., and Singer, A. (1980a). *J. Immunol.* **125,** 1340.
Boswell, H. S., Nerenberg, M. I., Scher, I., and Singer, A. (1980b). *J. Exp. Med.* **152,** 1194.
Boswell, H. S., Sharrow, S. O., and Singer, A. (1980c). *J. Immunol.* **124,** 989.
Bottomly, K., and Mosier, D. E. (1979). *J. Exp. Med.* **150,** 1399.
Braley-Mullen, H. (1974). *J. Immunol.* **113,** 1909.
Brown, A. R., Crandall, C. A., and Crandall, R. B. (1977). *J. Parasitol.* **63,** 950.
Butterworth, M., McClellan, B., and Allansmith, M. (1967). *Nature (London)* **214,** 1224.
Cambier, J. C., Uhr, J. W., Kettman, J. R., and Vitetta, E. S. (1977). *J. Immunol.* **119,** 2054.
Cameron, P. M., and Wood, D. D. (1978). *Cell. Immunol.* **38,** 176.
Chused, T. M., Kassan, S. S., and Mosier, D. E. (1976). *J. Immunol.* **116,** 1579.
Claflin, J. L., and Davie, J. M. (1974). *J. Exp. Med.* **140,** 673.
Claflin, J. L., and Davie, J. M. (1975). *J. Exp. Med.* **141,** 1073.
Claflin, J. L., Lieberman, R., and Davie, J. M. (1974). *J. Immunol.* **112,** 1747.
Clough, E. R., Levy, D. A., and Cebra, J. J. (1981). *J. Immunol.* **126,** 387.
Cohen, P. L., Scher, I., and Mosier, D. E. (1976). *J. Immunol.* **116,** 301.
Cohen, S., and McGregor, I. A. (1963). *In* "Immunity to Protozoa" (P. C. C. Garnham, A. E. Pierce, and I. Roitt, eds.). Blackwell, Oxford.
Cone, R. E., Huber, B., Cantor, H., and Gershon, R. K. (1978). *J. Immunol.* **120,** 1733.
Cooper, M. D., and Seligmann, M. (1977). *In* "B and T Cells in Immune Recognition" (F. Loor and G. E. Roelants, eds.), p. 377. Wiley, New York.
Cooper, M. D., Chase, H. P., Lowman, J. T., Krivit, W., and Good, R. A. (1968). *Am. J. Med.* **44,** 499.
Cooper, M. D., Keightley, R. G., and Lawton, A. R., III (1975). *In* "Membrane Receptors of Lymphocytes" (M. Seligmann, J.-L. Preud'homme, and F. M. Kourilsky, eds.), pp. 431–442. North-Holland Publ., Amsterdam.
Cosenza, H., and Köhler, H. (1972a). *Science* **176,** 1027.
Cosenza, H., and Köhler, H. (1972b). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2701.
Cowdery, J. S., Jr., Taurog, J. D., and Steinberg, A. D. (1980). *J. Immunol.* **12,** 499.
Cunningham, A. J. (1976). *Transplant. Rev.* **31,** 23.
Demant, P. (1979). *H-2 Registry Bull.* (16): *Mouse News Lett.* (61), 21.
Diener, E., Shortman, K., Russell, P., and Shortman, W. D. (1970). *Nature (London)* **225,** 731.
Dutton, R. W. (1966). *J. Exp. Med.* **123,** 665.
Feldmann, M., and Basten, A. (1971). *J. Exp. Med.* **134,** 103.
Feldmann, M., and Palmer, J. (1971). *Immunology* **21,** 685.
Felton, L. D. (1949). *J. Immunol.* **61,** 107.
Fernandez, C., and Möller, G. (1977). *J. Exp. Med.* **146,** 1663.
Festenstein, H. (1973). *Transplant. Rev.* **15,** 62.
Fidler, J. M., Morgan, E. L., and Weigle, W. O. (1980). *J. Immunol.* **124,** 13.
Finkelman, F. D., Smith, A. H., Scher, I., and Paul, W. E. (1975). *J. Exp. Med.* **142,** 1316.
Fleisher, T. A., White, R. M., Broder, S., Nissley, S. P., Blaese, R. M., Mulvihill, J. J., Olive, G., and Waldmann, T. A. (1980). *N. Engl. J. Med.* **302,** 1429.
Gardner, B., and Adinolfi, M. (1968). *Guy's Hosp. Rep.* **117,** 19.
Gearhart, P. J., Sigal, N. H., and Klinman, N. R. (1975). *J. Exp. Med.* **141,** 56.

Geha, R. S., Rosen, F. S., and Merler, E. (1973). *J. Clin. Invest.* **52,** 1726.
Gelfand, M. C., Asofsky, R., and Paul, W. E. (1974a). *Cell. Immunol.* **14,** 460.
Gelfand, M. C., Elfenbein, G. J., Frank, M. M., and Paul, W. E. (1974b). *J. Exp. Med.* **139,** 1125.
Gershon, R. K., and Kondo, K. (1976). *J. Immunol.* **117,** 701.
Gerson, J. M., Holden, H. T., and Herberman, R. B. (1980). *J. Natl. Cancer Inst.* **65,** 905.
Gitlin, D., and Craig, J. M. (1963). *Pediatrics* **32,** 517.
Glasebrook, A. L., Quintáns, J., Eisenberg, L., and Fitch, F. W. (1981). *J. Immunol.* **126,** 240.
Glode, L. M., and Rosenstreich, D. L. (1976). *J. Immunol.* **117,** 2061.
Golding, H., Tittle, T. V., Foiles, P. G., and Rittenberg, M. B. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 1056 (Abstr.).
Good, R. A. (1973). *Harvey Lect. Ser.* **67,** 1.
Goodman, M. G., and Weigle, W. O. (1979a). *J. Immunol.* **122,** 2548.
Goodman, M. G., and Weigle, W. O. (1979b). *J. Immunol.* **123,** 2484.
Goodman, M., Wainwright, R. L., and Weir, H. F. (1971). *Pediatrics* **48,** 907.
Greaves, M., and Janossy, G. (1972). *Transplant. Rev.* **11,** 87.
Greenwood, B. M., and Vick, R. M. (1975). *Nature (London)* **257,** 592.
Hämmerling, U., Chin, A. F., Abbott, J., and Scheid, M. P. (1975). *J. Immunol.* **115,** 1425.
Hämmerling, U., Chin, A. F., and Abbott, J. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2008.
Haughton, G., Lanier, L. L., and Babcock, G. F. (1978). *Nature (London)* **275,** 154.
Herzenberg, L. A., Tokuhisa, T., and Herzenberg, L. A. (1980). *Nature (London)* **285,** 664.
Hood, L., Gray, W. R., Sanders, B. G., and Dreyer, W. J. (1967). *Cold Spring Harbor Symp. Quant. Biol.* **32,** 133.
Howard, J. G., Christie, G. H., Courtenay, B. M., Leuchars, E., and Davies, A. J. S. (1971). *Cell. Immunol.* **2,** 614.
Hoyer, J. R., Cooper, M. D., Gabrielsen, A. E., and Good, R. A. (1968). *Medicine* **47,** 201.
Huber, B. T. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3460.
Huber, B. T., and Borel, Y. (1981). *In* "B Lymphocytes in the Immune Response: Functional, Developmental, and Interactive Properties" (N. Klinman, D. E. Mosier, I. Scher, and E. Vitetta, eds.), pp. 275–281. Elsevier, Amsterdam.
Huber, B. T., and Melchers, F. (1979). *Eur. J. Immunol.* **9,** 827.
Huber, B., Gershon, R. K., and Cantor, H. (1977). *J. Exp. Med.* **145,** 10.
Huber, B. T., Gathings, W. E., and Cooper, M. D. (1979). *In* "B Lymphocytes in the Immune Response" (M. Cooper, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 125–130. Elsevier, Amsterdam.
Huber, B. T., Jones, P. P., and Thorley-Lawson, D. A. (1981a). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4525.
Huber, B. T., Tokuhisa, T., and Herzenberg, L. A. (1981b). *Eur. J. Immunol.* **11,** 353.
Hunter, K. W., Jr., Finkelman, F. D., Strickland, G. T., Sayles, P. C., and Scher, I. (1979). *J. Immunol.* **123,** 133.
Izui, S., Zaldivar, N. M., Scher, I., and Lamberg, P-H. (1977). *J. Immunol.* **119,** 2151.
Jacobs, M. E., Steinberg, A. D., Gordon, J. K., and Talal, N. (1972). *Arthritis Rheum.* **15,** 201.
Janeway, C. A., Jr., and Barthold, D. R. (1975). *J. Immunol.* **115,** 898.
Janeway, C. A., Jr., Bert, D. L., and Mosier, D. E. (1980a). *Eur. J. Immunol.* **10,** 236.
Janeway, C. A., Jr., Bert, D. I., and Shen, F. W. (1980b). *Eur. J. Immunol.* **10,** 231.
Jayawardena, A. N., Janeway, C. A., Jr., and Kemp, J. D. (1979). *J. Immunol.* **123,** 2532.

Kaplan, R., and Quintáns, J. (1979). *J. Exp. Med.* **149,** 267.
Katz, D. H., Hamaoka, T., and Benacerraf, B. (1972). *J. Exp. Med.* **136,** 1404.
Kearney, J. F., Cooper, M. D., Klein, J., Abney, E. R., Parkhouse, R. M. E., and Lawton, A. R. (1977). *J. Exp. Med.* **146,** 297.
Kemp, J., Dohrman, L., Jayawardena, A., Huber, B., and Gershon, R. K. (1980). *Cell. Immunol.* **53,** 84.
Kenny, J. J., and Guelde, G. (1981). *In* "B Lymphocytes in the Immune Response: Functional, Developmental, and Interactive Properties" (N. Klinman, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 77–83. Elsevier, Amsterdam.
Kenny, J. J., Guelde, G., Lehrer, S. B., Claflin, J. L., and Scher, I. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 466 (Abstr.).
Kenny, J., Guelde, G., Claflin, J., and Scher, I. (1981). *J. Immunol.* **127,** 1629.
Kessler, S., Kim, J., and Scher, I. (1981). *J. Immunol.* **127,** 1674.
Kincade, P. W. (1977). *J. Exp. Med.* **145,** 249.
Kincade, P. W., and Paige, C. J. (1979). *In* "B Lymphocytes in the Immune Response" (M. Cooper, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 349–355. Elsevier, Amsterdam.
Kincade, P. W., Ralph, P., and Moore, M. A. S. (1976). *J. Exp. Med.* **143,** 1265.
Kincade, P. W., Moore, M. A. S., Lee, G., and Paige, C. J. (1978a). *Cell. Immunol.* **40,** 294.
Kincade, P. W., Paige, C. J., Parkhouse, R. M. E., and Lee, G. (1978b). *J. Immunol.* **120,** 1289.
Kirkland, T. N., Sieckmann, D. G., Longo, D. L., and Mosier, D. E. (1980). *J. Immunol.* **124,** 1721.
Kishimoto, T., Shigemoto, S., Watanabe, T., and Yamamura, Y. (1979). *J. Immunol.* **123,** 1039.
Köhler, H., Smyk, S., and Fung, J. (1981). *J. Immunol.* **126,** 1790.
Krüger, J., and Gershon, R. K. (1972). *J. Immunol.* **108,** 581.
Kurland, J. I., Kincade, P. W., and Moore, M. A. S. (1977). *J. Exp. Med.* **146,** 1420.
Lawton, A. R., and Cooper, M. D. (1974). *Contemp. Top. Immunobiol.* **3,** 193.
Lawton, A. R., Asofsky, R., Hylton, M. B., and Cooper, M. D. (1972). *J. Exp. Med.* **135,** 277.
Lee, J. C., and Ihle, J. N. (1981). *Nature (London)* **289,** 407.
Lee, W., Cosenza, H., and Köhler, H. (1974). *Nature (London)* **247,** 55.
Lemke, H., Coutinho, A., Gritz, H. G., and Gronowicz, E. (1975). *Scand. J. Immunol.* **4,** 707.
Lichtman, M. A., Vaughan, J. H., and Hamer, C. G. (1967). *Arthritis Rheum.* **10,** 204.
Lieberman, R., Potter, M., Mushinski, E. B., Humphrey, W., Jr., and Rudikoff, S. (1974). *J. Exp. Med.* **139,** 983.
Lindsten, T., and Andersson, B. (1979). *J. Exp. Med.* **150,** 1285.
Lindsten, T., and Andersson, B. (1981). *Cell. Immunol.* **61,** 386.
Litwin, S. D., Ochs, H., and Pollara, B. (1973). *Immunology* **25,** 573.
Lucas, S. J., Barry, D. W., and Kind, P. (1978). *Infect. Immun.* **20,** 115.
McGhee, J. R., Farrar, J. J., Michalela, S. M., Mergenhagen, S. E., and Rosenstreich, D. L. (1979). *J. Exp. Med.* **149,** 793.
McGuire, K. L., and Vitetta, E. S. (1981). *J. Immunol.* (in press).
McKearn, J. P., and Quintáns, J. (1980). *J. Immunol.* **124,** 77.
Marshall-Clarke, S., Cooke, A., and Hutchings, P. R. (1979). *Eur. J. Immunol.* **9,** 820.
Martin, S. E., and Martin, W. J. (1975). *J. Immunol.* **115,** 502.
Melchers, F., Braun, V., and Galanos, C. (1975). *J. Exp. Med.* **142,** 473.

Merchant, B., Snippe, H., Lizzio, E. F., and Inman, J. K. (1978a). *J. Exp. Med.* **147,** 1755.
Merchant, B., Snippe, H., Lizzio, E. F., and Inman, J. K. (1978b). *J. Immunol.* **120,** 1362.
Metcalf, D. (1976). *J. Immunol.* **116,** 635.
Metcalf, D., Nossal, G. J. V., Warner, N. L., Miller, J. F. A. P., Mandel, T. E., Layton, J. E., and Gutman, G. A. (1975). *J. Exp. Med.* **142,** 1534.
Metcalf, E. S., and Klinman, N. R. (1976). *J. Exp. Med.* **143,** 1327.
Metcalf, E. S., and Klinman, N. R. (1977). *J. Immunol.* **118,** 2111.
Metcalf, E. S., Sigal, N. H., and Klinman, N. R. (1977). *J. Exp. Med.* **145,** 1382.
Metcalf, E. S., Schrater, A. F., and Klinman, N. R. (1979). *Immunol. Rev.* **43,** 142.
Metcalf, E. S., Scher, I., and Klinman, N. R. (1980). *J. Exp. Med.* **151,** 486.
Mond, J. J., Lieberman, R., Inman, J. K., Mosier, D. E., and Paul, W. E. (1977). *J. Exp. Med.* **146,** 1138.
Mond, J. J., Scher, I., Mosier, D. E., Blaese, M., and Paul, W. E. (1978). *Eur. J. Immunol.* **8,** 459.
Mond, J. J., Sehgal, E., Sachs, D. H., and Paul, W. E. (1979a). *J. Immunol.* **123,** 1619.
Mond, J. J., Stein, K. E., Subbarao, B., and Paul, W. E. (1979b). *J. Immunol.* **123,** 239.
Mond, J. J., Mongini, P. K. A., Sieckmann, D., and Paul, W. E. (1980). *J. Immunol.* **125,** 1066.
Mongini, P., Friedman, S., and Wortis, H. (1978). *Nature (London)* **276,** 709.
Morgan, E. L., Fidler, J. M., and Weigle, W. O. (1980). *Cell. Immunol.* **50,** 445.
Morrissey, P. J., Boswell, H. S., Scher, I., and Singer, A. (1981). *J. Immunol.* **127,** 1345.
Mosier, D. E., Johnson, B. M., Paul, W. E., and McMaster, P. R. B. (1974). *J. Exp. Med.* **139,** 1354.
Mosier, D. E., Scher, I., and Paul, W. E. (1976). *J. Immunol.* **117,** 1363.
Mosier, D. E., Mond, J. J., and Goldings, E. A. (1977a). *J. Immunol.* **119,** 1874.
Mosier, D. E., Zitron, I. M., Mond, J. J., Ahmed, A., Scher, I., and Paul, W. E. (1977b). *Immunol. Rev.* **37,** 89.
Mozes, E., and Fuchs, S. (1974). *Nature (London)* **249,** 167.
Nariuchi, H., and Kakiuchi, T. (1981). *Cell. Immunol.* **61,** 375.
Nunn, M. E., and Herberman, R. B. (1979). *J. Natl. Cancer Inst.* **62,** 765.
O'Brien, A. D., Scher, I., Campbell, G. H., MacDermott, R. P., and Formal, S. B. (1979). *J. Immunol.* **123,** 720.
O'Brien, A. D., Rosenstreich, D. L., Scher, I., and Metcalf, E. S. (1980). *In* "Genetic Control of Natural Resistance to Infection and Malignancy" (E. Skamene, ed.). Academic Press, New York.
O'Brien, A. D., Scher, I., and Metcalf, E. S. (1981). *J. Immunol.* **126,** 1368.
O'Toole, M. M., and Wortis, H. H. (1980). *J. Immunol.* **124,** 2010.
Paige, C. J., Kincade, P. W., Moore, M. A. S., and Lee, G. (1979). *J. Exp. Med.* **150,** 548.
Parker, D. C. (1975). *Nature (London)* **258,** 361.
Pearl, E. R., Vogler, L. B., Okos, A. J., Crist, W. M., Lawton, A. R., and Cooper, M. D. (1978). *J. Immunol.* **120,** 1169.
Perlmutter, R. M., Hansburg, D., Briles, D. E., Nicolotti, R. A., and Davie, J. M. (1978). *J. Immunol.* **121,** 566.
Perlmutter, R. M., Nahm, M., Stein, K. E., Slack, J., Zitron, I., Paul, W. E., and Davie, J. M. (1979). *J. Exp. Med.* **149,** 993.
Plant, J., and Glynn, A. A. (1976). *J. Infect. Dis.* **133,** 72.
Press, J. L. (1981). *J. Immunol.* **126,** 1234.
Price, W. H., Newland, I. M., and Ferguson, T. (1974). *J. Immunogenet.* **1,** 221.
Puré, E., and Vitetta, E. (1980). *J. Immunol.* **125,** 1240.

Purtilo, D. T., Cassel, C., Yang, J. P. S., Stephenson, S. R., Harper, R., Landing, B. H., and Vawter, G. F. (1975). *Lancet* **1**, 935.
Purtilo, D. T., DeFlorio, D., Jr., Hutt, L. M., Bhawan, J., Yang, J. P. S., Otto, R., and Edwards, W. (1977). *N. Engl. J. Med.* **297**, 1077.
Quan, Z. S., Dick, R. F., Regueiro, B., and Quintáns, J. (1981). *Eur. J. Immunol.* **11**, 643.
Quintáns, J. (1977). *Eur. J. Immunol.* **7**, 749.
Quintáns, J. (1979). *Eur. J. Immunol.* **9**, 67.
Quintáns, J., and Kaplan, R. B. (1978). *Cell. Immunol.* **38**, 294.
Quintáns, J., McKearn, J. P., and Kaplan, D. (1979). *J. Immunol.* **122**, 1750.
Raff, M. C., Nase, S., and Mitchison, N. A. (1971). *Nature (London)* **230**, 50.
Raff, M. C., Megson, M., Owen, J. J. T., and Cooper, M. D. (1976). *Nature (London)* **259**, 224.
Reeves, J. P., Taurog, J. D., and Steinberg, A. D. (1981). *Clin. Immunol. Immunopathol.* **19**, 170.
Rhoades, K., Markham, R. L., Maxwell, P. J., and Monk-Jones, M. E. (1969). *Br. Med. J.* **3**, 437.
Romain, P. L., Cohen, P. L., Fish, F., Ziff, M., and Vitetta, E. S. (1980). *J. Immunol.* **125**, 246.
Rosen, F. S., Craig, J. M., Vawter, G., and Janeway, C. A., Jr. (1968). *In* "Immunologic Deficiency Disease in Man" (D. Bergsma and R. A. Good, eds.), pp. 67–70. National Foundation, New York.
Rosenberg, Y. J. (1979). *J. Exp. Med.* **150**, 1561.
Rosenstreich, D. L., Glode, L. M., Wahl, L. M., Sandberg, A. L., and Mergenhagen, S. E. (1977). *In* "Microbiology" (D. Schlesinger, ed.), p. 314. American Society of Microbiology, Washington, D.C.
Rosenstreich, D. L., Vogel, S. N., Jacques, A., Wahl, L. M., Scher, I., and Mergenhagen, S. E. (1978). *J. Immunol.* **121**, 685.
Rosenthal, A. S., and Shevach, E. M. (1973). *J. Exp. Med.* **138**, 1194.
Rosenwasser, L. J., and Huber, B. T. (1981). *J. Exp. Med.* **153**, 1113.
Sakamoto, K., Freed, H. J., and Purtilo, D. T. (1980). *J. Immunol.* **125**, 921.
Scher, I., Frantz, M. D., and Steinberg, A. D. (1973a). *J. Immunol.* **110**, 1396.
Scher, I., Strong, D. M., Ahmed, A., Knudsen, R. C., and Sell, K. W. (1973b). *J. Exp. Med.* **138**, 1545.
Scher, I., Ahmed, A., Strong, D. M., Steinberg, A. D., and Paul, W. E. (1975a). *J. Exp. Med.* **141**, 788.
Scher, I., Steinberg, A. D., Berning, A. K., and Paul, W. E. (1975b). *J. Exp. Med.* **142**, 637.
Scher, I., Sharrow, S. O., and Paul, W. E. (1976a). *J. Exp. Med.* **144**, 507.
Scher, I., Sharrow, S. O., Wistar, R., Jr., Asofsky, R., and Paul, W. E. (1976b). *J. Exp. Med.* **144**, 494.
Scher, I., Ahmed, A., and Sharrow, S. O. (1977a). *J. Immunol.* **119**, 1938.
Scher, I., Ahmed, A., Sharrow, S. O., and Paul, W. E. (1977b). *In* "Development of Host Defenses" (M. D. Cooper and D. H. Dayton, eds.), pp. 55–74. Raven, New York.
Scher, I., Zaldivar, N. M., and Mosier, D. E. (1977c). *In* "Microbiology—1977," pp. 310–313. American Society of Microbiology, Washington, D.C.
Scher, I., Berning, A. K., and Asofsky, R. (1979). *J. Immunol.* **123**, 477.
Scher, I., Berning, A. K., Kessler, S., and Finkelman, F. D. (1980). *J. Immunol.* **125**, 1686.
Schimpl, A., and Wecker, E. (1975). *Transplant. Rev.* **23**, 176.
Schirrmacher, V., Peña-Martinez, J., and Festenstein, H. (1975). *Nature (London)* **255**, 155.

Schroer, K. R., Kim, K. J., Prescott, B., and Baker, P. J. (1979). *J. Exp. Med.* **150,** 698.
Schwartz, R. H., David, C. S., Sachs, D. H., and Paul, W. E. (1976). *J. Immunol.* **117,** 531.
Scott, D. W., Layton, J. E., and Nossal, G. J. V. (1977). *J. Exp. Med.* **146,** 1473.
Sela, M., Mozes, E., and Shearer, G. M. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2696.
Sharon, R., McMaster, P. R. B., Kask, A. M., Owens, J. D., and Paul, W. E. (1975). *J. Immunol.* **114,** 1585.
Sher, A., and Cohn, M. (1972). *Eur. J. Immunol.* **2,** 319.
Sherr, D. H., Huber, B. T., Gershon, R. K., Benacerraf, B., and Dorf, M. E. (1981). *Eur. J. Immunol.* **11,** 241.
Shevach, E. M. (1978). *Springer Sem. Immunopathol.* **1,** 207.
Sieckmann, D. G. (1980). *Immunol. Rev.* **52,** 1.
Sieckmann, D. G., Asofsky, R., Mosier, D. E., Zitron, I. M., and Paul, W. E. (1978a). *J. Exp. Med.* **147,** 814.
Sieckmann, D. G., Scher, I., Asofsky, R., Mosier, D. E., and Paul, W. E. (1978b). *J. Exp. Med.* **148,** 1628.
Sieckmann, D. G., Scher, I., and Paul, W. E. (1979). *In* "Physical-Chemical Aspects of Cell Surface Events in Cellular Regulation" (R. Blumenthan and C. DeLisi, eds.), pp. 325–343. Elsevier, Amsterdam.
Sieckmann, D. G., Finkelman, F., and Scher, I. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 806 (Abstr.).
Sieckmann, D. G., Habbersett, R., Scher, I., and Paul, W. E. (1981). *J. Immunol.* **127,** 205.
Siegal, F. P., Pernis, B., and Kunkel, H. G. (1971). *Eur. J. Immunol.* **1,** 482.
Sigal, N. H., Gearhart, P. J., Press, J. L., and Klinman, N. R. (1976). *Nature (London)* **259,** 51.
Sigal, N. H., Pickard, A. R., Metcalf, E. S., Gearhart, P. J., and Klinman, N. R. (1977). *J. Exp. Med.* **146,** 933.
Singer, A., Cowing, C., Hathcock, K. S., Dickler, H. B., and Hodes, R. J. (1978). *J. Exp. Med.* **147,** 1611.
Singer, A., Hathcock, K. S., and Hodes, R. J. (1979). *J. Exp. Med.* **149,** 1208.
Singer, A., Morrissey, P. J., Hathcock, K. S., Ahmed, A., Scher, I., and Hodes, R. J. (1981). *J. Exp. Med.* **154,** 501.
Skarnes, R. C., and Harper, M. J. K. (1972). *Prostaglandins* **1,** 191.
Slack, J., Der-Balian, G. P., Nahm, M., and Davie, J. M. (1980). *J. Exp. Med.* **151,** 853.
Stein, K. E., Brennan, C. A., Mond, J. J., Mäkelä, O., and Paul, W. E. (1980a). *J. Immunol.* **124,** 1798.
Stein, K. E., Zopf, D. A., and Paul, W. E. (1980b). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 466 (Abstr.).
Steinberg, A. D., Taurog, J. D., Raveche, E. S., Cowdery, J. S., Smathers, P. A., and Reeves, J. P. (1981). *In* "B Lymphocytes in the Immune Response: Functional, Developmental, and Interactive Properties" (N. Klinman, D. Mosier, I. Scher, and E. Vitetta, eds.), pp. 345–354. Elsevier, Amsterdam.
Subbarao, B., Ahmed, A., Paul, W. E., Scher, I., Lieberman, R., and Mosier, D. E. (1979a). *J. Immunol.* **122,** 2279.
Subbarao, B., Mosier, D. E., Ahmed, A., Mond, J., Scher, I., and Paul, W. E. (1979b). *J. Exp. Med.* **149,** 495.
Sultzer, B. M. (1968). *Nature (London)* **219,** 1253.
Taurog, J. D., Moutsopoulos, H. M., Rosenberg, Y. J., Chused, T. M., and Steinberg, A. D. (1979). *J. Exp. Med.* **150,** 31.

Taurog, J. D., Raveche, E. S., Smathers, P. A., Glimcher, L. H., Huston, D. P., Hansen, C. T., and Steinberg, A. D. (1981). *J. Exp. Med.* **153,** 221.
Thomas, D. W., and Shevach, E. M. (1976). *J. Exp. Med.* **144,** 1203.
Thomas, D. W., Yamashita, U., and Shevach, E. M. (1977). *J. Immunol.* **119,** 223.
Thompson, D. J., Gezon, H. M., and Rogers, K. D. (1966). *Am. J. Epidemiol.* **84,** 314.
Tominaga, A., Takatsu, K., and Hamaoka, T. (1980). *J. Immunol.* **124,** 2423.
Tsu, T. T., and Herzenberg, L. A. (1980). *In* "Selected Methods in Cellular Immunology" (B. B. Mishell and S. M. Shiigi, eds.), p. 373. Freeman, San Francisco, California.
Vitetta, E. S., and Uhr, J. W. (1975). *Science* **189,** 964.
Vitetta, E. S., Melcher, U., McWilliams, M., Lamms, M. E., Phillips-Quagliata, J. M., and Uhr, J. W. (1975). *J. Exp. Med.* **141,** 206.
Vogler, L. B., Pearl, E. R., Gathings, W. E., Lawton, A. R., and Cooper, M. D. (1976). *Lancet* **2,** 376.
Volf, D., Sensenbrenner, L. L., Sharkis, S. J., Elfenbein, G. J., and Scher, I. (1978). *J. Exp. Med.* **147,** 940.
von Boehmer, H., and Sprent, J. (1974). *Nature (London)* **249,** 363.
Washburn, T. C., Medearis, D. N., and Childs, B. (1965). *Pediatrics* **35,** 57.
Watson, J., and Riblet, R. (1975). *J. Immunol.* **114,** 1462.
Watson, J., Kelly, K., Largen, M., and Taylor, B. A. (1978). *J. Immunol.* **120,** 422.
Whitlock, C. A., and Watson, J. D. (1979). *J. Exp. Med.* **150,** 1483.
Wood, C. B. S., Martin, W., Adinolfi, M., and Polani, P. E. (1970). *Atti Assoc. Genet. Ital* **15,** 229.
Yaffe, L., and Scher, I. (1981). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40,** 1112 (Abstr.).
Yaffe, L., Mond, J., Ahmed, A., and Scher, I. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 1057 (Abstr.).
Yoshimaga, M., Yoshimaga, A., and Waksman, B. H. (1972). *J. Exp. Med.* **145,** 101.
Yother, J., Gray, B., Baker, P., and Briles, D. (1981). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40,** 122 (Abstr.).
Zaldivar, N. M., and Scher, I. (1979). *Infect. Immun.* **24,** 127.

ADVANCES IN IMMUNOLOGY, VOL. 33

The Biology of Monoclonal Lymphokines Secreted by T Cell Lines and Hybridomas

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I. Introduction

The immune response involves complex interactions among various effector and regulatory cells of the reticuloendothelial system. Thymus-dependent T lymphocytes play a pivotal role in these interactions. Besides mediating several effector functions, e.g., lysis of target cells by cytotoxic T lymphocytes (CTL) *in vitro* or delayed-type hypersensitivity (DTH) reactions *in vivo*, T lymphocytes regulate humoral and cell-mediated immune responses in a negative or positive manner, mediated by T suppressor (Ts) or T helper (Th) cells, respectively. T cells are known to interact with B cells, with macrophages

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and among themselves (reviewed in 1). Ever since it was established that Th cells collaborate with B cells in antibody responses (2–4), immunologists have been searching for soluble factors which may mediate such interactions by acting as communication signals between the cells producing them and other cells carrying the relevant acceptor sites. The first report by Dutton *et al.* (5), concerning the ability of a mediator present in supernatant fluids of allogeneic mixed leukocyte cultures (MLC) to augment antibody responses against sheep red blood cells (SRBC) *in vitro*, was followed by numerous similar studies over the next 12 years. These studies have clearly documented that soluble mediators produced predominantly by activated T cells can have profound regulatory effects on the immune system, and others, of the multicellular organism.

To illustrate the complexity of this situation, a multitude of T cell-derived biologically active mediators, or lymphokines (LK), can be induced by stimulation with specific heterologous antigens, alloantigens, or mitogens; they suppress or augment immune responses or even affect nonimmunological systems; they are antigen-specific or nonspecific; they either lack or bear antigenic determinants encoded by the major histocompatibility complex (MHC) of the species, and they are MHC-restricted or unrestricted in their action on target cells.

Limitations of Conventional LK Preparations

Despite the overwhelming number of published studies on T cell-derived LK during the last 12 years, we are still facing serious difficulties in determining their structure–function relationship and biological significance. These difficulties reflect two major shortcomings of conventionally derived LK preparations, namely, the heterogeneity of the cell populations used for the production of these LK and the limited amounts available for structural analysis. T lymphocytes consist of distinct subpopulations identifiable on the basis of defined functions or cell surface markers. Even though it is possible to highly enrich for a given T-cell subset, this purification is never absolute. Moreover, since macrophages (or other types of antigen-processing cells) and their soluble products are required for T-cell activation in conventional cultures, it is practically impossible to exclude the contributions of cells other than T cells.

As a result, LK preparations derived by conventional means are likely to contain soluble products of different T-cell subsets as well as non-T cells. Hence, it is extremely difficult to assign a given biological activity to a distinct molecule. Numerous examples exist in the literature to illustrate the heterogeneity of conventional LK preparations. To

cite just a few, culture supernatants may contain mixtures of (a) antigen-specific (6,7) or nonspecific (8) helper and suppressor factors, (b) antigen-specific and nonspecific helper (9) or suppressor (10,11) factors, and (c) antigen-specific suppressor factors produced by at least two different subpopulations of Ts cells (12). Such heterogeneity may lead to incorrect conclusions and interpretations since, for example, an antigen-specific immunoregulatory LK may be obscured by the presence of an antigen-nonspecific one in the same crude supernatant.

Second, the amounts of a given LK available in conventional preparations are usually limited and too small to allow a precise biochemical analysis of the biologically active molecules. Since lymphocytes have a finite, and usually short, lifetime in culture it is impossible to obtain repeatedly uniform and homogeneous LK preparations in large enough quantities.

These two main limitations have impeded progress in the study of LK and introduced confusion into the field. Since all of these LK were defined by biological assays, the number of LK described has increased steadily as the biological assays used to measure their activities have diversified. These LK were given different acronyms and it is not clear at all whether, in many cases, two distinct biological activities are mediated by the same or different molecule(s).

Thus, it has become clear that developing homogeneous sources of large quantities of LK would greatly facilitate the study of these biologically active mediators and the elucidation of their biological role. As a result, a major effort has been directed in the past 4–5 years toward the solution of this problem. This effort has resulted in the establishment of stable and homogeneous T cell lines secreting distinct LK. Indeed, a large amount of information has already been gained within a relatively short time period from studies on the soluble products of such T cell lines. This review is intended to summarize the available information in this area. We will first review methodological aspects pertaining to the establishment of cloned, LK-secreting T cell lines and biological assays for such LK and then describe the products of these cell lines in two major groups, i.e. (a) antigen-specific immunoregulatory mediators, and (b) antigen-nonspecific LK, some of which act as immunomodulating agents while others act on target cells other than those directly involved in immunological responses. Immunoregulatory or other lymphokines (13–17) as well as cloning of antigen-reactive T cells (18) have recently been reviewed by others and this review is intended to cover strictly the production of LK by cloned T cell lines.

II. Production of LK-Secreting, Cloned T Cell Lines: Methodological Aspects

Homogeneous and stable production of large quantities of a soluble product requires at least two desirable properties of the producing cell, i.e., monoclonality and long life span. Accordingly, the two most important methodological aspects in the establishment of LK-secreting T cell lines relate to the cloning and immortalization of such cell lines. Cloning eliminates the potential problems raised by the existence of distinct LK species secreted by different T cells present in a heterogeneous cell population; moreover, it ensures stability of LK production since, by cloning a cell population, one obtains a single relevant cell free of other cells which do not produce the desired LK. Such contaminating cells may eventually outgrow the LK-secreting cells in an uncloned population, resulting in termination of LK production. However, it is important to realize that even a cloned T cell may secrete more than one distinct lymphokine. Thus, although cloning may restrict the number of LK being produced, it does not ensure that only one molecular species of LK will be produced.

Immortalization of LK production has been achieved with both malignant and normal T lymphocytes. In the first case, malignant T cells have either been selected for their own ability to produce and secrete LK or they have been fused with LK-producing normal T cells, using the somatic cell hybridization technology (see below). In the case of normal T cells, they were maintained in permanent culture either by supplementing the culture medium with T cell growth factor (TCGF; IL-2) or by repeatedly stimulating them with appropriate target cells (see below).

A. T-Cell Hybridization

The technique of somatic cell hybridization as refined by Kohler and Milstein for immunologically relevant cells (19) has provided a revolutionary tool for immunological studies (reviewed in 20–23). This technique allows the fusion of a single lymphocyte, producing a defined homogeneous product, to a selected tumor cell. The resulting hybrid is immortalized due to the property of the tumor cell to grow indefinitely and the secretory function of the normal lymphocyte is preserved. Single cell cloning techniques (see below) allow the selection of the relevant hybrid(s) which secrete the desired product(s). This technology, developed initially for the purpose of constructing B-cell hybridomas secreting antibodies of defined specificity, was later extended to the construction of T-cell hybridomas by fusing either normal or activated T lymphocytes with appropriate T lymphoma lines.

All of the reported studies on T-cell hybridomas have used a variant of the AKR/J-derived thymoma line BW5147 as a malignant cell partner for fusion, although some other T-cell lymphomas were used as well in earlier studies (24,25). This variant of the BW5147 thymoma was selected by Dr. R. Hyman (Salk Institute, La Jolla, CA) for resistance to 8-azaguanine and ouabain. The azaguanine resistance serves as a marker for the lack of the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT). An $HGPRT^-$ variant cannot survive in medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT), but a normal lymphocyte, which contains the HGPRT enzyme, will rescue the $HGPRT^-$ tumor cell upon fusion by providing the missing enzyme and such a hybrid cell will survive and grow indefinitely. The unfused normal lymphocytes, themselves, have a limited life span and will die within a few days in culture.

Although it appears that only few T-cell lymphomas are adequate for fusion, the choice of the BW5147 T lymphoma line has been fortunate since it has been successfully fused with either fresh or activated T cells, resulting in T-cell hybridoma lines which manifest a wide range of biological activities, namely, antigen-specific help or suppression (26), cell-mediated cytotoxicity (27,28), and LK production (see below).

The successful construction of T-cell hybridomas was demonstrated by the appropriate expression of cell surface markers (i.e., the Thy 1 and *H-2* alloantigens) or isozymes characteristic of the two partner cells used for fusion (24,29,30). In the earlier studies, T lymphoma cells were fused with T-cell populations primed by heterologous antigens, allogeneic cells, or the T cell mitogen concanavalin A (Con A). However, these studies failed to demonstrate, where analyzed, expression of T-cell functions (24,29,30). Subsequently, many laboratories have been successful in maintaining functional characteristics of the normal lymphocyte partner in such hybridoma lines (see below).

B. T-Cell Lymphomas

Spontaneous and experimentally induced lymphoid neoplasms have provided an extremely useful tool for studying lymphocyte biology. Such neoplasms represent distinct stages in the differentiation of normal lymphocytes and, furthermore, they can be obtained in a homogeneous form and in large quantities. In this respect, plasmacytomas which represent neoplasms of mature, immunoglobulin (Ig)-secreting B cells provided a unique tool to study the nature of the B-cell receptor for antigen after it has been found that a large number of such B-cell tumors can bind defined antigens or secrete Ig mole-

cules which specifically react with such antigens (31). Moreover, such B-cell neoplasms retain other functions of normal B cells in that they are responsive to a variety of activation and other regulatory signals (32).

The search for neoplastic T cells retaining antigen-specific functions has been more elusive. Roder *et al.* (33) described a virus-induced thymic lymphoma which provided antigen-specific help for a B cell-mediated anti-SRBC antibody response *in vitro* or *in vivo*. However, production of soluble helper factor(s) by this lymphoma has not been studied. On the other hand, earlier reports provided evidence about the ability of human lymphoid cell lines to secrete various antigen-nonspecific LK (34). A better definition of T lymphocytes by surface markers and other criteria allowed the identification of many neoplastic T cell lines in rodents and primates which secrete a variety of antigen-nonspecific LK (see below).

Since the search for antigen-specific neoplastic T cells has been so unsuccessful, in contrast to the many antigen-binding plasmacytomas which were discovered, immunologists have sought other ways for creating deliberately neoplastic T cells expressing antigen-specific function. Prominent among these is the method described by Finn *et al.* (35). Populations of T cells primed with keyhole limpet hemocyanin (KLH) were infected *in vitro* with radiation leukemia virus (RadLV) and the infected cells were injected into the thymus of syngeneic recipients. T-cell lymphomas developed in the majority of these mice and some of them were antigen-specific as demonstrated by the ability of the neoplastic T cells to serve as carrier-specific Th cells in the *in vitro* or *in vivo* antibody responses to dinitrophenyl (DNP)–KLH conjugates (35). The secretion of antigen-specific helper factors was not addressed in the aforementioned study, but a more recent study (see below) has established the validity of this experimental approach for creating T-cell lymphoma lines capable of secreting antigen-specific immunoregulatory mediators.

C. Nonmalignant T Cells

Earlier studies demonstrated that antigen-specific T lymphocytes can be expanded and enriched *in vitro* by repeated stimulation with antigen (36–39). However, such T cells stopped growing and lost their immunological function eventually, although in some cases permanent lines of alloreactive T cells have been established (38,40).

A major advance in this area was achieved when it was discovered in 1976 that the conditioned medium of mitogen-stimulated human T-cell populations contains growth factor(s) which allow the estab-

lishment of long-term human T cell lines (41,42). This technique has subsequently been applied to the establishment of antigen-specific murine CTL lines (43). The factor responsible for T cell growth has been termed T cell growth factor or TCGF (44) and more recently, interleukin-2 or IL-2 (45). TCGF has been characterized as protein possessing a molecular weight (MW) of approximately 15,000 in rats and humans, or 30,000 in mice (46–48). Its characterization has been facilitated by the development of a sensitive, short-term bioassay (44). By using TCGF and/or repeated antigen stimulation it has been possible to establish permanent lines of antigen-specific T cells manifesting cytotoxic, suppressor, or helper functions (18). While most CTL and Ts lines require TCGF for growth, Th lines usually require in addition continuous stimulation with the specific antigen in association with irradiated syngeneic lymphoid cells which most probably provide a source of antigen-presenting cells (APC). Such antigen presentation has been found to be MHC-restricted (49,50). Some of such Th lines do not require exogenous TCGF for growth and, in these cases, it was shown that the T cells produce TCGF and/or other LK species upon stimulation with antigen (50,51).

It is not clear to what extent such lines of growth factor-dependent, nonmalignant T cells represent normal T cells. Although chromosomal aberrations were found in some TCGF-dependent CTL cell lines which are grown in the absence of syngeneic filler cells (52), they are not overtly malignant *in vivo*. On the other hand, alloreactive Th cell lines which require constant stimulation by alloantigens for growth were found to display normal chromosomal patterns (51). Yet, a parental anti-F_1 Th line recognizing unique F_1 determinants (53) has been shown to produce a malignant tumor when injected into F_1 hybrid recipients of the appropriate stimulatory haplotype (54).

The fact that all of the nonmalignant T cell lines depend on TCGF and/or stimulation by antigen plus APC creates certain problems when one approaches the question of LK production by such cells. The conditioned medium supplied to the cells may very well contain, in addition to TCGF, other LK. Similarly, the irradiated filler cells may produce various LK. Two approaches have been used in order to determine whether the T cell line itself produces any LK. These approaches have been (a) to remove the exogenous growth factor(s) and LK by washing the cells, reculture them in fresh medium lacking exogenous LK, and assaying this short-term culture medium for LK activity, and (b) to stimulate the T cells with T cell mitogens, usually Con A, instead of antigen plus APC. In both situations, it has been possible to show LK production by such T cell lines (see below).

Since, in most cases, LK production does not require cell growth, as evidenced by its resistance to irradiation and other mitotic inhibitors, removal of exogenous growth factors would not prevent LK production by the cells.

D. Selection of Activated T Cells

Selection and enrichment of activated T cells is particularly important in the case of T cell lines manifesting antigen-specific functions. Since the proportion of antigen-specific T cells following primary antigenic stimulation is quite small, particularly when priming is done *in vivo*, it is desirable to select and enrich for antigen-specific T cells before immortalizing them by any of the methods discussed above. This is especially critical in the case of T-cell hybridomas since such enrichment will increase the proportion of relevant, antigen-specific T-cell hybridomas and reduce the amount of effort needed for screening and identification of the desirable hybridomas.

Antigen-specific Th cells or CTL have usually been enriched by repeated stimulation with antigen in the context of syngeneic APC in culture, following *in vivo* or *in vitro* priming (18,36–39). Such repetitive stimulation eliminates most of the irrelevant T cells and highly enriches for antigen-specific cells as demonstrated by the marked increase in function on a per cell basis (55,56).

In contrast to Th cells which were in general selected by priming with optimally immunogenic doses of antigen and repeated stimulation by antigen plus APC *in vitro*, preferential induction of Ts cells has been achieved by immunization with large doses of soluble antigens (i.e., in the absence of adjuvant) or hapten-modified syngeneic cells (25,57–59), often injected intravenously (59). Because of the high affinity of Ts cells for free antigen, these cells have been highly enriched by adsorption onto, and elution from, antigen-coated columns or plates (25,58–61). Furthermore, since murine Ts cells often express antigenic determinants encoded by the *I-J* subregion of the MHC, further selection and enrichment was achieved by staining the cells with fluorescent anti-*I-J* antibodies and sorting on the fluorescent-activated cell sorter (FACS; 25,60). However, such antigen-specific T cell lines were also successfully established without any special attempts at enrichment and selection.

T cell lines secreting antigen-nonspecific LK have been derived from fresh, unstimulated T cells (62), antigen-activated T cells selected as described above (50,51,63–66), or polyclonally activated T cells (67), in which case selection is not much of a problem since such polyclonal mitogens activate the majority of T cells.

E. T-Cell Cloning

The cloning of antigen-specific T cells has been a major goal of cellular immunologists for the past 6 years. Such cloning and long-term growth will allow the analysis of unresolved questions, e.g., the nature of the T-cell receptor for antigen. Pursuant to the earlier studies which demonstrated the propagation and enrichment of antigen-specific T cells by repeated antigenic stimulation, it became possible to clone mitogen-stimulated human (68) and murine (69) T cells in soft agar. The subsequent discovery and characterization of TCGF (IL-2) (41–48) made it possible to expand such cloned T cells and establish them as permanent lines. As a result of these methodological developments, cloned T cell lines manifesting various functions and antigenic specificity toward alloantigens (51–53,70–73), modified self antigens (74,75), particulate (76,77), or soluble (49,78) heterologous antigens were established in many laboratories (reviewed in 18).

As pointed out before, the ability to clone T cells is critical since it allows us to (a) pinpoint accurately the phenotype of a cell with a given function, and (b) obtain such cells free of other, irrelevant T cells. Ideally, cloning is carried out by controlled isolation of single cells using micromanipulation techniques. Although this method has been successfully used in one recent case for cloning mitogen-activated human T cells (79), almost all other studies have used other approaches for the cloning of T cells.

The experimental approach of choice for cloning T cells has been statistical in nature and is based on limiting dilution techniques and applying Poissonian statistics. This technique consists of plating the cells in liquid medium at such low concentrations that the probability of there being more than one cell per well is extremely unlikely. When one plates the cells in enough wells, it becomes a statistical certainty that the cells growing in a given well are the descendants of one progenitor cell. In the case of soft agar cloning, one can isolate a single colony with a high level of certainty by visually controlling the manipulation of the colonies growing in the semisolid medium. Because this technique is based on statistical considerations, it is essential to reclone such a cloned population at least once. Thus, if the cells in a given T cell line are indeed the derivatives of a single progenitor cell, then upon recloning all the resulting clones should display identical functional properties, whether it is an antigen-specific proliferation, secretion of a defined product, etc. Most, but not all, of the cell lines which will be reviewed here have been cloned in soft agar or in liquid medium.

T-cell cloning requires some kind of a growth support system. This

is usually provided by TCGF and/or antigen plus a source of syngeneic APC in the form of irradiated lymphoid cells. It is possible that besides serving as APC, such irradiated lymphoid cells also secrete other LK necessary for optimal cell growth, e.g., the macrophage product lymphocyte-activating factor (LAF; 80), termed more recently interleukin-1 (IL-1; 45). In this respect, it has recently been shown that LAF binds to T cells (81) and that it provides a differentiation signal for T cells, making them responsive to Con A stimulation as indicated by the consequent production of TCGF (81).

F. Stability of Cloned T Cell Lines

One of the greatest advantages of cloned cell lines in general is their stability. This stability is manifested by the retention of given cellular function for a long time in culture, thus providing a uniform source for studies over an extended period of time. Yet, functional T cell lines are not always stable, even after being cloned. Thus, although cloning is a very important step toward stabilization of a given function, it does not always ensure it. Perhaps to a larger extent than antibody-secreting B-cell hybridomas, some T-cell hybridomas tend to lose their function with time. In an earlier study describing the establishment of human γ-globulin (HGG)-specific Ts hybridomas (25), it was found that all clones lost chromosomes, cell surface *H-2* alloantigens, and biological activity within 3 months. Similarly, Watanabe *et al.* have reported the establishment of an antigen-nonspecific T hybridoma which suppresses immunoglobulin E (IgE) antibody responses in a class-specific manner (82). This hybridoma also lost its activity within ~20 weeks of culture and this functional loss was associated with chromosomal loss. However, biological activity was regained upon recloning of the cells (82). In practically all cases of T-cell hybridomas the hybrid clones are aneuploid, expressing different chromosome numbers ranging between the diploid and tetraploid numbers.

Instability and loss of function has also been a problem in the case of T-cell lymphomas and nonmalignant T cell lines. Such instability can result from differentiation or dedifferentiation events, somatic mutations followed by overgrowth of nonfunctional variants (65,83), or contamination of T-cell clones, e.g., by mycoplasma infection. Thus, although cellular instability and decline (or change) in function are unavoidable in certain instances, it is possible to ensure a good deal of stability by following some steps, i.e., freezing a large number of cloned cell samples early after cloning, recloning the cultured cells frequently to eliminate nonfunctional variants, and strictly observing sterile procedures. In general, growth and function can be recovered

after freezing various T cell lines. Using these techniques, it has been possible to maintain growth and function of many cloned T cell lines for a period of several years.

It is clear from the discussion presented above that although there is room for modifications and technical improvements, the most difficult barriers toward establishment of cloned, functional T cell lines have already been crossed. The present state of the art allows, therefore, a much more precise and meaningful experimental approach than has previously been possible to the fundamental questions underlying the mechanism of action and biological role of T cell-derived LK.

G. Biological Assays for LK Activity

Due to the limitations of conventional LK preparations as described above, it has not been possible to purify any of them to homogeneity. Furthermore, the assays for these LK activities are still, almost exclusively, biological in nature. We still do not know whether two distinct LK may possess the same biological activity or, conversely, whether one molecular species of LK may mediate different biological effects.

Most of the biological assays for LK activity are quite crude for two main reasons. First, since most LK preparations contain a mixture of distinct biologically active molecules it is difficult to assign a particular biological activity to a given LK. Second, the target cells used in such bioassays are, in the overwhelming majority of cases, heterogeneous cell populations; hence, it is almost impossible to pinpoint the direct target cell for LK action. Reliable binding studies, analogous to the ones conducted with hormones, are still not available for LK because these molecules have not been purified to homogeneity, with the possible exception of a very recently described binding assay for TCGF or IL-2 (83a). Thus, there is an acute need for cloned populations of cells that can be used as a reliable source of target cells in bioassays. The bioassay for IL-2 activity may serve as a good example for progress in this particular area (44). In this case, the activity of IL-2 is measured by its ability to promote the proliferation of cloned T cell lines and it is clear, therefore, that IL-2 acts directly on a T cell. Similarly, a clone of a murine T-cell lymphoma has recently been described, which responds to Con A stimulation by producing IL-2, but only when it is additionally stimulated with the macrophage product LAF or IL-1 (81). Such a cloned population provides therefore a well defined tool for an IL-1 bioassay.

The availability of antibodies reacting specifically with various LK would also be of tremendous importance to the field. Such antibodies will allow us to (a) affinity-purify LK from crude preparations, (b) de-

velop direct quantitative assays for such LK, and (c) assess their physiological role and biological significance. Unfortunately, obtaining antibodies against LK has proven to be quite difficult, mainly because of the lack of sufficient quantities of highly purified LK required for immunization and also, perhaps, due to conservation of primary structures of LK across species barriers which could foster varying degrees of immunological tolerance to them. Nevertheless, various studies within the past 7 years have described the preparation of such antibodies, obtained by immunizing heterologous species with partially purified LK preparations. These antibodies have reacted with macrophage migration inhibition factor (MIF; 84–88), lymphotoxin (89,90), lymphocyte mitogenic factor (91), inhibitor of DNA synthesis (92), leukocyte migration inhibitory factor or LIF (93), and LK enhancing *in vitro* antibody responsees, derived from an owl monkey T-cell leukemia (94). In some cases, such antibodies were shown to block biological responses *in vivo* (84,85), suggesting a physiological role of the relevant LK. However, due to the nature of the immunizing LK preparations, these antibodies do not appear to be monospecific and they are directed against more than one well-defined LK.

To overcome this problem, some investigators have recently used the somatic cell hybridization technique to construct cloned B-cell hybridoma lines secreting antibodies against distinct LK. Such B-cell hybridomas secreting monoclonal antibodies against human osteoclast activating factor (OAF; 95) or IL-1 (96), rat (97) or human (98) IL-2, and leukocyte interferon (IFNα; 99) have recently been described. These recent reports demonstrate the power of this experimental approach. In the case of human IFNα, it has been possible to achieve a 5000-fold purification and to develop a sensitive immunoradiometric assay, using a high-titered monoclonal antibody (99). However, some technical difficulties may still exist as, for example, in the case of a recently described monoclonal antibody against rat IL-2 (97). So far, it has not been possible to elute and recover IL-2 activity from insoluble immunoadsorbents coated with this antibody.

Thus, it seems that LK research is well on its way to develop tools which will enable a precise analysis of the mechanisms of action and physiological role of diverse LK. These tools will include, in addition to cloned LK-secreting T cell lines which are the subject of this article, anti-LK monoclonal antibodies and cloned populations of indicator cells to be used in bioassays.

Historically, the first bioassays for immunoregulatory LK were constructed to measure the effects on antibody responses *in vitro*. In the earlier studies, LK activity was assessed in cultures deliberately im-

munized with particulate or soluble antigens (5,100–107) by measuring the augmenting effect on unfractionated spleen cell populations, or the Th-replacing factor (TRF) activity in cultures of T cell-depleted spleen cells. Later on, these assays were expanded to measure the polyclonal activating properties of some LK. Such polyclonal activation is assessed by the proliferation of the responding B lymphocytes (108–110) or the total secretion of Ig molecules (108–112), measured typically in a reverse plaque assay (113).

In fact, the bioassays which measure the effects of LK preparations on Ig synthesis provide an excellent example for the two main problems inherent to this field, namely, the impurity of LK preparations on one hand, and of the target cell populations used in the bioassays on the other. Thus, it has been difficult to ascertain whether various "TRF" preparations actually replace Th cells (as their name implies) by acting directly on precursors of antibody-forming B cells or whether they act on residual Th cells or pre-T cells to amplify their helper activity. The latter argument has been recently supported experimentally by showing that IL-2-containing LK preparations will induce functional, antigen-specific T cells in athymic nude mice (114,115). Furthermore, it has been shown more recently that IL-2 derived from a T-cell hybridoma provides efficient help for T cell-depleted spleen cells immunized *in vitro* with SRBC (116). However, IL-2 most probably acted in this case on residual T cells which were not killed by treatment with anti-Thy 1 antibodies plus C. Indeed, a more rigorous depletion of T cells resulted in a cell population which did not respond to IL-2 alone (116), but responded efficiently to the synergistic stimulus provided by T-cell-specific (IL-2) and B-cell-specific (TRF) mediators derived from two different T cell lines, respectively (62,116). Studying the effects of T cell-derived LK on pure populations of cloned normal or neoplastic B cells might resolve these questions.

The bioassays developed for antigen-specific LK are similar to the ones described above and include *in vitro* as well as *in vivo* assays. In a typical helper assay, B-cell populations primed with a hapten, e.g., DNP, conjugated to a given protein carrier (e.g., carrier X), are tested, in culture or in an adoptive transfer system in irradiated recipients, for their ability to produce hapten-specific antibodies when stimulated with a DNP-carrier Y conjugate, in the presence of a putative helper LK specific for the carrier Y (13). In the case of a suppressive LK, it is assayed by its ability to suppress a Th-dependent antibody response (13). Antigen specificity of the biological response distinguishes between antigen-specific or -nonspecific LK.

Following the initial description of T cell-derived, antigen-

nonspecific LK and their effects on antibody responses it was found that similar preparations also modulate cell-mediated immune responses and several bioassays were constructed in this respect. These bioassays measure the ability of a given LK preparations to (a) potentiate suboptimal or undetectable antigen-specific CTL response (due to the lack of either sufficient Th cells or Ia-positive cells stimulating such Th cells; 117–120), (b) provide a direct mitogenic stimulus for T cells, (c) synergize with polyclonal T-cell mitogens to facilitate weak proliferative responses in the so-called costimulator assay (121), and (d) exert TCGF activity, using the bioassay briefly described above (44). To the extent that TCGF from several species has been purified, all of the above activities copurify and appear, therefore, to be mediated by a single class of T cell-specific LK (46,47).

Other bioassays were designed to measure the effects of T cell-derived LK on macrophages. Macrophages can express effector cell function toward a variety of intracellular bacterial and microbial pathogens and neoplastic cells (122). These activities depend on a series of reactions which result in the production of activated macrophages. This activation is accompanied by various changes in endocytic, biosynthetic, and secretory functions as well as others. The T cell-derived mediator responsible for these activities has been termed macrophage-activating factor or MAF. MAF, together with a second signal of undefined nature, induces macrophages to become cytocidal toward neoplastic cells and this effector activity, measured either by the release of radioisotope from lysed target cells or the terminal uptake of radioisotope by the remaining target cells, is the basis for a widely used assay for MAF activity (122). More recently, it has been shown that similar LK preparations are capable of recruiting Ia^+ macrophages into the peritoneal cavity of mice and the mediator responsible for this activity has been termed macrophage (Ia^+)-recruiting factor or MIRF (123). The relationship of this mediator to MAF or other T cell-derived LK is still unknown.

Other mediators secreted by activated T cells act on target cells which are not directly involved in immune responses and are, therefore, assayed in nonimmunological systems. These include (a) various factors which promote the differentiation of hematopoietic progenitor cells along different lineages, i.e., colony-stimulating factor (CSF) and erythroid-promoting activity (EPA) which act on granulocyte/macrophage and erythroid progenitors, respectively, (b) mast cell growth factor (MCGF), a mediator which appears to promote the growth of mast cells in culture, and (c) immune interferon (IFNγ) which is defined and assayed by its antiviral activity but which, in addition, may have profound immunoregulatory effects. Some of these biological

activities will be described in more detail below. The above are the most common biological activities ascribed to T cell-derived LK preparations. Some other activities, of less defined nature, will be discussed briefly below.

III. Monoclonal, Antigen-Specific Lymphokines

It is useful to classify T cell-derived immunoregulatory factors into those which are either antigen-specific or antigen-nonspecific. Antigen-specific factors (13,15–17) regulate immune responses exclusively to the antigen which induced their formation, they bind the respective antigen (or its specific idiotype; see below) and bear determinants encoded by the *I* region of the MHC. These factors have been discussed in detail in recent reviews (13,15–17). On the other hand, the antigen-nonspecific factors act in a polyclonal fashion, and many of them, although not all, do not bear detectable MHC gene products (15). Some of these factors possess, in fact, properties that place them in between the two classes of factors mentioned above, in that they are either generated by specific antigen stimulation and/or they carry MHC-encoded determinants, yet they are antigen-nonspecific in their biological activity. These factors may be of particular interest since they may form a link between the antigen-specific and nonspecific groups of factors (15).

Depending on their biological activity, the antigen-specific LK are divided into two major categories, namely, helper or suppressor factors (ThF or TsF, respectively). As clearly implied by their names, ThF replace Th cells and provide help to antigen-primed effector cell precursors, whether they are T or B lymphocytes. On the other hand, TsF, being the soluble products of Ts cells, act to inhibit or suppress the immune response. These two classes of biologically active molecules share two main properties, i.e., antigen-specificity and expression of Ia antigenic determinants (and more specifically, *I-A* subregion determinants by ThF vs *I-J* or *I-C* subregion antigens by TsF). The molecular basis for the biological activity of these mediators (help vs suppression) is still far from being clear (13,16,17). The various T cell lines secreting antigen-specific LK are listed in Table I.

A. Monoclonal, Antigen-Specific Helper Factors

T cell help has not been dissected to the same extent as T cell suppression (see below) in terms of the cellular subsets, their antigen vs idiotype specificities and soluble products. Nevertheless it appears that at least two Th subsets are involved in a secondary antibody response, either expressing or not Ia antigens (124). The interaction

TABLE I

T CELL LINES SECRETING ANTIGEN-SPECIFIC IMMUNOREGULATORY LK[a]

Designation of cell line	Antigen specificity	Comments[b]	Reference
A. Helper factors			
R-9	(T,G)-A--L	55,000–70,000 MW, reacts with anti-V_H, Id and *I-A* displayed on different chains	26,128,164,172
T85-109-45	CGG	MHC(*I-A*)-restricted, reacts with anti-V_H, hybridoma binds only Ia-associated antigen complex, not free antigen, *I-A*-restricted adsorption on unimmunized spleen cells	131,132
FL10	KLH	MHC-restricted, reacts with anti-V_H, requires Th cells, and inactive in T-depleted cultures, optimal activity when added on day 2–3	130b,135
E-9M(+)	(T,G)-A--L	TCGF-dependent line, reacts with anti-V_H	137
B. Suppressor factors			
A1	SRBC	200,000 MW. 85,000 (Id^+) and 25,000 (Ia^+) MW chains. Displays *I-E/C* determinants, MHC-unrestricted, adsorbed on B cells	144–148
Various lines	SRBC	Suppress induction or expression of DTH, MHC-unrestricted, <50,000 MW glycoprotein	150
S1.41	KLH	50,000–60,000 MW, MHC-unrestricted, acts on Th cells	57,170
Various lines	HGG	Fusion with EL4 or L5178, unstable, some suppress nonspecifically	25
9F181a 34S-704	KLH	MHC-restricted, reacts with anti-V_H, 70,000 MW. 45,000 (Id^+), and 25,000 ($I\text{-}J^+$) MW chains noncovalently linked in cytoplasm, covalently linked in secreted product, display unique *I-J* determinants, Id^- acceptor hybridoma (34S-281) identified	60,155–160
49A	Ova	—	161
C4#4	GAT	Single 24,000 MW chain displays both Id and *I-J* determinants, MHC unrestricted, may exist as a dimer	162,181

R-11	(T,G)-A--L	50,000–70,000 MW, probably 2 chains, reacts with anti-V_H	26,164
H7,Cl,G12	ABA	React with anti-ABA CRI, conventional TsF is a 92,000 MW, *I-J* negative single chain polypeptide	167
F12	ABA	Suppresses induction of DTH responses and priming for CTL responses, induces Ts_2 cells *in vivo*, i.e., factor is a TsF_1, reacts with anti-ABA CRI, Igh- but not MHC-restricted	59,169
CKB-17,CKB-39, B6-29	NP	React with anti-NP^b CRI, TsF_1, suppress the induction of DTH responses and induce Ts_2 cells, Igh- but not MHC-restricted (pseudorestriction), another hybridoma, CKB-59, is Id^- and suppresses DTH expression (Ts_2)	61,171
—	NP	TsF_1, acts on antiidiotypic T cells	135
—	NP	NP^b-positive, *I-J* determinants on TsF derived from BW5147, not from Ts haplotype	170
I82K54	MCA-1490 (BALB/c sarcoma)	Suppresses tumor-specific CTL responses, enhances tumor growth in immune mice or in Winn assay	177
$L_4(L_4H)$	HEL (lysozyme)	*In vitro* infection of HEL-specific Ts cells with RadLV and derivation of culture lines from *in vivo* growing thymomas, suppression of antigen-induced T cell proliferation	178
Cl.Ly23/4	SG (sheep glycophorin)	TCGF-dependent line, 70,000 MW, single chain polypeptide, p*I* 4.9–5.0, does not display Ia, inactivates Th cells, degraded into 45,000 MW, Id^- nonspecifically suppressive and 24,000 MW, Id^+ nonsuppressive products	179,180
C. No biological activity			
T34G6 T33D5	ABA	Inhibit binding of anti-ABA antibodies to ABA–BSA-coated plates (RIA), 62,000 MW single polypeptide chain, both $I\text{-}J^+$ and Id^+, unique peptides secreted by different clones	168

[a] Antigen-specific Th hybridomas whose soluble products have not been reported (35,136) are not included.

[b] All lines are constitutive producers and they or their soluble products bind antigen specifically. Unless otherwise indicated, all lines were derived by somatic cell hybridization with BW5147, their products display Ia determinants, and were assayed in culture or *in vivo* adoptive transfer systems of primary or secondary antibody responses.

between these two subsets may be mediated by an antigen-specific, *I-A*$^+$-soluble mediator (7). Other studies have demonstrated that, in addition to the "classical," antigen (carrier)-specific Th cell which is MHC-restricted in its action (125), another Th subset exists which is idiotype-specific (126) and MHC-unrestricted (127). The relationship between the Ia$^-$ and Ia$^+$ Th cells on one hand (124), and the antigen- or idiotype-specific Th cells on the other (125–127) is not clear.

As mentioned above, there has been only one isolated report on an antigen-specific Th line which was not derived by deliberate antigen immunization. The cell line involved is the culture line 485-2 derived from a virus-induced thymic lymphoma in CFW/D mice (33). Cells of this cell line, which apparently was not cloned, were capable of providing help for a primary anti-SRBC antibody response in an *in vivo* adoptive transfer system, or in *in vitro* cultures of T-cell-depleted splenocytes. The same malignant cells did not provide help for an antibody response against non-cross-reacting horse erythrocytes (HRBC). The soluble products of this cell line, either without or with antigen stimulation, were not studied (33).

The derivation of all the other T cell lines capable of secreting antigen-specific immunoregulatory factors involved deliberate immunization of the T cells with the relevant antigen. One approach, which was described earlier, has been to prime mice with an antigen (KLH) and then to induce malignant transformation of purified T-cell populations from such mice by RadLV virus infection *in vitro* (35). No particular efforts were made to enrich for antigen-specific Th cells in this study, but it may very well be that the virus preferentially infects antigen-activated T cells. This tentative conclusion is based on the finding that four out of five donor type thymic lymphomas induced in syngeneic C57BL/6 recipient mice by the intrathymic injection of the infected spleen cells manifested antigen-specific helper function following their establishment as permanent culture lines (35).

The antigen-specificity of these transformed Th lines was indicated by their ability to cooperate with DNP-primed B cells in an *in vivo* secondary anti-DNP antibody response only when the challenge antigen was a DNP–KLH conjugate, but not when DNP was conjugated to the unrelated carrier, chicken γ-globulin (CGG). Although no soluble products of these KLH-specific lymphoma lines were studied, this methodology is likely to be successful for the purpose of deriving similar cell lines secreting antigen-specific ThF. In fact, this experimental approach has resulted recently in the production of cloned T lymphoma lines secreting antigen-specific TsF (see below).

Eshhar *et al.* were the first to report the establishment of T hy-

bridoma cell lines secreting antigen-specific ThF (128). Thymocytes were adoptively primed to the synthetic polypeptide (T,G)-A--L in irradiated syngeneic C3H.SW (*H-2*^{b}) recipients and the educated T cells, removed from the spleens of these mice 1 week later, were further enriched for specific Th cells by a 24-hour culture on monolayers of (T,G)-A--L-pulsed adherent spleen cells. Following fusion with the BW5147 lymphoma line, growing hybridomas were selected for their biological activity and reactivity with a heterologous rabbit antiserum specific for the heavy chain variable region (V_H) of mouse Ig.

Since the same anti-V_H antiserum was used in several other studies of similar nature and because its use is directly related to the somewhat controversial question of the potential role of Ig determinants in the T-cell receptor, it is desirable to briefly describe this antiserum (129). It was prepared by immunizing rabbits with purified V_H fragments derived from the DNP-binding, murine IgA myeloma protein, MOPC-315. This antiserum was found to be specific for V_H determinants shared by many murine myeloma proteins or normal Ig of different classes, irrespective of antigen specificity, subclass, or allotype and it, therefore, most likely reacts with framework determinants of V_H (129). This is different from conventional anti-Ig antisera, prepared by immunization with whole Ig molecules, which are directed predominantly against constant regions of the Ig heavy or light chains. This antiserum has been extensively applied to the study of antigen-specific T cells and their soluble products and on the basis of these studies it has been concluded that V_H determinants are intimately associated with the antigen-specific T-cell receptor (reviewed in 13,16,17). These antigen-specific T cells or their soluble factors do not react, however, with conventional anti-Ig antibodies and lack, therefore, constant region determinants of Ig molecules.

Of 21 hybridoma lines screened (128), none reacted with anti-V_L or -Ig antibodies, whereas five expressed V_H determinants. Of these five, three lines modulated the antibody response to (T,G)-A--L but only one line, R-9, was capable of providing antigen-specific help. Help could be elicited in an *in vivo* adoptive transfer system or in a secondary *in vitro* antibody response. The R-9 line, which was subsequently cloned in agar, secreted constitutively a (T,G)-A--L-specific ThF. This ThF was affinity-purified on a (T,G)-A--L-Sepharose column and expressed V_H determinants.

In subsequent studies (26) it was found that the monoclonal (T,G)-A--L-specific ThF expressed idiotypic (Id) determinants shared with anti-(T,G)-A--L antibodies as well as *I-A*^{b} determinants. It is noteworthy that the R-9-derived ThF reacted with polyclonal, but not

with a monoclonal, anti-Ia^b antibodies. This may be due to the presence of T cell-specific Ia molecules on the ThF, not recognized by the monoclonal anti-Ia^b which could be directed to B cell-specific Ia antigens. The existence of such T cell-specific Ia molecules has been demonstrated by others (7,130,130a,130b). Biologically inactive effluents of R-9 supernatants from anti-Ia^b and anti-V_H (or anti-Id) columns, respectively, formed a biologically active ThF when mixed together (26). This implies that distinct ThF subunits expressing MHC products or idiotypic determinants exist in the secreted product in nonassociated form and that upon mixing they may reassociate with one another to form a biologically active molecule. The monoclonal ThF bound, in addition to (T,G)-A--L, some other structurally related synthetic polypeptides, indicating that it recognized an epitope common to all such polypeptides. The R-9-derived ThF was found to be a glycoprotein with a MW of 55,000–70,000 (26).

Another cloned T-cell hybridoma line, denoted T85-109-45, exhibiting CGG-specific Th function and secreting constitutively a ThF of the same antigen-specificity, was recently characterized by Lonai *et al.* (131,132). The hybrid cells as well as their soluble products expressed a CGG-binding receptor, V_H and *I-A* determinants, but not V_L determinants. This factor reacted with some, but not with other, monoclonal anti-Ia antibodies indicating a restricted expression of Ia antigens. In contrast to the R-9 hybridoma (or its factor) which bound free antigen, T85-109-45 hybridoma cells bound CGG only after it has been processed by syngeneic macrophages and presented in association with self-Ia antigens by the so-called Ia-associated antigen complex (IAC; 133). Furthermore, IL-1 was required for binding of the antigen to the hybridoma cells. Binding of antigen was specifically inhibited by pretreatment of the hybridoma cells with anti-V_H or -Ia antibodies. Unlike the (T,G)-A--L-specific conventional ThF, this monoclonal CGG-specific ThF acted in an *H-2*-restricted manner, namely, it cooperated only with antigen-primed, T cell-depleted B cells of the same *H-2* haplotype ($H\text{-}2^k$) as that expressed by the ThF. Similarly, the monoclonal ThF was adsorbed on unimmunized spleen cells in an *I-A*-restricted manner (132). Cooperation was measured in a secondary *in vitro* antibody response to a hapten–CGG conjugate, and this ThF had a marked biological activity even when diluted up to 1 : 1000 in the culture assay.

More recently, a T-cell hybridoma clone, denoted FL10, displaying antigen specificity for KLH was described by Hiramatsu *et al.* (130b). The primed T cells used for fusion were derived from A/J mice immunized twice with a high dose of soluble KLH. Although this im-

munization method is known to preferentially induce Ts cells (25,60), advantage was taken of the fact that A/J mice are genetically deficient and incapable of producing a KLH-specific TsF (134). Specific Th cells were further enriched by binding to KLH-coated plates, again a method routinely used for enrichment of Ts cells but not for Th cells which usually bind free antigen with low affinity. Ia^+ growing hybrids were selected by FACS analysis, expanded, and tested for biological activity following cloning by limiting dilution. Extracts of these clones were tested for their ability to augment *in vitro* secondary antibody (IgG) responses of unfractionated, DNP–KLH-primed spleen cells. The extracts of 3 out of 70 clones tested displayed stable antigen-specific augmenting activity. Like the (T,G)-A--L- (26,128) and the CGG- (131,132) specific ThF-secreting hybridomas described above, FL10 cells and their factor expressed $I\text{-}A^k$ determinants recognized by conventional, but not by monoclonal, antibodies as well as V_H determinants.

In contrast, some other properties of this monoclonal KLH-specific factor clearly distinguish it from the two monoclonal ThFs described earlier. In particular, the activity of the FL10-derived factor was dependent on the presence of KLH-specific Th cells in the culture and it had no biological activity in cultures of T cell-depleted, DNP–KLH-primed spleen cells. Thus, this factor did not act as a T cell-*replacing* factor but, rather, as a T cell-*augmenting* factor (TaF; 7). In addition, this TaF displayed optimal augmenting activity when added to the culture on day 2 or 3, rather than at initiation. Like the R-9-derived, (T,G)-A--L-specific ThF (26,128), but unlike the T85-109-45-derived, CGG-specific ThF (131,132), this KLH-specific TaF bound free antigen. On the other hand, the same monoclonal TaF was similar to the CGG-specific ThF, but differed from the (T,G)-A--L-specific ThF in its MHC-restricted biological activity. Thus, the $I\text{-}A^k$-bearing TaF displayed biological activity only in primed spleen cell cultures displaying *I-A* identity (135). The Ia specificities displayed by the FL10 clone and its TaF were T cell-specific since the reactivity of several anti-Ia antibodies with the clone or the soluble factor was removed by preadsorption on T, but not on B cells. In addition, monoclonal anti-Ia antibodies directed against private (Ia.2) or public (Ia.17) specificities of the B cell Ia molecules possessed by $H\text{-}2^a$ mice were unable to react with FL10 cells or the soluble TaF (130b).

Thus, although the three T-cell hybridoma-derived helper or augmenting factors described above share several important properties, i.e., expression of Ia and V_H determinants and specific antigen binding, they differ from each other in some other properties. These differences

will be discussed in more detail below, in relation to the mechanism of action of these factors and their molecular organization.

A rabbit erythrocyte (RE)-specific cloned Th hybridoma line was reported recently (136) although its soluble product was not studied. Th cells were derived from RE-primed mice and, following fusion, the hybridoma cells were cloned in soft agar. Cells of clone BP_1C_{36} expressed V_H determinants and receptors for the Fc portion of IgG molecules ($Fc\gamma R^+$) and were capable of providing antigen-specific help to cultures of RE-immunized athymic nude spleen cells in a primary antibody response (136).

A different experimental approach for generating continuous T cell lines producing antigen-specific ThF was reported recently (137). Instead of using the somatic cell hybridization technique, these authors have taken advantage of the property of TCGF (IL-2) to support the growth of activated T cells (41–48) to derive a TCGF-dependent, (T,G)-A--L-specific Th line (137). Spleen cells of C3H.SW mice, a strain which is a high responder to this synthetic polypeptide, were sensitized *in vitro* on antigen-pulsed adherent cell monolayers for 5–6 days, followed by a short-term second culture on such pulsed monolayers. Such secondary culture was found to enrich for antigen-specific Th cells (138). The cells were then transferred to TCGF-supplemented medium and established as a permanent line which remained stable for at least 18 months. The uncloned cells of one of these lines, E-9M(+), displayed potent antigen-specific Th activity *in vitro*. One to two hundred cells efficiently reconstituted secondary anti (T,G)-A--L antibody responses. To study specific ThF production, the culture supernatants of this line were harvested after 4–5 days of culture in the TCGF-supplemented medium. Such supernates were active at a dilution of 1 : 100, as indicated by their ability to replace Th cells in a secondary antibody response against a hapten–carrier conjugate in a carrier-specific manner. Interestingly, no nonspecific helper activity was detected in these supernatants despite the fact that they contained TCGF, at least at culture initiation. This may be due to complete consumption of TCGF by the actively growing T cells, or to the inability of TCGF to act on B-cell cultures depleted of potential Th precursors. Since this line was maintained in the presence of crude TCGF preparations, it is difficult to determine whether the secretion of the antigen-specific ThF was constitutive or induced by the residual Con A present in the crude TCGF preparations.

The antigen-specific ThF secreted by the E-9M(+) cell line bound (T,G)-A--L and in addition expressed *H-2* and V_H determinants as

well as Id determinants shared with anti-(T,G)-A--L antibodies. The E-9M(+) line differs from most TCGF-dependent, antigen-specific Th lines studied by others. Such lines were almost invariably found to be dependent on antigen plus APC, in addition to TCGF, for maintenance of growth and function in culture (49–51,78). Furthermore, such Th lines failed to produce, when examined, antigen-specific ThF (76), but secreted a number of antigen-nonspecific LK, including TCGF (50,51). A possible explanation for this discrepancy is that the antigen-specific ThF, but not the nonspecific LK, is secreted constitutively while, following stimulation with antigen plus APC, the antigen-specific ThF is masked by the nonspecific LK, particularly TCGF, which predominate in the culture supernatant. Alternatively, the E-9M(+) cells belong to a Th subset distinct from most cloned Th lines studied by others. The unique growth requirements of this cell line, i.e., its lack of dependence on antigen plus APC support this possibility.

Using a similar experimental approach to the one described above (137), it has recently been possible to establish lines of human (T,G)-A--L-specific Th cells. Human T cells were sensitized *in vitro* on (T,G)-A--L-pulsed monolayers of autologous adherent cells and subsequently cultured in the presence of human TCGF. Such lines maintained growth and function for several months and secreted a ThF which could replace (T,G)-A--L-specific Th cells in *in vitro* antibody-producing cultures of murine spleen cells. The active ThF bound antigen and reacted with antisera directed against HLA-DR (the human equivalent of the murine Ia) and human V_H determinants (Z. Bentwich, personal communication). A similar study about the establishment of uncloned, TCGF-dependent human Th cell lines secreting an antigen (influenza virus)-specific ThF has been reported very recently (138a). There is no doubt that antigen-specific human T cell lines and their soluble products will provide important tools to study antigen-specific immunoregulation in clinical situations.

B. Monoclonal, Antigen-Specific Suppressor Factors

Our thinking about the critical immunoregulatory role of Ts cells and their soluble products has evolved rapidly since the initial discovery of these cells by Gershon *et al.* (139,140). As a result of many subsequent studies, it has become clear that even Ts cells do not comprise a homogeneous T-cell subset and that multiple interactions among distinct Ts subsets and their soluble products combine to form a finely tuned immunoregulatory network (141). In the course of these studies, many seemingly contradictory results have been reported with

regard to issues such as the expression of MHC and/or V_H determinants, MHC restriction, antigen vs idiotype specificity, and the identity of the target cells for antigen-specific suppression.

Although adsorption of Ts populations on antigen-coated (25,58–61) or idiotype-coated (142) plates has allowed identification of antigen-specific and idiotype-specific Ts subsets, respectively, such separation cannot be regarded as absolute. Thus, the ultimate separation of various T-cell subsets involved in suppressor circuits requires cloning techniques. The development of these techniques together with methods for creating permanent T cell lines allow the study of Ts cells and their products in an extremely accurate manner. As a result of these and other developments, e.g., studies on T cell suppression in systems of defined haptens and their respective Id, it has been possible to incorporate the voluminous data on antigen-specific T cell suppression into one major pathway (143). According to this model, antigen triggers cyclophosphamide (CY)-sensitive, Lyt 1^+ or Lyt $1,2,3^+$, $I\text{-}J^+$ precursors to differentiate into first-order Ts cells (Ts_1). These Ts_1 cells secrete an Id^+, $I\text{-}J^+$, antigen-specific TsF_1 which acts in a MHC- and V_H-*un*restricted manner. TsF_1, either in the absence or presence of antigen, triggers the development of second-order Ts cells (Ts_2) from CY-insensitive, Lyt $1,2,3^+$, $I\text{-}J^+$ precursors. The mature, Lyt $2,3^+$, $I\text{-}J^+$ Ts_2 cell secretes in turn its soluble factor, TsF_2, which displays *I-J* (or *I-C*) determinants and antiidiotypic (or, possibly, antiantigen) specificity. The biological activity of TsF_2 is restricted by MHC- and, in the case of antiidiotypic TsF_2, V_H-linked genes. Finally, TsF_2 triggers an antigen-primed, Lyt $1,2,3^+$ and $I\text{-}J^+$, CY-sensitive precursors to become the final effectors of suppression, Ts_3, which may mediate suppression in an antigen-nonspecific manner (143).

To date, cloned Ts lines which display antigen-specific suppression and produce the corresponding TsF have been studied to a larger extent than Th lines. This may be due, in part, to the relative ease of preparing relatively pure Ts populations by adsorption on antigen-coated matrices. The overwhelming majority of permanent, antigen-specific Ts lines were derived by somatic cell hybridization with appropriate T lymphoma lines (reviewed in 26), although there have been other isolated reports on Ts lines immortalized by viral transformation or growth in TCGF-supplemented medium (see below). These Ts lines or their soluble products were shown to suppress various types of immune responses. Accordingly, we will first review in this section Ts hybridoma lines which suppress the responses to (a) particulate heterologous antigens (i.e., SRBC), (b) native or synthetic polypeptides, (c) defined haptenic determinants, and (d) tumor-specific anti-

gens, and conclude with the few studies on antigen-specific Ts lines derived by methods other than somatic cell hybridization.

1. Ts Hybridomas Specific for Particulate Antigens

Taussig *et al.* have studied an uncloned T hybridoma cell line, A1, which suppressed anti-SRBC antibody responses *in vitro* (144–148). SRBC-primed spleen cells were obtained from C57BL/10 mice primed with a high dose of SRBC. Following fusion with BW5147, supernatants harvested after 48 hours of culture were tested for suppressive activity and only 1 out of 58 growing hybridomas was found to secrete constitutively a factor which specifically suppressed the primary (IgM) or secondary (IgG) response to SRBC or TNP-SRBC conjugates in a Mishell–Dutton culture. Specific suppression was evident at a dilution of ~1 : 200 although some nonspecific suppression was found at higher concentration (>5% v/v). This TsF displayed suppressive activity even when added to the culture on day 3, and it expressed $H\text{-}2^b$, but not Ig determinants. Subsequent experiments, using adsorbed *H-2* antisera, indicated that the MHC determinants expressed by this TsF are encoded in the *I-E/C* subregion, in contrast to most other antigen-specific TsF which express *I-J* subregion antigens. The A1-derived TsF was not MHC- or species-restricted and its biological activity was removed following adsorption on unprimed populations of unfractionated or adherent spleen cells and nude spleen cells. On the other hand, nylon wool-purified T cells or peritoneal macrophages did not remove factor activity. Based on these results, it was concluded that B cells display an acceptor site for the factor, but Ia^+ macrophages, which are known to play a critical role in antigen presentation, cannot be excluded, particularly since their proportion among peritoneal exudate cells is relatively low (149). The expression of the acceptor site for this SRBC-specific TsF was found to be controlled by genes located to the right of the *I-J* subregion (147).

Taussig *et al.* have also studied the physicochemical properties of their TsF (145,147,148). By gel chromatography, the secreted factor had an apparent MW of approximately 200,000. Further characterization was carried out by internal labeling of the A1 hybridoma cells with [^{3}H]leucine, followed by adsorption of the culture supernatant on SRBC to selectively enrich for SRBC-specific labeled material. The SRBC were then lysed with a detergent and the soluble material was analyzed by polyacrylamide gel electrophoresis (PAGE) under reducing conditions. This analysis revealed two units with apparent MW of 25,000 and 85,000, respectively. The fact that a similar profile was obtained in nonreducing conditions indicates that these two units are

not linked covalently. The large subunit displayed the antigen-binding property while the small subunit was adsorbed by $H\text{-}2^b$ alloantisera. Similar analysis of labeled material extracted from A1 cells revealed that only the large subunit was adsorbed by SRBC, indicating that the $H\text{-}2^+$ and the SRBC-binding subunits exist intracellularly in a nonassociated form, and that they combine together before being secreted. A similar conclusion was derived from studies on hybridoma-derived, (T,G)-A--L-specific ThF (26) and KLH-specific TsF (see below).

Other T-cell hybridoma lines secreting a SRBC-specific TsF were described by Hewitt and Liew (150). The products of these hybridomas, in which the normal T cell partner was derived from CBA mice primed with a high dose of SRBC, suppressed DTH responses to SRBC. DTH responses were elicited by injecting mice subcutaneously with SRBC (DTH induction) and challenging them 5 days later in the footpad with the same antigen (DTH expression). The reactions were measured by the increase in footpad thickness 24 hours later. Four lines secreted a TsF which suppressed DTH induction (i.e., when injected before SRBC priming), but only one of these was antigen-specific. Six other lines secreted a factor which specifically suppressed the expression of DTH (i.e., when injected after antigen priming but before secondary challenge). The TsF which suppressed DTH expression was not MHC-restricted. The titer of the same hybridoma was $\sim 1:10^1$ in culture supernatants and $1:10^4$ in the serum of AKR mice bearing the growing hybridoma. The factor was found to have a molecular weight of less than 50,000, to express $I\text{-}J^k$ determinants and to bind to concanavalin A columns.

The Ts hybridoma lines suppressing either the induction or expression of DTH most probably represent two functionally distinct T-cell subsets. Thus, Ts_1 and Ts_2 cells were found to suppress the induction and expression, respectively, of DTH responses to the haptens azobenzenarsonate (ABA) and 4-hydroxy-3-nitrophenyl acetyl (NP) (151,152; see below).

2. *Ts Hybridomas Specific for Polypeptide Antigens*

Kontiainen *et al.* were the first to report the construction of functional, antigen-specific T hybridoma cell lines (57). KLH-specific Ts cells were induced *in vitro* by culturing unprimed CBA ($H\text{-}2^k$) T cells with a high concentration of KLH. Cells from this culture were fused with BW5147 and supernatants of growing hybrids were tested for biological activity. The culture supernatant of an uncloned line (which was later cloned), S1.41, suppressed primary or secondary *in vitro*

anti-DNP antibody responses only when the hapten was coupled to the specific carrier (KLH) but not to unrelated carriers. Suppression was evident with culture supernatant diluted up to 1 : 1000 or more. Biological activity was specifically adsorbed on KLH and anti-I-J^k, but not on anti-Ig, immunoadsorbents and could be recovered in the acid eluates. Furthermore, this monoclonal TsF reacted with rabbit or mouse (syngeneic) antisera raised against affinity-purified, KLH-specific conventional TsF. These antisera were shown before to react with constant (i.e., present in TsF, but not ThF, of different strain origins and antigen specificities) and variable-idiotypic (i.e., present only on KLH-specific TsF or ThF derived from the syngeneic mouse strain) regions of conventional KLH-specific factors, respectively (153). Like the conventional TsF (154), this S1.41-derived TsF displayed no MHC restriction in its biological activity and appeared to act directly on antigen-specific Th cells, in contrast to the SRBC-specific TsF which appeared to have B cells as its cellular targets (144–148). The apparent MW of this factor was 50,000–60,000 but the subunit structure of this TsF was not reported in this study (57).

HGG-specific Ts hybridoma lines were established by Taniguchi and Miller (25) by fusing HGG-tolerized CBA/H spleen cells, enriched on HGG-coated plates, with the T-cell lymphoma lines EL4 or L5178. Selection of hybrids expressing I-J^k determinants was made by positive sorting on the FACS. Several I-J^+ colonies growing in agar were found to have suppressive activity although it was not always antigen-specific. Thus, extracts of some hybridomas only suppressed the secondary adoptive anti-DNP–HGG antibody response while others suppressed the antibody response nonspecifically, as indicated by the suppression of the antibody response against HRBC. Extracts of some other I-J^+ hybridoma lines lacked suppressive *in vivo* activity. The main problem with these hybridoma lines was their instability. Thus, most lines progressively lost chromosomes, H-2^k gene products, and biological activity over a period of 3 months (25). This instability most probably reflected the inadequacy of the T lymphoma lines used for fusion in this study. The BW5147 line used for fusion in all other studies has proven to be a more successful choice.

An extensive series of studies which has shed light on the structure of antigen-specific monoclonal TsF was reported by Taniguchi, Tada, and their colleagues (60,155–160). Ts cells were derived from KLH-primed C57BL/6 mice. Enrichment of Ts cells before fusion and selection of I-J^+ hybrids after fusion were done as described above. Various I-J^+ lines were established by limiting dilution cloning or single cell manipulation techniques. KLH-specific suppression was evaluated by

studying the effects of extracted or secreted materials from such hybridoma lines on a secondary (IgG) *in vitro* antibody response to DNP–KLH conjugates (or DNP conjugates with other carriers as specificity controls). By these criteria some T hybridoma cell lines displayed antigen-specific suppression, others displayed nonspecific suppression while still others lacked suppressive activity.

Two cloned lines, 9F181a (60,156) and 34S-704 (155,158), producing KLH-specific TsF molecules, were studied in particular detail. These antigen-specific TsF molecules, either in their intracellular or secreted forms, displayed KLH binding, $I\text{-}J^b$ and V_H, but not V_L or Ig constant region determinants. Their biological activity was MHC-restricted and the restriction was mapped in the *I-J* subregion of the murine MHC. Some of these lines (9F181a) could be grown in *H*-2-compatible mice, retained their function and produced a high-titered TsF in the serum and ascites of the recipient mice. Expression of cell surface markers and biological activity were dependent on the cell cycle of the hybridoma. High levels of KLH-binding, $I\text{-}J^b$ expression, and TsF were all found during the M phase and decreased markedly during the S phase in synchronized cultures of the hybridoma cells (159).

The relationship between the antigen-binding and the $I\text{-}J^+$ moieties of the extracted (intracellular) or secreted forms of the KLH-specific TsF was studied by adsorption on KLH or anti-$I\text{-}J^b$ immunoadsorbent columns (155,158). The biological activity of the secreted TsF, derived from the ascites fluid of hybridoma-bearing mice, was adsorbed on each of these two types of column and could be recovered in the acid eluate. Combination of the effluents from the two columns did not reconstitute suppressive activity, indicating that free KLH-binding or $I\text{-}J^+$ moieties are not present in the secreted products. In contrast, the biological activity of the extracted product (which was also adsorbed on, and could be eluted from, either KLH or anti-*I-J* immunoadsorbents) could be reconstituted by mixing the effluents of the two columns (155). This result indicates that the TsF is composed of two subunits. In the intracellular product, both free subunits, i.e., the KLH-binding and the $I\text{-}J^+$ ones, as well as the associated form, coexist. On the other hand, the secreted product contains only the associated, biologically active form. Double adsorption experiments on the two respective immunoadsorbents eliminated the possibility that the suppressive activity in the mixture of the two effluent materials (of the extracted product) is due to additive effects of very low amounts of TsF remaining after each adsorption step.

More recently, it was found that the two polypeptide chains of the secreted product are covalently linked by disulfide bonds (158). Thus,

reduction of the TsF with 5 m*M* dithiothreitol (DTT) did not affect biological activity but subsequent alkylation with 10 m*M* iodoacetamide (which would prevent reformation of disulfide bonds) resulted in an inactive product. Moreover, mixing the effluent and acid eluate of a DTT-reduced material following passage on a KLH immunoadsorbent resulted in a biologically active TsF. Additional double adsorption experiments revealed that, indeed, cleavage by reduction with DTT separates the KLH-binding and $I\text{-}J^+$ moieties, respectively. Thus, the KLH-binding (idiotypic) and $I\text{-}J^+$ subunits could reassociate spontaneously to form the complete TsF (158).

The availability of functionally different T-cell hybridoma lines (i.e., nonsuppressor, antigen-specific or nonspecific suppressor hybridomas), all of which express *I-J* determinants, allowed Taniguchi *et al.* to reveal the heterogeneity of the *I-J* gene products (135,157,159). Conventional $I\text{-}J^b$ alloantisera, obtained from B10.A(5R) mice immunized with B10.A(3R) cells, were adsorbed on individual hybridoma clones and then tested for residual reactivity with other clones. Adsorption on an antigen-specific clone removed residual C-dependent cytotoxic activity against the homologous or other KLH-specific Ts hybridoma clones but left intact the reactivity against nonspecific suppressor or nonsuppressor clones. Conversely, anti-*I-J* serum adsorbed on cells of a nonspecific suppressor hybridoma retained their reactivity with KLH-specific or nonsuppressor clones. On the other hand, adsorption on a nonsuppressor hybridoma clone removed the cytotoxic activity against all T-cell hybridoma lines. These results indicate that the nonsuppressor hybridomas display two distinct *I-J* determinants. Only one of these is displayed on the antigen-specific Ts hybridoma clones whereas the other one is displayed by the antigen-nonspecific Ts hybridomas (157). Direct evidence for this conclusion was provided more recently by showing that each of two monoclonal anti-$I\text{-}J^b$ antibodies reacted either with the antigen-specific or nonspecific Ts hybridoma lines (159). These results imply that the *I-J* locus is composed of several subloci coding for distinct *I-J* determinants.

It was also reported that a cloned hybridoma line, denoted 49A, in which the normal T cell partner was derived from ovalbumin-tolerized mice, specifically formed rosettes with ovalbumin-coated red blood cells and secreted an antigen-specific TsF (161). However, no characterization of this TsF was given in this report.

Other studies have characterized monoclonal TsF specific for synthetic polypeptides or defined haptens. The use of such defined antigens is advantageous since the antibody response to them is usually

much more restricted, as indicated by the dominance of a particular Id. Kapp *et al.* (162) established a T-cell hybridoma clone, C4#4, which constitutively secreted TsF specific for the synthetic terpolymer L-glutamic acid^{60}-L-alanine^{30}- L-tyrosine^{10} (GAT). The antibody response to this antigen is controlled by *H*-2-linked immune response genes and it has been shown that lack of responsiveness to GAT in nonresponder strains results from the preferential development of GAT-specific Ts cells (163). Thus, GAT-specific Ts cells for fusion were obtained from nylon wool-purified spleen cells of GAT-rimed, DBA/1 (*H*-2^q) nonresponder mice. The culture supernatant of the C4#4 clone inhibited in an antigen-specific manner the (a) secondary plaque-forming cell (PFC) antibody response, and (b) T-cell proliferative responses of spleen cells from DBA/1 mice primed with the immunogenic form of GAT, i.e., GAT-methylated bovine serum albumin (MBSA) conjugate. this TsF, which had an apparent MW of 20,000–60,000, displayed Ia^q determinants and specific binding of GAT, but not Ig constant region determinants. The titer of this TsF in culture supernatants was extremely high and it displayed 50% suppression at dilutions of ~1 : 10,000 or more (162).

In their report on the (T,G)-A--L-specific Th hybridoma line, R-9, Eshhar *et al.* also noted another line, termed R-11, which specifically suppressed the antibody response to (T,G)-A)-L (128), and was derived from the same fusion experiment (26,164). The R-11-derived TsF reacted with (T,G)-A--L, anti-Id, -V_H, and -*H*-2^b sera, thus displaying properties similar to those of other TsF. This TsF specifically suppressed secondary anti-(T,G)-A--L antibody responses either in an adoptive transfer system *in vivo* or in culture. The fine specificity of this TsF, in terms of its ability to bind several other synthetic polypeptides which are structurally related to (T,G)-A---L, was similar, but not identical, to that of the monoclonal (T,G)-A--L-specific ThF.

3. *Hapten-Specific Ts Hybridomas*

The haptens ABA and NP have been used widely in studies on the regulation of antibody and cell-mediated, in particular DTH, responses. The antibody responses against these, as well as other, haptens by certain mouse strains consist of a dominant, cross-reactive idiotype (CRI). For example, it was found that 20–70% of anti-ABA antibodies produced by A/J mice share such a CRI which can be detected by a heterologous antiidiotypic (i.e., anti-CRI) antibody reagent (165). Thus, haptens, their respective CRI, and anti-CRI reagents provide potent tools for studying the relationship between B- and T-cell idiotypes present on antibody molecules or antigen-specific

T cell factors, respectively. Moreover, immunoregulatory Ts networks have been studied in detail in the ABA and NP haptenic systems and various Ts subsets and their soluble products have been well characterized (16,142,143,151,152). It is not surprising, therefore, that several independent groups have recently established ABA- (59,166–169) and NP- (61,135,170,171) specific T hybridoma lines. Except for one study (168) in which the biological activity of the hybridoma product was not evaluated, all the other lines secreted hapten-specific TsF.

In the ABA system, cells for fusion were obtained from A/J mice primed with either an ABA-mouse IgG conjugate (166,167) or ABA-conjugated syngeneic spleen cells injected intravenously (59,168,169), both of which have been shown to elicit ABA-specific Ts cells. Spleen cells were then fused with BW5147 lymphoma cells, either unfractionated (168) or following enrichment of Ts cells by adsorption on ABA-coated plates (59,166,167,169).

In the study of Pacifico and Capra (168), culture supernatants of growing hybridomas were selected for antigen-specific reactivity by testing their ability to inhibit the binding of affinity-purified mouse anti-ABA antibodies to ABA–BSA-coated polyvinyl microtiter plates in a solid phase radioimmunoassay (RIA). Binding of anti-ABA antibodies to the plate was detected with an ^{125}I-labeled rabbit anti-mouse Ig antibody. The constitutive soluble products of two clones, denoted T34G6 and T33D5, which inhibited anti-ABA antibody binding in the RIA were analyzed in particular detail. The soluble products of these hybridomas were tested extensively but found to lack detectable biologic activity, i.e., help or suppression (D. Capra, personal communication).

On the basis of biosynthetically incorporated radiolabeled amino acids it was estimated that the ABA-specific product comprised 0.1–0.2% of the total material in the serum-free culture supernatant. The ABA-specific radiolabeled material was affinity-purified on ABA–HGG columns by elution with free hapten. The radioactivity in the affinity-purified product was precipitated by rabbit anti-CRI and anti-Ia^k or *I-J*^{k} alloantisera but not by anti-mouse Ig, *-H-2K*, or *-H-2D* sera, indicating the presence of CRI and Ia determinants on this factor. Analysis of the same radiolabeled product by SDS–PAGE revealed that the affinity-purified material had a MW of 62,000 and this was not affected by reduction and alkylation, indicating that the ABA-specific hybridoma product is a single polypeptide chain (or multiple 62,000 MW subunits in noncovalent association). Tryptic peptide analysis of the affinity-purified material from two distinct clones by high-pressure liquid chromatography revealed that, while most of the tryptic pep-

tides were shared by the two products, few peptides were unique for each clone.

The structure of this antigen-specific product differs significantly from other monoclonal TsF or ThF. While the ABA-specific product is a single polypeptide which displays both Ia and Id determinants, studies on SRBC- and KLH-specific TsF (145,147,155,158) or (T,G)-A--L-specific ThF (26,128,172) have revealed a structure of two polypeptide chains, one of each displays either the Ia or idiotypic determinants. The overall MW in all cases is similar however (i.e., ~60,000–70,000). The reasons for this major discrepancy are not clear, particularly since no biological function has been assigned so far to this ABA-specific hybridoma product.

Goodman, Ruddle *et al.* have studied several uncloned T-cell hybridoma lines secreting an ABA-specific TsF (166,167). These products were detected by their ability to specifically inhibit primary anti-ABA antibody responses in culture. Fifty percent suppression was obtained with hybridoma supernatants diluted ~1 : 100. The hybridoma cells formed rosettes with ABA-coated SRBC and displayed the ABA CRI. Although the hybridoma-derived TsF was not characterized in detail, the conventional TsF of the same antigen specificity studied by this group also displayed the CRI but, unlike most other TsF, did not display *I-J* determinants. Furthermore, Goodman *et al.* have studied the biosynthetically labeled conventional product of enriched Ts populations following extraction from lysed cells, affinity purification on an ABA column and elution with free hapten. A protein with a MW of 92,000 by SDS–PAGE analysis mediated antigen-specific suppression of the antibody response. This protein was stabilized in the presence of protease inhibitors, suggesting that it is being degraded by intracellular proteases. The protein appeared to be composed of a single polypeptide chain (167). The apparent MW of the same material by gel filtration was lower, i.e., about 70,000.

It is noteworthy that an antigen (SRBC)-specific TsF recovered from culture supernatants of Con A-stimulated spleen cells also displayed a 92,000-MW component but, in addition, another polypeptide with a MW of 21,000 (167). Although not proven directly, this 21,000-MW protein could possibly be the Ia^+ subunit of the TsF. If the TsF subunits existed in the cell predominantly in dissociated form and in culture supernatants in a covalently linked form, this could explain why such a 21,000-MW protein or *I-J* determinants were not identified on the product obtained from cell lysates by affinity purification on ABA columns. However, if this were the case, one would have to assume that the $I\text{-}J^+$ subunit is not necessary for biological activity. The

reasons for major discrepancies in the structures of antigen-specific LK studied by different groups will be discussed in more detail below.

A similar ABA-specific TsF derived from a cloned T-cell hybridoma line, F12, was characterized more recently (59,169). The F12-derived TsF specifically suppressed induction of DTH responses and *in vivo* priming for ABA-specific CTL responses, normally induced by the injection of ABA-coupled syngeneic spleen cells subcutaneously (173). Furthermore, this study went one step beyond other studies on monoclonal TsF, in characterizing the F12-derived TsF as a product of the first-order suppressor cells, Ts_1. This conclusion was based on the finding that the F12 supernatant induced, when administered into A/J mice repeatedly, Ts_2 cells defined by their ability to suppress the expression, but not the induction, of DTH responses. Like the conventional TsF_1, the biological activity of the monoclonal TsF was restricted by Igh-1 genes but not by the *H-2* complex. The F12-derived TsF was retained on immunoadsorbent columns composed of ABA, anti-CRI, or anti-$I\text{-}J^k$ and could be recovered in the acid eluates of such columns (169). It is quite likely that cloned T-cell hybridomas representing other distinct Ts subsets will be available soon and these will enable us to analyze precisely the chain of events in the antigen-specific suppression circuit regulating the DTH response to this particular hapten.

Okuda *et al.* have very recently characterized cloned T-cell hybridoma lines secreting TsF molecules which regulate DTH responses against the hapten NP (61,171). Recent studies have shown that NP-specific Ts cells, induced by the intravenous injection of NP-coupled spleen cells into syngeneic C57BL/6 mice, express the NP^b idiotype which is also the predominant idiotype (>85%) among C57BL/6 anti-NP antibodies (174). The V_H gene coding for this NP^b idiotype is closely linked to the $Ig\text{-}1^b$ heavy chain allotype linkage group. Moreover, these antigen-binding, idiotype-positive Ts_1 cells were found to trigger the development of anti-idiotypic Ts_2 cells in the presence of NP-derivatized syngeneic cells (151).

Enriched populations of NP-specific Ts cells from CKB ($H\text{-}2^k$) or C57BL/6 ($H\text{-}2^b$) mice were fused with the BW5147 T lymphoma. Growing hybrids were screened for the presence of *I-J* and NP^b idiotypic determinants. Only 29% of the $I\text{-}J^+$ hydrids displayed also the NP^b idiotype and three lines, CKB-17, CKB-39, and B6-29, of which one (B6-29) was cloned, were established. The free NP hapten inhibited the C-dependent lysis of the hybridoma cells by both anti-*I-J* or anti-NP^b Id antibodies (61). All three hybrids secreted into their culture supernatants NP-specific TsF which were capable of (a) suppress-

ing the induction, but not the expression, of a DTH response, and (b) inducing effector phase Ts cells, i.e., Ts_2 cells when administered into naive syngeneic recipients for 4–5 consecutive days. These Ts_2 cells could suppress expression of NP-specific DTH responses in NP-primed mice or secondary *in vitro* anti-NP antibody responses (171). The biological activity of these TsF could be removed by adsorption on immunoadsorbents composed of the appropriate anti-*I-J* alloantiserum or guinea pig anti-NP^b Id serum, and was recovered in the acid eluate. No Ig constant region determinants were displayed by these monoclonal TsF. By all of the above criteria, these three cell lines represent hybrids of the first-order suppressor cell, Ts_1.

The hybridoma-derived TsF were further tested for genetic restrictions of their biologic activity by using congenic strain combinations at either the *H-2* or the Igh loci (171). The TsF could suppress DTH responses in Igh-identical and *H-2*-incompatible mice but not in the reverse situation, indicating that TsF activity is Igh- but not *H-2*-restricted. Furthermore, the use of strains in which genetic recombination occurred between the Igh-C and Igh-V loci indicated that this genetic restriction maps to the Igh-V locus. However, in a subsequent experiment it was demonstrated that this was in fact a pseudorestriction. Thus, although the TsF did not suppress DTH responses in Igh-incompatible strains, it nevertheless induced in these strains Ts_2 cells. Such Ts_2 cells could effectively suppress the expression of DTH responses in strains of mice which expressed the same Igh allele as that expressed by the TsF-producing strain. Therefore, only the Ts_2 population, but not the hybridoma-derived TsF itself, is Igh-restricted in its action. In addition, the TsF-induced Ts_2 cell population was also *H-2*-restricted in its biologic activity (171).

Interestingly, the hybrid line CKB-59, which was not functional when tested for inhibition of DTH induction and did not display the NP^b Id was subsequently found to inhibit the expression of the response, i.e., when injected into primed mice before the elicitation of the DTH response. This $I\text{-}J^+$, NP^b-negative cell line presumably represents a hybrid of Ts_2 cells. It remains to be determined if the CKB-59 hybridoma displays antiidiotypic specificity, as would be expected for Ts_2 cells.

NP-specific T-cell hybridomas secreting antigen-specific TsF have recently been studied by others (135,170). In one of these studies (170) it was found that the TsF derived from a fusion product between the BW5147 ($H\text{-}2^k$) lymphoma and C57BL/10 ($H\text{-}2^b$) Ts cells expressed $I\text{-}J^k$ determinants, indicative of an association between an $H\text{-}2^b$-derived idiotypic polypeptide chain and a lymphoma ($H\text{-}2^k$)-derived $I\text{-}J^+$

polypeptide in the biologically active product. Such a finding supports the model according to which the V_H- and *I*-gene products are expressed on separate polypeptide chains which become covalently linked upon secretion.

4. Tumor-Specific Ts Hybridomas

It is well known today that cell-mediated immune responses against tumor-specific antigens are also regulated by different subsets of Ts cells and their soluble products, as well as by Th cells (175). Deliberate manipulation of these immunoregulatory circuits in favor of the tumor-bearing host is of obvious potential importance in clinical situations. Thus, cloned lines of tumor-specific regulatory T cells should allow more precise definition of the regulation of tumor-specific immunity.

Nelson *et al.* have fused thymocytes of BALB/c mice bearing the methylcholanthrene-induced sarcoma, MCA-1490, which are known to contain Ts cells (176), with the BW5147 T lymphoma line. The supernatants of one hybrid clone, denoted I82K54, displayed tumor-specific suppression (177). Suppression was assessed in three different experimental systems, namely (a) suppression of specific tumor target cell lysis by CTL populations, (b) enhancement of tumor growth in tumor-immunized mice which would normally reject an inoculum of the immunizing tumor, and (c) enhancement of tumor growth in the tumor neutralization (Winn) assay. Suppression was mediated even by very high dilutions ($\sim 1:10^6$) of the hybridoma culture supernatants and it was specific in that the responses to a non-cross-reactive sarcoma, MCA-1511, were not affected by the same TsF. The physico- or immunochemical properties of the TsF were not described in this study (177).

5. Non-Hybridoma-Derived Monoclonal TsF

Although the somatic cell hybridization technique has been the experimental method of choice for establishing antigen-specific Ts cell lines, two alternative approaches were recently described. One involves the *in vitro* neoplastic transformation of enriched, antigen-specific Ts populations by RadLV, followed by intrathymic injection of the infected Ts cells (178), as originally described by Finn *et al.* (35). The other method is to establish TCGF-dependent Ts lines in growth factor-supplemented culture medium (179,180).

In the first case (178), Ts cells from C57BL/6 mice, tolerized to hen egg-white lysozyme (HEL), were purified on antigen-coated plates and infected with RadLV. These infected cells were injected intrave-

nously into syngeneic recipients. Thymomas developed within 6 months in 6 out of 20 injected mice, and these were subsequently established as permanent culture lines. Cells of one of these lymphomas, L_4, or its culture-established line, L_4H, expressed Lyt 2 and $I\text{-}J^b$ determinants and their extracts or culture supernatants were capable of inhibiting, in a dose-dependent manner, the *in vitro* T-cell proliferative responses of HEL-primed T cells. The proliferative response to an unrelated protein antigen, purified protein derivative (PPD) of *Mycobacterium tuberculosis*, was not affected by the same extract. Extracts of cell lines lacking $I\text{-}J^b$ determinants did not display suppressive activity (178).

Fresno *et al.* have reported recently (179,180) the establishment of a TCGF-dependent T cell line which displayed suppressive activity specific for a protein derived from SRBC, sheep glycophorin (SG). Antigen-specific T cells were obtained from SRBC-primed C57BL/6 mice. Lyt $2,3^+$ Ts cells were enriched by treatment with anti-Lyt 1 antibodies plus C, followed by adsorption on SG-coated plates. Adherent cells were removed and cultured in microtiter plates in medium supplemented with crude TCGF. One clone that was characterized in detail, denoted C1.Ly23/4, was derived by micromanipulation of a single cell from a growing colony (179). Cells of this clone bound SG, but not glycophorin from erythrocytes of other species, and inhibited in a specific manner anti-SRBC PFC responses in cultures of SRBC-primed, Lyt 1^+ T cells (as a source of Th cells) and unprimed B cells. Cells of this clone were biosynthetically labeled with [^{35}S]methionine and a labeled protein with a MW of 70,000 (by SDS–PAGE analysis) was found to bind specifically to SRBC. This binding was inhibited specifically by soluble SG. Biological activity was removed on SG-immunoadsorbents and could be recovered in the acid eluate.

By preincubation of the SG-specific, 70,000-MW protein with either Lyt 1^+ T cells or B cells it was concluded that the target for this TsF is the Lyt 1^+ Th cell and inactivation of these Th cells required the presence of antigen. The TsF was further purified (180) to virtual homogeneity by fractionation of internally labeled secreted material on Sephacryl S-200 and DEAE-cellulose columns, followed by isoelectrofocusing (IEF). The resulting protein was >95% homogeneous according to SDS–PAGE analysis and represented two peptides which were distinguished by their p*I* values (~4.9 and 5.0, respectively). These purified proteins were retained on columns coated with lentil lectin (indicating their glycoprotein nature) or SG but not on columns coated with anti-mouse Ig or -Ia antibodies. Thus, this TsF is distinct

from most other TsF in its lack of expression of Ia determinants. Preincubation of SRBC-primed Th cells with the purified peptide for 24 hours in the presence of antigen completely inactivated these Th cells. Thus, the Ts cells producing this TsF most probably represent effector-phase suppressors, i.e., Ts_2 (or Ts_3) cells and not Ts_1, cells which act to induce Ts_2 cells.

In more recent experiments, two peptides which represent degradation products of the 70,000-MW glycoprotein were identified. One (MW = 45,000) suppressed antibody responses nonspecifically and did not bind antigen and the other (MW = 24,000) bound antigen but lacked biological activity. It was also concluded that the 70,000-MW TsF is composed of a single polypeptide chain since the size was not affected by using reducing vs nonreducing conditions during purification (180).

It is noteworthy that, with the exception of the above studies (179,180), all other TCGF-dependent T cell lines were those of CTL or Th cells. Thus, it seems that establishment of TCGF-dependent Ts lines has been more difficult than T cells manifesting other functions. It is possible that some Ts cells have some yet undefined requirements for long-term growth in addition to TCGF. It remains to be seen whether TCGF-dependent lines of other Ts subsets (i.e., Ts_1 cells) can be established.

C. Structure and Mechanism of Action of Antigen-Specific Monoclonal LK

Questions such as the molecular organization of the antigen-specific T cell factors, the relationship of the idiotypic determinants on such factors to the T-cell receptor or the antibody-combining site, the role of MHC gene products displayed by such factors and their exact mechanism of action have been discussed in detail by others (13,16,17,26,143) and are not the subject of this review. However, we would like to point out the power of cloned, LK-secreting, antigen-specific T cell lines as tools for analyzing such questions.

In general, two types of structurally distinct factors have been inferred from studies on conventional or monoclonal antigen-specific molecules derived from activated T cells. In one category are those factors which are composed of two subunits. One of these binds antigen and the other displays Ia determinants. The SRBC- and KLH-specific monoclonal TsF (145,147,148,155,158,159) and the (T,G)-A--L-specific monoclonal ThF (26,172) are representative of this group. The other category includes factors with a MW of approxi-

mately 70,000, composed of a single chain and lacking Ia determinants, as exemplified by the ABA- and SRBC (SG)-specific TsF (167,180). However, not all factors conform to this scheme. The 62,000-MW ABA-specific monoclonal product with unknown function studied by Pacifico and Capra (168) and the 24,000-MW GAT-specific monoclonal TsF studied by Kapp *et al.* (162,181; J. A. Kapp, personal communication) are composed of a single polypeptide chain which displays both idiotypic (antigen-binding) and Ia determinants.

It is not known whether these two groups of factors as well as the "exceptions" represent products of different T-cell subsets in the complex pathways of help or suppression, or whether they represent secreted regulatory products vs cell-bound T cell receptors. The SG-specific, 70,000-MW single chain product studied by Fresno *et al.* (180) has been reported to be degraded into a nonspecifically suppressive 45,000- and a 24,000-MW antigen-binding (but nonsuppressive) subunits. This is different from the SRBC-specific (145,147,148) or KLH-specific (155,158,159) TsF in which the large, 85,000-MW (145) or 45,000-MW (159) subunit binds antigen and the small, 25,000-MW subunit displays Ia determinants but no antigen binding.

An antigen-specific TsF, very similar to the one studied by Fresno *et al.* (180), has very recently been purified by hapten affinity chromatography of supernatants from conventional cultures of cells tolerized *in vivo* to the haptens DNP, TNP, or oxazolone (182). This TsF which existed as noncovalently linked dimers or oligomers was composed of a single 68,000-MW polypeptide chain which bound antigen but lacked MHC or Ig constant region determinants. This purified TsF was bound to Fc-like receptors on macrophages and was degraded into 45,000–50,000 and 25,000–30,000 MW products, reminiscent of the TsF studied by Fresno *et al.* (180). On the basis of these data and previous findings that have documented the obligatory intermediary role of macrophages in transmitting suppressor signals (183), it was suggested that this antigen-binding factor represents the antigen receptor of the effector Ts cell and that for biological activity (i.e., suppression) it has to be covalently associated with a separate, Ia^+ peptide. This Ia^+ peptide can be the product of the same Ts cell or it can be acquired from macrophages. Thus, according to this hypothesis the MHC determinants are not an integral part of the T-cell receptor but, rather, become covalently associated with it in order to confer upon it biological activity.

Another apparent discrepancy exists between the KLH-specific TsF and the (T,G)-A--L-specific ThF. While the secreted product of the first one does not contain free subunits (155), the secreted form of the other

(26) appears to contain free subunits since the mixture of effluents from anti-Id and anti-Ia columns, respectively, is biologically active (26).

Although it seemed that contradictory results obtained by different groups studying antigen-specific immunoregulatory LK were largely due to the heterogeneity of conventional preparations which contain products of distinct T-cell subsets, the study of monoclonal factors has not yet solved all of these contradictions. For example, the monoclonal, KLH-specific TsF studied by Taniguchi *et al.* appears to represent the product of the first-order, Ts_1 cell since it binds antigen (60) and induces another set of Ts cells to release a non-antigen-binding, KLH-specific TsF (159). Nevertheless, this TsF_1, which, according to the unifying scheme suggested recently (143), should be MHC-*un*restricted in its biological activity, was in fact MHC-restricted (157,159,160). In order to accommodate these findings within the unifying scheme (143), it was postulated that under certain conditions Ts_2 cells may have antiantigen, rather than antiidiotype, specificity and that the *I-J*-restricted, KLH-specific TsF is in fact a TsF_2.

Thus, it may well be that immunoregulatory pathways differ after all from one system to the next depending on various factors such as the antigen used (simple hapten vs complex protein, soluble vs particulate), the effector response measured (antibody, DTH) etc. Analysis of the homogeneous, monoclonal TsF and ThF should resolve this and other questions. The following is a list (which is by no means complete) of some forms of experimental analyses which are now feasible as a result of the availability of monoclonal, antigen-specific factors:

1. Using monoclonal TsF or ThF together with cloned populations of target cells for such factors, it should be possible to reconstruct precisely the sequence of events in the complex pathways of help or suppression and to pinpoint the target for a given factor. The idiotypic and antiidiotypic NP-specific Ts hybridoma lines (61,171) represent two distinct links in this chain of events. Similarly, the product of a KLH-specific Ts hybridoma line was found to be adsorbed on another, biologically inactive $I\text{-}J^+$ hybridoma line (159). Furthermore, interaction of the TsF induced the second hybridoma, 34S-281, which apparently lacked biological activity, to release an $I\text{-}J^+$ TsF which did not bind KLH. Thus, the 34S-281 hybridoma may represent a Ts_2 cell with antiidiotypic specificity.

2. The use of monoclonal products and target cells will enable the accurate definition of the site(s) of MHC restriction along the immunoregulatory pathway. Thus, the recent study by Okuda *et al.* (171) demonstrated that an apparent Igh gene restriction in the induction of

Ts_2 by a NP-specific monoclonal TsF reflected in fact restriction which operates at a subsequent step, namely, during the expression of the DTH response.

3. These monoclonal cell lines will allow a detailed analysis of T cell-specific Ia molecules. It has been shown that the Ia determinants of Ts hybridomas differ from those present on B cells or macrophages (130,130a,130b) and, moreover, that Ts hybridomas with different functions (i.e., no suppression, antigen-specific or nonspecific suppression) each display unique Ia determinants (157). Such unique Ia molecules can be obtained in large quantities from homogeneous T cell lines for purification and structural analysis.

4. The role of Ia determinants in distinct biological activities can now be analyzed. ThF or TsF display *I-A* or *I-J* subregion determinants, respectively. Since (a) it is possible to obtain separately the Ia^+ and antigen-binding subunits of the active molecules and reasssociate them into an active product (26,155,158), and (b) Ts and Th hybridomas of the same antigen specificity are now available (26,128), one can experimentally test whether recombination of an $I\text{-}J^+$ subunit from a (T,G)-A--L-specific TsF with an Id^+ subunit from a (T,G)-A--L-specific ThF will now result in a suppressive or helper (or inactive) material. Similarly, by combining Ia^+ and antigen-binding moieties from two factors with different antigen specificities, respectively, it will be possible to ascertain whether the Ia^+ chain contributes in some way to the antigen specificity or affinity of a given factor.

5. Sufficient quantities of homogeneous, antigen-specific cells and molecules should be available in the near future for the purification and sequencing of these biologically active factors and for the preparation of specific cDNA probes.

6. Finally, such homogeneous materials which can be obtained in essentially unlimited quantities have a therapeutic potential. It is not difficult to imagine alloantigen- or autoantigen-specific TsF being used to prevent allograft rejection or cure an autoimmune disease, respectively.

It can be predicted that information pertaining to most of the above experimental areas should be forthcoming in the very near future.

IV. Monoclonal, Antigen-Nonspecific Factors

The T cell-derived antigen-nonspecific LK comprise a very heterogeneous group of molecules in many aspects. They vary widely in

structure as well as in function—some displaying clear immunoregulatory activities and others acting on target cells outside the immune system. They act on T or B lymphocytes, on macrophages, various progenitor cells in the hematopoietic system, and other cell types. It is important to realize that despite the fact that all of these LK act in an antigen-nonspecific manner, the production of some or most of them can be induced by stimulation with specific antigens, including soluble antigens, heterologous erythrocyte antigens, and alloantigens. In addition, polyclonal T-cell mitogens and some other agents have been used to stimulate LK production by normal T cells. Among the T-cell hybridoma and lymphoma lines, some secrete their LK constitutively while others require an inductive signal. The question of constitutive vs induced LK production will be discussed in more detail below.

The discussion in this section will be divided into four parts, namely (a) immunoregulatory LK which display suppressive activities, (b) those with potentiating/enhancing activity, (c) interferon (IFN), and finally (d) all other LK which do not participate directly in immunoregulation and act, for the most part, on target cells not directly involved in immune responses. The reasons for discussing IFN as a separate category are 2-fold; first, although IFN has clearly been shown to regulate immune responses, its strict (and original) definition is based on its antiviral properties. Second, even as an immunoregulatory factor, IFN displays a whole spectrum of activities from suppression to enhancement and cannot, therefore, be clearly placed among either the suppressive or helper factors.

A. Suppressive Factors

1. Immunoglobulin E-Selective Suppressor Factor (IgE-TsF)

The IgE antibody response is of obvious interest to basic and clinical immunologists because of its role in allergic diseases. It is well known today that immunoregulatory mechanisms which act selectively on antibody responses of this particular class exist (reviewed in 184). Kishimoto *et al.* have demonstrated the existence of IgE-specific Th(185) and Ts (186) cells and their soluble products. These IgE-selective factors were not antigen-specific. Similar observations were subsequently made by others (184).

The IgE–TsF was subsequently characterized by Kishimoto *et al.* (187). This factor was released in cultures of spleen cells from mice primed with DNP–*Mycobacterium*. Its characteristics were as follows: it (a) required stimulation with the specific antigen for *in vitro* produc-

tion but was antigen-nonspecific in its action, (b) only suppressed antibody responses of the IgE class, (c) appeared to be MHC-restricted in its action, (d) displayed Ia determinants, and (e) was adsorbed by primed B, but not by T, cells.

In order to better define this IgE–TsF and study its mechanism of action, Watanabe *et al.* have subsequently established T-cell hybridoma lines which constitutively secrete IgE–TsF (82). Normal T cells for fusion with BW5147 were obtained from nylon wool-purified splenocytes of DNP–*Mycobacterium*-primed BALB/c mice. Culture supernatants of one hybridoma clone, 26-M10, suppressed *in vivo* secondary IgE responses in an adoptive transfer system, while having no effect on the IgG response. Suppression occurred only when hapten-primed B cells, but no carrier-primed T cells, were preincubated with the monoclonal IgE–TsF, indicating that B cells were the targets of this factor. The cloned hybridoma cells had an average number of 50 chromosomes and were unstable in that they gradually lost chromosomes and biological activity over a ~5 month period. However, the activity could be recovered upon recloning the hybridoma cells (82). The serological and physicochemical properties of the monoclonal IgE–TsF were not reported in this study (82).

2. *Suppressive Immunoglobulin-Binding Factor (IBF)*

Gisler and Fridman have reported the constitutive production of a LK, termed immunoglobulin-binding factor (IBF), in short-term (~2 hours) cultures of *in vivo*-alloactivated T cells (188). As its name implies, IBF binds to the Fc portion of IgG (but not other Ig classes). IBF was found to suppress primary IgM and IgG *in vitro* antibody responses to T-dependent and -independent antigens in an antigen-nonspecific manner. Pretreatment of spleen cells before culture or addition to culture on day 4 had no suppressive effect while maximal suppression (80–90%) was achieved when crude or affinity-purified IBF was added to the antibody culture system on day 3. The presence of Fc receptor on IBF enabled its affinity purification using immunoadsorbents coated with IgG. It was concluded that IBF acted on antigen-primed B cells by interfering with their terminal differentiation into antibody-forming cells (188). It was shown subsequently that IBF represents a soluble form of the Fcγ receptor shed by activated T cells (189).

Conventionally derived IBF was further characterized by affinity purification on IgG-coated immunoadsorbents, iodination of the eluted material, and SDS–PAGE analysis under reducing conditions (190). Two peaks of radioactivity, corresponding to MW of 18,000 and 38,000,

were identified and both of these were adsorbed on anti-Ia immunoadsorbents. By these, and other, criteria IBF appears to be an Ia^+ glycoprotein in which the 38,000–40,000 MW chain is necessary for binding to IgG. Whether the 18,000-MW moiety is a second, distinct subunit of IBF or simply a degradation product of the larger moiety has not been established (190).

Various T-cell lymphoma lines which express Fc receptors were subsequently screened for IBF production in view of the fact that IBF appeared to represent a soluble Fcγ receptor. One such lymphoma, L5178Y, maintained as an ascitic tumor in DBA/2 mice, was found to release IBF upon short-term culture (191). This tumor-derived IBF was found to be very similar to the conventional IBF by both functional (191) and structural (192) properties. Analysis of internally labeled material from L5178Y cells by gel filtration indicated suppressive activity associated with 140,000 and >300,000 MW molecules. SDS–PAGE analysis revealed an 80,000-MW biologically active material which dissociated under reducing conditions into 20,000- and 40,000-MW subunits. These data suggest that IBF is composed of two covalently linked chains and that it may exist in polymeric forms (192).

Another homogeneous source of IBF was provided by cloned T-cell hybridoma lines (193–195). The T cells used for fusion were activated *in vivo* to alloantigens (T2 series) or SRBC (T1 series). These two series of hybridomas, which were selected for the presence of Fcγ receptors, were cloned and found to lose their Fcγ receptors upon short-term incubation in serum-free balanced salt solution (BSS) and, in parallel, to release constitutively into the culture medium biologically active IBF (193–195). The slight enhancing activity found sometimes in crude culture supernatants but not in affinity-purified and eluted material suggests the presence of an enhancing factor (193) and this point will be discussed in further detail below. The hybridoma-derived IBF displayed similar properties to those of the conventional factor.

3. *Haplotype-Specific Suppressor Factor (TsF-H)*

Sorensen and Pierce have very recently described an *H-2*-restricted, antigen-nonspecific Ts cell induced by the injection of semiallogeneic, $(AXB)F_1$ hybrid spleen cells into neonatal A type mice (196). These long-lived Ts cells were activated by *in vitro* exposure to B type macrophages (i.e., macrophages displaying the alloantigens encountered originally by the neonates). The activated Ts cells suppressed primary, but not secondary, *in vitro* antibody responses to a variety of soluble or particulate antigens. Interestingly, despite the fact that these treated

mice were partially tolerant toward the inducing alloantigens, their Ts cells did not suppress *in vitro* allogeneic mixed leukocyte or CTL responses (196). The activation as well as the effector function of these Ts cells were restricted by genes located in the *I-A* subregion of the murine MHC. These haplotype-specific Ts cells are reminiscent of the MLR-Ts cells described by Rich and Rich which are induced in a similar way, are restricted by the *I-E/C* subregions, and act in an antigen-nonspecific manner to suppress allogeneic MLRs (197).

It was found subsequently (198) that the haplotype-specific Ts cells manifest their function via the release of a soluble factor, TsF-H, which displayed similar properties to the Ts cells in that its production and effector function required *I-A* identity with the activated macrophages or with the target spleen cells used in the suppression assay, respectively. Moreover, the TsF-H was found to display determinants identical to, or cross-reactive with, *I-A* antigens (198).

T-cell hybridomas which constitutively secrete TsF-H were constructed by fusing populations of haplotype-specific Ts cells with the BW5147 lymphoma (198). Growing hybrids were selected for suppression of primary antibody responses in culture, cloned by limiting dilution and one clone, 267.A4.A1, was characterized in detail. The soluble product of this clone and of conventional Ts cells were very similar. Both were found to be $I\text{-}A^+$ molecules, with a MW of 45,000–68,000, which were sensitive to low pH (3.5), trypsin and heat (60°C, 30 minutes). This monoclonal TsF-H lacked *I-J* or Ig constant region determinants (198).

This TsF as well as the conventional one studied by Rich and Rich (197) are similar to antigen-specific TsF in that they display Ia antigens and are MHC-restricted. However, while most antigen-specific TsF display *I-J* determinants, the antigen-nonspecific ones display *I-A* (198) or *I-E/C* (197) determinants. The monoclonal TsF-H appears to be similar to antigen-specific TsF in another respect, namely, its ability to induce another T-cell population to secrete a second TsF which displays MHC-*un*restricted, antigen-nonspecific suppressive activity in primary antibody responses (198). By analogy with the antigen-specific Ts systems, the antigen-nonspecific TsF-H would appear to be the product of a Ts_2 cell.

4. Other Monoclonal Suppressor Factors

Other groups have described the production of suppressive factors by T lymphoma, hybridoma, or TCGF-dependent lines in murine (199), owl monkey (200,201), or human (202–205) systems. Most of

these suppressed antigen-induced or polyclonal antibody responses in culture and almost all were secreted constitutively.

Stocker *et al.* described a cloned murine T lymphoma line, WEH1-22.1, which secreted a TsF capable of suppressing primary (but not secondary) IgM antibody responses induced *in vitro* by T cell-dependent, but not by T-independent, antigens. This TsF was found to be an Ig molecule secreted by the WEH1-22.1 thymoma (199). MacDonald *et al.* have studied a factor, produced by owl monkey-derived leukemic T cell lines, capable of *enhancing* antibody responses (94,206). This enhancing factor (OMEF) will be described in more detail below; however, pertinent to this section is the finding that the enhancing factor could be converted into an owl monkey-derived suppressor factor (OMSF) by repeated cycles of freezing and thawing or by dialysis against Tris–glycine buffer, pH 8.3 (200,201). OMSF reacted with an antiserum raised against OMEF and was characterized as a non-covalently linked dimer of OMEF, possessing a MW of 66,000 and a p*I* value of 6.5. It suppressed primary anti-SRBC PFC responses by 50–70% when added to the cultures at initiation (200,201).

Of particular interest are the immunoregulatory LK secreted by human T cells, since their study might shed light on immunoregulation and its abnormalities in human disease. Several human TsF have been described. Takada *et al.* reported that a human T cell leukemic line, MOLT-4, secreted a TsF which suppressed primary antibody responses against heterologous erythrocytes by mouse spleen cells (202). This TsF was secreted only when MOLT-4 cells were cocultured with SRBC, but not when they were cultured either alone or with chicken red blood cells. This property was correlated with the ability of MOLT-4 cells to form rosettes with SRBC (a well known property of human T cells) but not with chicken RBC. Thus, TsF production was most probably triggered by ligand–receptor interaction and TsF production cannot be regarded as constitutive in this case. The physicochemical properties of this TsF were not described in their report (202).

Grillot-Courvalin *et al.* have recently constructed a human T-cell hybridoma line by fusing peripheral blood lymphocytes (PBL) from a patient with a variable agammaglobulinemia with a thymidine kinase-deficient (and, therefore, HAT-sensitive) clone, D1R11, derived from a human acute lymphocytic leukemia line, KE37 (203). The majority of PBL used for fusion had the phenotype of activated Ts cells. The yield of growing hybrids was low but one hybridoma, which expressed surface markers of both parental T cells used for fusion, was

studied in more detail. The culture supernatant of this hybridoma clone, DE × D1R11, suppressed the pokeweed mitogen (PWM)-induced polyclonal activation of normal PBL, measured by the percentage of PBL displaying cytoplasmic Ig after 7 days in culture. Maximal suppression (50–75%) was obtained at supernatant dilutions of ~1 : 10, and the titer of this monoclonal TsF was not augmented by stimulation of the hybrid cells with Con A (203). Preliminary studies indicated that (a) the hybrid cell did not express Fcγ receptors and did not produce IFN, (b) the monoclonal TsF had to be added during the first 36 hours of culture in order to suppress B-cell differentiation, and (c) it did not inhibit PWM-induced mitogenesis, nor did it affect the polyclonal activation of human B cells induced by *Nocardia*, a relatively T-independent polyclonal activator of B cells (203). This study constitutes the first known report about a functional human T-cell hybridoma line and it is hoped that, with improved methodologies, more successes will be achieved in this particular area in the near future.

A monoclonal TsF termed cultured T cell-derived soluble immune suppressor supernate of B cells (CTC-SISS-B) has recently been described (204). The T cells producing this factor were derived from the peripheral blood of a patient with a nonleukemic form of mycosis fungoides. By their reaction with several monoclonal antibodies, these cells displayed the phenotype of activated suppressor cells and, indeed, they were shown before to suppress PWM-induced *in vitro* Ig production by human PBL (206a). These T cells were established as a permanent line by growing them in the presence of human TCGF.

After being washed and placed in fresh medium lacking TCGF, this CTC line secreted within 8–24 hours a TsF which, like the producer cell, suppressed PWM-induced Ig production measured in a reverse hemolytic plaque assay. This TsF suppressed (a) Ig production in all classes, and (b) polyclonal activation induced by Epstein–Barr virus (EBV), a T-independent B-cell activator. Fifty percent suppression was obtained with supernatant dilution of ~1 : 500 but cell-mediated immune responses, i.e., allogeneic MLR and CTL responses, were not affected at all. This TsF displayed its biological activity when added during the first 24–48 hours of culture, and suppression was evident only when late (5–7 days), rather than early (3–4 days), PFC responses were measured. In addition, CTC-SISS-B production was radioresistant and partially dependent on the presence of fetal calf serum (FCS) in the culture medium (204).

Physicochemical characterization indicated that this TsF bound the sugar L-rhamnose, was acid stable and partially heat labile at 56°C, and had a MW of 60,000–90,000. By all its biological and

physicochemical properties, this LK is very similar to SISS-B produced conventionally by Con A-stimulated human PBL, which was studied before by the same group (207). Parenthetically, conventional cultures produced in addition a second TsF, termed SISS-T, which was specific for T cell targets (208).

In contrast to the above TsF which suppress B cell responses, Wolf *et al.* described a lipid suppressor substance (LSS) secreted constitutively by an uncloned human cell line established from a cutaneous T-cell lymphoma (205). A similar material was secreted by antigen-activated conventional cultures of human T cells. The supernatant of this cell line suppressed mitogen- and antigen-induced proliferation of human T cells *in vitro* at dilutions of up to 1 : 1000. However, it was not clear whether this factor was actually secreted by T cells, since this cell line displayed a stable phenotype composed of 70% null cells and only 30% T cells (205).

It is important to note that in most of the cases where TsF (or ThF and TRF to be discussed in detail below) appeared to have specificity for B cell responses, it was not established directly that the LK molecules actually acted on B cells. The assays were conducted with uncloned target B cells and although the B cells were enriched by removing T cells, the contribution of residual T cells or macrophages cannot be discounted.

B. T Cell Helper or Replacing Factors

As a group, T cell-derived LK which augment or facilitate various kinds of immune responses have probably been studied more than any other group of LK. These LK have been induced by a variety of methods and shown to affect both humoral and cell-mediated immune responses. In the absence of direct, quantitative assays, numerous bioassays were established to detect the activity of these factors. Almost all such bioassays are deficient in that they measure an end effect, using heterogeneous target cell populations. However, as a result of several developments during the last 4 years, a somewhat clearer picture begins to emerge. This is due largely to the (a) establishment of permanent T cell lines secreting these immunoregulatory molecules, and (b) use of cloned target cells as indicators in bioassays.

Thus, it appears at the present as if the collection of T cell helper or replacing factors can be divided into at least two major categories. The first one includes a class of closely related molecules which act specifically on T target cells. TCGF (IL-2) is the prototype of this class. The second group includes factors which appear to act on B target cells, either directly or through some intermediary cells, e.g., macrophages.

Such factors have been termed T cell-replacing factors or TRF. This term implies that the particular LK actually replaces Th cells and completely obviates the need for them in antibody responses. However, the possibility was raised that, even though tested on T cell-depleted target populations, TRF (or contaminating TCGF present in most crude preparations) may act on some residual T cells or even on pre-T cells which do not express Thy 1 alloantigens and cause the differentiation and expansion of such cells into functional Th cells which then cooperate with B cells (46,209). Yet, the development of more refined bioassays and biochemical methods indicate that TRF molecules do act directly on B cells and, thus, are T cell replacing factors in the true sense. This conclusion is based on the biochemical separation of T cell-specific (TCGF) from B cell-specific (TRF) molecules (209), as well as the demonstration of direct TRF effects on cloned, homogeneous populations of neoplastic (112) or normal (210) B lymphocytes.

Finally, another mediator, termed allogeneic effect factor (AEF), defined originally by its ability to replace Th cells in *in vitro* antibody responses (107), is distinct from the two groups of LK mentioned above in that it displays Ia determinants (211). Such Ia determinants are displayed by most antigen-specific ThF and TsF molecules (see above) as well as by some antigen-nonspecific TsF (195,197,198), but not by other antigen-nonspecific helper factors.

1. Allogeneic Effect Factor (AEF)

AEF is a soluble mediator secreted in supernatants of short-term secondary MLC of *in vivo* alloantigen-activated T cells. It has originally been characterized as a 45,000-MW glycoprotein composed of two subunits, bearing Ia determinants and capable of activating B cells to develop antibody responses *in vitro* to particulate or soluble antigens in the absence of Th cells (107,211–214). More recent studies in our laboratory have focused on some T-cell activating properties of AEF (215–219). These latter studies have shown that AEF(1) is highly mitogenic for unprimed T cells, (2) can trigger the differentiation of unprimed T cells, in the absence of exogenous antigen, into CTL which display the Lyt 2^+ phenotype, preferentially lyse *H-2*-identical target cells and are directed against antigen(s) determined by the *K* region of the murine major histocompatibility complex (MHC), and (3) stimulate the differentiation of unprimed T cells into responding cells of the Lyt 1^+ phenotype which can be restimulated in secondary syngeneic mixed lymphocyte reactions (SMLR) that are directed against antigen(s) determined by the *I* region of the *H-2* complex

(215–219). These latter properties of AEF, namely, its ability to trigger differentiation of *unprimed* T cells into functional effector cells, distinguish this biologically active mediator from those lymphokines, such as TCGF (IL-2); TCGF is characterized biologically by its ability to support long-term growth of *primed,* fully mature T lymphocytes previously activated by antigen or otherwise (46,47).

This heterogeneity of biological activities displayed by AEF-containing MLC supernatants raised the question of whether the same or different molecules mediate these various effects. Recent biochemical analysis has shown that the AEF-mediated B cell- and T cell-activating properties cochromatograph on sizing gels and coelectrophorese when subjected to IEF (Fig. 1). Although suggestive, such biochemical criteria are still inadequate to allow conclusions concerning molecular heterogeneity or homogeneity with regard to the distinct biological activities of AEF. In order to address this and other questions more directly, and to provide a more uniform source of AEF molecules for further purification and analysis, we have utilized the technique of somatic cell hybridization to construct T-cell hybridomas secreting AEF (220).

T-cell blasts ($H\text{-}2^{d}$) activated to alloantigen ($H\text{-}2^{k}$) *in vivo* and secondarily sensitized *in vitro,* as done routinely for the preparation of conventional AEF, were fused with the BW5147 thymoma line. After fusion, hybrids were detected in 115 of 288 wells plated, and the *unstimulated* supernatants from these positive wells were screened for B-cell-activating capacities. Approximately half of these supernatants manifested little or no positive biological effects, about one-third displayed low-to-moderate biological activity, and a small number demonstrated rather potent biological effects when tested for their ability to restore the *in vitro* antibody responses of T cell-depleted B cells following stimulation with SRBC or TNP–SRBC (220). The hybridoma supernatants tested were obtained in serum-free conditions by washing the cultured hybridoma cells, replating them in serum-free medium and harvesting the culture supernatants after 24 hours.

Culture supernatants from the unfused parental line, BW5147, failed to display any B cell-activating properties in these cultures. Two hybridomas, 27 and 34, which constitutively secreted higher levels of helper activity, were selected for further experimentation, and were found to be true somatic cell hybrids as evidenced by their expression of the relevant Thy 1 and *H-2* alloantigens characteristic of both partner cell types used for fusion. The B-cell activating property of these hybridoma supernatants was demonstrated by their capacity to stimulate T cell-depleted spleen cells to respond *in vitro* to T-dependent

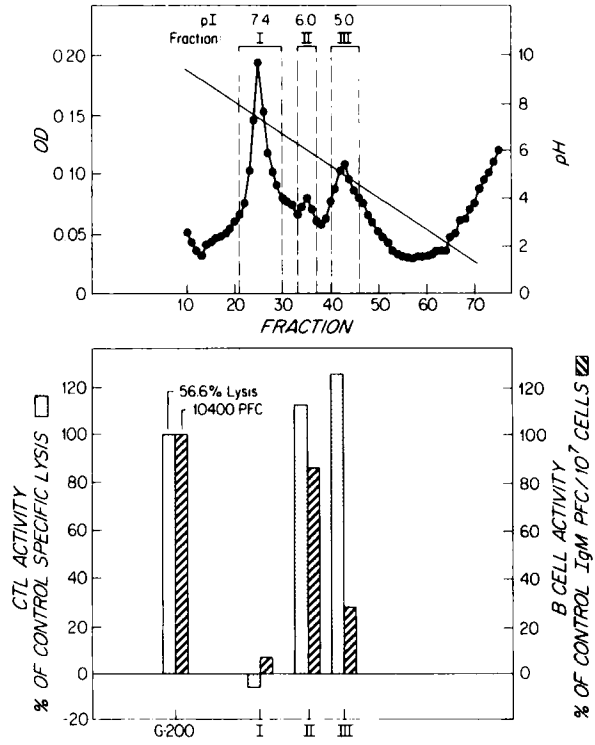

FIG. 1. Biological activity of fractions obtained by isoelectrofocusing (IEF) of an active Sephadex G-200 AEF peak. Thirty milliliters AEF (DBA/2 → $C3D2F_1$) was concentrated by Amicon filtration and chromatographed on a Sephadex G-200 column. Biologically active fractions coeluting with an insulin marker (37,000 MW) were pooled, dialyzed against 1% glycine buffer to remove salts, and then subjected to column IEF (24 hours at 400 V followed by 20 hours at 800 V). Fractions of 1.5 ml were pooled into 3 separate pools as shown in the top panel, dialyzed against PBS, and tested for biological activities at 5% v/v.

antigens. The T-cell-activating properties of these hybridoma supernatants were verified by their capacity to stimulate autonomous development of self-specific cytotoxic T lymphocytes and by their capacity to exert mitogenic effects on unprimed T cells. The biologically active molecules secreted by these hybridomas were, like conventional AEF, inhibitable by specific anti-Ia antibodies thus indicating the presence of Ia determinants on the relevant hybridoma products. In addition, these AEF-secreting hybridomas could be stimulated to proliferate and secrete increased quantities of AEF when exposed to the specific alloantigen-bearing target cells to which the T-cell blasts had been originally sensitized (220).

Hybridoma 34 was subsequently cloned by limiting dilution and the soluble products of several clones were found to be biologically active. It is noteworthy that hybridoma 34 did not produce TCGF, either constitutively or following Con A stimulation. On the other hand, other T-cell hybridoma lines derived from the same fusion were subsequently found to produce TCGF, but not AEF, measured by its ability to replace Th cells in primary antibody responses (see below). These findings indicate that the biologically active molecules secreted by these hybridoma lines are distinct from TCGF. Further analysis of these hybridoma lines, using a series of anti-Ia antibodies and other tools, is currently underway.

2. *T Cell-Replacing and Related Factors (TRF)*

A prototype of TRF molecules studied independently by several groups is the factor described originally by Schimpl and Wecker (105,221). This factor was produced by mitogen- or alloantigen-activated Lyt 1^+2^-, Fc receptor-negative T cells. TRF replaced Th cells in T cell-depleted normal, or athymic nude, spleen cultures responding to SRBC. It displayed maximal stimulatory activity when added after 2 days of culture. On the basis of this and other findings it was concluded that TRF provides a late differentiation signal to antigen-stimulated, proliferating B cells, which causes them to mature into antigen-secreting cells. The conventional product was found to be a glycoprotein possessing a MW of approximately 30,000, a p*I* value of 5.2, and lacking MHC-encoded determinants. The activity could be inhibited by a heterologous goat anti-TRF serum, and preliminary data indicated the feasibility of obtaining goat anti-TRF monoclonal antibodies (209,222).

The question of whether TRF acts through the stimulation of residual mature T cells or pre-T cells, or conversely, whether contaminating TCGF may contribute to the effects observed, was addressed subsequently (209,223). By a variety of biochemical and serological criteria it was possible to separate TRF from TCGF. Hamaoka *et al.* have reached a similar conclusion using TRF produced conventionally by specific antigen stimulation or by a T-cell hybridoma (224). In this case, the separation between the two activities was based on a number of genetic, serological, and biological criteria (224). Another unresolved question is whether TRF preparations provide to B cells proliferation or differentiation signals, or both.

The need to address these questions in a direct manner resulted in the establishment of (a) T cell lines secreting TRF without contaminating TCGF or LAF, and (b) homogeneous B cell lines which can be used as targets in TRF bioassays. Schreier *et al.* established cloned

lines of SRBC- or HRBC-specific Th cells in serum-free medium (225). These lines were maintained by constant stimulation with antigen plus irradiated syngeneic spleen or peritoneal cells in the absence or presence of TCGF. When stimulated with antigen and filler cells, the cloned Th cells secreted a variety of distinct LK which affected hematopoietic progenitor cells, T and B lymphocytes (50,225). Using these cloned Th cells, Schreier *et al.* observed a "bystander effect," i.e., when cloned Th cells specific for a given antigen, e.g., SRBC, were added to cultures of athymic nude spleen cells together with SRBC and non-cross-reactive HRBC, similarly high levels of PFC responses against both antigens were obtained (225,226). This finding indicated that an antigen-nonspecific TRF which allowed an antibody response to an unrelated antigen was produced as a result of specific antigenic stimulation of the cloned Th cells. Stimulation for LK production was MHC-restricted (225,226).

The mechanism of action of TRF secreted in these cultures in relationship to the stage of differentiation of B cells was analyzed in more detail (109,110,227). Factor production was induced by stimulating RBC-specific Th cells with antigen and irradiated syngeneic spleen cells. To test the effects of factors produced in these cultures on B cells, resting or bacterial lipopolysaccharide (LPS)-activated B cells were either added directly to the Th cultures (109,110) or supernatants from the Th cultures were added to such B cells (227). Small, resting B cells or activated B-cell blasts were prepared by velocity sedimentation of fresh or LPS-stimulated athymic nude spleen cells, respectively. Polyclonal or antigen-induced B-cell activation was evaluated by measuring cellular proliferation and PFC responses.

The results of these experiments can be summarized as follows: factors produced in cultures of antigen-stimulated cloned Th cells acted on both resting and activated B cells, but in a different manner. B-cell blasts were stimulated by TRF to proliferate and secrete Ig polyclonally (i.e., in the absence of antigen) and about one in three B cells responded in this manner. This help was not MHC-restricted, i.e., TRF derived from Th cells of a given *H-2* haplotype stimulated LPS blasts of other *H-2* haplotypes. Thus, TRF provided an MHC-unrestricted proliferative and differentiative signals to B-cell blasts (227).

In contrast, the same TRF preparations did not stimulate small, resting B cells to proliferate at a detectable level, even in the presence of specific antigen. However, such B cells differentiated within 1 day into large, blast-like cells which subsequently secreted Ig without cell division. Despite the fact that the cell number remained constant in

these cultures, the proportion of blasts and Ig-secreting cells increased and peaked on day 5, by which time about one in three cells secreted Ig. This polyclonal activation without cell division was also MHC-unrestricted. Furthermore, such polyclonally activated B cells lost their capacity to replicate as indicated by their failure to respond subsequently to mitogenic stimuli provided by LPS or antigen-specific help, i.e., HRBC-specific Th cells plus HRBC and syngeneic macrophages (227).

Proliferation was measured in these experiments by the uptake of tritiated thymidine (227) and it is quite likely that this method is not sensitive enough to detect the clonal proliferation of the resting B cells specific for the same antigen against which the cloned Th cells are directed. In fact, by measuring the frequency of B cells secreting specific, anti-HRBC antibody (in the presence of HRBC-specific cloned Th cells, HRBC, and syngeneic fillers) it was concluded that the antigen-specific B cells do proliferate under these conditions in an *H-2*-restricted manner (110). Thus, in the presence of antigen-specific help, the overwhelming majority of B cells mature polyclonally without replication while the antigen-specific B cells divide in addition.

These studies demonstrated that cloned T cells can secrete factors which affect B cells in the absence of Th cells and can, therefore, operationally be termed TRF. Two distinct effects, namely, proliferation and differentiation, were observed and resting or activated B cells responded in a different way to these factors. These studies did not determine whether the different effects are mediated by one or more distinct B cell-specific molecules, whether other LK present in these supernatants (e.g., TCGF) play a role and, moreover, whether the biologically active mediators are in fact products of T cells (since irradiated syngeneic spleen cells were required in these cultures for stimulation of Th cells). The LK detected in this system were, therefore, termed collectively B cell replication and maturation factors or BRMF (227).

Further insight into the nature of B cell-specific TRF was provided by other recent studies which described an alloreactive T cell line (116) and a T-cell hybridoma (228) secreting TRF but not TCGF or LAF. Thus, the possible contribution of these two other LK to the effects measured could definitely be ruled out.

The alloreactive T cell line C.C3.11.75 established by Dennert and Raschke (40) was maintained by repeated stimulation with specific allogeneic cells for the last 6 years or so. The uncloned cells displayed the Lyt 1^+2^- phenotype and specificity for *I-A* subregion determinants in both their proliferative and cytotoxic activities (229). This cell line

provided help for B cells in a primary anti-SRBC antibody response *in vitro* (230). However, this help was alloantigen-specific in that only B cells of the same *H-2* haplotype originally used to select this cell line were activated to produce antibody. On the other hand, supernatants of the allo-stimulated cell line provided help to B cells of different haplotypes, indicating that stimulation of TRF production is antigen-specific but the TRF itself acts in a nonspecific and MHC-unrestricted manner (230).

Interestingly, the supernatant fluids of the stimulated cell line were found to lack TCGF activity (116). This finding is an exception to the "rule" that TCGF is the universal mitogen for activated T cells (231,232). Obviously, the C.C3.11.75 cells proliferate continuously in the presence of specific alloantigen but in the absence of demonstrable TCGF production. The lack of TCGF production by this cell line allowed an analysis of its TRF activity in comparison with that of a T-cell hybridoma line, FS6-14.13, which, conversely, secretes TCGF but not TRF (62, and see below). T cells were rigorously depleted from the assay cultures by pretreating the spleen cell donors *in vivo* with a rabbit anti-mouse thymocyte serum, followed by an *in vitro* treatment of the spleen cells with anti-Thy 1 antibodies plus C (116).

Under these conditions the C.C3.11.75-derived TRF or the FS6-14.13-derived TCGF were relatively incapable of allowing B cells to respond to SRBC in a primary culture. However, a marked synergism was observed when supernatants of the two cell lines were mixed (116). These findings were interpreted to indicate that, in addition to antigen, at least two additional signals are required to trigger antibody production by B cells. One is provided by TRF which acts directly as a differentiation signal on B cells and the other—by TCGF which may act either directly on B cells or on residual mature T cells or immature pre-T cells. Although the TRF-producing T cell line used in this experiment was uncloned, similar data were obtained with clones of the C.C3.11.75 line (G. Dennert, personal communication). Characterization of the soluble products of this cell line by IEF and SDS–PAGE indicates the existence of three distinct molecular species, two of which stimulate B cells polyclonally into Ig secretion without proliferation while the third one induces both polyclonal activation and proliferation (G. Dennert, personal communication). In this respect, it should be emphasized that factors which presumably induce differentiation without proliferation may in fact trigger one or more critical rounds of proliferation which is below the level of detection and such a possibility has yet to be ruled out or validated.

Although these experiments serve to distinguish between TRF and

TCGF, the fact that TRF secretion by the C.C3.11.75 cell line requires allogeneic stimulator cells raises a problem. The stimulator cells which include T cells may secrete their own LK. The fact that TRF was produced following stimulation with a hamster–mouse hybridoma line, displaying the stimulatory $I\text{-}A^k$ antigen(s), addresses this problem but does not eliminate it altogether.

On the other hand, the TRF-secreting T-cell hybridoma line, B151K12, being a constitutive producer, does not require coculture with a stimulatory cell population (228). The normal T cells used to establish this cloned hybridoma were derived from BALB/c mice primed and boosted with *M. tuberculosis*. Such T cells were found earlier to release TRF into their culture supernatants upon *in vitro* stimulation with the specific antigen, PPD (233).

Supernatants of this clone augmented (a) *in vitro* secondary (IgG) antibody responses to a number of haptens and SRBC by splenic B cells of normal mice, and (b) primary (IgM) anti-SRBC responses of athymic nude mice-derived spleen cells (228). The antibody response of the nude spleen cells to SRBC was not accompanied by cellular proliferation, in contrast to LPS-induced responses. Supernatants were active at dilutions of ~1 : 5000 and displayed optimal activity when added to 2-day-old cultures, similar to the late action of the TRF studied by Schimpl *et al.* (222,223). Hapten-primed B cells from DBA/2Ha or (DBA/2Ha × BALB/c)F_1 male hybrid mice, which were found before to be genetically defective in the expression of a TRF receptor (234), did not respond to this monoclonal TRF and, moreover, an alloantiserum against the TRF receptor blocked the biological activity of this TRF (228). These findings provide compelling evidence that this constitutive product of the B151K12 T hybrid clone is indeed a B cell-specific LK displaying late differentiation activity.

More recent experiments have demonstrated directly that the B151K12-derived soluble product lacks TCGF activity (224). Moreover, it was shown that conventionally derived TRF was adsorbed on hapten-primed B cells but not on activated T cells and, vice versa, TCGF present in the same conventional preparations was adsorbed by the latter, but not by the first, cell population. The antiserum directed against the TRF receptor did not have any effect on the biological activity of TCGF (224). These results clearly distinguish TRF from TCGF and support similar findings by others (209,223).

The biologically active TRF secreted by the C.C3.11.75 (116) and B151K12 (228) T cell lines as well as conventional Con A-stimulated culture supernatants were analyzed comparatively (112). The soluble products of the two T cell lines, but not the conventional Con A super-

natant which is also expected to contain TRF, stimulated polyclonal Ig secretion in the absence of detectable cellular proliferation by neonatal and mature normal B cells, and by the leukemic B cell line, BCL_1, which displays the phenotype of an immature B cell (112). The demonstration of an effect of these monoclonal TRF preparations on cloned neoplastic B cells is a direct proof that TRF acts directly on B target cells. This finding has very recently been confirmed by Howard *et al.* who found that conventionally derived TRF preparations activated a homogeneous population of a long-term normal B cell line into Ig secretion (210).

An alloreactive cloned Th cell line, termed L2, and maintained by repeated stimulation with allogeneic stimulator cells, has been found to secrete a number of biologically active LK (51,63–65). Among these is a mediator termed polyclonal B cell stimulating factor or BCSF which activated T cell-depleted spleen cells into polyclonal Ig secretion. BCSF was distinct from TCGF since (a) TCGF levels in culture supernatants declined below the level of detection 48–72 hours after stimulation of the cloned L2 cells while high BCSF levels persisted throughout 8 days of culture, and (b) a spontaneously arising variant of L2, termed L2V, did not produce TCGF but secreted normal levels of BCSF (65). It was not determined in this study whether BCSF was a B-cell mitogen. The finding that L2 or L2V cells could be stimulated to produce BCSF by Con A, instead of specific alloantigen, directly proves that the cloned T cells themselves secrete the mediator(s).

Nabel *et al.* have recently described a method for establishing cloned lines of T and natural killer (NK) cells at discrete stages of cellular differentiation from normal, not intentionally primed mice (235). Such lines were established by culturing cells from various lymphoid organs in TCGF-containing conditioned medium and the initial stimulus to activate the cells may have been provided by residual Con A present in the crude TCGF preparations. The lines were cloned by limiting dilution or single cell manipulation and they displayed phenotypes of T cells at various differentiation stages. Several clones of the Lyt $1^+,2^-$ phenotype were isolated and all of them were found to augment the number of Ig-secreting cells during a 4 day culture of splenic B cells in the absence of antigen. Thus, these clones of which one, Cl.Ly1^+2^-/9, was studied in detail, activated B cells polyclonally (235,236). The supernatants of this clone had a similar effect, displaying biological activity at dilutions of up to 1 : 1000. This supernatant was not mitogenic, however, for resting B cells.

The molecules synthesized by this inducer T cell line were studied by internal labeling of the cells with [^{35}S]methionine and/or

[^{3}H]leucine followed by gel chromatography and SDS–PAGE. The molecules which activated B target cells had an apparent MW of 45,000–50,000 and a p*I* value of ~6.0. This activity was separated from 30,000-MW TCGF which was also secreted by the same clone, but not from another biological activity present, namely, the induction of macrophage/granulocyte colonies from bone marrow progenitors (235,236).

Schrader *et al.* have established a cloned T-cell hybridoma line, denoted 123, by fusing a population of KLH-primed T cells with BW5147 lymphoma cells (66). Following Con A stimulation, these hybridoma cells secreted a number of distinct LK, including TRF, colony-stimulating factor or CSF (66), TCGF (237), and a mast cell growth factor or MCGF (238). TRF activity was tested by assaying the effects of Con A-stimulated hybridoma supernatants on spleen cells of athymic nude mice cultured without or with heterologous RBC as an antigen (66). The supernatants had clear TRF activity as indicated by the marked increase of antigen-specific PFC responses, including those to a T-independent antigen. Although it was claimed that TRF activity required the presence of antigen in culture, some polyclonal activation in the absence of antigen was also observed (66). This biological activity was separated from the factor(s) affecting hematopoietic or mast cells but its structural characteristics have not been documented.

Rubin *et al.* found that coculture of two human B leukemic cell lines, RPMI-1788 and RPMI-4098, but not the culture of either of these alone, resulted in a secretion of a factor which augmented primary (IgM) anti-SRBC antibody responses in cultures of unfractionated murine spleen cells (239,240). This human enhancing factor (HEF) which, apparently, was not tested for TRF activity in T cell-depleted cultures proved to be a 35,000–38,000 MW glycoprotein whose biological activity was destroyed by heating to 80°C for 30 minutes but not by heating to 56°C, or treating with RNase or DNase (239,240).

A factor with similar characteristics which displayed biological activity in cultures of T cell depleted splenocytes was later isolated from supernatants of unstimulated leukemic T cell lines induced by inoculation of owl monkeys with *Herpesvirus saimiri* (206). A 2600-fold purification of this owl monkey-enhancing factor (OMEF) was obtained by ion-exchange and gel chromatographies followed by SDS–PAGE (94). Antibodies were raised in goats against this purified OMEF and an RIA was developed to quantitate the levels of ^{125}I-labeled OMEF. It was found that OMEF is a 39,000-MW glycoprotein with a p*I* value of 6.5. It was active in nanogram levels when added to

SRBC-stimulated spleen cell cultures on the second day but not on day 0. The goat antibodies blocked the biological activity of OMEF (94) and, in addition, OMEF reacted with an antiserum raised against HEF (240, and see above) but not with another antiserum raised against a murine-enhancing factor.

As mentioned briefly earlier, repeated freezing and thawing or dialysis against Tris buffer (pH 8.3) of OMEF preparations resulted in a suppressive activity (201). In contrast to OMEF, the suppressive factor (OMSF) displayed its activity when added to spleen cell cultures at initiation but not on the second day. It was found to be a 66,000 glycoprotein composed of 39,000-MW subunits which comigrated with OMEF molecules. Furthermore, OMSF bound antibodies raised against OMEF and inhibited the binding of OMEF to such antibodies, indicating a very close structural relationship between the enhancing and suppressive activities (200,201).

Rubin *et al.* have established an uncloned T cell line by culturing T cells from FCS-primed mice in the continuous presence of FCS and irradiated syngeneic spleen cells (241). This cell line (line 12) consisted of about 70% T cells and 30% non-T cells, some of which displayed surface Ig, and maintained a stable diploid karyotype for over a year. When stimulated with FCS and syngeneic spleen cells, this cell line secreted biologically active factors which could (a) activate a polyclonal CTL response from unprimed precursors, (b) provide help for the induction of alloantigen-specific CTL, and (c) induce a polyclonal B cell response, as indicated by the development of anti-SRBC PFC in cultures of unfractionated spleen cells not exposed to antigen (241). Since the assay cultures contained T cells it is not known whether the biologically active factor is truly a TRF. Moreover, it has not been reported whether culture supernatants of this cell line contain TCGF, or whether the different supernatant activities are mediated by one or more distinct LK.

Finally, Vesole *et al.* described the constitutive production of a lymphocyte mitogenic factor by several human lymphoid cell lines, including the T cell line, CCRF-CEM (242). The 15,000–30,000 MW stimulatory factor was directly mitogenic to human PBL and also augmented mitogenic responses induced by phytohemagglutinin (PHA) or PWM. The same supernatant contained also an inhibitor of cell proliferation. B cells appeared to be the main targets of the stimulatory factor (242).

To summarize this section, TRFs are operationally defined as mediators which replace Th cells in antibody responses by acting either directly on B cells or via some intermediary cells. In conven-

tional assay culture, the contributory role of residual mature T cells or of pre-T cells which are not eliminated by conventional treatment with anti-Thy 1 antibodies plus C, cannot be easily dismissed. The same applies also to cultures of spleen cells from athymic nude mice since these are also known to contain pre-T cells (243) and mature Thy 1^+ cells (244). Nevertheless, the use of biochemical, genetic, and serological criteria provided compelling evidence that TRF is distinct from T cell-specific LK (TCGF) and acts directly on B cells (209,223,224). The identity of TRF as a B cell-specific LK has finally been established in a direct manner by identifying monoclonal cellular sources of TRF which are free of other LK (e.g., TCGF or LAF) and establishing assay systems which use homogeneous B cell lines as target populations (65,112,116,210,228).

In agreement with the original definition of TRF as a late-acting, B cell differentiation factor, several monoclonally derived TRF preparations have been shown to behave in a similar manner (94,228). Among TRF-like molecules, a distinction has to be made between those which provide a differentiation signal vs others which are mitogenic. Moreover, the relationship between TRF which activate B cells polyclonally vs those which replace Th cells in a specific, antigen-induced antibody response is not fully understood. While differentiation effects are usually exerted on both resting and activated B cells, proliferative effects are evident, for the most part, only with activated B-cell blasts (109,110,227). Inasmuch as these B cell-specific mediators have been characterized in structural terms, they appear to have MW in the range of 30,000–50,000 (94,209,222,235,236, and G. Dennert, personal communication).

It is probably fair to state that TRF and TRF-like molecules comprise a heterogeneous class of B cell-specific immunoregulatory molecules which provide differentiation and proliferation signals to B lymphocytes at distinct differentiation stages. These molecules appear to fulfill an essential role in T–B cell collaborations by either turning on or shutting off B-cell responses. Our understanding of these TRF and their biological role will undoubtedly improve when additional monoclonal cellular sources of these molecules, improved assay procedures using cloned B target cells, and better purification methods become available in the near future.

3. *T Cell Growth Factor (TCGF, IL-2)*

The large number of studies on TCGF or IL-2 since its original discovery as a promoter of the long-term growth of ligand-activated T cells (41,42) reflects the high level of interest that this particular LK

has aroused recently. Studies on the nature of TCGF have greatly been aided by the development of a short-term and sensitive bioassay (44). This is essentially a lymphocyte proliferation assay which measures the TCGF-dependent cellular proliferation of cloned T cell lines (usually CTL) by the uptake of [^{3}H]TdR. This assay has three important characteristics: (a) cloned T cells are used as targets, (b) it is a short-term (24 hour) assay, and (c) the data can be semiquantitated and the relative titers of different preparations can be compared using a probit analysis (44). Moreover, this assay measures uniquely the activity of TCGF. Other LK, antigens, or polyclonal T cell activators such as Con A or PHA have no effects in this assay.

Similar results obtained independently by different groups have allowed the formulation of a generally accepted model regarding the mechanisms of production and action of TCGF (231,232,245). According to this model, stimulation by antigen/lectin activates macrophages to produce LAF or IL-1 (45,80). The production of LAF itself appears to be dependent on T cells, at least when its production is induced by T-cell mitogens. This dependence may form the basis for the observed requirement for Ia$^+$ macrophages in TCGF production (246). The same antigen/lectin induces a subset of T cells to differentiate into TCGF-responsive cells via the expression of TCGF receptors and, together with LAF, another T-cell subset to produce TCGF. Once the circuit of TCGF production has been completed, the antigen/lectin or LAF are not necessary anymore for T-cell proliferation. Binding of TCGF molecules to their respective cell surface receptors will trigger the activated T cells into proliferation which will continue as long as TCGF is available. It follows that antigens or lectins are mitogenic for T cells because they induce such cells to both release, and become responsive to, TCGF.

While mature T cells produce TCGF, immature (as well as mature) T cells respond to it. Thus, thymocytes (46,247) or spleen cells of athymic nude mice (114,115,248–253) can respond to TCGF in the presence of antigen by differentiating into mature, functional T cells. Although it has been postulated that TCGF is mitogenic only for activated T cells, some very recent studies indicated that partially purified TCGF is mitogenic for normal, unstimulated cells of both nude and normal mice (254–256). These findings may indicate that TCGF stimulates normal as well as activated T cells or, alternatively, that some endogenously activated T cells present in a normal T-cell population are stimulated to proliferate by TCGF.

Under some circumstances, the same T cell may be capable of producing, and responding to, TCGF simultaneously. Thus, although

TCGF-dependent (i.e., responsive) Ts and CTL lines do not produce TCGF, some (if not all) cloned Th line both respond to TCGF and produce it when stimulated with the specific antigen in the context of MHC-identical antigen-presenting cells (51,63,225). Some mechanisms may exist which limit the potentially uncontrolled proliferation of Th cells which produce TCGF and respond to it at the same time (257,258). The Th cell may, therefore, play a central role according to this scheme in regulating the clonal expansion of Ts or CTL, and indirectly affect the level of help available for cell-mediated and humoral responses.

TCGF molecules in different species have similar biological properties. While human TCGF stimulates the growth of mouse T cells, the reverse is not true (231). Despite the similarity of function, TCGF from human, rat, and mouse differ to some extent in their molecular characteristics. Murine TCGF has a MW of 23,000–30,000 with several p*I* values being reported in the range of 3.9–4.9 (46,231,232,256) and it is eluted from ion-exchange chromatography columns by salt concentrations of 0.1–0.2 *M*. In one recent study it was reported that following denaturation by SDS, murine TCGF exists as a biologically active 16,000-MW polypeptide (259) and, thus, the larger molecule identified by most others may represent a dimer. Rat and human TCGF both have a molecular weight of 14,000–16,000, are eluted by 0.05 *M* salt and have typical p*I* values in the general ranges of 5.4–5.5 and 6.0–7.0, respectively (47,48,231,232). The existence of discrete peaks with different p*I* values appears to be due to various levels of glycosylation of the same polypeptide (232). The structural relationship between TCGF molecules of different species is indicated by their cross-reactions with monoclonal antibodies raised against rat (97) or human (98) TCGF.

A significant portion of recent studies on TCGF focused on the establishment and identification of TCGF-producing T cell lines which would provide the large amounts of material necessary for biochemical characterization. As a result, more lines secreting TCGF than any other nonspecific LK have been described within the last 2 years. These lines are listed in Table II. Since the functional and biochemical properties of TCGF produced by these various cell lines appear to be very similar (if not identical) to each other and also to conventionally derived TCGF, there is no point in discussing separately each of these cell lines in detail. Rather, we will provide a general overview of these T cell lines and their secreted TCGF molecules and describe in more detail only the new information which emerged from their study. It is also important to note that many of these cell lines secrete, in addition

TABLE II
TCGF-SECRETING T CELL LINES[a]

Designation of cell line	Species	Comments	Reference
A. Normal (nonmalignant) lines			
—	Mouse	SRBC- or HRBC-specific Th lines maintained in TCGF or by repeated stimulation with antigen plus syngeneic filler cells, antigen presentation required for factor production is specific and *I-A*-restricted	225
L2	Mouse	Mls gene product-specific alloreactive Th line, requires alloantigen or Con A stimulation for growth and LK production, spontaneous variant (L2V) does not secrete TCGF	51,63,65
Cl.Ly1^{+}2^{-}/9	Mouse	TCGF-dependent line established from a normal spleen, constitutive producer, 30,000 MW	236
B. T-cell leukemias			
EL4	Mouse	PMA-induced TCGF secretion, 30,000 MW p*I* 3.8–4.4	269
LBRM-33 (and clones)	Mouse	Con A or PHA-induced secretion augmented by PMA, peak production accompanied by cell death, 30,000 MW, p*I*s 4.3–4.7 and 4.9–5.1, eluted by 0.1–0.2 *M* salt (DEAE), sensitive to proteases, 8 *M* urea, 70°C (30 minutes), resistant to pH 2–10, 4 *M* urea, 56°C (1 hour) or 70°C (15 minutes)	270,271
EL4 azg^{r}	Mouse	Secretion is radiation (7000 rad)-resistant, 30,000 MW	272
WEH1-7	Mouse	LAF(IL-1) augments Con A-dependent secretion	273
4SP	Mouse	—	Altman and Haas (submitted)

Jurkat-FHCRC	Human	PMA alone stimulates production or augments mitogen-induced production	274
—	Human	Short-term lines from patients with Sezary syndrome	275
HUT-78, HUT-102	Human	Cell lines from patients with cutaneous T-cell leukemias, TCGF required for establishment and maintenance but a few lines lost requirement for exogenous TCGF and secrete their own TCGF constitutively	232,245 279a
CTCL-2			
MLA 144	Gibbon ape	Constitutive production at high level, 21,500 MW, eluted by 0.04–0.06 *M* salt (DEAE) p*I* 6.45, resistant to pH 2–9 or 60°C (60 minutes)	276
C. T-cell hybridomas			
FS6-14.13	Mouse	Normal, unimmunized T cells used for fusion, 30,000–40,000 MW	62
Various	Mouse	Fusion of TCGF-producing hybridoma FS6-14.13 with TCGF-propagated, antigen-specific Th cells. TCGF production requires *I-A*-restricted specific antigen presentation	266
123	Mouse	KLH-primed T cells used for fusion, 35,000–40,000 MW	237
97	Mouse	Alloantigen-activated T cells used for fusion, 34,000 MW	264
1A8C3C10	Mouse	Fusion of PHA-stimulated LBRM-33 cells (see above) with BW5147, constitutive production	265
24A10	Mouse	Alloantigen-activated T cells used for fusion, 30,000–40,000 MW, eluted by 0.03–0.15 *M* salt (DEAE). Some clones produce constitutively, augmented by mitogen	267,268

[a] Unless otherwise indicated, all cell lines require mitogen (Con A or PHA) stimulation for production. Many lines secrete in addition other LK, e.g., TRF (BCGF), CSF, MCGF, and hematopoietic growth factors. Activity assayed usually in TCGF bioassay but also in costimulator assay or by help for induction of CTL and antibody responses.

to TCGF, other biologically active factors, including B cell-specific molecules described earlier and factors acting on hematopoietic progenitor cells, mast cells, etc. (50,51,64–66,110,225,227,235–238, 260–263, and see below).

TCGF-secreting T cell lines were identified or established in mice (51,62,63,65,225,236,237,264–273; Altman and Haas, in preparation), humans (232,245,274,275,279a), and gibbon apes (276). In most of the studies such cell lines have been cloned by limiting dilution or soft agar techniques. The cell lines were obtained using three different experimental approaches:

1. Establishment of antigen-specific, normal Th cell lines which depend on stimulation by antigen (51,63,65,225) or TCGF (236). Obviously, in the case of TCGF-dependent lines, the cells are removed from the TCGF-containing conditioned medium and placed in fresh medium in order to distinguish secreted TCGF from exogeneously supplied TCGF (236).
2. Screening of established leukemic T cell lines for constitutive or induced TCGF production (245,269–276,279a). All non-T neoplastic cell lines screened in such studies were found to be negative (270,274).
3. Construction of T-cell hybridoma lines. T cells used for fusion were derived from normal, unstimulated spleens (62) or from spleens of mice sensitized to the soluble antigen KLH (237) or allogeneic cells (264,267,268). In one case, mitogen-stimulated, TCGF-producing lymphoma cells, rather than normal T cells, were used for fusion with BW5147 (265) and, in another study, a TCGF-secreting T-cell hybridoma was used as a vehicle for fusion with TCGF-propagated, antigen-specific Th lines (266). This resulted in a hybridoma which is inducible by the specific antigen to secrete TCGF. This situation is analogous to that of normal Th cell lines which are stimulated by the specific antigen to secrete TCGF (51,63,65,225). In both cases, irradiated syngeneic lymphoid cells are required, most probably for appropriate MHC-restricted antigen presentation to the cloned Th cells.

While most of the TCGF-producing T cell lines require an inductive signal, some secrete TCGF constitutively (245,264,265, 267,268,276,279a). Among the constitutive producers, production can be augmented in some cases by mitogen stimulation (264, 267, 268). The inducing signals required for TCGF secretion by T-cell lymphomas or hybridomas have, almost exclusively, been polyclonal

T cell mitogens, i.e., Con A and PHA. However, in a few studies the tumor promoter, phorbol myristate acetate (PMA), has been found to be an efficient inducer of TCGF production (269,274) or to act synergistically with suboptimal mitogen concentrations (270,274). In the case of normal Th lines, production of TCGF is induced by stimulation with antigen plus syngeneic filler cells as mentioned earlier (51,63,65,225) or by Con A in the absence of filler cells (65).

The question of constitutive vs mitogen-dependent production of TCGF (or other LK) is intriguing from several aspects. From a practical point of view, it would be advantageous to have lectin-free TCGF preparations since the contaminating lectins may interfere with, or otherwise modify, the activity of TCGF. Second, this question is of obvious importance in the general realm of cell biology in terms of understanding the cellular processes which are set in motion by an inducing ligand, culminating in the secretion of a defined product.

It is interesting to note that all of the T cell lines secreting antigen-specific LK do so constitutively, as described above. At least two possibilities should be considered to explain this difference. One is that the bioassays for antigen-specific factors are much more sensitive than for the nonspecific ones and, thus, antigen-nonspecific LK may be secreted constitutively, but at levels too low to be detected in a bioassay. This possibility is supported by the findings that, in general, the biological activity of antigen-specific LK is of a much higher titer than that of nonspecific LK in culture supernatants. It is not uncommon to get significant biological responses to antigen-specific ThF or TsF when the active supernatants are diluted 10^3- to 10^4-fold (131,132,162,178).

The other possibility is that the requirement for an inductive signal or the lack of it reflect the stage of differentiation of the producing cell. Thus, some T cell lines may have been "frozen" in a state of differentiation which requires additional differentiation provided by mitogen in order to secrete their products; other lines may have been immortalized at a fully differentiated stage where they do not require any additional stimulus for LK production. This is analogous to the situation with neoplastic B cell lines where mature plasmacytomas secrete Ig constitutively whereas immature lines, such as BCL_1, require a TRF signal (112). This possibility is supported by the finding that some lines obtained by subcloning of the TCGF-producing lymphoma LBRM-33 lost their ability to produce TCGF upon mitogenic stimulation (270) but regained the ability to secrete high levels of TCGF when the macrophage product LAF (IL-1) was added to mitogen-stimulated cultures (81). Most likely, LAF (which in itself did not stimulate TCGF production) provided a differentiation signal to the

cells and converted them to a state of responsiveness to the inductive signal provided by PHA or Con A. In another case, the addition of LAF to cultures of the murine T lymphoma line, WEHI-7, augmented the Con A-induced TCGF secretion by about 7-fold (273). Parenthetically, these findings suggest that at least some cell lines which were classified as nonproducers may in fact turn out to be producers provided the right stimulatory signals can be defined.

The fact that various leukemic T cell lines secrete TCGF raises the question whether the relationship between malignancy and TCGF production is purely coincidental or causal. Theoretically, if a T cell produces, and at the same time responds to, its own TCGF this may result in an uncontrolled proliferation of the cell, i.e., malignancy. In an analogous manner, malignant sarcoma lines were found to produce their own growth factors (277,278). In order to establish such a causal relationship it will be necessary to demonstrate direct binding of TCGF to the leukemic T cell and a consequent biological response. Such binding has been inferred from adsorption experiments and, more recently, binding of radiolabeled TCGF to primate leukemic T cell lines which constitutively secrete TCGF was demonstrated directly, using the newly described TCGF binding assay (83a).

Recent findings by Poiesz *et al.* (279) and Gootenberg *et al.* (279a) are relevant to this discussion. They have established TCGF-dependent T cell lines from several patients with cutaneous T-cell lymphoma (CTCL) and demonstrated by various criteria that the cultured cells derive from the malignant lymphoma. A few of these cell lines became independent of exogenous TCGF after several weeks of culture and were found to constitutively secrete their own TCGF at a low level (232,245,279,279a). Thus, the potential role of TCGF in the maintenance of T cell malignancies is an interesting question which awaits further studies.

Studies on biological and structural properties of monoclonally derived TCGF revealed great similarity to TCGF molecules derived from conventional cultures. From a functional point of view, monoclonal as well as conventional TCGF can (a) support the growth of activated T cells, (b) provide a costimulator effect (121) to murine thymocytes stimulated by suboptimal mitogen doses, (c) provide help for alloantigen-specific CTL responses of thymocytes or nude spleen cells, and (d) facilitate anti-SRBC PFC responses of nude spleen cells *in vitro* (62,237,265,268,269,271). In addition, monoclonal and conventional TCGF share similar MW, charge, and p*I* values (62,232,264,267–269,271,276). While conventional preparations of

human TCGF contain discrete molecular entities which can be distinguished by IEF and SDS–PAGE and are due to different degrees of glycosylation of the same polypeptide, the monoclonal TCGF derived from the human leukemic T cell line, Jurkat, focuses as a single peak with a MW of 14,600 and p*I* value of 8.2, both corresponding to the asialo form of the conventional human TCGF (232).

The availability of cloned T cell lines secreting large quantities of TCGF opens the way to molecular studies aimed at preparing cDNA probes which can be used to elucidate the regulation of TCGF synthesis in normal and transformed T cells. Indeed, the feasibility of this approach has recently been demonstrated, using a gibbon ape leukemic T cell line, MLA144, which secretes constitutively large amounts of TCGF (276). Poly(A)-containing RNA, enriched for mRNA molecules, was microinjected into *Xenopus laevis* oocytes. Translation of the TCGF-specific mRNA was evidenced by the secretion, into the oocyte culture medium, of biologically active TCGF which reacted with monoclonal antibodies specific for human TCGF (H. Rabin, personal communication). Similar results concerning the *in vitro* translation of murine TCGF-specific mRNA, obtained from the EL4 T cell leukemia (269), into a biologically active product were recently obtained in another laboratory (279b).

In summary, the use of monoclonal TCGF sources and cloned target cells have clearly established that TCGF is a T-cell product which, in turn, acts on T-cell targets. TCGF constitutes a class of closely related molecules which serve as growth (and perhaps differentiation) factors for T cells. The various biological effects of TCGF appear to be related to its ability to bind to specific receptors on activated T cells and stimulate them to proliferate. The availability of cell lines producing large quantities of TCGF and of monoclonal antibodies specific for TCGF (97,98) enable the development of direct quantitative assays for this LK and the evaluation of its physiological role and therapeutic potential.

4. *Interleukin-3 (IL-3)*

Ihle *et al.* have recently found that culture supernatants of mitogen or antigen-activated T cells contain a LK, which they proposed to term interleukin-3 (IL-3), defined by its ability to induce *in vitro* the appearance of the enzyme 20α-hydroxysteroid dehydrogenase (20αSDH) in spleen cells of athymic nude mice (280). This enzyme has been shown to be a specific marker of mature T lymphocytes. Spleen cells of nude mice contain low levels of this enzyme which increase slowly

with age. The majority of cells displaying 20αSDH in spleens of nude mice, in contrast to normal mice, are not sensitive to treatment with anti-Thy 1 antibodies plus C (280). Conditioned media induce a proliferation-dependent increase of 5- to 10-fold in enzyme levels over a period of 20–30 hours, and the phenotype of both the precursor and induced cell populations is Thy 1.2- and Lyt 1,2-negative.

The factor responsible for this activity has been partially purified from conditioned media and found to have a MW of 30,000–50,000. Separation of this activity from TCGF (IL-2) was achieved by ion-exchange chromatography. IL-3 did not bind to such columns and was eluted in the breakthrough fraction while TCGF activity was eluted by 0.12 *M* NaCl. Furthermore, neither TCGF nor thymosin fraction V had any activity in the 20αSDH induction assay and, conversely, partially purified IL-3 had no activity in the TCGF assay (280). In addition, IL-3 has been distinguished by a variety of criteria from other mediators, e.g., LAF, CSF, and IFNγ. Another biological activity recently found to be associated with IL-3 is its direct mitogenic activity for Thy 1^- spleen cells or nylon wool-purified normal spleen cells. Thus, IL-3 may serve as a differentiation and growth factor for pre-T cells or immature T cells, perhaps acting at an earlier differentiation step than TCGF.

Using partially purified, TCGF-free IL-3 preparations, Happel *et al.* have recently established cloned lines of Thy 1^+, Lyt 1^+2^- cells from normal, unstimulated, nylon wool-purified splenocytes (281). Once established, these cell lines became independent of exogenously added IL-3 and, moreover, were found to secrete constitutively high levels of IL-3, measured by the induction of 20αSDH in nude spleen cells. The levels of IL-3 in supernatants of these cell lines were ~100-fold higher than in conventional, Con A-stimulated spleen cell culture supernatants. The same T cell lines which secreted IL-3 constitutively could be induced by PMA to secrete also TCGF. IL-3 secreted by these cell lines did not support the growth of a TCGF-dependent T cell line, indicating that more than one distinct LK may be involved in regulating the growth of T cells (281).

Preliminary experiments suggested that, in fact, T cell lines established in IL-3 may be specific for FCS in the culture medium since both their establishment and IL-3 secretion are highly FCS-dependent (281). If that is the case, then the production of IL-3 cannot be regarded as constitutive but, rather, FCS-induced and these cell lines may be related to the FCS-dependent cell lines described by Rubin *et al.* (241). Thus, although IL-3 appears to be distinct from TCGF, many more studies are required to determine its exact nature, im-

munoregulatory activities, relationship to other LK, and biological role.

C. The Relationship between Monoclonal Antigen-Nonspecific TsF and ThF

Despite the fact that antigen-specific ThF and TsF display opposite biological activities, they appear to be closely related in general structure and expression of Ia and V_H determinants as discussed above. While antigen-nonspecific immunoregulatory factors are a much more heterogeneous group of molecules, one may nevertheless demonstrate similarity between certain suppressive and enhancing factors. Two examples will serve to delineate the close relationship between some antigen-nonspecific immunoregulatory LK with opposing biological activities, all of which are produced by permanent T cell lines.

The first one involves IBF (188–195) and AEF (107,211–220). These two mediators with opposing biological activities are similar in several aspects, namely, they are both (a) composed of two subunits (38,000–40,000 and 18,000–20,000 MW for IBF, 30,000–40,000 and 12,000 MW for AEF), (b) induced by *in vivo* "education" of thymocytes against allogeneic cells, and (c) Ia-positive. IBF is released within 2 hours by cells cultured in serum-free BSS while AEF is released in cultures maintained in serum-free Dulbecco's MEM (DMEM).

Since hybridomas secreting IBF (193) and AEF (220) became available recently, we and Fridman exchanged hybridoma cell lines producing these two respective factors in an effort to comparatively analyze them. The results of this comparative analysis were as follows (W. H. Fridman, personal communication): AEF-producing hybridomas 27 and 34, like the IBF-producing clone T2D4, were found to be $Fc\gamma R^+$. The $Fc\gamma R$ was shed in BSS but not in DMEM. Incubation of all three hybrid cell lines in DMEM resulted in supernatants displaying typical AEF activity, namely, augmentation of anti-SRBC responses *in vitro*, especially when added to cultures at initiation, while incubation of the same cell lines in BSS resulted in an IBF-like suppressive activity which was most pronounced upon late addition into culture. These findings demonstrate that, upon careful analysis, factors which appear to display opposite biological activity may in fact possess similar activities, depending on the experimental conditions used for their preparation. Thus, it is possible that crude AEF and IBF preparations may both contain a mixture of suppressive and stimulatory activities and only the relative proportions of the two in each crude preparation are different. A high proportion of the suppressive material will result in a predominant IBF activity while a shift in the balance toward the

stimulatory material will be manifested by a predominant AEF activity. This question may be amenable to experimental analysis in view of the availability of the IBF- and AEF-secreting hybrid cell lines.

The other example involves the owl monkey-derived leukemic T cell lines which constitutively secrete factors with two opposing activities, namely, enhancing OMEF and suppressive OMSF (94,200,201, and see above). OMSF was generated by repeated freezing and thawing or Tris buffer (pH 8.3) dialysis of OMEF-containing culture supernatants. The two biological activities were structurally related since both possessed the same basic 39,000-MW polypeptide and reacted with the same antiserum. Further analysis of other antigen-nonspecific immunoregulatory LK with opposing activities, which can now be obtained in large quantities from permanent T cell lines, may elucidate the relationship between such activities.

D. Immune Interferon (IFNγ)

Interferons (IFN) have been defined as proteins which exert virus-nonspecific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein (282). They have been classified, mainly on the basis of antigenic specificities, into three types, i.e., leukocyte, fibroblast, and immune IFN, termed more recently IFNα, IFNβ, and IFNγ, respectively (282). Although IFN were described originally as antiviral substances, it is clear now that IFN preparations display antiproliferative and immunoregulatory effects in many systems (reviewed in 283,284). The immunoregulatory effects cover a whole spectrum from suppression to enhancement (285–288). We will limit our discussion in this section to IFNγ since it is the predominant IFN type produced by populations of T lymphocytes which are stimulated immunologically, namely, by antigens or polyclonal mitogens.

IFNγ has not been characterized to the same extent as IFNα or IFNβ. It is distinguished from the latter two types by its sensitivity to pH 2 and antigenicity. Since its production by activated T lymphocyte populations is macrophage-dependent it has been difficult to identify the producer cell (289,290). Moreover, because IFNγ is produced during antigen or mitogen stimulation of T-cell populations, a procedure which also leads to the production of other immunoregulatory LK, and since this type of IFN has not in general been purified to the same extent as other IFN types, it becomes difficult to ascribe a given immunoregulatory activity to IFNγ. Particular interest in IFNγ derives from studies which indicate that IFNγ displays greater antiproliferative effects on neoplastic cells than IFNα or IFNβ (291). For these and

other reasons it has become necessary to identify permanent cell lines which produce IFNγ in large quantities.

Marcucci *et al.* have recently described the production of IFNγ by TCGF-dependent cloned T cell lines (292). The T cells were derived from mice sensitized by skin application of the antigen, picryl chloride, grown in TCGF-containing culture medium and cloned by limiting dilution. No constitutive production of IFNγ was observed and, moreover, production did not occur in the TCGF-containing conditioned medium. IFNγ was secreted only when the cells were transferred to fresh, TCGF-free medium and stimulated with 5–10 μg/ml PHA or Con A. Production occurred in serum-free medium and titers of up to 1000 international units (IU) per ml were obtained. The antiviral activity in the culture supernatants was measured in a conventional IFN bioassay which is based on the reduction of viral-induced cytopathic plaques in monolayers of appropriate cell lines (in this case, vesicular stomatitis virus and L cell monolayers). The identity of the antiviral activity as IFNγ was established by various conventional criteria, including the sensitivity to pH 2, requirement for intact RNA synthesis by the target cells, and loss of biological activity after incubation with anti-IFNγ, but not with anti-IFNα or -IFNβ, sera. T cell clones varied in the level of IFNγ secreted (292).

In a human system, Nathan *et al.* reported the constitutive production of IFNγ by a malignant cell line (293). This T cell line, Mo, was established from the spleen of a patient with a T cell variant of hairy cell leukemia (294). The same cell line was found to produce several other LK (295–298, and see below). Although Mo cells constitutively produce low levels of IFNγ, production was augmented up to ~500-fold by induction with the T-cell mitogen, PHA, or even better, by a combination of PHA and PMA. IFN secretion did not require serum. The IFN secreted by this cell line was found to be entirely of the γ type by its acid (pH 2) lability, lack of production upon stimulation with inducers of other IFN types such as Sendai virus or polyinosinic–polycytidylic acid, and lack of reactivity with anti-IFNα sera.

In physicochemical terms, the IFNγ produced by the Mo cell line was inactivated by heat (56°C, 1 hour), 8 *M* urea, 6 *M* guanidine–HCl, 0.1% SDS and pronase. IFNγ was separated from the bulk of contaminating proteins on a Con A-Sepharose column, with a concomitant 20-fold increase in specific activity and very high recovery. This indicates that this IFNγ is a glycoprotein. A ~50-fold purification was obtained by hydrophobic chromatography. Gel chromatography studies revealed that the biologically active product had a MW of ~40,000.

IFNγ secreted by Mo cells is distinct from other LK products of the same cell line by its physicochemical properties (293).

In addition, production of IFNγ by the alloreactive T cell line, L2 (65), and by clones of the T-cell hybridoma line, 24A10 (267), has also been described very recently. Production was dependent on specific allogeneic stimulation in the first case and on Con A stimulation in the second. The availability of IFNγ-producing T cell lines will make it possible to purify this LK, and analyze its immunoregulatory activities and therapeutic potential with greater precision.

E. Other Nonspecific Lymphokines

1. Macrophage-Specific Factors

Activated T lymphocyte populations are known to secrete a number of factors which affect macrophage function, including those which inhibit macrophage migration (MIF), activate macrophages to become cytotoxic, and display other differentiated functions (122), and, finally, recruit Ia^+ macrophages into the peritoneal cavity of mice (123). Since macrophages, in particular those which display Ia determinants, are critical components of immune responses, it is quite likely that LK affecting macrophage function can indirectly modulate immune responses but such an immunoregulatory role for macrophage-specific LK has not yet clearly and directly been shown.

Secretion of LK affecting macrophage function by cloned T-cell hybridoma lines has recently been reported. In one case, such hybridomas were derived from the fusion of the T-cell lymphoma, BW5147, with Con A-activated spleen cells (67). One active hybridoma, W38, was cloned by limiting dilution and some clones were found to secrete constitutively factor(s) which (a) inhibited the migration of macrophages, a property used to define MIF, (b) enhanced the production of the second complement component, C2, by macrophage monolayers, and (c) caused a 3- to 4-fold increase in the amount of elastase released by macrophage monolayers into a serum-free culture medium. The same hybridoma supernatants did not contain IFN activity (67). It was not established whether the various biological effects were mediated by the same molecule or whether the hybridoma supernatants also contained MAF, as defined by its ability to cause macrophages to become cytotoxic.

The T-cell hybridomas constructed in our laboratory, by fusing BW5147 lymphoma cells with alloantigen-activated normal T cells (220,267,268), were screened for constitutive or mitogen-induced production of MAF as well as IFN. To screen for the presence of MAF in

T-cell hybridoma supernatants, we have used a ^{51}Cr-release tumoricidal assay. In this assay 2×10^5 adherent peptone-elicited peritoneal exudate macrophages are incubated with MAF sources and excess amounts of heat-killed *Listeria monocytogenes* as a source of second signal. After 4 hours, the stimuli are washed off and 2×10^4 ^{51}Cr-labeled P815 mastocytoma cells added. After incubation for 18 hours at 37°C, the specific amount of ^{51}Cr released into the supernatant is determined. One unit of MAF is defined as the amount of MAF required to effect 50% of maximal ^{51}Cr release.

Four parental hybridomas were screened for their ability to release MAF (267). Only one of the four, hybridoma 24, could be identified as a MAF producer. Production was dependent on stimulation by Con A or PHA and MAF activities could be demonstrated and titrated out between dilutions of 1/100 to 1/500. The amount of MAF released was dependent on the Con A concentration used during the stimulation; maximal release was found between Con A concentrations of 10 and 20 μg/ml. Compared to a standard MAF preparation, the uncloned T-cell hybridoma 24 displayed 20–30% of LK activity. Stimulated culture supernatants of this hybridoma also contained IFN activity (267; Schreiber *et al.*, submitted).

Upon cloning by limiting dilution, 27 clones were obtained. None released MAF or IFN constitutively. Of these, 24 clones produced MAF when stimulated with 10 μg/ml Con A. At least 7 of the clones produced higher MAF levels than the MAF standard. Ten clones did not produce IFN. One of these clones, G1, produced 55,000 units of MAF/ml which is 25 times greater than the MAF standard. No correlation was found between IFN levels and MAF levels of the various clones; of particular importance is the fact that clone G1 failed to secrete IFN despite the extraordinarily high quantities of MAF it produced. Taken together, these results indicate that MAF and IFN can be distinguished on the basis of differential production by clones of the T-cell hybridoma.

The biochemical analysis of MAF produced by the T-cell hybridoma clones 24/G1 has just begun. T-cell hybridoma MAF is being produced at the rate of 2 liters per week and represents the equivalent in conventional MAF preparations of 4000 mouse equivalents per liter. A comparison of the heat and pH sensitivities of conventional and T-cell hybridoma MAF have shown them to be identical; both are stable to incubation for 1 hour at 56°C but destroyed upon incubation at 65°C or upon incubation at or below pH 4 for 18 hours at 4°C. The MW of the hybridoma-derived MAF was found to be 50,000, identical to conventional MAF, as determined by gel filtration chromatography. The p*I* of

this monoclonal MAF is 5.3 although it exhibited some charge heterogeneity. It is anticipated that this unique reagent will be the source of sufficient amounts of starting material to achieve the purification and identification of macrophage activating factor.

Ely *et al.* have recently reported (65) that culture supernatants of the alloantigen-stimulated cloned T cell line, L2 (51,63), contain two factors which affect macrophage function, namely, migration inhibition factor (MIF; 84,85) and macrophage (Ia^+)-recruiting factor (MIRF; 123), in addition to other LK which were described earlier.

2. *Mast Cell Growth Factor (MCGF)*

Mast cells play a central role in IgE-mediated immediate hypersensitivity reactions via the release of histamine and other pharmacologically active substances. In addition, there are several lines of evidence that indicate a close relationship between mast cells and T lymphocytes. Mast cells have been shown to differentiate from precursors in mouse (299) or rat (300) thymus *in vitro* and it has been suggested that at least some mast cells may derive from T lymphocytes (301). T cells were shown to possess histamine receptors (302–305) and histamine can inhibit induction or expression of cell-mediated cytotoxicity (306,307). Finally, mast cells were found to participate in DTH responses and their degranulation appears to be T cell-dependent (308).

The mutual relationship between mast cells and T lymphocytes was further indicated by more recent observations about the ability of T cell-derived soluble mediators to support the long-term growth of mast cells *in vitro* (238,260,261,263,309–311). Cultures of mast cells were established from spleens of normal (238) or nude (309) mice, bone marrow of normal or nude mice (309–311), and fetal liver (260). Schrader *et al.* (238,310,311), Tertian *et al.* (309), and Nabel *et al.* (260) have independently found that it is possible to select for the growth of mast cells in initially heterogeneous cultures derived from such cellular sources by growing them in medium conditioned by Con A-stimulated spleen cells. Since such medium also contains TCGF which will support the growth of T cells it was necessary to select against T cell growth in the cultures. This was achieved by heating the conditioned medium at 65°C for 30 minutes (238,263), a procedure which was found to decrease TCGF activity without affecting the mast cell-specific activity, or by separation between adherent and nonadherent cells (309) since T cells were found to be more adherent to plastic than mast cells.

Using such conditions, pure populations of cells displaying the characteristics of mast cells were established, propagated for over a

year and cloned in soft agar or by micromanipulation (238,260,311). These cells were termed by Schrader persisting or P cells (238), to distinguish them from tissue mast cells. The cells growing in such cultures were identified as mast cells on the basis of their typical morphology, the presence of basophilic granules, positive reactions with toluidine blue or alcian blue, lack of phagocytic activity, presence of Fc receptors for IgG or IgE, and the presence of histamine, serotonin, and other typical mediators (238,260,309,311). The cells were found to lack the typical T-cell markers, i.e., Thy 1 and Lyt (238,260,309) but displayed the Ly 5 marker which is characteristic of lymphocytes and hematopoietic cells (260,309) and Ia determinants (238,309). The growth factor(s) responsible for this biological activity were termed P cell-stimulating factor or PSF (263,311) and mast cell growth factor or MCGF (260,309) but, for the sake of simplicity, will all be referred to herein as MCGF.

Subsequent to the discovery of MCGF activity in conventional culture supernatants it was found that several cloned T cell lines secrete this LK. These include the T-cell hybridoma 123 (66) and lymphoma LBRM-33 (270) which require mitogen stimulation for MCGF secretion (238,261,263,310,311) or the TCGF-dependent inducer T cell clone, Cl.Ly1$^+$2$^-$/9 (235,236), which secretes MCGF constitutively (260). These three lines were shown to produce other nonspecific LK such as TRF and TCGF (see above). In addition, a non-T, myelomonocytic leukemia line, WEHI-3, secreted MCGF constitutively (261). Like the conventional product, these monoclonally derived factors supported the growth of mast cells in long-term culture or short-term bioassays. Since the target cells used in these bioassays were cloned mast cells (238,260,311) it is clear that the mast cell is indeed the target for this LK.

MCGF was partially purified from supernatants of conventional cultures (261,263) or cloned T cell lines (260,263) and was compared to two other LK secreted by the same cell lines, i.e., TCGF and granulocyte–macrophage colony-stimulating factor (GM-CSF). Nabel *et al.* reported that MCGF purified to apparent homogeneity had a MW of 45,000 and a p*I* of ~6.0 (260) and in this respect it was not separable from TRF or GM-CSF which had very similar properties (235,236), but could be dissociated from the 30,000 MW TCGF.

The conventionally derived MCGF characterized by Yung *et al.* (261) had a similar MW of 35,000–40,000, higher than that of TCGF and in addition it could be dissociated from TCGF on DEAE-cellulose columns. While MCGF eluted in the breakthrough fraction (~0.005 *M* PBS, pH 8.3), TCGF eluted at higher salt concentrations of ~0.1 *M*.

Moreover, as discussed above, the WEHI-3 cell line secreted constitutively MCGF but no TCGF. On the other hand, WEHI-3 also secretes G-CSF which has size and charge properties similar to MCGF. DEAE-Sephadex fractions of WEHI-3-conditioned medium enriched for G-CSF activity also contained MCGF activity. In contrast, fractions of Con A-conditioned medium enriched for MCGF activity following Sephadex G-150 or DEAE-cellulose chromatography had no or very little CSF activity, respectively (261). Thus, it appears that the predominant CSF activity in Con A-stimulated spleen culture supernatants did not copurify with MCGF although further biochemical analyses will be necessary to dissociate these two LK.

The MCGF produced by two T-cell hybridoma lines, 123 and T6, differed in several aspects from the biologically active materials described above (263). Its MW appeared to be smaller, i.e., 29,000 or 23,000 by Sephadex G-75 chromatography in PBS or 6 *M* guanidine–HCl, respectively. Furthermore, it did not elute from DEAE-Sepharose in the breakthrough fraction but, rather, at higher salt concentrations of 0.03–0.08 *M* NaCl (pH 8.5). This MCGF was inactivated by trypsin, protease, 2-mercaptoethanol, and 85°C (30 minutes), but not by chymotrypsin, RNase, neuraminidase, guanidine–HCl, or 56°C. Its glycoprotein nature was indicated by its binding to the mannose-specific lectins, lentil lectin and Con A. Upon IEF, the activity was found across a broad range between p*I*s of 4 and 8. However, neuraminidase treatment or MCGF production in the presence of tunicamycin, a glycosylation inhibitor, resulted in a biologically active material which focused between p*I* 6 and 8, indicating that the charge heterogeneity is due, at least in part, to glycosylation.

MCGF activity could be dissociated from TCGF by several criteria. TCGF bound more strongly to hydrophobic phenyl-Sepharose column, was almost completely inactivated by 65°C (30 minutes) and did not bind to Con A-Sepharose. In contrast, GM-CSF shared size, heat stability, 2-ME sensitivity, and lectin-binding properties with MCGF. However, the two could be separated by their p*I* values. After neuraminidase treatment, the major peak of GM-CSF was at p*I* 4.7 whereas the major fraction of MCGF had a p*I* between 6 and 8 (263). The relationship between this MCGF and two other factors secreted by the same T cell hybridoma, i.e., TRF and a factor stimulating the *in vitro* growth of pluripotential stem cells, is not clear. MCGF may also be related to IL-3 (280) since both have a similar MW and elute from DEAE-cellulose columns in the breakthrough fraction although they act on different target cells.

The system studied by Schrader *et al.* (238,263,311) differs in one important aspect from those described by Nabel *et al.* (260) and Tertian *et al.* (309). In the latter systems, the mast cells were found to be absolutely dependent on MCGF and did not survive in its absence (260,309). In contrast, the factor(s) studied by Schrader *et al.* appears to regulate the growth of P cells but is not an absolute requirement since growing P cells were identified in MCGF-free, long-term bone marrow cultures (263). This discrepancy may reflect differences between the growth factors and/or the mast cells studied. Mast cells are a heterogeneous group of cells and the lines established by different investigators may reflect cells at distinct differentiation stages. However, the amounts of histamine in the cells studied were all in a similar range of 200–1400 ng/10^6 mast cells, less than the amount detected in mature peritoneal mast cells. In fact, it was suggested in all of the above studies that the cultured cells represent immature mastocytes (260,309,311).

In summary, the existence of T cell-derived LK which regulate the growth of mast cells *in vitro* appears to be established. The apparent heterogeneity of these factors may reflect distinct molecules acting at different stages of mast cell differentiation. However, the exact biochemical nature of these factors, their relationship to other LK, in particular those which regulate hematopoietic progenitor cells, and physiological role remain to be determined.

3. *Monoclonal LK Acting on Hematopoietic Cells*

Hematopoiesis is regulated by complex mechanisms which are still poorly understood. It is known, however, that culture supernatants of T cell mitogen-stimulated splenocytes contain a variety of CSF-stimulating progenitors of megakaryocytes, eosinophils, erythrocytes, granulocytes, macrophages (312), and T cells (313). The dissection of these soluble mediators has just begun but the recent discovery that they can be produced by cloned T cell lines (a) establishes a direct regulatory role of T cells in *in vitro* hemopoiesis, and (b) allows the preparation of such LK in large quantities for biochemical and functional studies. The biological activity of these hemopoiesis-regulating LK is measured in soft agar cultures by the differentiation and growth of morphologically and functionally identifiable cells from more primitive progenitors.

Schrader *et al.* reported that supernatants of the T-cell hybridoma line, 123, contain a factor which promoted the *in vitro* growth of hemopoietic pluripotential stem cells, measured in the *in vivo*

colony-forming unit-spleen (CFU-S) assay (237,263). This factor was not separated from MCGF since both had similar MW (25,000–30,000), p*I* (6–8), and heat stability.

The other LK studied act on more differentiated progenitor cells. Prominent among this is GM-CSF whose characteristics and production by the T-cell hybridoma, 123, the T lymphoma line, LBRM-33, and the TCGF-dependent inducer clone, Cl.Lyl$^+$2$^-$/9, have already been described earlier (236,261,263). The CSF studied by Nabel *et al.* could not be separated from MCGF or TRF by its size or p*I* value (236,260). Similarly, complete separation of CSF from MCGF could not be achieved by Yung *et al.* (261) or by Clark-lewis and Schrader (263). On the other hand, CSF could more readily be dissociated from TCGF in these studies (236,261–263) based on both functional and biochemical criteria. CSF and TCGF molecules produced by the murine T leukemia line, EL4, could be separated by hydrophobic chromatography on phenyl-Sepharose columns (262).

Schreier *et al.* have reported the production of CSF promoting the growth of granulocyte or macrophage colonies and burst-promoting activity (BPA;314) for early committed erythroid cells in cultures of cloned, antigen-specific Th cell stimulated with antigen and syngeneic spleen cells (50,225). Similarly, Ely *et al.* found that the cloned alloreactive T cell line, L2, which was described in detail earlier, releases CSF upon alloantigen stimulation (65). However, the properties of these LK were not described in these reports.

The human T cell line, Mo (294), has been described before with respect to its ability to secrete IFNγ (293). In fact, the same cell line was found in earlier studies to secrete constitutively two distinct LK which affected human hematopoietic progenitor cells. One of these is erythroid-promoting activity (EPA) measured by its ability to enhance the proliferation of erythroid progenitors from human peripheral blood or bone marrow *in vitro* (297). This EPA was found to be an acidic (p*I* 3.5–4.8) glycoprotein with a MW of ~45,000 which was resistant to 15 minutes of boiling, eluted from DEAE-Sephadex columns at 0.15–0.25 *M* NaCl (pH 7.2), and bound Con A. This EPA was species-specific in that it did not affect murine erythroid progenitors (297).

The same human cell line released in addition CSF which stimulated the growth of human granulocyte/macrophage colonies in methyl cellulose. Production of this CSF, which was also species-specific, was augmented by stimulation with PHA. The CSF was a 34,000-MW acidic glycoprotein (p*I* 4.5–5.3), it resisted denaturing agents and heating (70°C but not 80°C for 40 minutes), and eluted from DEAE-Sephadex columns at 0.18–0.22 *M* NaCl (295,296). Another LK se-

creted by the same line was termed neutrophil migration inhibition factor (T-derived) or NIF-T (298). This Con A-binding, 35,000–45,000 MW glycoprotein was heat stable (80°C, 30 minutes) and sensitive to pronase. The availability of this human cell line which secretes at least two distinct LK, i.e., CSF and EPA, acting on hematopoietic progenitor cells should facilitate studies on the regulation of hematopoiesis in humans.

F. Restricted Production of Nonspecific LK by T Cell Lines

As mentioned before, one of the problems that has hampered studies on various LK is related to the presence of heterogeneous LK species in conventional culture supernatants. Thus, one of the advantages of the cloned T cell lines is that such heterogeneity can be reduced. However, even though the producer T cell is cloned, it may nevertheless secrete more than one distinct LK. Numerous examples have been given in this review of cloned T cell lines which secrete at the same time different antigen-nonspecific LK, e.g. hybridoma 123 (66,237,238,263,310,311) and the inducer T cell clone, Cl.Ly1$^+$2$^-$/9 (235,236,260), both secrete TCGF, TRF, CSF, and MCGF simultaneously. The alloreactive T cell line, L2 (51,63–65), secretes the first three of these LK and a few others while the human T cell lines, Mo, secrete four distinct biological activities, i.e., IFNγ, EPA, CSF, and NIF-T (293–298), to cite just some representative examples.

This is not much of a problem in the case of T cell lines which secrete antigen-specific LK. First, such lines do not appear to produce nonspecific LK and, second, even if they did, it would be relatively easy to purify the antigen-specific LK by affinity chromatography on immunoadsorbents coated with the relevant antigen. In the case of antigen-nonspecific LK, it may be much more difficult to achieve absolute separation between distinct molecular species. Therefore, methods which favor a more restricted pattern of LK production by such "oligopotent" T cell lines would be of obvious importance.

At least two different methods appear to have this potential. The first one involves repeated subcloning of a T cell line. We have successfully applied this method in the case of our T-cell hybridoma lines constructed by fusing alloantigen-activated T cells with the T-cell lymphoma BW5147 (267; Schreiber *et al.*, submitted). One of the uncloned lines, hybridoma 24, was found to secrete upon Con A stimulation at least three distinct biological activities, namely, TCGF, MAF, and IFNγ. Upon cloning we obtained clones which displayed a more restricted pattern of LK production (Figs. 2 and 3). For example, clone

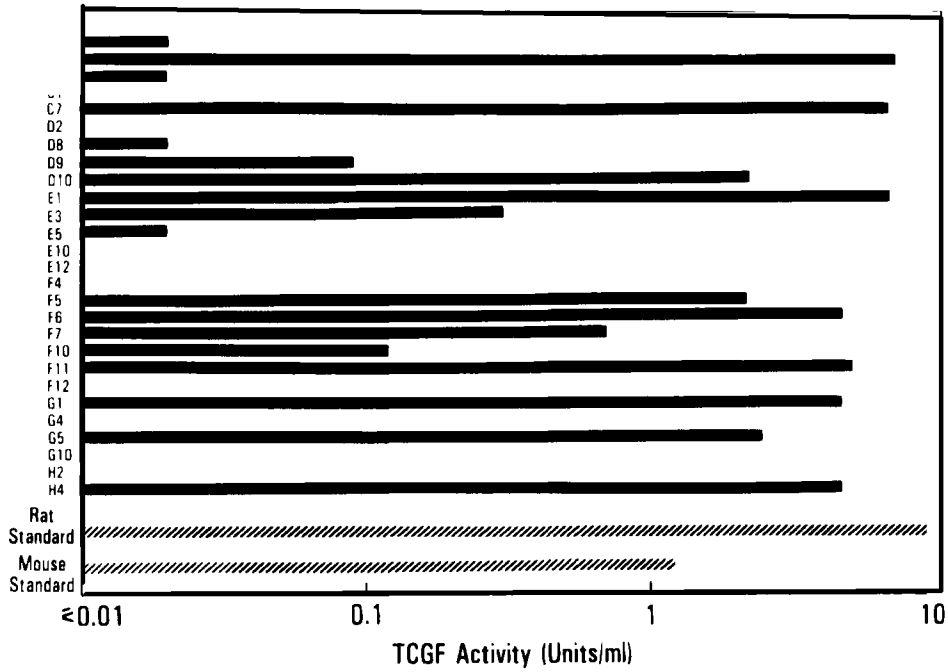

FIG. 2. T cell growth factor (IL-2) production by clones of T-cell hybridoma 24. Hybridoma cells were cloned at 0.5 cells/well in the presence of 2×10^5 irradiated (2500 rads) BALB/c thymocytes in flat-bottom microtiter wells (200 μl/well). Growing clones were transferred to, and expanded in, 16 mm wells. Confluent cultures were split into two new cultures, one serving as a control and the other one stimulated for 20 hours with 10 μg/ml Con A. Supernatants were then titrated for TCGF activity.

F4 secreted MAF activity, very little IFN (antiviral) activity, and no TCGF, clone G1 secreted high levels of TCGF and MAF activity but no detectable IFN; still another clone, G4, secreted IFN, very little MAF activity, and no TCGF. However, it should also be noted that the majority of clones that did not produce a given LK were also negative for the other two (e.g., clones C1, D2, E10, E12, F12, G10, H2). Thus, it may well be that once the biosynthetic machinery of a cell is set in motion by an inductive signal, it will produce various biologically active molecules in concert in the majority of cases. This method for obtaining a more restricted LK production may be particularly helpful when the T cell line in question is heterogeneous, i.e., not really a derivative of a single cell.

Another method which may be more successful when dealing with a true clone is based on the selection of variants which have lost the

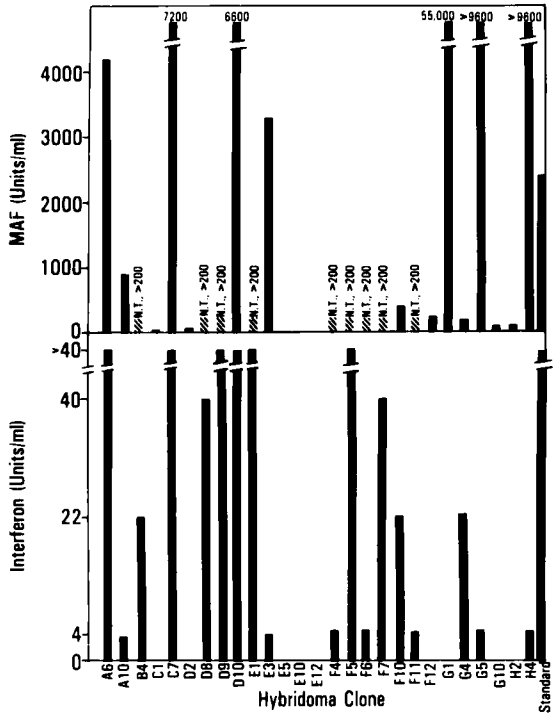

FIG. 3. MAF and interferon production by T-cell hybridoma clones. Levels represent those following stimulation of cultures with 10 μg/ml concanavalin A for 24 hours. Neither MAF nor IFN was detectable in any of the unstimulated culture supernatants. Broken bars represent MAF-containing supernatants identified by screening but not titrated. These clones secreted at least 200 units/ml.

ability to produce a given LK. Such variants may arise spontaneously by somatic mutation but the rate of such mutations can be increased by deliberate mutagenesis. Ely *et al.* have identified a spontaneously arising variant, L2V, of the alloreactive T cell line, L2, which had ceased to produce TCGF but continued to secrete two other LK species, i.e., CSF and BCGF (65). Similarly, functional changes have been induced in CTL lines by using the mutagen ethyl methanesulfonate (52,83). Such mutagenized lines have acquired resistance to several drugs (52) or lost their cytolytic function (83). It is conceivable that, by using a similar approach, it may be possible to select mutated clones which secrete a more restricted number of, or ideally only one, LK. Such clones may serve as useful tools, not only from the practical point of view as producers of a given LK free of other, contaminating mole-

cules, but also in molecular studies on the regulation of cell activation along distinct and defined pathways.

V. Concluding Remarks

The field of lymphokine research has been plagued with phenomenology. This has largely been due to the lack of sufficiently large quantities of defined LK, to the heterogeneity of LK species in conventional preparations, and to the ambiguity of the assay systems. However, it appears that this field is now entering a more rigorous and analytical phase due to recent methodological developments in two major areas, namely (a) the ability to immortalize functional T lymphocytes by either somatic cell hybridization or viral transformation, and (b) cloning of such functional T cell lines. Combination of these two methodologies enable us to establish cloned T cell lines which secrete only one or very few LK species in practically unlimited quantities.

With these basic tools in our hands, we can now approach a number of major questions regarding the structure and function of various LK. Several areas which are amenable to experimental analysis and promise to be rewarding can be illustrated:

1. The purification of LK to homogeneity both on an analytical scale to study their structure and on a preparative scale to study their biological role and therapeutic potential. Purification may be easier in the case of antigen-specific LK using affinity chromatography on antigen-coated immunoadsorbents. In the case of antigen-nonspecific LK, it may be necessary to use different approaches (see below).
2. Construction of B-cell hybridoma lines secreting monoclonal antibodies specific for various LK. Since such an antibody should be specific for one distinct LK species it could in turn be used as a reagent for the affinity purification of the respective LK, particularly in the case of antigen-nonspecific LK. In addition, such antibodies can be used to study the physiological role of a given LK. If such a LK is produced by the intact animal and plays an obligatory role in normal physiological responses, then it should be possible to perturb immune (or other) responses *in vivo* by using antibodies to that particular LK.
3. The availability of purified LK and specific monoclonal antibodies should allow development of quantitative assays, i.e., radioimmunoassays and enzyme-linked immunosorbent assays. Such assays would have a significant advantage over the currently used bioassays which are, in many cases, too ambiguous. Moreover, these assays will allow us to evaluate the physiology of LK by measuring

their levels in body tissues and fluids in health and disease. Most of the LK reviewed herein have not been shown to be produced *in vivo* by the intact individual and sensitive quantitative assays should afford the opportunity to detect them *in vivo*.

4. Development of cloned populations of target cells for various LK, e.g., T and B cells, macrophages, and various hematopoietic progenitors. Cloned cells will allow us to define with precision the cellular target of a given LK, to develop more reliable bioassays, and to synthesize the complete pathway of cellular interactions which culminate in a defined end effect such as in the case of suppressor immunoregulatory circuits.

5. Along more basic lines, the availability of purified LK will allow detailed studies on their molecular organization and mechanisms of action. In the case of antigen-specific factors, their relationship to T and B cell idiotypes can be analyzed as described in more detail in Section III. Purified LK can be radiolabeled, their interactions with cell surface receptors can be studied by direct binding experiments, and their fate following interaction with the respective receptor sites can be analyzed.

6. Finally, and perhaps most importantly, purified LK have a tremendous potential as therapeutic tools. This potential should first be explored in experimental models and, subsequently, translated into clinical situations. It almost seems as if the number of potential therapeutic uses is limited only by the imagination of the investigator. The use of soluble products, rather than the cloned T-cell populations producing them, in clinical studies is advantageous since allogeneic clones will most probably be rejected fairly quickly by the recipient and the establishment of autologous clones for each diseased individual appears to be highly impractical.

Suppression of immunity in an antigen-specific or nonspecific manner may be beneficial in autoimmune diseases, organ transplantation, and IgE-mediated allergic responses. On the other hand, potentiation of immune responses would be desirable in all kinds of diseases which result in generalized immunosuppression, e.g., cancer, viral disease, and parasitic infections. Various agents can be considered in this respect. TCGF could be used to boost T cell function, either alone or in combination with antigen (e.g., tumor)-specific T cell clones. IFNγ may activate NK cells or act via other mechanisms to augment resistance to malignant and viral diseases. MAF may be capable of activating macrophages *in vivo* to become cytocidal toward tumor cells or toward normal cells harboring pathogens and antigen-specific ThF may be used to augment immune responses in a selective manner. LK

which regulate hematopoiesis may be of great benefit in diseases which result from deficiency of certain progenitor cells. These examples are enough to illustrate the great promise which LK appear to have.

Thus, it can be safely predicted that important information in all of the above areas as well as in others should be forthcoming in the immediate future. The best evidence for the intensity and productivity of research on LK production by cloned T cell lines should probably come in the form of a long list of related reports that will certainly appear before this review is published and the short time span before an updated review becomes necessary.

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REFERENCES

1. Katz, D. H. (1977). "Lymphocyte Differentiation, Recognition and Regulation." Academic Press, New York.
2. Claman, H. N., Chaperon, E. A., and Triplett, R. F. (1966). *Proc. Soc. Exp. Biol. Med.* **122,** 1167.
3. Davies, A. J. S., Leuchars, E., Wallis, V., Marchant, R., and Elliott, E. V. (1967). *Transplantation* **5,** 222.
4. Mitchell, G. F., and Miller, J. F. A. P. (1968). *J. Exp. Med.* **128,** 821.
5. Dutton, R. W., Falkoff, R., Hirst, J. A., Hoffman, M., Kappler, J. W., Kettman, J. R., Lesley, J. F., and Vann, D. (1971). *Prog. Immunol.* **1,** 355.
6. Kilburn, D. G., Talbot, F. O., Teh, H.-S., and Levy, J. G. (1979). *Nature (London)* **277,** 474.
7. Tokuhisa, T., Taniguchi, M., Okumura, K., and Tada, T. (1978). *J. Immunol.* **120,** 414.
8. Kishimoto, T., and Ishizaka, K. (1974). *J. Immunol.* **112,** 1685.
9. Mudawwar, F. B., Yunis, E. J., and Geha, R. S. (1978). *J. Exp. Med.* **148,** 1032.
10. Tada, T., Taniguchi, M., and Okumura, K. (1977). *Prog. Immunol.* **3,** 369.
11. Taniguchi, M., and Tokuhisa, T. (1980). *J. Exp. Med.* **151,** 517.
12. Hirai, Y., and Nisonoff, A. (1980). *J. Exp. Med.* **151,** 1213.
13. Tada, T., and Okumura, K. (1979). *Adv. Immunol.* **28,** 1.
14. Rocklin, R. E., Bendtzen, K., and Greineder, D. (1980). *Adv. Immunol.* **29,** 55.
15. Altman, A., and Katz, D. H. (1980). *J. Immunol. Methods* **38,** 9.
16. Germain, R. N., and Benacerraf, B. (1980). *Springer Semin. Immunopathol.* **3,** 93.

17. Taussig, M. J. (1980). *Immunology* **41,** 759.
18. Moller, G., ed. (1980). *Immunol. Rev.* **54.**
19. Kohler, G., and Milstein, C. (1975). *Nature (London)* **256,** 495.
20. Moller, G., ed. (1979). *Immunol. Rev.* **47.**
21. Kennett, R. H., McKearn, T. J., and Bechtol, K. B., eds. (1980). "Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analysis." Plenum, New York.
22. Diamond, B. A., Yelton, D. E., and Scharff, M. D. (1981). *N. Engl. J. Med.* **304,** 1344.
23. Liu, F.-T., and Katz, D. H. (1981). *In* "Monoclonal Antibodies and T Cell Products" (D. H. Katz, ed.). CRC Press, Boca Raton, Florida, in press.
24. Hammerling, G. J. (1977). *Eur. J. Immunol.* **7,** 743.
25. Taniguchi, M., and Miller, J. F. A. P. (1978). *J. Exp. Med.* **148,** 373.
26. Eshhar, Z. (1981). *In* "Monoclonal Antibodies and T Cell Products" (D. H. Katz, ed.). CRC Press, Boca Raton, Florida, in press.
27. Nabholz, M., Cianfriglia, M., Acuto, O., Conzelman, A., Haas, W., von Boehmer, H., McDonald, H. R., Pohlit, H., and Johnson, J. P. (1980). *Nature (London)* **287,** 437.
28. Kaufman, Y., Berke, G., and Eshhar, Z. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2502.
29. Goldsby, R. A., Osborne, B. A., Simpson, E., and Herzenberg, L. A. (1977). *Nature (London)* **267,** 707.
30. Kohler, G., Lefkovits, I., Elliott, B., and Coutinho, A. (1977). *Eur. J. Immunol.* **7,** 758.
31. Eisen, H. N., Little, J. R., Osterland, C. K., and Simms, E. S. (1967). *Cold Spring Harbor Symp. Quant. Biol.* **32,** 75.
32. Moller, G., ed. (1979). *Immunol. Rev.* **48.**
33. Roder, J. C., Tyler, L., Singhal, S. K., and Ball, J. K. (1978). *Nature (London)* **273,** 540.
34. Glade, P. R., and Hirschorn, K. (1970). *Am. J. Pathol.* **60,** 483.
35. Finn, O. J., Boniver, J., and Kaplan, H. S. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4033.
36. MacDonald, H. R., Engers, H. D., Cerottini, J. C., and Brunner, K. T. (1974). *J. Exp. Med.* **140,** 718
37. Ben-Sasson, S. Z., Paul, W. E., Shevach, E. M., and Green, I. (1975). *J. Immunol.* **115,** 1723.
38. Dennert, G., and De Rose, M. (1976). *J. Immunol.* **116,** 1601.
39. Watanabe, T., Fathman, C. G., and Coutinho, A. (1977). *Immunol. Rev.* **35,** 3.
40. Dennert, G., and Raschke, W. (1977). *Eur. J. Immunol.* **7,** 352.
41. Morgan, D. A., Ruscetti, F. W., and Gallo, R. C. (1976). *Science* **193,** 1007.
42. Ruscetti, F. W., Morgan, D. A., and Gallo, R. C. (1977). *J. Immunol.* **119,** 131.
43. Gillis, S., and Smith, K. A. (1977). *Nature (London)* **268,** 154.
44. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. (1978). *J. Immunol.* **120,** 2027.
45. Aarden, L. A. *et al.* (1979). *J. Immunol.* **123,** 2928.
46. Watson, J., Gillis, S., Marbrook, J., Mochizuki, D., and Smith, K. A. (1979). *J. Exp. Med.* **150,** 849.
47. Gillis, S., Smith, K. A., and Watson, J. (1980). *J. Immunol.* **124,** 1954.
48. Mier, J. W., and Gallo, R. C. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6134.
49. Sredni, B., Tse, H. Y., Chen, C., and Schwartz, R. H. (1981). *J. Immunol.* **126,** 341.
50. Schreier, M. H., and Iscove, N. N. (1980). *Nature (London)* **287,** 228.

51. Glasebrook, A. L., Sarmiento, M., Loken, M. R., Dialynas, D. P., Quintans, J., Eisenberg, L., Lutz, C. T., Wilde, D. B., and Fitch, F. W. (1981). *Immunol. Rev.* **54,** 225.
52. Nabholz, M., Conzelman, A., Acuto, O., North, M., Haas, W., Pohlit, H., von Boehmer, H., Hengartner, H., Mach, J.-P., Engers, H., and Johnson, J. P. (1980). *Immunol. Rev.* **51,** 125.
53. Fathman, C. G., and Hengartner, H. (1978). *Nature (London)* **272,** 617.
54. Fathman, C. G., and Weissman, I. L. (1980). *Nature (London)* **283,** 404.
55. Schrier, R. D., Skidmore, B. J., Kurnick, J. T., Goldstine, S. N., and Chiller, J. M. (1979). *J. Immunol.* **123,** 2525.
56. Watson, J., and Mochizuki, D. (1980). *Immunol. Rev.* **51,** 257.
57. Kontiainen, S., Simpson, E., Bohrer, E., Beverley, P. C. L., Herzenberg, L. A., Fitzpatrick, W. C., Vogt, P., Torano, A., McKenzie, I. F. C., and Feldmann, M. *Nature (London)* **274,** 477.
58. Taniguchi, M., and Miller, J. F. A. P. (1977). *J. Exp. Med.* **146,** 1450.
59. Whitaker, R. B., Nepom, J. T., Sy, M.-S., Takaoki, M., Gramm, C. F., Fox, I., Germain, R. N., Nisonoff, A., Greene, M. I., and Benacerraf, B. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6441.
60. Taniguchi, M., Saito, T., and Tada, T. (1979). *Nature (London)* **278,** 555.
61. Okuda, K., Minami, M., Ju, S.-T., and Dorf, M. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4557.
62. Harwell, L., Skidmore, B., Marrack, P., and Kappler, J. (1980). *J. Exp. Med.* **152,** 893.
63. Glasebrook, A. L., and Fitch, F. W. (1980). *J. Exp. Med.* **151,** 876.
64. Glasebrook, A. L., Quintans, J., Eisenberg, L., and Fitch, F. W. (1981). *J. Immunol.* **126,** 240.
65. Ely, J. M., Prystowsky, M. B., Eisenberg, L., Quintans, J., Goldwasser, E., and Fitch, F. W. *J. Immunol.* **127,** 2345.
66. Schrader, J. W., Arnold, B., and Clark-Lewis, I. (1980). *Nature (London)* **283,** 197.
67. Jones, C. M., Braatz, J. A., and Herberman, R. B. (1981). *Nature (London)* **291,** 502.
68. Rozenszajn, L. A., Shoham, D., and Kalechman, I. (1975). *Immunology* **29,** 1041.
69. Sredni, B., Kalechman, Y., Michlin, H., and Rozenszajn, L. A. (1976). *Nature (London)* **259,** 130.
70. Nabholz, M., Engers, H. D., Collavo, D., and North, M. (1978). *Curr. Top. Microbiol. Immunol.* **81,** 176.
71. Glasebrook, A. L., and Fitch, F. W. (1979). *Nature (London)* **278,** 171.
72. Baker, P. E., Gillis, S., and Smith, K. A. (1979). *J. Exp. Med.* **149,** 273.
73. Bach, F. H., Inouye, H., Hank, J. A., and Alter, B. J. (1979). *Nature (London)* **281,** 307.
74. von Boehmer, H., Hengartner, H., Nabholz, M., Lernhardt, W., Schreier, M. H., and Haas, W. (1979). *Eur. J. Immunol.* **9,** 592.
75. Braciale, T. J., Andrew, M. E., and Braciale, V. L. (1981). *J. Exp. Med.* **153,** 910.
76. Watson, J. (1979). *J. Exp. Med.* **150,** 1510.
77. Schreier, M. H., and Tees, R. (1980). *Int. Arch. Allergy Appl. Immunol.* **61,** 227.
78. Kimoto, M., and Fathman, C. G. (1980). *J. Exp. Med.* **152,** 759.
79. Zagury, D., Morgan, D. A., and Fouchard, M. (1980). *Behring Inst. Mitteil.* **67,** 201.
80. Gery, I., Gershon, R. K., and Waksman, B. H. (1972). *J. Exp. Med.* **136,** 218.
81. Gillis, S., and Mizel, S. B. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1133.
82. Watanabe, T., Kimoto, M., Maruyama, S., Kishimoto, T., and Yamamura, Y. (1978). *J. Immunol.* **121,** 2113.

83. Dialynas, D. P., Loken, M. R., Glasebrook, A. L., and Fitch, F. W. (1981). *J. Exp. Med.* **153,** 595.
83a. Robb, R. J., Munck, A., and Smith, K. A. (1981). *J. Exp. Med.* **154,** 1455.
84. Yoshida, T., Bigazzi, P. E., and Cohen, S. (1975). *J. Immunol.* **114,** 688.
85. Geczy, C. L., Friedrich, W., and de Weck, A. L. (1975). *Cell. Immunol.* **19,** 65.
86. Onozaki, K., Haga, S., Muira, K., Ichikawa, M., and Hashimoto, T. (1979). *Cell. Immunol.* **48,** 258.
87. McLeod, T. F., Baskin, B. L., Meltz, S. K., Glade, C. S., and Glade, P. R. (1977). *Cell. Immunol.* **32,** 370.
88. Block, L. H., Jaksche, H., Bamberger, S., and Ruhenstroth-Bauer, G. (1978). *J. Exp. Med.* **147,** 541.
89. Gately, M. K., Gately, C. L., Henney, C. S., and Mayer, M. M. (1975). *J. Immunol.* **115,** 817.
90. Granger, G. A., Shimizu, I., Harris, L., Anderson, J., and Horn, P. (1978). *Clin. Immunol. Immunopathol.* **10,** 104.
91. Geczy, C. L. (1977). *J. Immunol.* **119,** 2107.
92. Namba, Y., and Waksman, B. H. (1977). *Immunochemistry* **14,** 143.
93. Bendtzen, K. (1977). *Cell. Immunol.* **29,** 382.
94. Mulcahy, H. L., Rubin, A. S., and MacDonald, A. B. (1981). *Immunology* **42,** 25.
95. Luben, R. A., Mohler, M. A., and Nedwin, G. E. (1979). *J. Clin. Invest.* **64,** 337.
96. Buhler, H. P., and Blaser, K. (1980). *Behring Inst. Mitteil.* **67,** 226.
97. Gillis, S., and Henney, C. S. (1981). *J. Immunol.* **126,** 1978.
98. Stadler, B. M., Berenstein, E. H., Siraganian, R. P., and Oppenheim, J. J. (1981). In "Mechanisms of Lymphocyte Activation" (H. Kirchner and K. Resch, eds.), p. 570. Elsevier, Amsterdam.
99. Secher, D. S., and Burke, D. C. (1980). *Nature (London)* **285,** 446.
100. Rubin, A. S., and Coons, A. H. (1972). *J. Exp. Med.* **136,** 1501.
101. Harwell, L., Kappler, J. W., and Marrack, P. (1976). *J. Immunol.* **116,** 1379.
102. Waldmann, H., and Munro, A. (1975). *Transplant. Rev.* **23,** 213.
103. Andersson, J., Moller, G., and Sjoberg, O. (1972). *Eur. J. Immunol.* **2,** 99.
104. Hunter, P., and Kettman, J. R. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 512.
105. Schimpl, A., and Wecker, E. (1975). *Transplant. Rev.* **23,** 176.
106. Watson, J. (1973). *J. Immunol.* **111,** 1301.
107. Armerding, D., and Katz, D. H. (1974). *J. Exp. Med.* **140,** 19.
108. Geha, R. S., Mudawwar, F., and Schneeberger, E. (1977). *J. Exp. Med.* **145,** 1436.
109. Schreier, M. H., Andersson, J., Lernhardt, W., and Melchers, F. (1980). *J. Exp. Med.* **151,** 194.
110. Andersson, J., Schreier, M. H., and Melchers, F. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1612.
111. Primi, D., Hammarstrom, L., Moller, G., Smith, C. I. E., and Uhr, J. (1979). *Scand. J. Immunol.* **9,** 467.
112. Pure, E., Isakson, P. C., Takatsu, K., Hamaoka, T., Swain, S. L., Dutton, R. W., Dennert, G., Uhr, J. W., and Vitetta, E. S. (1981). *J. Immunol.* **127,** 1953.
113. Gronowicz, E., Coutinho, A., and Melchers, F. (1976). *Eur. J. Immunol.* **6,** 588.
114. Wagner, H., Hardt, C., Heeg, K., Rollinghoff, M., and Pfizenmaier, K. (1980). *Nature (London)* **284,** 278.
115. Stotter, H., Rude, E., and Wagner, H. (1980). *Eur. J. Immunol.* **10,** 719.
116. Swain, S. L., Dennert, G., Warner, J. F., and Dutton, R. W. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2517.

117. Altman, A., and Cohen, I. R. (1975). *Eur. J. Immunol.* **5,** 437.
118. Plate, J. M. D. (1976). *Nature (London)* **260,** 329.
119. Finke, J. H., Orosz, C. G., and Battisto, J. R. (1977). *Nature (London)* **267,** 353.
120. Sopori, M. L., Alter, B. J., Bach, F. H., and Bach, M. L. (1977). *Eur. J. Immunol.* **7,** 323.
121. Paetkau, V., Mills, G., Gerhart, G., and Monticone, V. (1976). *J. Immunol.* **117,** 1320.
122. Nathan, C. F., Murray, H. W., and Cohn, Z. A. (1980). *N. Engl. J. Med.* **303,** 622.
123. Scher, M. G., Beller, D. I., and Unanue, E. R. (1980). *J. Exp. Med.* **152,** 1684.
124. Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. (1978). *J. Exp. Med.* **147,** 446.
125. Katz, D. H., Graves, M., Dorf, M. E., DiMuzio, H., and Benacerraf, B. (1975). *J. Exp. Med.* **141,** 263.
126. Woodland, R., and Cantor, H. (1978). *Eur. J. Immunol.* **8,** 600.
127. Bottomly, K., and Mosier, D. E. (1981). *J. Exp. Med.* **154,** 411.
128. Eshhar, Z., Apte, R. N., Lowy, I., Ben-Neriah, Y., Givol, D., and Mozes, E. (1980). *Nature (London* **286,** 170.
129. Ben-Neriah, Y., Wuilmart, C., Lonai, P., and Givol, D. (1978). *Eur. J. Immunol.* **8,** 797.
130. Murphy, D. B., Herzenberg, L. A., Okumura, K., Herzenberg, L. A., and McDevitt, H. O. (1976). *J. Exp. Med.* **144,** 699.
130a. Hayes, C. E., and Bach, F. H. (1978). *J. Exp. Med.* **148,** 692.
130b. Hiramatsu, K., Miyatani, S., Kim, M., Yamada, S., Okumura, K., and Tada, T. (1981). *J. Immunol.* **127,** 1118.
131. Lonai, P., Puri, J., Hammerling, G. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 549.
132. Lonai, P., Puri, J., Bitton, S., Ben-Neriah, Y., Givol, D., and Hammerling, G. J. (1981). *J. Exp. Med.* **154,** 942.
133. Puri, J., and Lonai, P. (1980). *Eur. J. Immunol.* **10,** 273.
134. Taniguchi, M., Tada, T., and Tokuhisa, T. (1976). *J. Exp. Med.* **144,** 20.
135. Tada, T., Taniguchi, M., Okumura, K., Hayakawa, K., Hiramatsu, K., and Suzuki, G. (1980). *Proc. Conv. Immunol. Immunobiol. Major Histocompat. Complex, 7th* p. 1.
136. Misfeldt, M. L., and Hanna, E. E. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1813.
137. Apte, R. N., Lowy, I., De Baetselier, P., and Mozes, E. (1981). *J. Immunol.* **127,** 25.
138. Apte, R. N., Dayan, M., and Mozes, E. (1981). *Cell. Immunol.* **61,** 104.
138a. Fischer, A., Beverley, P. C. L., and Feldmann, M. (1981). *Nature (London)* **294,** 166.
139. Gershon, R. K., and Kondo, K. (1971). *Immunology* **21,** 903.
140. Gershon, R. K. (1974). *Contemp. Top. Immunobiol.* **3,** 1.
141. Cantor, H., and Gershon, R. K. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 2058.
142. Sy, M.-S., Dietz, M. H., Germain, R. N., Benacerraf, B., and Greene, M. I. (1980). *J. Exp. Med.* **151,** 1183.
143. Germain, R. N., and Benacerraf, B. (1981). *Scand. J. Immunol.* **13,** 1.
144. Taussig, M. J., Corvalan, J. R. F., Binns, R. M., and Holliman, A. (1979). *Nature (London)* **277,** 305.
145. Taussig, M. J., and Holliman, A. (1979). *Nature (London)* **277,** 308.
146. Taussig, M. J., Corvalan, J. R. F., Binns, R. M., Roser, B., and Holliman, A. (1979). *Eur. J. Immunol.* **9,** 768.
147. Taussig, M. J., Corvalan, J. R. F., and Holliman, A. (1979). *Ann. N.Y. Acad. Sci.* **332,** 316.

148. Taussig, M. J. (1980). "Protides of the Biological Fluids" (H. Peeters, ed.), Vol. 28, p. 529. Pergamon, Oxford.
149. Cowing, C., Schwartz, B. D., and Dickler, H. B. (1978). *J. Immunol.* **120,** 378.
150. Hewitt, J., and Liew, F. Y. (1979). *Eur. J. Immunol.* **9,** 572.
151. Weinberger, J. Z., Germain, R. N., Benacerraf, B., and Dorf, M. E. (1980). *J. Exp. Med.* **152,** 161.
152. Dietz, M. H., Sy, M.-S., Benacerraf, B., Nisonoff, A., Greene, M. I., and Germain, R. N. (1981). *J. Exp. Med.* **153,** 450.
153. Kontiainen, S., and Feldmann, M. (1979). *Thymus* **1,** 59.
154. Kontiainen, S., and Feldmann, M. (1978). *J. Exp. Med.* **147,** 110.
155. Taniguchi, M., Takei, I., and Tada, T. (1980). *Nature (London)* **283,** 227.
156. Tada, T., Hayakawa, K., Okumura, K., and Taniguchi, M. (1980). *Mol. Immunol.* **17,** 867.
157. Taniguchi, M., Takei, I., Saito, T., Hiramatsu, S., and Tada, T. (1980). *In* "Biochemical Characterization of Lymphokines" (A. L. de Weck, F. Kristensen, and M. Landy, eds.), p. 577. Academic Press, New York.
158. Taniguchi, M., Saito, T., Takei, F., and Tokuhisa, T. (1981). *J. Exp. Med.* **153,** 1672.
159. Taniguchi, M., Saito, T., Takei, I., Kanno, M., Tokuhisa, T., and Tomioka, H. (1981). *In* "Lymphokine Reports" (M. Feldmann and M. H. Schreier, eds.), Vol. 5. Academic Press, New York.
160. Taniguchi, M., Saito, T., Takei, I., and Tada, T. (1979). *In* "T and B Lymphocytes: Recognition and Function" (F. H. Bach and B. Bonavida, eds.), p. 667. Academic Press, New York.
161. Whitaker, R. B., and Ruddle, N. H. (1980). *Cell. Immunol.* **55,** 56.
162. Kapp, J. A., Araneo, B. A., and Clevinger, B. L. (1980). *J. Exp. Med.* **152,** 235.
163. Kapp, J. A., Pierce, C. W., Schlossman, S., and Benacerraf, B. (1974). *J. Exp. Med.* **140,** 648.
164. Eshhar, Z., Apte, R., Lowy, I., and Mozes, E. (1981). *In* "Monoclonal Antibodies and T Cell Hybridomas" (G. Hammerling and U. Hammerling, eds.), p. 469. Elsevier, Amsterdam.
165. Nisonoff, A., Benacerraf, B., and Greene, M. I. (1982). *J. Immunol.* **128,** 49.
166. Ruddle, N. H., Beezley, B. B., Lewis, G. K., and Goodman, J. W. (1980). *Mol. Immunol.* **17,** 925.
167. Goodman, J. W., Lewis, G. K., Primi, D., Hornbeck, P., and Ruddle, N. H. (1980). *Mol. Immunol.* **17,** 933.
168. Pacifico, A., and Capra, J. D. (1980). *J. Exp. Med.* **152,** 1289.
169. Takaoki, M., Sy, M-S., Whitaker, B., Nepom, J., Finberg, R., Germain, R. N., Nisonoff, A., Benacerraf, B., and Greene, M. I. (1982). *J. Immunol.* **128,** 49.
170. Kontiainen, S., Cecka, J. M., Culbert, E., Simpson, E., and Feldmann, M. (1980). *In* "Protides of the Biological Fluids" (H. Peeters, ed.), Vol. 28, p. 451. Pergamon, Oxford.
171. Okuda, K., Minami, M., Sherr, D. H., and Dorf, M. E. (1981). *J. Exp. Med.* **154,** 468.
172. Apte, R. N., Eshhar, Z., Lowy, I., Zinger, H., and Mozes, E. (1981). *Eur. J. Immunol.* **11,** 931.
173. Sherman, L. A., Burakoff, S. J., and Benacerraf, B. (1978). *J. Immunol.* **121,** 1432.
174. Weinberger, J. Z., Germain, R. N., Ju, S.-T., Greene, M. I., Benacerraf, B., and Dorf, M. E. (1979). *J. Exp. Med.* **150,** 761.
175. Naor, D. (1979). *Adv. Cancer Res.* **29,** 45.
176. Hellstrom, I., Hellstrom, K. E., and Bernstein, I. D. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5294.

177. Nelson, K., Cory, J., Hellstrom, I., and Hellstrom, K. E. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2866.
178. Ricciardi-Castagnoli, P., Doria, G., and Adorini, L. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3804.
179. Fresno, M., Nabel, G., McVay-Boudreau, L., Furthmayer, H., and Cantor, H. (1981). *J. Exp. Med.* **153,** 1246.
180. Fresno, M., McVay-Boudreau, L., Nabel, G., and Cantor, H. (1981). *J. Exp. Med.* **153,** 1260.
181. Webb, D. R., Krupen, K., Araneo, B. A., Kapp, J. A., Nowowiejski, I., Healy, C., Wieder, K., and Stein, S. (1981). *In* "Potential Utilization of Lymphokines and Thymic Factors in Cancer Therapeutics" (A. L. Goldstein, ed.). Raven, New York, in press.
182. Rosenstein, R. W., Murray, J. H., Cone, R. E., Ptak, W., Iverson, G. M., and Gershon, R. K. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5821.
183. Ptak, W., Zembala, M., and Gershon, R. K. (1978). *J. Exp. Med.* **148,** 424.
184. Katz, D. H. (1980). *Immunology* **41,** 1.
185. Kishimoto, T., and Ishizaka, K. (1973). *J. Immunol.* **111,** 1194.
186. Suemura, M., Kishimoto, T., Hirai, Y., and Yamamura, Y. (1977). *J. Immunol.* **119,** 149.
187. Kishimoto, T., Hirai, Y., Suemura, M., Nakanishi, K., and Yamamura, Y. (1978). *J. Immunol.* **121,** 2106.
188. Gisler, R. H., and Fridman, W. H. (1975). *J. Exp. Med.* **142,** 507.
189. Neauport-Sautes, C., Dupuis, D., and Fridman, W. H. (1975). *Eur. J. Immunol.* **5,** 849.
190. Joskowicz, M., Rabourdin-Combe, C., Neauport-Sautes, C., and Fridman, W. H. (1978). *J. Immunol.* **121,** 777.
191. Fridman, W. H., Guimezanes, A., and Gisler, R. H. (1977). *J. Immunol.* **119,** 1266.
192. Neauport-Sautes, C., and Fridman, W. H. (1977). *J. Immunol.* **119,** 1269.
193. Neauport-Sautes, C., Rabourdin-Combe, C., and Fridman, W. H. (1979). *Nature (London)* **277,** 656.
194. Teillaud, J.-L., Rabourdin-Combe, C., Stanislawski, M., Neauport-Sautes, M., and Fridman, W. H. (1981). *Cell. Immunol.* **63,** 349.
195. Fridman, W. H., Rabourdin-Combe, C., Neauport-Sautes, C., and Gisler, R. H. (1981). *Immunol. Rev.* **56,** 51.
196. Sorensen, C. M., and Pierce, C. W. (1981). *J. Exp. Med.* **154,** 35.
197. Rich, S., and Rich, R. (1976). *J. Exp. Med.* **143,** 672.
198. Sorensen, C. M., and Pierce, C. W. (1981). *J. Exp. Med.* **154,** 48.
199. Stocker, J. W., Marchalonis, J. J., and Harris, A. W. (1974). *J. Exp. Med.* **139,** 785.
200. Rubin, A. S., Mulcahy, H. L., and MacDonald, A. B. (1980). *In* "Biochemical Characterization of Lymphokines" (A. L. de Weck, F. Kristensen, and M. Landy, eds.), p. 493. Academic Press, New York.
201. Mulcahy, H. L., Rubin, A. S., and MacDonald, A. B. (1981). *Immunology* **42,** 37.
202. Takada, A., Takada, Y., and Minowada, J. (1974). *J. Exp. Med.* **140,** 538.
203. Grillot-Courvalin, C., Brouet, J.-C., Berger, R., and Bernheim, A. (1981). *Nature (London)* **292,** 844.
204. Fleisher, T. A., Greene, W. C., Uchiyama, T., Goldman, C. K., Nelson, D. L., Blaese, R. M., and Waldmann, T. A. (1981). *J. Exp. Med.* **154,** 156.
205. Wolf, R. L., Pincus, S. H., Merler, E., and Rosen, F. S. (1981). *Clin. Immunol. Immunopathol.* **18,** 351.
206. MacDonald, A. B., Fraser, C. E. O., and Rubin, A. S. (1978). *Immunology* **34,** 137.

206a. Uchiyama, T., Broder, S., Bonnard, G. D., and Waldmann, T. A. (1980). *Trans. Assoc. Am. Phys.* **93,** 251.
207. Fleisher, T. A., Greene, W. C., Blaese, R. M., and Waldmann, T. A. (1981). *J. Immunol.* **126,** 1192.
208. Greene, W. C., Fleisher, T. A., and Waldmann, T. A. (1981). *J. Immunol.* **126,** 1185.
209. Schimpl, A., Hubner, L., Wong, C., and Wecker, E. (1980). *In* "Immunology 80, Progress in Immunology IV" (M. Fougereau and J. Dausset, eds.), p. 403. Academic Press, New York.
210. Howard, M., Kessler, S., Chused, T., and Paul, W. E. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5788.
211. Armerding, D., Sachs, D. H., and Katz, D. H. (1974). *J. Exp. Med.* **140,** 1717.
212. Katz, D. H., Armerding, D., and Eshhar, Z. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 541. Academic Press, New York.
213. Eshhar, Z., Armerding, D., Waks, T., and Katz, D. H. (1977). *J. Immunol.* **119,** 1457.
214. Armerding, D., Eshhar, Z., and Katz, D. H. (1977). *J. Immunol.* **119,** 1468.
215. Altman, A., Bechtold, T. E., Cardenas, J. M., and Katz, D. H. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3477.
216. Altman, A., Cardenas, J. M., Bechtold, T. E., and Katz, D. H. (1980). *J. Immunol.* **124,** 105.
217. Altman, A., Cardenas, J. M., Bechtold, T. E., and Katz, D. H. (1980). *J. Immunol.* **124,** 114.
218. Altman, A., and Katz, D. H. (1980). *Immunol. Rev.* **51,** 3.
219. Altman, A., and Katz, D. H. (1980). *J. Immunol.* **125,** 1536.
220. Katz, D. H., Bechtold, T. E., and Altman, A. (1980). *J. Exp. Med.* **152,** 956.
221. Schimpl, A., and Wecker, E. (1972). *Nature (London) New Biol.* **237,** 15.
222. Hubner, L., Schimpl, A., and Wecker, E. (1980). *Mol. Immunol.* **17,** 591.
223. Schimpl, A., Hubner, L., Wong, C. A., and Wecker, E. (1980). *Behring Inst. Mitteil.* **67,** 221.
224. Hamaoka, T., Takatsu, K., Okuno, K., and Tsuchida, T. (1981). *J. Immunol.* **126,** 659.
225. Schreier, M. H., Iscove, N. N., Tees, R., Aarden, L., and von Boehmer, H. (1980). *Immunol. Rev.* **51,** 315.
226. Schreier, M. H. (1980). *Behring Inst. Mitteil.* **67,** 185.
227. Melchers, F., Andersson, J., Lernhardt, W., and Schreier, M. H. (1980). *Eur. J. Immunol.* **10,** 679.
228. Takatsu, K., Tanaka, K., Tominaga, A., Kumahara, Y., and Hamaoka, T. (1980). *J. Immunol.* **125,** 2646.
229. Dennert, G., Swain, S. L., Waterfield, J. D., Warner, J. F., and Dutton, R. W. (1981). *Eur. J. Immunol.* **11,** 62.
230. Waterfield, J. D., Dennert, G., Swain, S. L., and Dutton, R. W. (1979). *J. Exp. Med.* **149,** 808.
231. Watson, J., Mochizuki, D., and Gillis, S. (1980). *Immunol. Today,* **1,** 113.
232. Smith, K. A., and Ruscetti, F. W. (1981). *Adv. Immunol.* **31,** 137.
233. Takatsu, K., Tominaga, A., and Hamaoka, T. (1980). *J. Immunol.* **124,** 2414.
234. Tominaga, A., Takatsu, K., and Hamaoka, T. (1980). *J. Immunol.* **124,** 2423.
235. Nabel, G., Fresno, M., Chessman, A., and Cantor, H. (1981). *Cell* **23,** 19.
236. Nabel, G., Greenberger, J. S., Sakakeeny, M. A., and Cantor, H. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1157.
237. Schrader, J. W., and Clark-Lewis, I. (1981). *J. Immunol.* **126,** 1101.
238. Schrader, J. W. (1981). *J. Immunol.* **126,** 452.

239. Rubin, A. S., MacDonald, A. B., and Coons, A. H. (1974). *J. Exp. Med.* **139,** 439.
240. Rubin, A. S., and MacDonald, A. B. (1976). *Immunology* **30,** 69.
241. Rubin, B., Cooley, M. A., Le Borgne de Kaouel, C., Taylor, R. B., and Golstein, P. (1980). *Scand. J. Immunol.* **12,** 401.
242. Vesole, D. H., Goust, J.-M., Fett, J. W., and Fudenberg, H. (1979). *J. Immunol.* **123,** 1322.
243. Loor, F., and Kindred, B. (1973). *J. Exp. Med.* **138,** 1044.
244. MacDonald, H. R., Lees, R. K., Sordat, B., Zaech, P., Maryanski, J. L., and Bron, C. (1981). *J. Immunol.* **126,** 865.
245. Ruscetti, F. W., and Gallo, R. C. (1981). *Blood* **57,** 379.
246. Larsson, E.-L., Iscove, N. N., and Coutinho, A. (1980). *Nature (London)* **283,** 664.
247. Wagner, H., Rollinghoff, M., Schawaller, R., Hardt, C., and Pfizenmaier, K. (1979). *Nature (London)* **280,** 406.
248. Gillis, S., Union, N. A., Baker, P. E., and Smith, K. A. (1979). *J. Exp. Med.* **149,** 1460.
249. Hunig, T., and Bevan, M. J. (1980). *J. Exp. Med.* **152,** 688.
250. Wagner, H., Hardt, C., Bartlett, R., Pfizenmaier, K., Rollinghoff, M. and Heeg, K. (1980). *Behring Inst. Mitteil.* **67,** 105.
251. Ando, I., and Hurme, M. (1981). *Nature (London)* **289,** 494.
252. Gillis, S., and Watson, J. (1981). *J. Immunol.* **126,** 1245.
253. Reimann, J., and Diamantstein, T. (1981). *Clin. Exp. Immunol.* **43,** 641.
254. Bodeker, B. G. D., van Eijk, R. V. W., and Muhlradt, P. F. (1980). *Eur. J. Immunol.* **10,** 702.
255. Lipsick, J. S., and Kaplan, N. O. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2398.
256. Granelli-Piperno, A., Vassali, J.-D., and Reich, E. (1981). *J. Exp. Med.* **154,** 422.
257. Wagner, H., Hardt, C., Heeg, K., Pfizenmaier, K., Solbach, W., Bartlett, R., Stockinger, H., and Rollinghoff, M. (1980). *Immunol. Rev.* **51,** 215.
258. Hardt, C., Rollinghoff, M., Pfizenmaier, K., Mosmann, H., and Wagner, H. (1981). *J. Exp. Med.* **154,** 262.
259. Caplan, B., Gibbs, C., and Paetkau, V. (1981). *J. Immunol.* **126,** 1351.
260. Nabel, G., Galli, S. J., Dvorak, A. M., Dvorak, H. F., and Cantor, H. (1981). *Nature (London)* **291,** 332.
261. Yung, Y. P., Eger, R., Tertian, G., and Moore, M. A. S. (1981). *J. Immunol.* **127,** 794.
262. Hilfiker, M. L., Moore, R. N., and Farrar, J. J. (1981). *J. Immunol.* **127,** 1983.
263. Clark-Lewis, I., and Schrader, J. W. (1981). *J. Immunol.* **127,** 1941.
264. Pfizenmaier, K., Rollinghoff, M., and Wagner, H. (1980). *Behring Inst. Mitteil.* **67,** 68.
265. Stull, D., and Gillis, S. (1981). *J. Immunol.* **126,** 1680.
266. Kappler, J., Skidmore, B., White, J., and Marrack, P. (1981). *J. Exp. Med.* **153,** 1198.
267. Altman, A., Schreiber, R. D., and Katz, D. H. (1981). *In* "Monoclonal Antibodies and T Cell Products" (D. H. Katz, ed). CRC Press, Boca Raton, Florida, in press.
268. Altman, A., Sferruzza, A., Weiner, R. G., and Katz, D. H. (1982). *J. Immunol.* **128,** 1365.
269. Farrar, J. J., Fuller-Farrar, J., Simon, P. L., Hilfiker, M. L., Stadler, B. M., and Farrar, W. L. (1980). *J. Immunol.* **125,** 2555.
270. Gillis, S., Scheid, M., and Watson, J. (1980). *J. Immunol.* **125,** 2570.
271. Mochizuki, D., Watson, J., and Gillis, S. (1980). *J. Immunol.* **125,** 2579.
272. Shimizu, S., Konaka, Y., and Smith, R. T. (1980). *J. Exp. Med.* **152,** 1436.
273. Smith, K. A., Gilbride, K. J., and Favata, M. F. (1980). *Nature (London)* **287,** 853.
274. Gillis, S., and Watson, J. (1980). *J. Exp. Med.* **152,** 1709.

275. Solbach, W., Barth, S., Graubner, M., Rollinghoff, M., and Wagner, H. (1980). *Behring Inst. Mitteil.* **67,** 265.
276. Rabin, H., Hopkins, R. F., Ruscetti, F. W., Neubauer, R. H., Brown, R. L., and Kawakami, T. G. (1981). *J. Immunol.* **127,** 1852.
277. Todaro, G. J., De Larco, J. E., Marquardt, H., Bryant, M. L., Sherwin, S. A., and Sliski, A. H. (1979). *In* "Hormones and Cell Culture" (G. H. Sato and R. Ross, eds.), p. 323. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
278. De Larco, J. E., and Todaro, G. J. (1980). *J. Cell. Physiol.* **102,** 367.
279. Poiesz, B. J., Ruscetti, F. W., Mier, J. W., Woods, A. M., and Gallo, R. C. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6815.
279a. Gootenberg, J. E., Ruscetti, F. W., Mier, J. W., Gazdar, A., and Gallo, R. C. (1981). *J. Exp. Med.* **154,** 1403.
279b. Bleackley, R. C., Caplan, B., Havele, C., Ritzel, R. G., Mosmann, T. R., Farrar, J. J., and Paetkau, V. (1981). *J. Immunol.* **127,** 2432.
280. Ihle, J. N., Pepersack, L., and Rebar, L. (1981). *J. Immunol.* **126,** 2184.
281. Happel, A. J., Lee, J. C., Farrar, W. L., and Ihle, J. N. (1981). *Cell* **25,** 179.
282. Stewart, II, W. E., Blalock, J. E., Burke, D. C., Chany, C., Dunnick, J. K., Falcoff, E., Friedman, R. M., Galasso, G. J., Joklik, W. K., Vilcek, J. T., Youngner, J. S., and Zoon, K. C. (1980). *J. Immunol.* **125,** 2353.
283. Gresser, I. (1977). *Cell. Immunol.* **34,** 406.
284. Stewart II, W. E. (1979). "The Interferon System" Springer-Verlag, Berlin and New York.
285. Lindahl-Magnusson, P., Leary, P., and Gresser, I. (1972). *Nature (London) New Biol.* **237,** 120.
286. Sonnenfeld, G., Mandel, A. D., and Merrigan, T. C. (1977). *Cell. Immunol.* **34,** 193.
287. Simon, P. L., Farrar, J. J., and Kind, P. D. (1979). *J. Immunol.* **122,** 127.
288. Heron, I., and Berg, K. (1979). *Scand. J. Immunol.* **9,** 517.
289. Neumann, C., and Sorg, C. (1977). *Eur. J. Immunol.* **7,** 719.
290. Kirchner, H., Zawatzky, T., Engler, H., Schirrmacher, V., Becker, H., and von Wussow, P. (1979). *Eur. J. Immunol.* **9,** 824.
291. Bloom, B. R. (1980). *Nature (London)* **284,** 593.
292. Marcucci, F., Waller, M., Kirchner, H., and Krammer, P. (1981). *Nature (London)* **291,** 79.
293. Nathan, I., Groopman, J. E., Quan, S. G., Bersch, N., and Golde, D. W. (1981). *Nature (London)* **292,** 842.
294. Saxon, A., Stevens, R. H., and Golde, D. W. (1978). *Ann. Intern. Med.* **88,** 323.
295. Golde, D. W., Quan, S. G., and Cline, M. J. (1978). *Blood* **52,** 1068.
296. Lusis, A. J., Quan, D. H., and Golde, D. W. (1981). *Blood* **57,** 13.
297. Golde, D. W., Bersch, N., Quan, S. G., and Lusis, A. J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 593.
298. Weisbart, R. H., Golde, D. W., Spolter, L., Eggena, P., and Rinderknecht, H. (1979). *Clin. Immunol. Immunopathol.* **14,** 441.
299. Ginsburg, H., and Sachs, L. (1963). *J. Natl. Cancer Inst.* **31,** 1.
300. Ishizaka, T., Okudaira, H., Mauser, L. E., and Ishizaka, K. (1976). *J. Immunol.* **116,** 747.
301. Burnet, F. M. (1977). *Cell. Immunol.* **30,** 358.
302. Shearer, G. M., Melmon, K. L., Weinstein, Y., and Sela, M. (1972). *J. Exp. Med.* **136,** 1302.
303. Plaut, M., Lichtenstein, L. M., and Henney, C. S. (1973). *Nature (London)* **244,** 284.

304. Roszkowski, W., Plaut, M., and Lichtenstein, L. M. (1977). *Science* **195,** 683.
305. Ogden, B. E., and Hill, H. R. (1980). *Immunology* **41,** 107.
306. Plaut, M., Lichtenstein, L. M., Gillespie, E., and Henney, C. S. (1973). *J. Immunol.* **111,** 389.
307. Schwartz, E., Sutton, S., Askenase, P. W., and Gershon, R. K. (1980). *Immunopharmacology* **2,** 179.
308. Askenase, P. W., Burstajn, S., Gershon, M. D., and Gershon, R. K. (1980). *J. Exp. Med.* **152,** 1358.
309. Tertian, G., Yung, Y. P., Guy-Grand, D., and Moore, M. A. S. (1981). *J. Immunol.* **127,** 788.
310. Schrader, J. W., and Nossal, G. J. V. (1980). *Immunol. Rev.* **53,** 61.
311. Schrader, J. W., Lewis, S. J., Clark-Lewis, I., and Culvenor, J. G. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 323.
312. Burgess, A. W., Metcalf, D., Russell, S. H. M., and Nicola, N. A. (1980). *Biochem. J.* **185,** 301.
313. Jones-Villeneuve, E. V., Rusthoven, J. J., Miller, R. G., and Phillips, R. A. (1980). *J. Immunol.* **124,** 597.
314. Iscove, N. N. (1978). *In* "Hematopoietic Cell Differentiation" (D. W. Golde, M. J. Cline, D. Metcalf, and C. F. Fox, eds.), p. 37. Academic Press, New York.

ADVANCES IN IMMUNOLOGY, VOL. 33

Autoantibodies to Nuclear Antigens (ANA): Their Immunobiology and Medicine

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I. Introduction

The presence of one or more circulating serum autoantibodies to nuclear antigens (ANA) is a hallmark of systemic rheumatic diseases. This group of systemic rheumatic diseases is not homogeneous in clinical manifestations and there are gradations in variety and severity of multisystem involvement. Systemic lupus erythematosus (SLE) is the prototype autoimmune disease with many organ systems such as joints, skin, kidney, central nervous system, serous membranes, lungs, heart, and skeletal muscle all potentially susceptible to disease involvement. Others in this group, such as mixed connective tissue disease (MCTD), Sjögren's syndrome, rheumatoid arthritis (RA), and scleroderma, are of an intermediate order in the range and variety of organ systems involved. At the other end of the spectrum is dermato/polymyositis where the main target organs are skin and muscle. In spite of the apparent heterogeneity of clinical manifestations, there are reasons for considering the relatedness of these diseases. The clinician is often confronted with the patient who has symptoms of not one disease but of two or more systemic rheumatic diseases occurring at

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the same time. This combination can involve any of the diseases described above and has been called "overlap" systemic rheumatic disease (Ziff, 1961). Further, there is an unusually high frequency of "clusters" of different rheumatic diseases in the same family, affecting different family members.

ANAs occur in extraordinarily high frequency in many of these systemic rheumatic diseases. Interest in this area has increased in recent years because of two reasons. ANAs have become useful aids in the diagnosis and treatment of systemic rheumatic diseases and have an important place in the armamentarium of diagnostic laboratory tests. Second, analysis of the nuclear autoantigens with the new technologies in biochemistry, immunology, and molecular and cell biology has provided interesting new information on the nature of these nuclear macromolecules and pointed to their possible functions in the regulation and control of cellular biological processes.

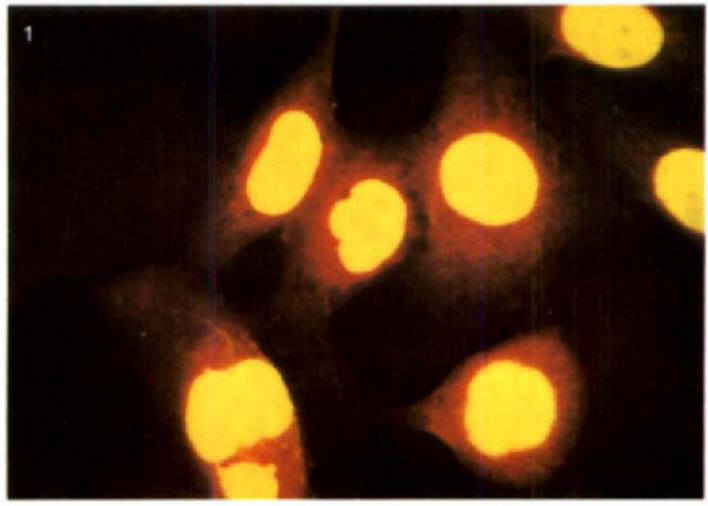
II. Immunofluorescence and ANAs

A widely used technique for the detection of ANAs is indirect immunofluorescence and, in most diagnostic laboratories, this is the initial screening test. Some of the earlier studies were performed using sections of cryopreserved tissues or organs as substrates. Recently tissue culture cell lines have seen increasing use. Some of the advantages of certain cell lines are larger nuclei and nucleoli than those in sections of differentiated tissues and organs. Some examples of nuclear staining patterns obtained with different sera are shown in the color illustrations (Figs. 1–6). Figure 1 shows a homogeneous pattern of staining produced by the serum of a patient with SLE. In this and other illustrations, the somewhat reddish hue of the cytoplasm is due to counter-staining with Evans blue. The relatively negative staining of the nucleolus is in contrast to the staining of the nucleoplasm. The homogeneous pattern of nuclear staining is given by antibodies to histones (Tan *et al.*, 1976), although it is not definitely known if it is

FIG. 1. Homogeneous nuclear staining produced by serum from a patient with SLE in indirect immunofluorescence. The substrate was a cytocentrifuged preparation of tissue culture cell line (HEp-2). The cells were counterstained with Evans Blue, which stained cytoplasm reddish. ×675.

FIG. 2. Speckled nuclear staining produced by serum with antibody to nuclear RNP (U1-RNP). Note that nucleoli were completely unstained. No Evans Blue counterstain. ×675.

FIG. 3. A pattern of nucleolar staining demonstrated by sera from some patients with scleroderma. ×675.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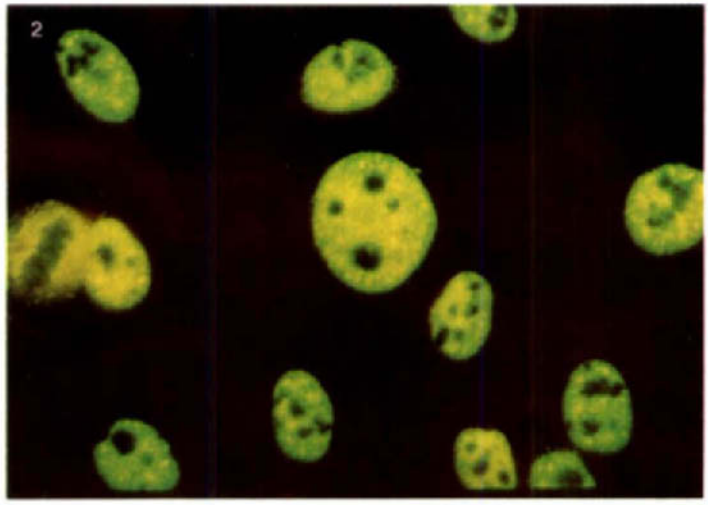
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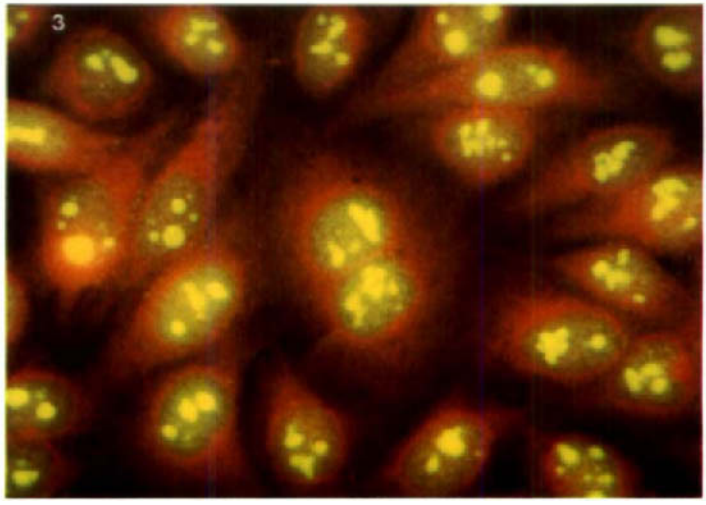
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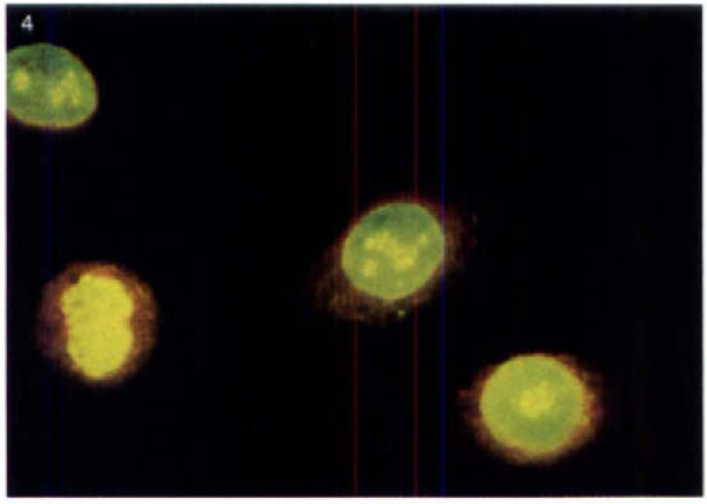
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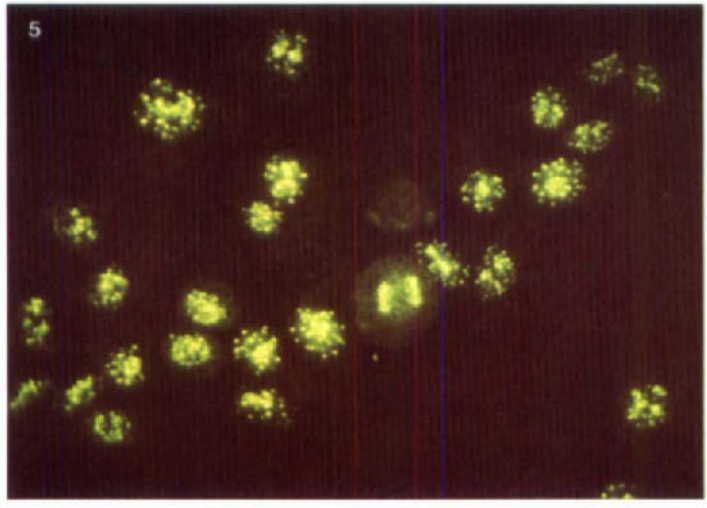
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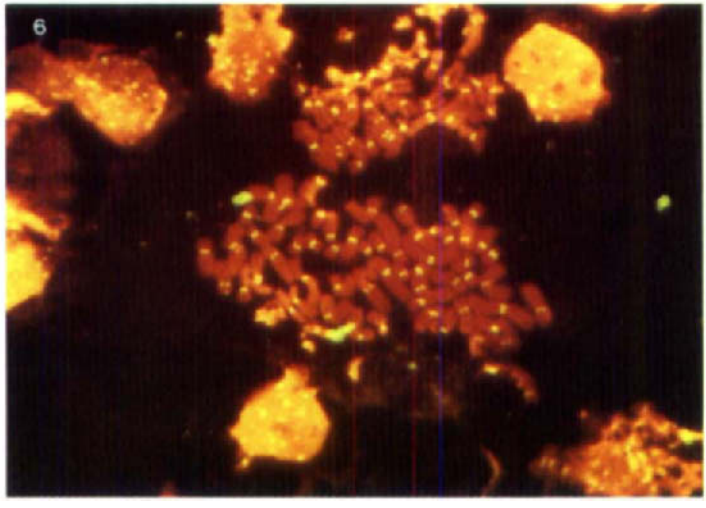
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restricted only to this antibody specificity. Figure 2 shows speckled nuclear staining with negative nucleoli. The preparation was not counterstained with Evans blue and the negative cytoplasm is not seen in this picture. This pattern with variations in size and density of speckles is given by sera containing antibodies to nonhistone nuclear antigens (Nakamura and Tan, 1977). Figure 3 depicts the pattern observed with antinucleolar antibodies. The large size of nucleoli in certain cell lines facilitates the detection of these antibodies. Figure 4 shows a homogeneous nucleoplasmic pattern associated with nucleolar staining. This is an example of mixed patterns of ANA commonly observed with rheumatic disease sera, a reflection of the simultaneous occurrence of multiple ANAs in the same serum. Figure 5 shows a large speckled pattern which is known to be associated with antibody to centromere/kinetochore. The clue to the nature of the antibody specificity was the observation that the reactive antigen was segregating with condensed chromosomes in the cell undergoing mitosis in the center of the picture (Moroi *et al.*, 1980). The association with chromosomes was consistently observed whether the cells were in prophase, metaphase, or anaphase. When a chromosome spread was used as substrate (Fig. 6), the localization of the antigen at the centromeric region of the chromosome was clearly visualized. In subsequent studies, the antigenic determinants have been localized by immunoelectromicroscopy to the inner and outer plates of the trilaminar structure of the kinetochore (Brenner *et al.*, 1981).

It is not difficult to make the conclusion that the different patterns of nuclear staining were produced by ANAs reacting with different nuclear or nucleolar antigens. The immunofluorescence technique lends itself readily to localizing autoantigens in nucleus, nucleolus, or cytoplasm. However, some important factors have to be taken into consideration. First, some nonhistone nuclear antigens are highly soluble even in physiological buffer solutions. Examples are nuclear ribonucleoprotein (nRNP) (Northway and Tan, 1972) and the SS-B/La antigen (Tan, unpublished observations) which are readily leached from nuclei

FIG. 4. A serum showing combined pattern of staining with nucleolar and homogeneous nucleoplasmic staining. ×675.

FIG. 5. Centromere/kinetochore staining produced by CREST syndrome patient on cytocentrifuged spread of a human B lymphocyte cell line. Of interest is the cell undergoing mitotic division in the center of the figure. The nuclear antigen segregated with the dividing chromosomes. ×675.

FIG. 6. Centromere/kinetochore staining as in Fig. 5 but on a chromosome spread, showing the distinct nature of reactive antigen in relation to chromosomes. Counterstaining with ethidium bromide stained DNA of chromosomes red. ×675.

TABLE I
AUTOANTIBODIES TO DNA AND HISTONES

Antibody reactive with	Clinical association
Double-strand DNA only	SLE. Rare cases reported.
Double/single-strand DNA with reaction of immunological identity	SLE (60–70%). Rarely in other diseases where it is usually in low titer
Single-strand DNA only. Antigenic determinants related to exposed purines and pyrimidines	SLE, other rheumatic diseases and certain nonrheumatic diseases
Histones (H1, H2A, H2B, H3, H4)	Drug-induced LE (95–100%), rheumatoid arthritis (15–20%), and SLE (30%)

during washing procedures. Therefore, sera containing antibodies to nRNP and SS-B/La are sometimes reported to be negative for ANA. A second feature is that certain nuclear antigens may be destroyed by some fixatives while other antigens maintain reactivity. This factor is particularly important since it is not known what fixatives are used by manufacturers to "preserve" the activity of commercial ANA kits. These considerations may account in part for reports of sera being positive for ANA in one laboratory and negative in another.

III. Spectrum and Profiles of ANAs

Table I gives a list of antinuclear antibodies which react with DNA and histones. Antibodies to DNA fall into three broad classifications. The first group are antibodies which are reactive with double-strand DNA and are not cross-reactive with single-strand DNA (Arana and Seligmann, 1967; Carr, 1969; Gilliam *et al.*, 1980). This type of antibody appears to be rare and only a few cases have been recognized. The nature of the antigenic determinant site on double-strand DNA is not known. The second class of antibodies is reactive with determinants possessed in common between double- and single-strand DNA (Tan *et al.*, 1966; Arana and Seligmann 1967; Carr 1969; Tan and Natali, 1970; Koffler *et al.*, 1971; Cohen *et al.*, 1971b; Stollar, 1973; Gilliam *et al.*, 1980). In the literature, this is commonly called antibody to native DNA. The third class of antibodies is reactive only with single-strand DNA and does not cross-react with double-strand DNA (Stollar *et al.*, 1962; Tan *et al.*, 1966; Tan and Natali, 1970; Koffler *et al.*, 1971; Cohen *et al.*, 1971b). The antigenic determinants have been shown to be related to purines and pyrimidines exposed on single-strand DNA. These antibodies have been called antibodies to denatured DNA.

TABLE II
AUTOANTIBODIES TO NONHISTONE NUCLEAR PROTEINS AND RNA-PROTEIN COMPLEXES

Antibody reactive with	Clinical association
1. Sm antigen	SLE (30–40%). Marker antibody
2. Nuclear ribonucleoprotein (nRNP) or U1-RNP	MCTD (95–100%). Lower frequency in SLE, discoid LE, and scleroderma
3. SS-A/Ro antigen	Sjögren's syndrome (60–70%), SLE (30–40%)
4. SS-B/La antigen	Sjögren's syndrome (50–60%), SLE (10–15%)
5. Scl-70	Scleroderma (15–20%). Marker antibody
6. Centromere/kinetochore	CREST (70–90%). Marker antibody
7. RANA (rheumatoid arthritis associated nuclear antigen)	RA (90–95%)
8. Ma antigen	SLE (20%)
9. PCNA (proliferating cell nuclear antigen)	SLE (5–10%)
10. PM-1	Polymyositis/scleroderma overlap (87%). Dermatomyositis (17%)
11. Mi-1	Dermatomyositis (11%)
12. Jo-1	Polymyositis (31%)
13. Ku	Polymyositis/scleroderma overlap (55%)

Antibodies to histones also have multiple specificities and have been shown to react with all the five major classes of histones, H1, H2A, H2B, H3, and H4 (Kunkel *et al.*, 1960; Stollar, 1971; Fritzler and Tan, 1978; Rubin *et al.*, 1982). In addition, complexes of H2A-H2B and H3-H4 appear to have unique antigenic determinants not present on the individual histone subclasses (Rubin *et al.*, 1982).

A major class of ANAs which has received increasing attention recently are those reactive with nonhistone nuclear antigens (Table II). The list is already long and will unquestionably become longer as the work on ANAs progresses. Of great interest is the observation that some autoantibodies are disease "markers" and are therefore restricted to certain diseases while others are more widely distributed. The marker antibodies are those against Sm, Scl-70, and kinetochore antigens which are diagnostic antibodies for SLE, scleroderma, and the CREST subset (see Section IX) of scleroderma, respectively (Tan and Kunkel, 1966a; Tan *et al.*, 1980; Moroi *et al.*, 1980). Examples of autoantibodies with wider distribution are anti-nRNP and anti-SS-A/Ro (Sharp *et al.*, 1972, 1976; Northway and Tan, 1972; Mattioli and Reichlin, 1973; Scopoletis *et al.*, 1980). It is possible that the autoantibodies found in dermato/polymyositis may also be disease-specific but since these have been more recently studied, it remains to be clearly established

TABLE III
AUTOANTIBODIES TO NUCLEOLAR ANTIGENS

Antibody reactive with	Clinical associations
4 S–6 S Nucleolus-specific RNA (? U3 RNA)	Scleroderma and certain overlap connective tissue diseases
Other nucleolar RNAs and RNA–protein particles	Primarily scleroderma

in further studies (Wolfe *et al.*, 1977; Nishikai and Reichlin, 1980a,b; Mimori *et al.*, 1981). In spite of the wide range and complexity of antibodies to nonhistone nuclear antigens, the picture is emerging of characteristic profiles of ANAs being related to specific disease states.

Table III presents a list of autoantibodies to nucleolar antigens. Very few antinucleolar antibodies have been characterized with regard to their immunological specificities. One in this category is the antibody reacting with a small RNA which is specific to the nucleolus (Pinnas *et al.*, 1973; Miyawaki and Ritchie, 1973). This may be related to U-3 RNA described by Busch and his colleagues several years ago (Ro-Choi and Busch, 1974). There are probably many other antinucleolar antibodies which have not been chemically characterized since many distinct nucleolar staining patterns can be differentiated by immunofluorescence. If it is possible to draw parallel conclusions from

TABLE IV
AUTOANTIBODIES TO OTHER NUCLEAR ANTIGENS

Antibody reactive with	Clinical association
Mitotic spindle apparatus (MSA) (McCarty *et al.*, 1981)	Variety of rheumatic diseases including carpal-tunnel syndrome
Variable large speckles (VLS) (Klipple *et al.*, 1981)	Undifferentiated connective tissue disease. IgM antibody
SL antigen (Harmon *et al.*, 1981)	Overlap of Sjögren's syndrome and SLE
Granulocyte-specific nuclear antigen (Elling *et al.*, 1968; Wiik *et al.*, 1974)	Rheumatoid arthritis
LANA (leukemia-associated nuclear antigen). (Klein *et al.*, 1974; Steiner *et al.*, 1975)	Acute and chronic leukemia
NPC (nasopharyngeal carcinoma)-associated nuclear antigens. (Yoshida *et al.*, 1975; Lamelin *et al.*, 1978)	Multiple autoantibodies in patients with NPC
Melanoma-associated antigens (Morton *et al.*, 1968; McBride *et al.*, 1972)	Multiple autoantibodies in melanoma patients
Liver-disease associated antigens (Ziegler *et al.*, 1976)	Chronic aggressive hepatitis

nuclear immunofluorescence staining, these staining patterns may be related to antibodies reactive with different nucleolar macromolecules.

There are many other ANAs (Table IV) which are presently under investigation in different laboratories and reports of these have appeared in preliminary communications. They include ANAs found in systemic rheumatic disease (Klipple *et al.*, 1981; Harmon *et al.*, 1981; McCarty *et al.*, 1981). ANAs have also been found in other diseases such as malignancy and chronic liver disease (Klein *et al.*, 1974; Yoshida *et al.*, 1975; Lamelin *et al.*, 1978; Morton *et al.*, 1968; McBride *et al.*, 1972). ANAs found in certain malignancies and liver disease have been known for some time, but generally, the nature of the reactive nuclear antigens has not been well characterized. A granulocyte-specific nuclear antigen–antibody system has been described in rheumatoid arthritis (Elling *et al.*, 1968; Wiik *et al.*, 1974).

Table V describes the synonyms of some nuclear antigen–antibody systems. Antibody to Sm and nRNP have been called antibodies to extractable nuclear antigen(s) (ENA) (Sharp *et al.*, 1972). Since many nonhistone nuclear antigens are readily extractable in physiological saline or other buffer solutions, the use of ENA only for Sm and nRNP is incorrect. According to biochemical studies (Lerner and Steitz, 1979) Sm and nRNP associate in particulate complexes and it is probably more correct to refer to these antigens as an ENA complex. The SS-B antigen is immunologically identical to the La and Ha nuclear antigens and the SS-A antigen is immunologically identical to Ro antigen (Tan *et al.*, 1977; Alspaugh and Maddison, 1979). Certain problems which remain to be resolved will be discussed later (see Section VII). Antibody to rheumatoid arthritis-associated nuclear antigen (RANA) is similar to the antigen reactive with the RANA precipitin (RAP)

TABLE V
SYNONYMS OF SOME NUCLEAR ANTIGEN–ANTIBODY SYSTEMS

Nuclear antigen	Synonyms	Nature of antigen
Sm	ENA complex	Nonhistone protein(s)
nRNP or U1 RNP		Complex of U1 RNA and protein(s)
SS-A	Ro	Nuclear (? cytoplasmic) antigen
SS-B	La, Ha	Nonhistone protein complexed to small RNAs
RANA (RA-associated nuclear antigen	Antigen for RA precipitin (RAP)	Epstein–Barr virus-induced nuclear antigen
Scl-70	Scl-1	70,000 MW chromatin-associated nonhistone protein

(Alspaugh *et al.*, 1978) and is a nuclear antigen induced in EBV-infected cells. The Scl-70 and Scl-1 nuclear antigens are similar, being a 70,000-molecular weight, basic, nonhistone nuclear protein (Douvas *et al.*, 1979a; Tan *et al.*, 1980). These nuclear antigens were studied in different laboratories and assigned different nomenclatures, and were later found to be immunologically identical antigens.

When one analyzes the spectrum of ANAs in different rheumatic diseases, it becomes apparent that each disease has a distinct profile of ANAs. In SLE, the profile is one of multiplicity with each ANA present in different frequency (Fig. 7). In contrast, in diseases such as MCTD and drug-induced lupus erythematosus, the profiles are characterized by less heterogeneity. In MCTD, antibody to nRNP is present in almost all cases and in drug-LE, the relevant autoantibody is against histone. The vertical profiles in this figure are also of interest. Antibody to Sm is present only in SLE and hence its usefulness as a

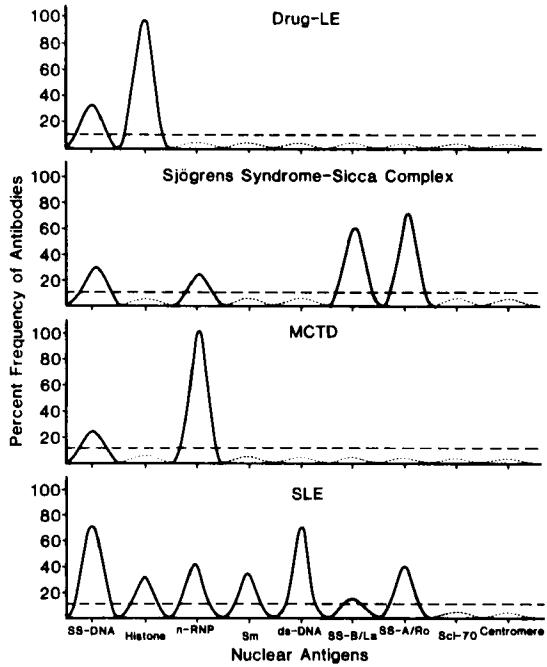

FIG. 7. Distinctive profiles of ANAs are seen with various autoimmune diseases. SLE is characterized by multiplicity of ANAs occurring in different frequencies. Anti-Sm is present in 35% of SLE but in no other diseases and is a diagnostic marker. Anti-double-strand DNA is also diagnostic of SLE when in high titer. MCTD is characterized by anti-nuclear RNP without other ANAs except for anti-single-strand DNA which is present in many rheumatic diseases. Sjögren's syndrome sera contain anti-SS-A and anti-SS-B with a low frequency of anti-nRNP. Drug-induced LE is characterized by presence of antihistone antibody. The broken lines indicate relative absence of antibodies.

serological and diagnostic marker. However, it is present in approximately 35% frequency and not in all patients. Antibody to nRNP is present in MCTD, SLE, and Sjögren's syndrome-sicca complex but its frequency should be noticed, being present in almost all cases of MCTD and in lower frequency in the other two. Antibody to SS-DNA is present in all the four diseases analyzed in Fig. 7 and therefore is of limited value as a diagnostic parameter. The antibodies related to scleroderma (Scl-70 and centromere) were generally absent in these four diseases. To the clinician, these profiles have been of great help in differential diagnosis especially in difficult clinical situations. The important feature of ANA profiles in rheumatic diseases is not only the presence but also the absence of certain ANA specificities. The data on ANA profiles in other rheumatic diseases are not complete, but it appears that there are also characteristic profiles for scleroderma, dermato/polymyositis, and RA.

IV. Autoantibodies to DNA

It was interesting that before anything was known concerning ANAs, Hargraves *et al.* (1948) discovered a phenomenon in SLE which was called the LE cell. This was a polymorphonuclear leukocyte which had ingested a large amorphous basophilic inclusion body and was demonstrable in smears made from bone marrow or clotted blood. Subsequent studies showed that this phenomenon was related to a serum factor reacting with nuclear material (Haserick *et al.*, 1950; Holman and Kunkel, 1957; Lachmann, 1961). Thus, the LE cell phenomenon was the reaction of an antinuclear antibody with autologous nuclear material. The recognition of antibody reactive with DNA occurred almost concurrently in several laboratories. Robbins *et al.* (1957) and Cepellini *et al.* (1957) demonstrated this by complement fixation, Seligmann (1957) by immunoprecipitation, and Miescher and Strassle (1957) by agglutination. Many important biochemical and key clinical observations concerning antibody to DNA followed these studies. Biochemical observations demonstrated the presence of antibodies reactive with "native" and "denatured" DNA and clinical observations established the importance of antibody to native DNA in SLE and its significance in immune complex-mediated vasculitis.

1. Antigenic Determinants on DNA Reactive with Autoantibodies

Autoantibodies to DNA in certain SLE sera are present in concentrations sufficient to form immunoprecipitin lines in double diffusion in agar or agarose. The study in Fig. 8 illustrates that immunodiffusion analysis can readily identify different populations of antibodies. Two SLE sera in the central wells were tested against native (double-

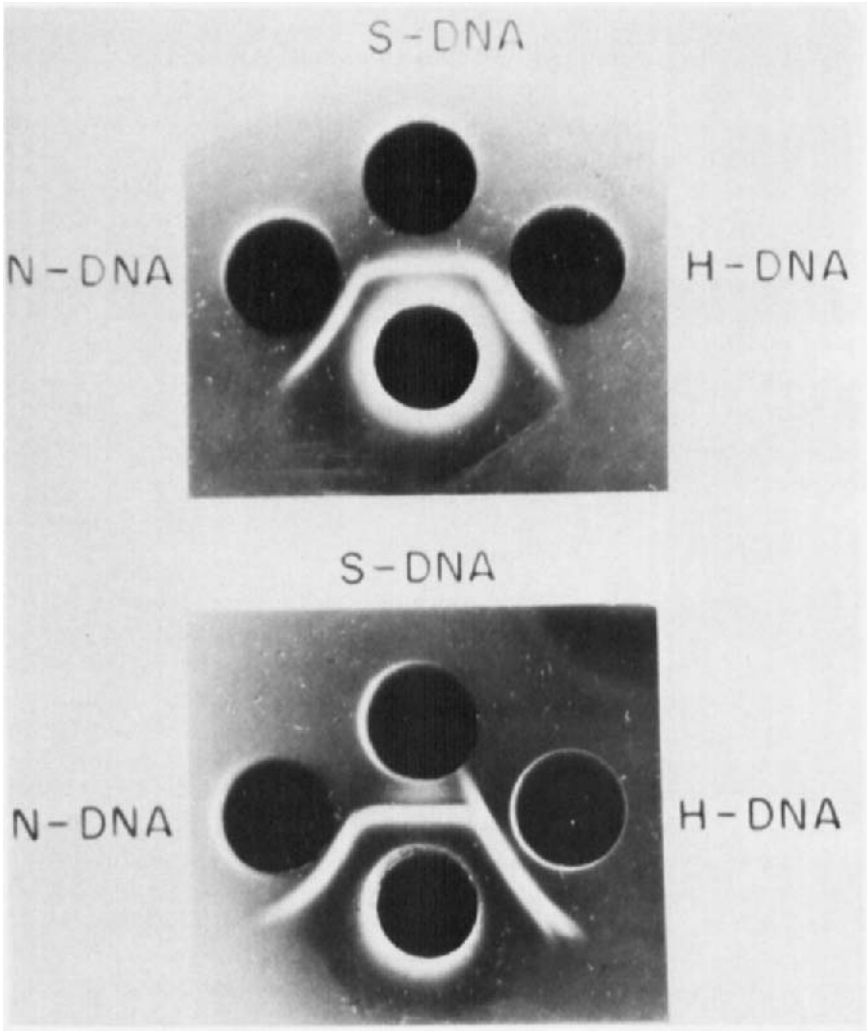

FIG. 8. Reactions of 2 SLE sera with native DNA (N-DNA), sonically disrupted DNA (S-DNA), and heat-denatured DNA (H-DNA). Serum in upper frame gave identical precipitin line with the different preparations of DNA. Serum in lower frame gave one type of precipitin line similar to upper well serum and another which reacted only with H-DNA (Tan *et al.*, 1966).

strand) DNA, sonicated DNA (shorter segments of double-strand DNA with denatured ends), and heat-denatured single-strand DNA. The serum in the upper frame reacted with all three forms of DNA to demonstrate precipitin lines of immunological identity. Thus the antibody was reacting with identical antigenic determinants present in all

three forms of DNA. The serum in the lower frame contained two precipitating antibodies, one with properties similar to the first serum. The other antibody was reactive only with heat-denatured DNA. The latter reaction could only be produced by an antibody recognizing determinants unique to single-strand DNA and not available on sonicated or double-strand DNA. In the reaction with heat-denatured DNA, the second serum actually gave two precipitin reactions. In the light of recent studies by Stollar and Papalian (1980), this is probably related to the observation that folding-back of single-strand DNA produces some secondary helical structures, so that a certain amount of "antigenically native" DNA was also present in heat-denatured DNA.

In addition to these two major classes of DNA antibodies, Arana and Seligmann (1967), Carr (1969), Tan and Natali (1970), Koffler *et al.* (1971), and Gilliam *et al.* (1980) have described antibodies which react solely with native, double-strand DNA and do not cross-react with single-strand DNA. This type of DNA antibody occurs with lower frequency than the other types just described.

Many investigators have analyzed the antigenic specificity of antibodies to native or double-strand DNA. The reaction was not inhibited by oligonucleotides (Stollar, 1975). However, synthetic double-strand polynucleotides such as poly(dA-dT) · poly(dA-dT) and poly-(dG) · poly(dC) were good inhibitors (Picazo and Tan, 1975). For some sera, pd(AT) · pd(AT) was as potent an inhibitor as double-strand DNA. These synthetic polynucleotides were also directly reactive with anti-native DNA antibody but double-strand RNA forms such as poly(A) · poly(U) and poly(I) · poly(C) were nonreactive (Stollar, 1970; Steinman *et al.*, 1976). From these and other studies (Samaha and Irwin, 1975) it appeared that the common feature of the antigenic forms was not the base/s but the deoxyribose-phosphate backbone. Clearly, however, heterogeneity exists in those antibodies reactive with double-strand DNA. Gilliam *et al.* (1980) purified antibodies from the serum of a patient with SLE and showed that one population bound solely to double-strand DNA in the form of the circular plasmid pBR322 while another which bound to double-strand poly(dA-dT) also bound well to single-strand DNA. In the latter situation, Stollar and Papalian (1980) advanced evidence that folding back of single-strand DNA to form secondary helical structures creates the reactive antigenic determinant and that a single strand of the deoxyribose-phosphate backbone is not sufficient for antigenicity. Studies to determine the size of reactive DNA fragments showed a wide range but it appeared that 40–50 base pairs obtained from limited digests of DNA was the optimum length (Papalian *et al.*, 1980).

Antibodies in human sera reactive with single-strand DNA and not

cross-reactive with double-strand DNA have been clearly shown to have specificity for the bases of nucleic acids. Here again, a great diversity of antigenic determinants has been found. When pyrimidine oligonucleotides of varying chain lengths were isolated by DEAE chromatography and tested for inhibition, tetra- or pentathymidylic acid were the most potent inhibitors for certain sera (Stollar *et al.*, 1962). Other sera were inhibited by deoxyadenylic acid and adenylic acid and, in some cases, the monomer was as potent as polyadenylic acid (Stollar and Levine, 1963). In addition, Koffler *et al.* (1971) and Natali and Tan (1972) demonstrated direct immunoprecipitation between SLE sera and synthetic single-strand polymers of adenylic, inosinic, and guanylic acids. No precipitation was observed with polycytidylic acid. In immunodiffusion, it could be clearly demonstrated that reactive RNA homopolymers were antigenically deficient compared to single-strand DNA (Fig. 9). The heterogeneity of determinants on single-strand DNA, using purified SLE antibodies as

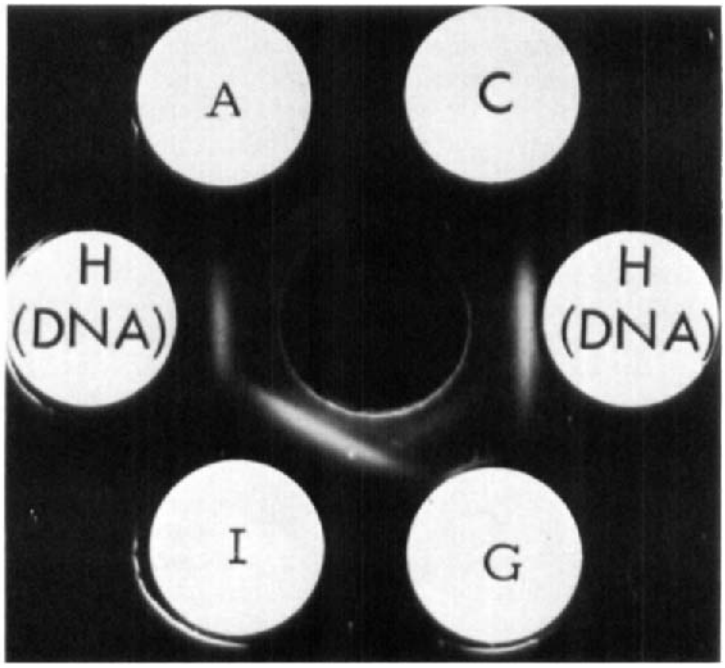

FIG. 9. SLE serum in center well reacted with heat-denatured single-strand DNA (H-DNA) and with poly(A), poly(I), and poly(G) but not with poly(C). DNA precipitin lines spurred over lines of polynucleotides showing antigenic deficiency of polynucleotides compared to DNA (Natali and Tan, 1972).

probes, was recently reported by Manak and Voss (1978). An interesting feature of antibodies to native DNA is that although it occurs in animal models of SLE like the NZB/NZW, MRL/1, and BXSB strains (Lambert and Dixon, 1968; Steinberg *et al.*, 1969; Andrews *et al.*, 1978), it has been extremely difficult to induce antinative DNA, *de novo*, in other strains of mice or in other experimental animals. In contrast, antibodies to single-strand DNA can be readily induced in experimental animals by immunization. The subject of experimentally induced DNA antibodies has been extensively reviewed by several authors (Lacour *et al.*, 1973; Stollar, 1973).

2. *Clinical Significance of Anti-DNA Antibodies*

In the late 1950s and early 1960s the extensive research into pathogenic mechanisms associated with immune complex vasculitis and the application of the immunofluorescence technique set the stage for the realization of the important role of anti-DNA antibody in SLE. Dixon and his associates (1958,1961) were dissecting the step-by-step pathogenetic events leading to glomerulonephritis and vasculitis in experimental models of serum sickness. This could be produced not only by the host's response with antibody production to the immunizing antigen (Dixon *et al.*, 1958, 1961) but also by the injection of preformed soluble antigen–antibody complexes (McCluskey *et al.*, 1960). When tissues from patients with SLE were examined with the newly introduced immunofluorescence technique, deposits of γ-globulin were found in the glomerular capillary loops of diseased kidneys and in the vascular lesions of other organs (Vazquez and Dixon, 1957; Mellors *et al.*, 1957; Paronetto and Koffler, 1965). Further investigation by Lachmann *et al.* (1962) showed the presence of C3 in association with deposited γ-globulin. These findings strongly suggested that immune complex-mediated mechanisms could be playing an important role in tissue injury in SLE.

In 1966, there was a report concerning the sequential appearance of DNA antigen and antibody in the blood of patients with SLE (Tan *et al.*, 1966). In the patient shown in Fig. 10, antibody to DNA had been detected on four successive occasions in the serum prior to an acute exacerbation of his disease. During the period of exacerbation which was characterized by prolonged high fever, generalized skin rash, and increased proteinuria, antibody to DNA disappeared from the serum and DNA antigen was detected on seven successive occasions spanning a period of 6 weeks. After intensive treatment with large doses of corticosteroids and eventual recovery from the acute episode, DNA antigen disappeared from serum without any reappearance of anti-

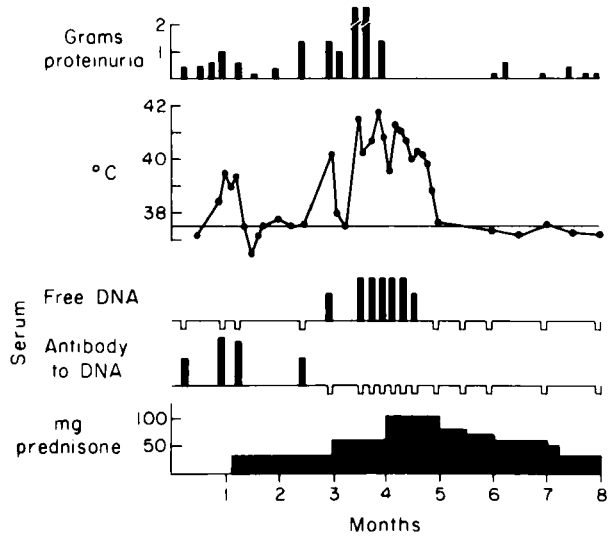

FIG. 10. A patient with SLE initially showing DNA antibody in four consecutive serum samples. Exacerbation of illness with high fever and increased proteinuria coincided with appearance of DNA antigen in serum and disappearance of antibody (Tan *et al.*, 1966).

body. It could be shown that serum taken from the earlier period of his illness (Fig. 11, 2–19) which contained precipitating antibody to DNA, was autoreactive with the DNA present in later sera (4–18 and 4–26) taken during the period of exacerbation. In studies with other SLE sera from similar patients, it was shown that the reactions between DNA antibody and antigen in serum had the capacity to fix complement. In this patient, the event which incited the release of DNA antigen into the circulation was probably related to an intense sunburn received from falling asleep under a sunlamp. In other patients, the inciting events were not evident.

Now that one of the antigen–antibody systems suspected of causing immune complex-mediated disease in SLE was demonstrated, it remained to be proven that DNA antigen and/or antibody could be localized in damaged tissue. This was demonstrated in the kidneys of SLE patients (Koffler *et al.*, 1967). DNA antigen was demonstrated, *in situ*, on the glomerular basement membrane by an immunofluorescence technique, and elution of immunoglobulin from kidney homogenates showed enrichment of DNA antibody many fold over the concentration in serum, suggesting specific trapping of DNA antibody presumably with the antigen in kidney.

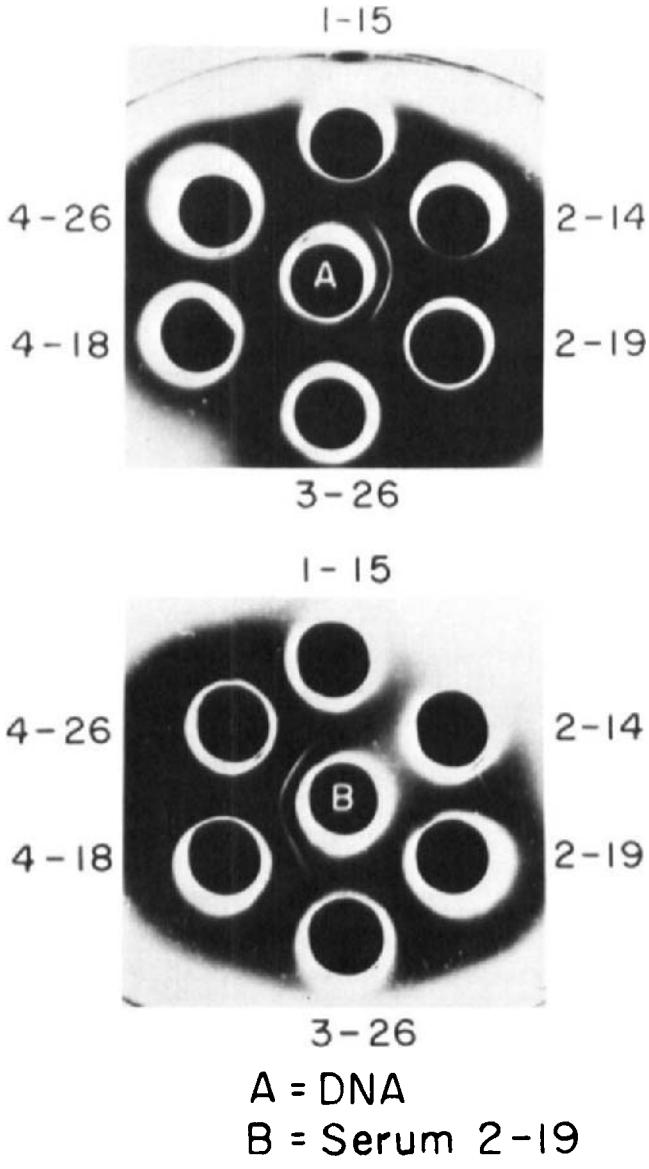

FIG. 11. Immunodiffusion studies on sera from patient in Fig. 10, showing early sera (February 14 and 19) with DNA antibody and later sera (April 18 and 26) containing DNA antigen. Serum of 2–19 was autoreactive with sera of 4–18 and 4–26 (Tan *et al.*, 1966).

Since these initial reports, there have been many studies confirming the important relationship between antibody to DNA and clinical disease in SLE (Schur and Sandson, 1968; Carr *et al.*, 1969; Pincus *et al.*, 1969; Koffler *et al.*, 1971, 1974; Robitaille and Tan, 1973; Raptis and Menard, 1980). However, it is important *not* to accept a simplistic relationship between titers of DNA antibody and disease activity and it is necessary to think in terms of immunological mechanisms. Persistently high titers of antibody may not always be correlated with deposition of immune complexes, particularly if antibody circulates in the absence of antigen. Low titers of DNA antibody may be of great significance if this is due to removal of antibody by formation of antigen–antibody complexes and not a result of decreased production. Conversely, the presence of free circulating DNA may not connote a situation of antigen excess if DNA antibody were not present concurrently, since extremely high levels of circulating DNA have been observed in liver and other diseases (Tan *et al.*, 1966) without associated immune complex vasculitis.

Several investigators have been able to demonstrate directly the presence of circulating immune complexes of DNA antibody and antigen (Harbeck *et al.*, 1973; Nydegger *et al.*, 1974; Bruneau and Benveniste, 1979). Others have shown that cryoprecipitates obtained from serum of patients with SLE contain both DNA antigen and antibody (Winfield *et al.*, 1975a; David *et al.*, 1978). These cryoprecipitates form in part from circulating immune complexes which precipitate at lower temperatures. Sano and Morimoto (1981) have used polynucleotide kinase to radiolabel the DNA moiety of immune complexes with ^{32}P so that they could be detected by the radioisotope marker and have shown that some immune complexes may consist of DNA fragments of 30 to 40 base pairs. These small polynucleotides could be protected from total degradation by circulating deoxyribonucleases because of their complexing with antibody as suggested by the preliminary communication of Fidelus *et al.* (1981).

Most investigators agree that antibody to native DNA has a special relationship with SLE. The antibody is occasionally encountered in other diseases but if present, it is in low titer. Figure 12 depicts a study comparing DNA antibody titers between SLE and other rheumatic diseases. Included were many controls consisting of normal persons, a random population of ANA-negative sera, and nonrheumatic disease controls (DCS). It can be appreciated that sera with high binding for DNA by radioimmunoassay were primarily among SLE patients. Certain other rheumatic disease sera showed abnormal DNA binding but with few exceptions, these were all in the low range. This picture

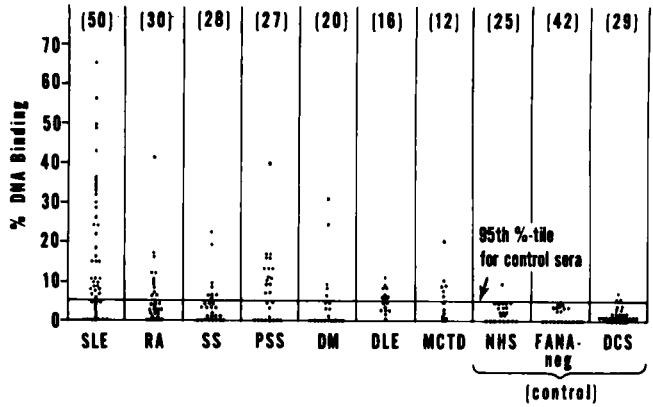

FIG. 12. Antibody to native DNA was measured by a radioimmunoassay method. The numbers in parentheses indicated the number of patients tested. SLE, Systemic lupus erythematosus; RA, rheumatoid arthritis; SS, Sjögren's syndrome; PSS, progressive systemic sclerosis; DM, dermatomyositis; DLE, discoid lupus; MCTD, mixed connective tissue disease; NHS, normal human serum; FANA-neg, serum negative for fluorescent antinuclear antibody; DSC, disease control serum. With few exceptions, high binding for native DNA was present in the SLE group (Notman *et al.*, 1975).

contrasts with that found for antibody to single-strand DNA, where this antibody is present in SLE and other rheumatic diseases such as rheumatoid arthritis and in nonrheumatic diseases such as chronic active hepatitis and infectious mononucleosis (Koffler *et al.*, 1971). As stated previously, for serodiagnostic purposes, antibody to single-strand DNA has not been found to be useful, but this should not be equated with nonpathogenicity. Andres *et al.* (1971) and Koffler *et al.* (1974) have shown that material with singel-strand deoxynucleotide determinants were present in glomerular capillaries of SLE kidneys and the evidence is convincing that they were in the form of immune complexes.

The importance of antibody to native DNA in serodiagnosis of SLE and in following response to treatment has given impetus to many attempts to develop sensitive and reliable assays. Wold *et al.* (1968) developed a radioimmunoassay, using ^{3}H-labeled *B. subtilis* DNA and ammonium sulfate precipitation of antibody-bound DNA. This was further developed by Pincus *et al.* (1969). Carr *et al.* (1969) used [^{3}H]actinomycin D-labeled DNA as the radiolabeled antigen. Ginsberg and Keiser (1973) developed a filter assay to separate antibody-bound DNA from free DNA. A criticism which has been raised against many assays for antinative DNA antibody concerns contamination of native DNA with single-strand forms. This would com-

plicate interpretation of DNA binding activity since antibodies to both double- and single-strand DNA would be represented. Methods have been advocated using adsorbant columns to separate double- from single-strand DNA (Tan and Natali, 1970; Winfield and Davis, 1974) and S1 nuclease to digest single-strand forms (Medoff *et al.*, 1976). Recently, the synthetic double-strand polymer poly(dA-dT) has been introduced as the antigen in radioimmunoassay (Steinman *et al.*, 1977). Aarden *et al.* (1975) used the kinetoplast of *Crithidia lucilliae* as the source of native DNA in an immunofluorescence assay. This appears to have gained wide acceptance because of the relative ease of the assay, but some question remains concerning its sensitivity vis-à-vis radioimmunoassays and whether the kinetoplast contains only native DNA without association with nuclear proteins (Sontheimer *et al.*, 1978; Deegan *et al.*, 1978; Ballou and Kushner, 1979).

V. Autoantibodies to Histone

Histones are basic nuclear proteins containing high molar ratios of positively charged amino acids, lysine and arginine. Recent studies have clarified the *in situ* organization of histones in the nucleus (Kornberg, 1977; Isenberg, 1979; McGhee and Felsenfeld, 1980), and have shown that they comprise a fundamental component of the nucleosome. The monomer nucleosome structure is an octameric association of two molecules each of histones H2A, H2B, H3, and H4. Wrapped around this octamer is a 146-base pair length of DNA and connecting one nucleosome with the next is a variable length of linker DNA. Histone H1 is not an intrinsic constituent of the nucleosome itself but appears to play a role in linkage between nucleosomes.

Early in the studies on autoantibodies in human sera, some SLE patients were found to have antibodies to histones by a complement fixation technique (Kunkel *et al.*, 1960). Investigations along this line were extended (Stollar, 1971) and SLE sera were shown to have antibodies to histone fractions H2A, H2B, H3, and H4. In general, sera with these antibodies were detected infrequently and antibody titers were relatively low. This may have been due in part to the fact that complement fixation was used, and since histones themselves tend to be anticomplementary (Hekman and Sluyser, 1973), only sera with relatively high concentrations of antibodies may have been examined.

An immunofluorescence method has been used for the detection of antibodies specific to histones (Tan *et al.*, 1976). This is based on the principle that histones and many nonhistone nuclear proteins are extractable from tissue sections with dilute hydrochloric acid (0.1 *N*) but

DNA is not. When such HCl-extracted sections are incubated with purified histones, the latter reassociates with DNA remaining in nuclei, and the histone-reconstituted section may be used as substrate in immunofluorescence. The criteria for a reaction denoting antihistone antibody is based on a three-step assay as shown in Fig. 13. On the control tissue section, the serum showed a homogeneous/patchy pattern of nuclear staining. On HCl-extracted tissue, the ANA became negative and on histone-reconstituted tissue, the ANA became positive again. The immunofluorescence method circumvents problems of anticomplementarity of histones and is readily adaptable in a laboratory doing routine ANA immunofluorescence. Also, purified histones are commercially available. However, it has been observed recently that antihistone antibody in certain types of drug-induced LE (see below) are not detected with this method.

Solid-phase immunoassays have also been developed for detecting antihistone antibodies. These methods make use of binding of total histones or histone subfractions to polystyrene surfaces and detection of bound antibody with isotope-labeled (Romani *et al.*, 1980; Blankstein *et al.*, 1980; Rubin *et al.*, 1981) or enzyme-labeled reagent (Romac *et al.*, 1981). This review does not cover the field of antihistone antibodies induced in experimental animals, but there has been extensive work in this area. These induced antibodies have been produced against distinct histone subclasses and have been used as reagents to probe histone organization and structure in the nucleosome (Bustin, 1978; Stollar, 1978).

Clinical Significance of Antihistone Antibodies

Autoantibodies to nuclear histones are found in three systemic rheumatic diseases, drug-induced LE, SLE, and rheumatoid arthritis. The high prevalence of antibodies to histones in drug-induced LE was first observed with the immunofluorescence assay (Fritzler and Tan, 1978). In the study, depicted in Fig. 14, there were 23 patients whose symptoms were related to procainamide, two to isoniazid, and two to nitrofurantoin. In the immunofluorescence assay, all 23 sera became ANA negative on HCl-extracted tissue and 22 of 23 (96%) became positive again on histone-reconstituted tissue. This could be contrasted with the diversity of patterns produced by sera from 20 patients with SLE (Fig. 14B). Many sera were negative on HCl-extracted tissue but some remained positive or dropped in titer. Of those which became negative, a few regained ANA positivity but many did not. Further analysis of the meaning of these results revealed that ANAs in drug-LE were almost all against histones, whereas in SLE, histone was

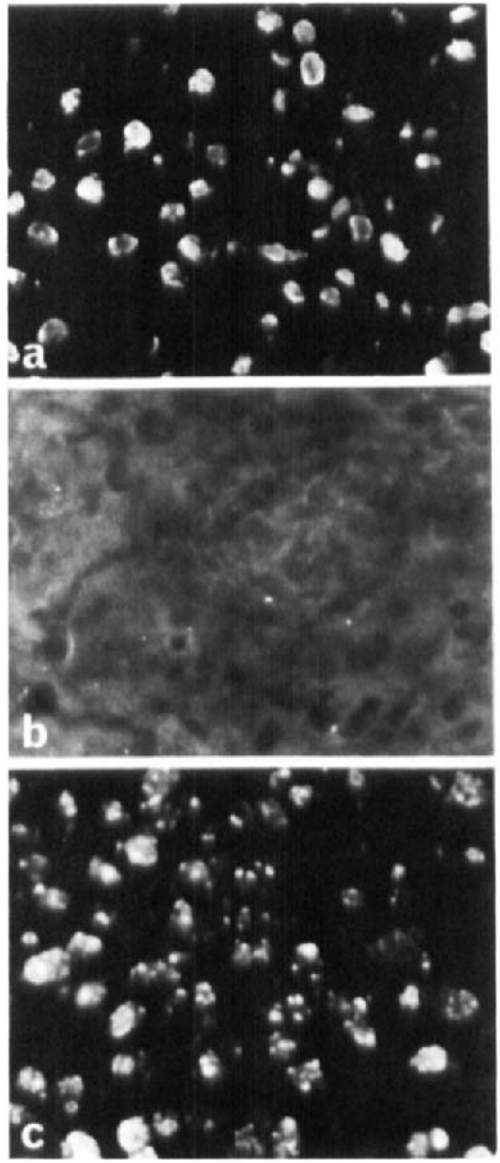

FIG. 13. Immunofluorescence method for detecting antihistone antibodies. A serum with antihistone antibody shows homogeneous/patchy pattern of nuclear staining on control tissue (a). On HCl-extracted tissues, ANA is negative (b) and on histone-reconstituted tissue (c), ANA becomes positive again (Fritzler and Tan, 1978).

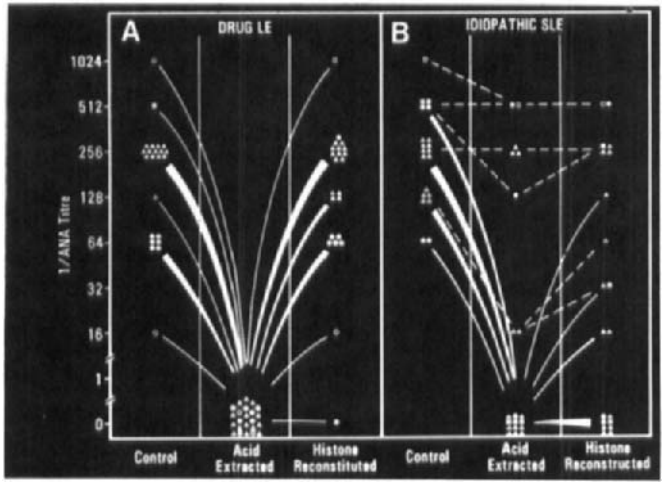

FIG. 14. Examination of drug-induced LE (A) and SLE (B) sera in the histone-reconstitution immunofluorescence assay. Drug-induced LE (A) showed pattern of reactivity suggesting antibodies to histones whereas SLE (B) had strikingly variable patterns related to diversity of antibodies (see text). (From Fritzler and Tan, 1978.)

one of many other nuclear antigens (Table VI). In drug LE, antibody to single-strand DNA was the only other ANA specificity detected and antibodies to double-strand DNA, Sm, and nuclear RNP were absent. These studies, in addition to demonstrating the high prevalence of antihistone antibodies in drug-LE, also highlight the distinct serologic differences between drug-LE and SLE.

These studies have recently been extended, using solid phase radioimmunoassay to detect antihistone antibodies (Rubin *et al.*, 1982). This has confirmed the presence of antihistone antibodies in drug-LE, SLE, and rheumatoid arthritis. An interesting feature is the observation that the bimolecular complex of H2A-H2B is the dominant target antigen of antihistone antibodies in SLE and also in

TABLE VI
ANTIBODY PROFILES IN IDIOPATHIC SLE AND DRUG-INDUCED LE

	Antibody to				
	Histones	Sm	nRNP	dsDNA	ssDNA
Idiopathic SLE (20)	7	9	3	7	4
Drug-LE (23)	22	0	0	0	3

procainamide-induced LE (Rubin *et al.*, 1982; Portanova *et al.*, 1982). In hydralazine-induced LE however, the complex of H2A–H2B is an important target antigen but the complex of H3–H4 appears to be even more reactive (Table VII). In both types of drug-LE (hydralazine and procainamide), IgM antibodies were present in higher concentration than IgG antibodies. The difference between procainamide- and hydralazine-induced ANAs was particularly noticeable in the histone-reconstituted immunofluorescence assay (Grossman and Barland, 1981; Portanova *et al.*, 1982). For reasons which may only in part be related to decreased sensitivity of immunofluorescence compared to radioimmunoassay, hydralazine-induced ANA was not detected in the former but detected in the latter technique.

A rheumatic disease syndrome resembling but not entirely similar to SLE can be produced by certain drugs such as procainamide, hydralazine, and a family of anticonvulsants (Alarcon-Segovia, 1969; Blomgren, 1973; Lee and Chase, 1975; Weinstein, 1980). The clinical picture of this syndrome has been extensively reported (Blomgren *et al.*, 1972; Perry, 1973; Whittingham *et al.*, 1972). In addition to serological differences between drug-LE and SLE described above, there are very distinct clinical differences. The major ones are the rare occurrence of renal disease and central nervous system diseases in drug LE. Malar erythema is very uncommon in drug-LE although other types of skin rash may be frequent. Nevertheless enough similar-

TABLE VII
ACTIVITIES OF ANTIHISTONE ANTIBODIES INDUCED BY PROCAINAMIDE (Pr) AND HYDRALAZINE (Hy)[a]

Ig class	Drug	Mean antibody activity to histones (cpm)		
		H1	H2A-H2B	H3-H4
IgM	Pr	741*	2348**	805
	Hy	141*	478**	705
IgG	Pr	225	1212**	0
	Hy	167	151**	0

[a] Values shown are the mean cpm of 10 procainamide and 10 hydralazine sera. The mean binding of 8 normal human sera plus 2 standard deviations was subtracted from all values for each antigen tested. The p values were calculated by Student's t test (two-tailed).

* $p < 0.05$.

** $p < 0.01$.

ities such as arthritis, pleuritis with pleural effusion, and serum LE cells exist between drug LE and SLE so that investigators have been stimulated into determining the mechanisms involved in drug-LE with the anticipation that such studies might provide some clues to SLE.

An important finding concerned the role of hepatic acetyltransferase, an enzyme capable of acetylating drugs such as hydralazine and procainamide. Perry *et al.* (1970) showed that patients with phenotypically low levels of this enzyme were more prone to develop ANA and clinical symptoms when treated with hydralazine than patients with phenotypically high levels of acetyltransferase. However, patients who were fast (high level) acetylators were not immune to the development of ANA—it took longer and a larger cumulative dose of hydralazine. These key findings have been confirmed in other studies, including patients treated with procainamide (Henningsen *et al.*, 1975; Woosley *et al.*, 1978). In prospective studies on patients newly started on treatment with procainamide for treatment of arrhythmias, 50% of all patients developed ANAs after 1 year of treatment (Blomgren, 1969) and when acetylator phenotypes were determined, the slow acetylators developed ANAs more quickly than the fast acetylators (Woosley *et al.*, 1978). In the latter study, it was further shown that almost all patients irrespective of acetylator phenotype developed positive ANAs after prolonged treatment.

Some of the molecular interactions which might play a role in ANA induction have been studied. It was shown that hydralazine could bind to soluble deoxyribonucleoprotein in a physicochemical interaction which markedly increased the viscosity of nucleoprotein (Tan, 1974). This has been confirmed (Dubroff and Reid, 1980) and similar interactions have been observed between procainamide and nucleoprotein (Eldredge *et al.*, 1974; Gold *et al.*, 1977). Furthermore, the complexing of hydralazine with nucleoprotein partially protected the latter from digestion with trypsin, and it was suggested that this might enhance nucleoprotein immunogenicity (Tan, 1974). Further mechanisms may involve the reactive amino group of procainamide since it has been shown that patients treated with acetyl-substituted procainamide have much lower prevalence of ANA than those treated with the unacetylated form (Lahita *et al.*, 1979; Stec *et al.*, 1979).

In addition to drug-LE and SLE, sera from patients with rheumatoid arthritis contain antibodies to histones. This was initially observed as a cross-reaction of polyclonal rheumatoid factor with cell nuclei (Hannestad and Johannessen, 1976). Extension of these studies showed that the reactive nuclear antigen was present in nucleosomes (Hannestad

and Stollar, 1978). Johnson (1979), Aitcheson *et al.* (1980), and Agnello *et al.* (1980) have added to these studies. Aitcheson showed that 10/19 (53%) of isolated polyclonal rheumatoid factors demonstrated cross-reacting ANA. There were other equally high-titered isolated rheumatoid factors which did not show ANA activity. Six of the ANA-positive rheumatoid factors were analyzed for antigenic specificity and 5/6 reacted with total histones in the immunofluorescence test. Agnello *et al.* (1980) have carefully analyzed isolated monoclonal rheumatoid factor from a patient with Waldenstrom's macroglobulinemia and showed cross-reactivity with DNA-histone by both immunoprecipitation and immunofluorescence. It was interesting that rheumatoid factors isolated from SLE and idiopathic cryoglobulinemia did not cross-react with nuclei. These preliminary findings raise interesting questions concerning the nature of the cross-reaction. Is the dual reactivity of some rheumatoid factors a true cross-reaction in the sense that IgG and nuclear histones share common antigenic determinants or is it an example of multispecificity of an antibody for different antigens that are structurally unrelated (Richards *et al.*, 1975; Johnston and Eisen, 1976)? These questions remain to be elucidated by further studies.

VI. Autoantibodies to Sm and Nuclear Ribonucleoprotein (nRNP)

Autoantibodies to many nuclear antigens, with the notable exception of histones, can be detected by immunoprecipitation in double diffusion. This type of reaction is dependent on many factors, including solubility of antigen and high concentration and precipitating nature of the antibody. In spite of these limitations, immunodiffusion analysis has been one of the key instruments in the discovery of many autoantibodies to nuclear antigens which are either nonhistone proteins or associated with nonhistone proteins. This section deals with antibodies to Sm and nuclear ribonucleoprotein (nRNP) together, because although the antigenic determinants are probably on different molecules, there is strong evidence that some Sm and nRNP are associated in multimolecular complexes called nuclear ribonucleoprotein particles (Sekeris and Guialis, 1981).

Figure 15 depicts an immunodiffusion analysis with saline extract of rabbit thymus in the center well and sera containing antibodies to Sm, nRNP, and SS-B/La in the peripheral wells. Several features are of interest. The saline extract contained multiple nuclear antigens showing that these nuclear components, at least in part, are loosely bound and can be dissociated readily from the nucleus. The SS-B/La precipitin line intersects both Sm and nRNP lines, which in this system

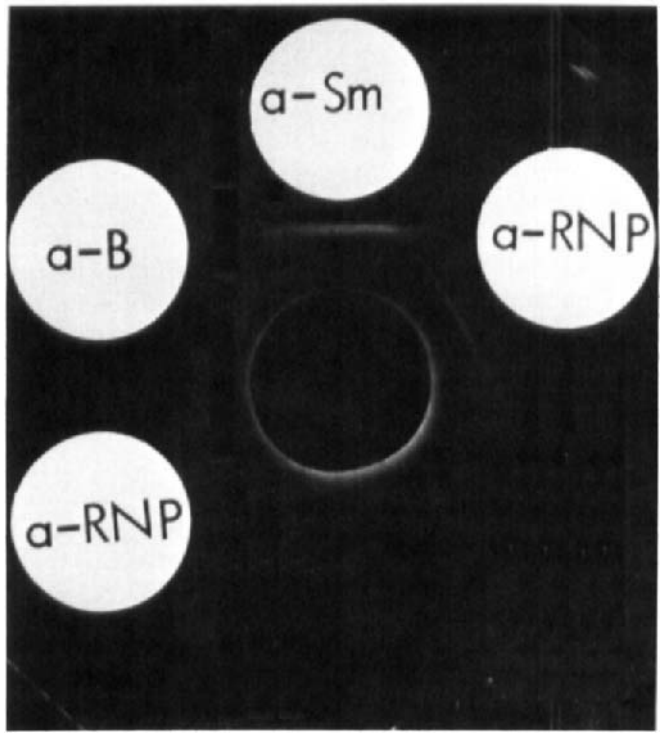

FIG. 15. Immunodiffusion analysis of Sm, nRNP, and SS-B/La precipitating systems. Center well contains saline extract of rabbit thymus and peripheral wells sera with different precipitating antibodies. Note that the Sm line forms a spurious "spur" formation over the nRNP line which in this system does *not* represent partial immunological identity (see text). (From Deng *et al.*, 1982.)

signifies immunological nonidentity of SS-B/La from Sm and nRNP. The reactions between Sm and nRNP lines are different, with the Sm line giving what appears to be a "spur" formation over the nRNP line. This is commonly observed, although, under certain conditions, the Sm and nRNP lines can be seen to intersect (Northway and Tan, 1972; Barque *et al.*, 1981). The "spur" formation has been interpreted by many investigators to represent partial immunological identity of Sm and nRNP with nRNP being deficient to Sm in antigenic determinants. It is important to note that this is an oversight in interpretation because a true immunological spur formation representing partial identity may be seen when a single antiserum is analyzed against antigens in several peripheral wells (Ouchterlony, 1964). The system depicted in Fig. 15 is an analysis of a complex antigenic extract against

different antisera in peripheral wells. The apparent "spur" formation has been called by Ouchterlony a reaction of inhibition. The interpretation, therefore, is that the nRNP line is somehow inhibited from intersecting the Sm line. It will be appreciated from evidence presented below that this inhibition is related to the fact that all nRNP particles are complexed with Sm, whereas there are some Sm molecules which are free of nRNP and are able to produce the "spur" formation.

In 1972, biochemical studies were reported which differentiated the properties of Sm and nRNP antigens (Northway and Tan, 1972). These studies were performed with saline extracts of purified calf thymus nuclei. Figure 16 is a composite of DEAE chromatography, G-200 filtration, and sucrose density gradient ultracentrifugation of the nuclear extract. Fractions from these different procedures were analyzed for content of Sm or nRNP antigens by immunodiffusion against standard reference sera. It can be seen that in each of the three fractionation procedures, there was a region of nRNP antigenicity which was always associated with Sm activity. Another contiguous region in each fractionation analysis showed only Sm activity. Particularly interesting was the G-200 filtration which showed that the two regions of Sm activity were separated by a trough of low activity suggesting that the heterodispersity of Sm was not a continuum but represented two distinct regions. In subsequent studies, Mattioli and Reichlin (1973) also presented evidence for a physical association of nRNP with Sm and also for free Sm in extracts of calf thymus.

In addition, certain other characteristics differentiate Sm from nRNP. Sm is stable at 56°C for 1 hour whereas the antigenicity of nRNP is totally lost. Sm is stable at a pH range of 3 to 11 and nRNP at pH 7 to 9. More importantly, Sm is resistant to ribonuclease treatment whereas nRNP is not. Sm is resistant or only partially sensitive to trypsin while nRNP is extremely sensitive (Northway and Tan, 1972; MacGillivray *et al.*, 1981).

Lerner and Steitz (1979) have used the technology of molecular biology to study ANAs and have opened an area of research which might provide new insights into our understanding of autoimmune diseases. They labeled mouse Ehrlich ascites tumor cells with ^{32}P or [^{35}S]methionine and analyzed the nuclear RNAs and proteins precipitated by antibodies to Sm and nRNP. Figure 17 shows a urea–acrylamide gel electrophoresis of the precipitated RNAs. Anti-nRNP precipitated U1 RNA while anti-Sm precipitated U1, and four other RNAs, U2, U4, U5, and U6. These investigators have shown that the RNAs belong to the small nuclear RNA species known for some years

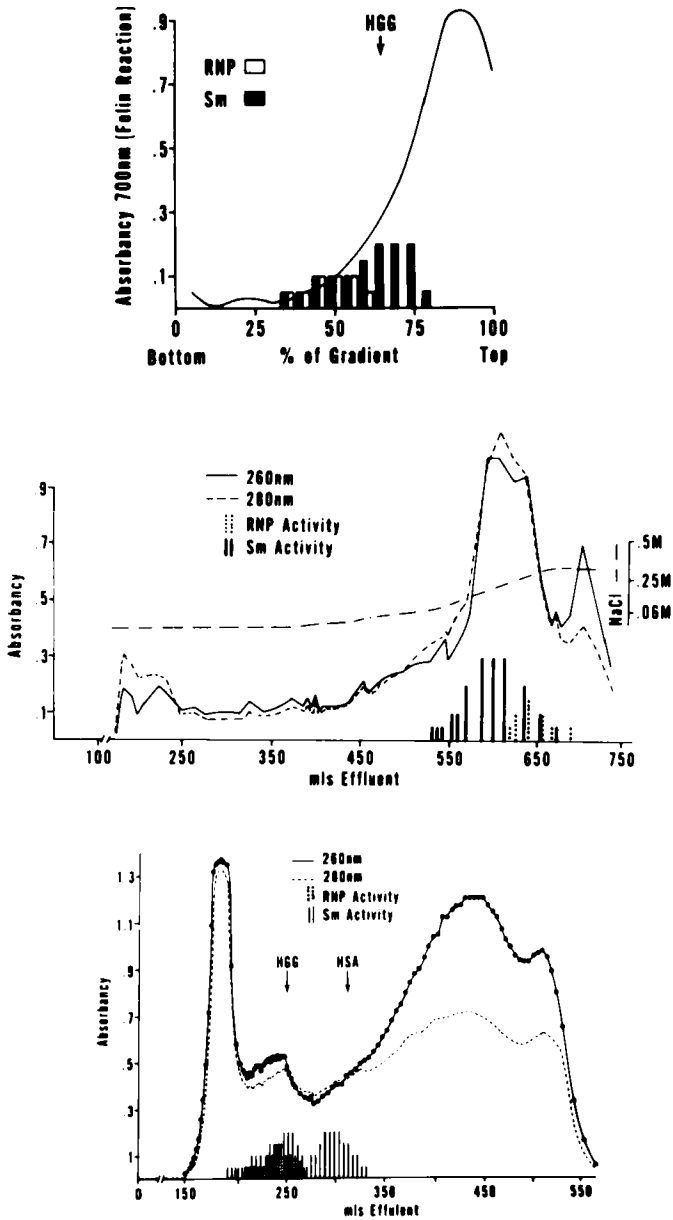

FIG. 16. Biochemical studies on Sm and nRNP antigens in calf thymus nuclei. Top panel was 5–20% sucrose gradient ultracentrifugation, middle panel DEAE chromatography, and bottom panel Sephadex G-200 gel filtration. In each procedure, there is a region of Sm activity associated with nRNP but also another region of free Sm activity. In G-200, the two regions of Sm activity are separated by a trough of lower activity. (Adapted from Northway and Tan, 1972.)

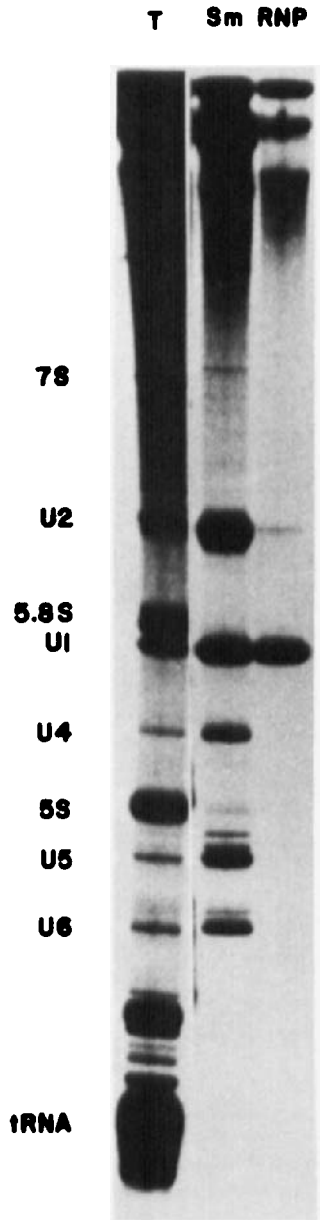

FIG. 17. HeLa cells were labeled with ^{32}P and extract of whole cells immune-precipitated with anti-Sm and anti-nRNP sera. Labeled RNA in immune precipitates were extracted and analyzed on acrylamide–urea gels. Left track shows total cell RNA, middle track shows that anti-Sm precipitated U1, U2, U4, U5, and U6 RNA, and right track that anti-nRNP precipitated only U1 RNA. (Courtesy of J. A. Steitz.)

(Reddy and Busch, 1981; Weinberg and Penman, 1968; Hellung-Larsen, 1977). Of particular interest was U1 RNA where the nucleotide sequence has been established previously. Lerner and Steitz called attention to the fact that the nucleotide sequence of U1 RNA showed a high degree of homology with nucleotide sequences across splice junctions of HnRNA (Lerner *et al.*, 1980) and proposed that U1 RNA with associated proteins or the U1 RNP particle may play a role in the processing of HnRNA to messenger RNA. Preliminary evidence has been reported that anti-Sm and anti-nRNP do inhibit splicing in an *in vitro* system which transcribes mature mRNA from the adenoviral genome (Yang *et al.*, 1981). These observations need to be confirmed, but in any event, they have provided an opening into the molecular biology of the nonhistone autoantigens. The protein moieties precipitated by anti-Sm and anti-nRNP were shown to consist of seven polypeptides in each case, varying from 12,000 to 35,000 molecular weight.

Many other groups of investigators have also analyzed the RNA and/or polypeptide composition of Sm and nRNP antigens. Some have used Staph protein A precipitation of immune complexes like Lerner and Steitz but others have used spontaneous antigen–antibody precipitation. Affinity columns constructed with IgG antibody have also been used to specifically purify the antigens. The findings are summarized in Table VIII. It can be seen that there is a wide diversity of results for either the RNA or protein components of Sm and nRNP. In spite of this diversity, there appears to be agreement that U1 RNA is precipitated by anti-nRNP but there is little consensus concerning the protein components. At the present time, it is difficult to understand the reasons for these different results. An important explanation may relate to the fact that Sm exists free and also in association with nRNP. These antigens may be in dynamic states of association and dissociation with each other and with other molecules, and in the immune precipitates, one obtains the antigen itself together with other molecules associated with the antigen.

The dynamic states of Sm and nRNP can best be observed in synchronized cells, where at any one time the majority of cells are in a limited phase of the cell cycle. In Figs. 18 and 19, synchronized cells were stained in immunofluorescence with anti-Sm and anti-nRNP (Deng *et al.*, 1981). It can be observed that the intracellular locations of the antigens change at different phases of the cell cycle. For Sm, the antigen is intranuclear at G_1 and S but in late G_2 and M, Sm is located outside the nucleus or away from the region of chromosome condensation. The nRNP antigen remains intranuclear throughout the

TABLE VIII
REPORTED RNA AND PROTEIN NATURE OF Sm AND nRNP ANTIGENS

References	Sm		nRNP	
	RNA	Protein	RNA	Protein
Douvas *et al.* (1979a)				13 and 30 K
Lerner and Steitz (1979)	5 snRNAs (U1, U2, U4, U5, U6)	7 polypeptides (12–35K)	2 snRNAs (U1, U6)	7 polypeptides (12–35K)
Takano *et al.* (1980)			5 snRNAs (40, 60, >77, >77)[a]	6 polypeptides (13–65K)
Agelli *et al.* (1980)		55K		
Waelti and Hess (1980)		110 and 28K		
Barque *et al.* (1981)	5 snRNAs (U1, U6, and a, b, c)	4 polypeptides (10–30K)	U1, U2, U5	4 polypeptides (10–30K)
White *et al.* (1981)	U1	3 polypeptides (8.8–10.8K)	U1	3 polypeptides (8.8–10.8K)
P. J. White and S. O. Hoch (personal communication)		13K		4 polypeptides (13, 40, 55K)
Takano *et al.* (1981)		4 polypeptides (12–13K)	1 snRNA (99)[a]	3 polypeptides (30, 65K)
MacGillivray and Carroll (1982)	10 RNAs (4–5 S)	3 or 4 polypeptides (10 and 90K)	10 RNAs (4–5 S)	5 polypeptides (10, 12, 30K)

[a] Numbers in parentheses indicate nucleoside lengths of RNAs.

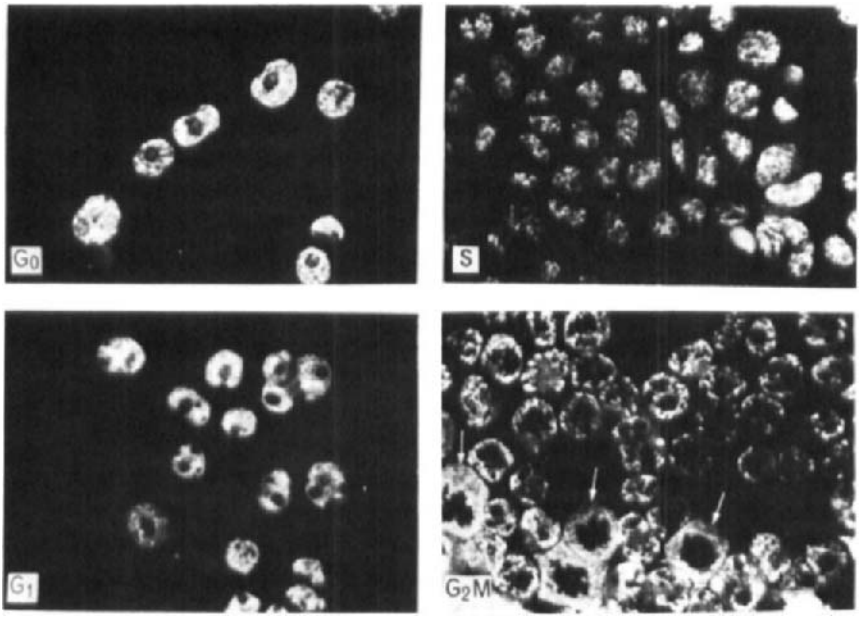

FIG. 18. Synchronized human B lymphoid cell line (WiL_2) was used as substrate for anti-Sm serum. In G_0 phase of the cell cycle, staining is densely speckled nucleoplasmic with some nucleolar stain. In G_1, staining is somewhat similar with apparent absence of nucleolar staining. In S phase, the staining is more speckled in nature. In G_2/M, Sm antigen appeared to be segregating in peripheral areas of the cell and seen more already in dividing cells (arrows), the antigen was peripheral to area of condensing chromosomes (Deng *et al.*, 1982).

cell cycle except during metaphase itself when it appears as clumps at the periphery of condensing chromosomes. It is possible that at different phases of the cell cycle, antigens such as Sm may be associated with different RNP particles. If nonsynchronized cells are used as starting material for immunoprecipitation, different proteins or RNAs might be coprecipitated, depending on where the majority of cells happen to be in the cell cycle. Furthermore, cells of different origins were used as sources of nuclear antigens and although some snRNAs like U1 RNA are highly conserved in different species, other RNAs and nuclear proteins precipitating with ANAs may be dissimilar in different species.

Clinical Significance of Anti-Sm and Anti-nRNP Antibodies

Antibody to Sm antigen has been shown to be a seriological marker for SLE. In 1966, Tan and Kunkel partially purified the Sm antigen

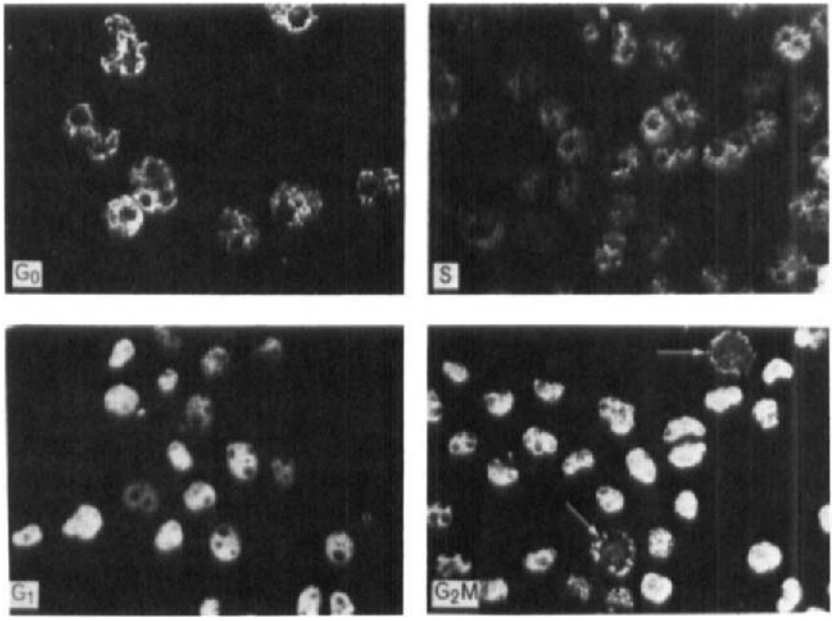

FIG. 19. Similar study as in Fig. 18, but using anti-nRNP. In G_0, G_1, and S, staining was nucleoplasmic without nucleolar staining. In G_2/M, nRNP continues to be seen throughout the nucleoplasm except in mitotic cells (arrows), where it appeared to be in clumps surrounding the area of condensing chromosomes (Deng *et al.*, 1981).

and showed that it was a nonhistone nuclear component whose antigenicity was not destroyed by DNase or RNase treatment. Antibody was present in SLE sera but absent in a variety of other diseases examined. In acute SLE, antibody was present in 75% of cases. These initial observations have been substantiated in many subsequent studies (Northway and Tan, 1972; Parker, 1973; Notman *et al.*, 1975; Barland *et al.*, 1975; Farber and Bole, 1976; Kurata and Tan, 1976; Jonsson and Norberg, 1978). The specificity of anti-Sm for SLE is illustrated in Fig. 20, where hemagglutination was used to detect anti-Sm in many rheumatic disease sera. With the exception of one patient with mixed connective tissue disease (MCTD), all other sera with anti-Sm were from SLE patients. There have been scattered observations of patients with rheumatoid arthritis and scleroderma with anti-Sm antibodies, but in these situations they appeared to be patients with overlapping symptoms of SLE. In a general population of patients with SLE (both acute and chronic cases), the frequency of this antibody is between 30 and 40%. It is interesting that in the MRL/1 mouse, a strain with

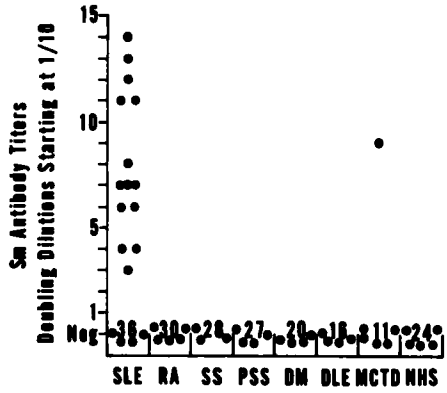

FIG. 20. Antibody to Sm antigen was determined by hemagglutination in different rheumatic diseases. With the exception of one patient with mixed connective tissue disease, all other sera with anti-Sm were from patients with SLE. (From Notman *et al.*, 1975.)

lymphoproliferative disease and features of SLE, the frequency of anti-Sm antibody is similar to that in man (Eisenberg *et al.*, 1978). On the other hand, other mouse strains like NZB/NZW and BXSB with SLE features do not have anti-Sm antibody.

Unlike antibody to Sm, antibody to nRNP is not a serologic marker for any disease although it is often misinterpreted to be diagnostic of MCTD. In 1971, Sharp and colleagues called attention to an unusual rheumatic disease associated with antibody to a nuclear antigen which was sensitive to RNase digestion. This disease entity was described in detail later (Sharp *et al.*, 1972) and called mixed connective tissue disease (MCTD) because it had features of SLE, scleroderma, and dermato/polymyositis. These patients did not have sufficient criteria to be classified as SLE according to the preliminary classification criteria of the American Rheumatism Association (Cohen *et al.*, 1971a). Other unusual features included absence of renal disease, and unusually high frequency of Raynaud's phenomenon and edematous swelling of hands and fingers. Patients with this constellation of clinical features also had high titers of antibody to a nuclear antigen which, in addition to RNase sensitivity, was also sensitive to protease digestion (Northway and Tan, 1972). The sera of these patients were characterized uniformly by high titers of speckled nuclear staining in immunofluorescence. At the same time as these studies were reported, Mattioli and Reichlin (1971) described a precipitating antibody in SLE against a soluble nuclear antigen which was also RNase and protease sensitive and which they called Mo. This antibody was pres-

ent in 30 to 50% of SLE patients. In a subsequent multicenter study, the nRNP and Mo antigens were shown to be immunologically identical (Tan *et al.*, 1977). Thus, in the historical perspective, antibody to nRNP was detected in different laboratories and in two disease conditions. Further studies have shown that although its frequency is highest in MCTD and SLE, it is also present in somewhat lower frequency in discoid lupus, scleroderma, rheumatoid arthritis, and Sjögren's syndrome (Notman *et al.*, 1975; Parker, 1973). Figure 21 illustrates some of these findings. Of note is the demonstration that high titers of antibody can be observed in rheumatoid arthritis, scleroderma, and discoid LE, but at a much lower frequency than in MCTD and SLE.

A question of clinical interest concerns the existence of MCTD as a separate clinical entity. This question is important particularly in differentiating MCTD from scleroderma and dermato/polymyositis, since treatment with corticosteroids has been beneficial in MCTD but generally ineffective in the other two diseases. Thus, if patients with MCTD are mistakenly diagnosed as scleroderma or dermato/polymyositis, corticosteroid treatment may be withheld to the detriment of such patients. In evaluating the question concerning MCTD

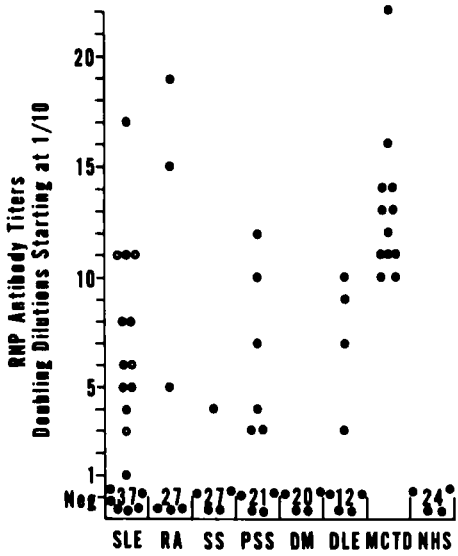

FIG. 21. Antibody to nRNP was determined by hemagglutination with the same sera as in Fig. 20. Note that all patients with MCTD had uniformly high titers. However, antibody was also present in other diseases, and sometimes in relatively high titers also. (From Notman *et al.*, 1975.)

as a clinical entity, one needs to consider how this clinical syndrome was recognized (Sharp *et al.*, 1972). It was based in part on the presence of high titers of antibody to an RNase-sensitive antigen, as determined by a hemagglutination technique. These sera were not examined at that time for antibodies to native DNA, histones, other nonhistone antigens, or nucleolar antigens. Thus it was possible that some of the 25 original patients described (Sharp *et al.*, 1972) might have had other ANAs besides antibody to nRNP. This might in part account for what has been shown to be a heterogeneous group of diseases, including scleroderma and SLE in the follow-up report of these 25 patients (Brody *et al.*, 1977). The recent information concerning the serology of MCTD shows that it is characterized by the exclusive presence of antibody to nRNP, in contrast to SLE, scleroderma, and polymyositis, the three diseases with which it shares some common features (Tan and Peebles, 1980). These points are depicted in Table IX. By serological analysis, many investigators have shown that there are patients whose sera contain only antibodies to nRNP. When these patients were analyzed, there appeared to be a clustering of clinical features, including Raynaud's phenomenon, myositis, swollen hands and fingers, serositis, and paucity of renal disease (Parker, 1973; Farber and Bole, 1976; McCain *et al.*, 1978; Jonsson and Norberg, 1978; Bennett and O'Connell, 1980). This cluster of clinical features and the exclusive presence of anti-nRNP have persuaded these investigators to support the existence of MCTD as a distinct clinical syndrome. There are other investigators who have considered MCTD to be a variant or subset of SLE (Reichlin and Mattioli, 1974; Maddison *et al.*, 1978). In the latter reports, patients were identified as MCTD on the basis of high titers of antibody to nRNP without strict exclusion of other ANAs. This might have resulted in the inclusion of patients with SLE, a disease noted for the presence of multiple autoantibodies (Kunkel, 1977), including anti-nRNP. The exclusion of other ANAs in sera should be examined not only by immunodiffusion which detects precipitating antigen–antibody systems but also by immunofluorescence which detects nonprecipitating antinucleolar antibodies and radioimmunoassay and hemagglutination to detect anti-DNA and other antibodies. The present evidence suggests that there is a group of patients with clinical features of MCTD as described above who are characterized by high titers of antibody to nRNP but without antinative DNA, Sm, or nucleolar antibodies.

An interesting observation concerning patients with autoantibodies to Sm and nRNP is the presence of nuclear deposits of immunoglobulins in cells of the epidermis and dermis in their skin biopsies (Kunkel

TABLE IX

SEROLOGICAL DIFFERENTIATION BETWEEN MCTD AND OTHER DISEASES

	Antibodies to								
	nRNP	dsDNA	Histone	Sm	SS-B/La	Scl-70	Centromere	Nucleolus	DM/PM[a] antigens
MCTD	+	−	−	−	−	−	−	−	−
SLE	+	+	+	+	+	−	−	−	−
Scleroderma	+	−	−	−	−	+	+	+	±
DM/PM	+	−	−	−	−	−	−	−	+

[a] DM/PM, Dermatomyositis/polymyositis.

and Tan, 1964; Tan and Kunkel, 1966; Tuffanelli, 1975; Gilliam and Prystowsky, 1977). Recent studies support the opinion that this was an *in vivo* phenomenon, i.e., circulating ANAs reacting with host cell nuclei and not an *in vitro* or postbiopsy artifact (Shu *et al.*, 1977; Izuno, 1978). Some authors have suggested that this may be due to the effect of a permeability factor in the circulation of some patients causing increased permeability of cell membranes to IgG-ANA (Izuno, 1978) while others suggest this may be due to penetration of IgG-ANA by way of cell membrane Fc receptors (Alarcon-Segovia *et al.*, 1978).

Antibodies to Sm and nRNP have been detected by immunodiffusion (Tan and Kunkel, 1966; Mattioli and Reichlin, 1971; Northway and Tan, 1972), passive hemagglutination (Sharp *et al.*, 1972; Notman *et al.*, 1975), counterimmunoelectrophoresis (CIE) (Kurata and Tan, 1976; Bresnihan *et al.*, 1977), and more recently by analysis of precipitated snRNAs (Lerner and Steitz, 1979). The immunodiffusion technique is easily performed but requires the use of standard reference sera for identification, a situation similarly required in the CIE technique. As a result of the diagnostic usefulness of these antibodies, standard reference sera have been in great demand. The passive hemagglutination technique has found a significant role among diagnostic tests because of the capability to titer the antibodies semiquantitatively. This system does not detect antibodies to other nuclear nonhistone antigens such as SS-B/La (Jonsson *et al.*, 1976) but antibodies to cytoplasmic RNP are detected by this test (Miyachi and Tan, 1979) and may be a source of confusion. Because of technical problems sometimes encountered with hemagglutination, some laboratories have adapted the CIE technique in a semiquantitative assay. It might be anticipated that when Sm and nRNP antigens are purified, further tests may be developed to directly assay these antibodies both qualitatively and quantitatively.

VII. Autoantibodies to SS-A/Ro and SS-B/La

Historically, one of the earliest studies which might have been related to autoantibodies of the SS-A/Ro and SS-B/La specificities could have been described by Anderson and his associates (1962). These investigators used double diffusion methods, testing sera against extracts of human thyroid tissue. In Sjögren's syndrome sera, they detected two precipitating antibodies which were called SjD and SjT. Anti-SjT was present in highest frequency in patients with Sjögren's syndrome and occasionally in patients with SLE and RA. Anti-SjD was more widely distributed, being present in Sjögren's syndrome, SLE, RA,

and scleroderma. In the light of current data, the SjD system could be equivalent to SS-A/Ro and SjT to SS-B/La. Contact with one of the investigators in this study (W. W. Buchanan) revealed that sera used in these studies were not available and it has not been possible to compare the Anderson data with present studies.

In 1969, Clarke *et al.* reported the finding of a serum antibody reacting with a cytoplasmic antigen extracted from human tissues obtained at autopsy. The antigen was named Ro and was present in spleen, liver, kidney, lung, and lymph node and was also present in dog spleen. It was presumed to be cytoplasmic because it was present in the cytoplasmic fraction of homogenized cells but attempts to localize it to the cytoplasm by immunofluorescence were unsuccessful. Antigenicity was not destroyed by treatment with proteolytic enzymes trypsin, chymotrypsin, and pepsin or with DNase and RNase. In an analysis of 45 patients with SLE, 11 (24%) were found to have this antibody. This was followed by a study of Mattioli and Reichlin (1974) describing the presence of a cytoplasmic RNA–protein antigen (La) not associated with ribosomes, which precipitated with antibodies in some SLE sera. The antigen–antibody reaction was analyzed by double diffusion and the antigen was present in the cytoplasmic fraction of homogenized calf thymus but absent from the nuclear fraction. The La antigenic activity was lost after RNase or trypsin treatment. Sera with this antibody stained nuclei of mouse liver weakly but showed strong staining of the cytoplasm of thymus cells with absence of nuclear or nucleolar staining in the latter. Of nine patients with anti-La antibody, five patients had SLE, one discoid LE, and three undefined connective tissue disease.

Although the Ro and La antigens were initially thought to be cytoplasmic antigens, recent evidence raises the possibility that the La antigen may be predominantly nuclear in location and Ro may also be partially if not mostly nuclear. In an interlaboratory study between Alspaugh and Maddison (1979), it was demonstrated that the Ro and SS-A antigens were immunologically identical by double diffusion analysis, using several different sera and antigens prepared from different tissues. Similarly, the La and SS-B antigens were immunologically identical. The SS-A and SS-B immune systems were found in a study of patients with Sjögren's syndrome (hence the designation SS). Saline-soluble extracts were prepared from a human B lymphocyte cell line (WiL_2) and sera from patients with Sjögren's syndrome-sicca complex showed two precipitating antibodies in double diffusion. In a subsequent study (Alspaugh *et al.*, 1976), it was demonstrated that sera with anti-SS-A and anti-SS-B both showed positive nuclear fluores-

cence. Anti-SS-B sera showed nuclear staining on both human WiL_2 cell substrate and mouse kidney sections but anti-SS-A was positive only on human WiL_2 cells. The antigenicity of both SS-A and SS-B was destroyed by trypsin but unaffected by DNase or RNase.

Studies on one of these immune systems (SS-B) was extended by Akizuki and associates (1977a) who prepared extracts from nuclei of calf thymus, calf liver, and rat liver and showed the presence of a nuclear antigen (Ha) which precipitated with antibody in sera of patients with Sjögren's syndrome-sicca complex (30%) and SLE (13%). The antigen was localized by immunofluorescence to nuclei of rat and mouse liver, kidney, and thyroid, using antibody isolated from immune precipitates. The antigen was immunologically identical in double diffusion analysis with SS-B and like the latter, the antigenicity was destroyed by trypsin but not by DNase or RNase.

The main reason for considering SS-A and Ro and similarly SS-B and La to be related antigens is their immunological identity. The studies of Alspaugh and Tan (1975, 1976) and Akizuki *et al.* (1977a,b) point to a predominance of the autoantibodies in Sjögren's syndrome and to a lesser extent in SLE. The findings of Clarke *et al.* (1969) and Mattioli and Reichlin (1974) are not at variance with Alspaugh and Akizuki. The former investigators analyzed selected sera containing these autoantibodies and since this was performed in an SLE clinic, an overrepresentation of SLE patients might be expected. However, important differences remain to be resolved and these include the nuclear or cytoplasmic location of the antigens and their chemical nature. However, recent data derived from analysis of RNA in immunoprecipitates (Hendrick *et al.*, 1981) further strengthen the opinion of related identities of SS-A with Ro and SS-B with La.

Akizuki *et al.* (1977b) have employed antibody affinity columns to isolate the SS-B/La antigen and claim purification of a 1000-fold from the crude thymus nuclear extract. This material was radiolabeled with ^{131}I and in G-100 Sephadex filtration had a molecular weight of 43,000. Recently Teppo *et al.* (1981) using biochemical separative procedures claimed that a material possessing SS-B/La antigenicity had a molecular weight of 68,000 on SDS–polyacrylamide gel electrophoresis. The nature of the antigen, whether it is a protein or a RNA–protein complex, and the size remain to be elucidated. There has been no further characterization of the SS-A/Ro antigen.

In studies from the laboratory of J. A. Steitz, Hendrick *et al.* (1981) have recently examined the species of small RNAs precipitated by anti-SS-A/Ro and anti-SS-B/La sera. This was an extension of studies which had shown U1 RNP to be precipitated by anti-nRNP sera and

other small nuclear RNAs to be precipitated by anti-Sm sera. The small RNAs precipitated by anti-Sm and anti-nRNP were extremely similar in gel profiles and nucleotide fingerprints when cells from different animal species were used, suggesting that these RNAs were highly conserved in different species through the course of evolution. In contrast, small RNAs precipitated by anti-SS-A/Ro and anti-SS-B/La were quite different between mouse and human cells, suggesting that these classes of RNAs were not highly conserved species. From mouse Ehrlich ascites tumor cells, anti-SS-B/La precipitated two species of 4.5 S RNAs previously sequenced (Ro-Choi *et al.*, 1972; Harada and Kato, 1980) and a species 5 S rRNA-like molecule. The latter was also precipitated from human (HeLa) cells but the two 4.5 S RNA species were not. On the other hand, other small RNA species, not previously described, were precipitated from HeLa cells by anti-SS-B/La sera. We have confirmed these findings, using labeled rat Novikoff hepatoma cells as source of cellular antigens. In Fig. 22, is depicted the characteristic RNA profiles obtained in 10% acrylamide, 7 *M* urea gels for anti-Sm (lane 6 showing U1, U2, U4, U5, and U6 RNAs) and for anti-SS-B/La (lane 2) and anti SS-A/Ro (lane 3). Lanes 1 and 8 show the total RNA of the cell extract. The 4.5 S RNA species precipitated by anti-SS-B/La from mouse cells appear also to be precipitated from rat cells (labeled A and B in lane 2).

These findings from immunoprecipitation should not be taken to mean that the precipitated RNAs are the reactive antigens. In fact, the evidence from immunological studies cited previously suggest strongly that the cellular antigen(s) reactive with the autoantibodies are protein in nature. Also, the work of Steitz and colleagues repeatedly show that deproteinized RNAs do not form immune precipitates with autoantibodies. Thus, it is reasonable to assume that the precipitated RNAs may be brought down because of complexing with protein carriers which possess specific antigenic determinants. Even if this is the case, the exciting findings are the highly selective spectra of RNAs associated with different autoantigens. The significance of these autoantigen–RNA relationships is at present unknown. Of further interest is the observation that anti-SS-B/La sera precipitate adenovirus VA RNAs in adenovirus-infected cells and Epstein–Barr virus encoded RNAs (EBER) in EB virus-infected cells (Lerner *et al.*, 1981a,b). VA RNA and EBER are not precipitated by anti-Sm, anti-nRNP, or anti-SS-A/Ro. Another interesting feature of SS-B/La antigen was observed in studies using synchronized cells (Deng *et al.*, 1981). In G_0 phase of the cell cycle, the antigen was nucleoplasmic in location but during G_1 and early S, there was strong staining for SS-B/La in the

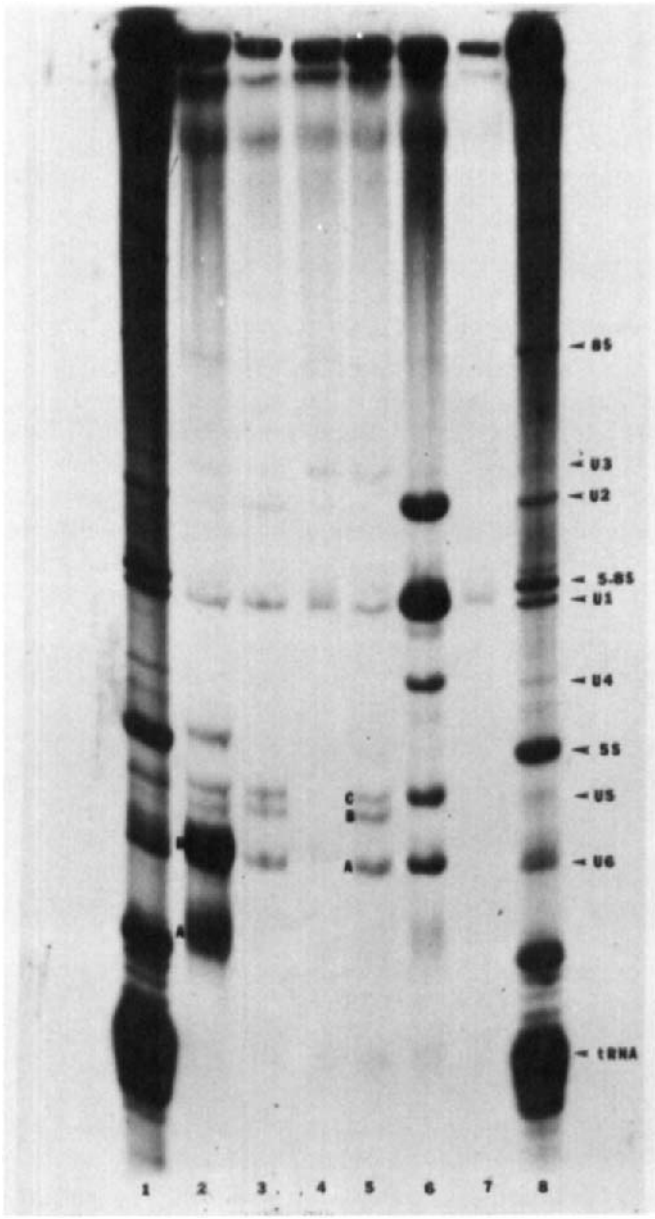

FIG. 22. Novikoff hepatoma cells of the rat were labeled with ^{32}P and total cell extract prepared. Immune precipitates were obtained with anti-Sm (lane 6), anti SS-B/La (lane 2), anti-SS-A/Ro (lane 3), and normal human serum (lane 7). RNA was extracted from immune precipitates and run on 10% acrylamide, 7 *M* urea gels. Lanes 1 and 8 represent total RNA of the cell extract. The RNAs precipitated by anti-Sm (lane 6, U1, 2, 4, 5, 6) can be compared with RNAs precipitated by anti-SS-B/La (lane 2) and anti-SS-A/Ro (lane 3). Lanes 4 and 5 are RNAs precipitated by other sera.

nucleolus (Fig. 23). During late S and G_2, nucleolar staining disappeared to be replaced by nucleoplasmic staining. The explanation for these findings is not evident at the present time and it is not clear whether nucleolar localization at G_1/S signifies new synthesis of antigen or redistribution and concentration at this site. Against the latter is the fact that there was no diminution of nucleoplasmic staining at the G_1/S phase of the cell cycle.

Clinical Significance of Anti-SS-A/Ro and Anti-SS-B/La

The studies of Alspaugh and Tan (1976), Kassan *et al.* (1977), Akizuki *et al.* (1977b), Provost (1979), and Scopelites *et al.* (1980) all point to the use of anti-SS-B/La as a serological marker for Sjögren's syndrome-sicca complex. In general, the antibody appears to be detected in approximately 60% of such patients. Since the method used was immunoprecipitation in double diffusion, it is possible that a more sensitive technique might increase the frequency. Attempts have been

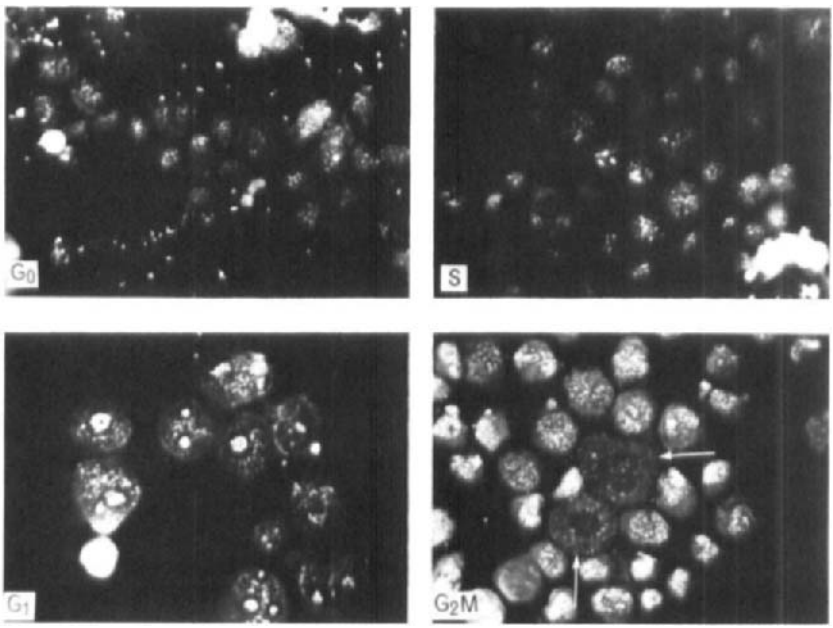

FIG. 23. Fluorescent photomicrography of anti-SS-B/La staining on synchronized WiL_2 cells. In G_0, fine speckles were present in the nucleoplasm. In G_1, there was prominent nucleolar staining associated with speckled nucleoplasmic staining. In the S phase, nucleolar staining had almost disappeared but nucleoplasmic staining persisted. In G_2, the antigen was still present in nucleoplasm but in two mitotic cells (arrows), the antigen was sparsely distributed in cell periphery. (From Deng *et al.*, 1981.)

made to develop a hemagglutination assay for the antibody but without success (Akizuki *et al.*, 1977a; Jonsson *et al.*, 1976). Table X depicts the studies showing frequencies of anti-SS-B/La in different disease conditions. SLE patients with sicca symptoms appeared to be the only other disease with this antibody and if patients with sicca symptoms were excluded, the incidence in SLE became vanishingly small. If the test system for detection of SS-B/La antibody could be increased in sensitivity, it is possible that this autoantibody could become a potent diagnostic aid to the clinician for the diagnosis of Sjögren's syndrome-sicca complex. Already, Martinez-Lavin *et al.* (1979) have shown that demonstration of SS-B/La antibody was instrumental in increased awareness in the diagnosis of Sjögren's syndrome. In an analysis of a group of patients with Sjögren's syndrome, 40% were not initially considered to have Sjögren's syndrome because of paucity of sicca symptoms and prominence of other systemic symptoms. The detection of SS-B/La antibody in their sera led to further studies confirming this diagnosis. In another context, a significant percentage of patients presenting to an ophthalmology clinic for keratoconjunctivitis were found to have SS-B/La antibody and other serological abnormalities such as hypergammaglobulinemia and rheumatoid factor (Forstot *et al.*, 1982). The likely possibility that these patients may represent early subclinical forms of Sjögren's syndrome should allow design of prospective studies to examine the natural history of the disease.

A recent finding of great interest concerning anti-SS-A/Ro antibody is its association with neonatal lupus. Franco *et al.* (1981) initially reported their findings on three such infants and this study has been expanded by the same group of investigators (W. L. Weston *et al.*,

TABLE X
FREQUENCY OF ANTI-SS-B/La ANTIBODIES

	Alspaugh *et al.* (1976) (%)	Kassan *et al.* (1977) (%)	Akizuki *et al.* (1977b) (%)	Provost *et al.* (1979) (%)
SS-Sicca	48	68	73	30
SS-RA	3	5	—	—
SLE	0	2	3	10–15
SLE-sicca	—	73	85	—
Rheumatoid arthritis	0	0	0	—
Scleroderma	1	0	0	—
Polymyositis	—	0	0	—
MCTD	0	0	—	—
Discoid LE	0	—	—	—

personal communication) to include eight infants. Five of the infants had discoid lupus skin lesions, one had discoid lesions and congenital heart block, and the remaining two had congenital heart block alone. Seven of the eight infants had anti-SS-A/Ro antibody which was totally absent in 71 age-matched control infants. All mothers of the affected infants had anti-SS-A/Ro antibody. The transplacental passage of autoantibody was supported by follow-up studies in four infants in whom antibody titer decreased and disappeared by 6 months of age. In the eight mothers of the affected infants, four had no clinical symptoms, two had xerostomia, one had Sjögren's syndrome, and one SLE. These findings have been confirmed by Kephart *et al.* (1981) who described two additional infants with neonatal lupus who had SS-A/Ro antibody in their sera. At the present time the serological association between SS-A/Ro antibody and neonatal lupus appears quite strong. It would seem reasonable that all pregnant women with SLE or Sjögren's syndrome should be analyzed for presence of SS-A/Ro antibody as should all infants with discoid LE or congenital heart block with a view to considerations for prophylactic intervention.

Provost and colleagues (1977) have reported that adult SLE patients with SS-A/Ro antibody have increased prevalence of photosensitivity, renal disease, Sjögren's syndrome, and rheumatoid factor and that 75% of these patients have SS-B/La antibody. This group of patients may well be similar to those reported by Kassan *et al.* (1977) and Akizuki *et al.* (1977b) as SLE patients with sicca symptoms. In addition a group of patients described to be SLE patients but ANA negative (by standard immunofluorescence) generally have SS-A/Ro antibody (Provost, 1979). However, these investigators (Provost *et al.*, 1979) also state that these patients may demonstrate a positive ANA when human tissue is utilized as a nuclear substrate. With the increasing awareness that certain antigens such as SS-A/Ro may be present in lower concentration in some tissues than in others, the use of more than one tissue substrate may be in order to examine the sera of ANA-negative SLE patients. This may help to clarify the conflicting issue concerning the existence of ANA-negative SLE. With increasing experience in immunofluorescence and the judicious use of tissue substrates, it is possible that ANA-negative SLE may become a gradually vanishing species.

VIII. Autoantibody to Proliferating Cell Nuclear Antigen (PCNA)

The nonhistone nuclear antigens, Sm, nRNP, and SS-B/La, are present in the nuclei of cells in interphase, which are representative of

cells in organs like kidney and liver and are also present in nuclei of cells which are actively proliferating. Because of this feature, tissue sections of liver or kidney and preparations of tissue culture cells can be used interchangeably to demonstrate antibodies to these nuclear antigens. However, with the accumulating information concerning ANAs it is becoming apparent that certain nuclear antigens reactive with autoantibodies may have restricted expression in some tissues. This is particularly true of autoantibody to proliferating cell nuclear antigen (PCNA) seen in some patients with SLE (Miyachi *et al.*, 1978).

This autoantibody was initially detected by immunoprecipitation in double diffusion when the serum of a patient with SLE showed a precipitin reaction against an extract of human tissue culture cells which was different from precipitin lines to DNA, Sm, nRNP, or SS-B/La. When this serum was analyzed by immunofluorescence on mouse kidney sections (Fig. 24) it was observed that in addition to nuclear staining of all cells, a few scattered cells in the interstitial areas of the kidney showed stronger fluorescence. When the serum was successively diluted, the majority of nuclear staining which involved tubular and glomerular cells disappeared and only interstitial cell nuclear staining remained. These findings suggested that the serum contained two populations of ANAs, one present at lower concentration

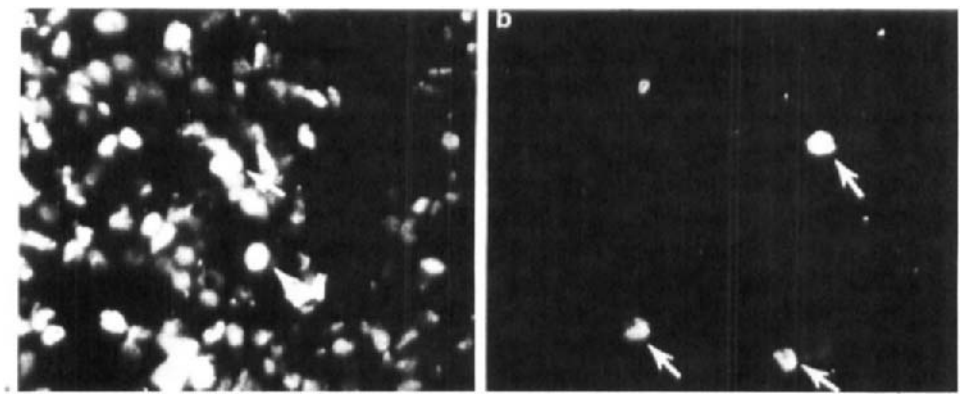

FIG. 24. Serum containing anti-PCNA was examined by immunofluorescence, using mouse kidney sections as substrate. At low dilutions of serum (a), two different intensities of nuclear staining were detected, with a few cells (arrows) showing stronger nuclear staining than the majority of cells. At high dilutions (b), the difference became more apparent, with the majority of cells showing faint or negative nuclear staining and a few cells (arrows) continuing to show strong nuclear staining. Although it is not readily apparent from this micrograph, these cells were not in renal tubules or glomeruli, but were in the interstitial tissue (Miyachi *et al.*, 1978).

which was reactive with nuclei of tubular and glomerular cells and another present at higher concentration, which was reactive only with nuclei of interstitial cells. By coincidence, the antibody present in higher concentration and staining interstitial cells was the antibody demonstrating the precipitin reaction in immunodiffusion. This was determined by a series of absorption experiments correlating the activities of precipitating and immunofluorescence reactions. These absorption experiments also rendered the serum "monospecific" in relationship to antinuclear antibody. The absorbed serum did not stain nuclei of peripheral blood lymphocytes but after mitogen stimulation (with PHA, Con A, or pokeweed), nuclei of blast-transformed cells were reactive. Also, continuous tissue culture cell lines were stained with absorbed serum. Since the antibody was not reactive with nondividing interphase cells but reactive with rapidly proliferating or mitogen-stimulated cells, the nuclear antigen was called proliferating cell nuclear antigen (PCNA).

PCNA was not restricted to cell type but was present in what appeared to be activated cells of lymphoid (T and B cell) origin, epithelial cells and germ line cells. This is illustrated in the study depicted in Table XI (Miyachi *et al.*, 1978). Since PCNA was not detectable in mature lymphocytes, either because the antigen was absent or present in amounts below the sensitivity of immunofluorescence, it was possible to determine the kinetics of antigen appearance after mitogen stimulation. In such a study, PCNA was first detected in the nucleolus after PHA or Con A stimulation and subsequently appeared to migrate to nucleoplasm with disappearance from the nucleolus in the blast-transformed lymphocyte (Takasaki *et al.*, 1981).

TABLE XI
DISTRIBUTION OF CELLS STAINING WITH ANTIBODY TO PCNA

	Nuclear staining by immunofluorescence	
	Positive	Negative
Thymus	Cortical area	Medulla
Spleen	White pulp, including marginal zone	Red pulp, small lymphocytes
Lymph node	Germinal center	Paracortical area, medulla
Small intestine	Luminal epithelial cell, lymphoid follicle	Cells of lamina propria and submucosa
Testis	Undifferentiated spermatogonia	Mature sperm, Sertoli cell

By immunodiffusion analysis, anti-PCNA was detected in 4% (3/70) of patients with SLE but was not detected in patients with RA, scleroderma, or polymyositis. In the three patients with this antibody, no unusual clinical features were detected which would clearly separate them from other SLE patients, with the possible exception that there was evidence of greater lymphoproliferation. Obviously more patients will be needed to establish this preliminary observation of relationship with lymphoproliferation. Antibody to PCNA might be a useful reagent for detecting activated or blastoid cells and initial studies in this laboratory have demonstrated the capability of identifying leukemic patients undergoing blast crisis since these patients demonstrate increased numbers of cells in peripheral blood staining for PCNA (Takasaki *et al.*, in preparation).

IX. Autoantibodies in Scleroderma (Scl-70, Centromere/Kinetochore and Nucleolar Antigens)

Scleroderma or progressive systemic sclerosis is a generalized disorder of connective tissue characterized by fibrosis and sclerosis of skin and a number of internal organs, and pathological changes in digital and other small blood vessels (Rodnan, 1979; Korn and Leroy, 1979). The disease is of unknown etiology and varies in severity and progression. One form is characterized by generalized cutaneous involvement (diffuse scleroderma) which in certain patients leads to rapidly progressive and fatal involvement of the kidneys resulting in renal failure. There is a subset called CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) which tends to have a prolonged and often relatively benign course of illness but which may lead to severe pulmonary arterial hypertension as an internal organ complication. This form tends to have more limited fibrosis of the skin confined to the fingers (sclerodactyly) and face. Recently, it has been shown that ANAs occur in high frequency in scleroderma and that diffuse scleroderma and the CREST variant can be distinguished serologically by the specificities of their respective ANAs (Tan *et al.*, 1980).

It had long been recognized that ANAs were present in scleroderma but the reported frequencies varied between 40 and 90% with the low number being the more widely accepted figure (Beck, 1963; Burnham *et al.*, 1966; Rothfield and Rodnan, 1968; Ritchie, 1970). This was probably related to the fact that various tissue preparations were used as substrates in indirect immunofluorescence, with lower frequencies observed on organ sections and higher frequencies on touch imprints

of tumor tissue. With the use of continuous tissue culture cell lines as substrate, ANAs were detected in almost all cases of scleroderma.

Many continuous cell lines are suitable as immunofluorescence substrate for the detection of antinuclear and antinucleolar antibodies in scleroderma sera, but cell lines with rapid doubling times and large numbers of mitotic cells are more desirable. Such cell lines facilitate the identification of antinucleolar and anticentromere/kinetochore antibodies. Figure 25 depicts the variety of nuclear staining which was observed with scleroderma sera (Bernstein *et al.*, 1982). Figure 25A is representative of centromere staining which in interphase cells consist of a finite number of large round or ovoid granules but in mitotic cells are segregated with condensed chromosomes. Fine speckled staining is illustrated in Fig. 25B where the speckles are too tiny and too many to be individually resolved. In dividing cells, the condensing chromosomes are not stained but staining appears in the peripheral cell areas. This serum contains another antibody producing nuclear "dots." Figure 25C shows coarse speckles which are clearly larger than those in Figure 25B but more numerous than centromere patterns. Coarse speckles also do not segregate with condensing chromosomes in dividing cells. Figure 25D shows a diffusely grainy pattern of staining which appears to involve both nucleoplasm and nucleoli. Two other characteristics were observed—there was a brighter rim of perinucleolar staining and chromosomal staining was present in dividing cells. Not illustrated in Fig. 25 is a homogeneous pattern of nucleoplasmic staining also observed with scleroderma sera.

Three nucleolar patterns of staining could be distinguished. Illustrated in Fig. 26A is speckled nucleolar staining consisting of small discrete speckles within a dark border. This dark halo delineated the nucleolar staining from the surrounding fine speckled nuclear staining which coexisted in all cases. Homogeneous nucleolar staining is illustrated in Fig. 26B. This lacked a dark perimeter and appeared to be associated with fine or coarse speckled nucleoplasmic staining. Clumpy nucleolar staining (Fig. 26C) consisted of tightly clustered granules which in some instances, perhaps related to density of the granules, had the appearance of homogeneous nucleolar staining. This serum also gave nuclear "dot" staining. It should be stated that these different patterns of nucleolar staining were apparent although there might be some cell-to-cell variations on the same slide. These variations may reflect changes in nucleolar ultrastructure in different phases of the cell cycle as demonstrated by electron microscopy (Busch and Smetana, 1970; Goessens and Lepoint, 1974).

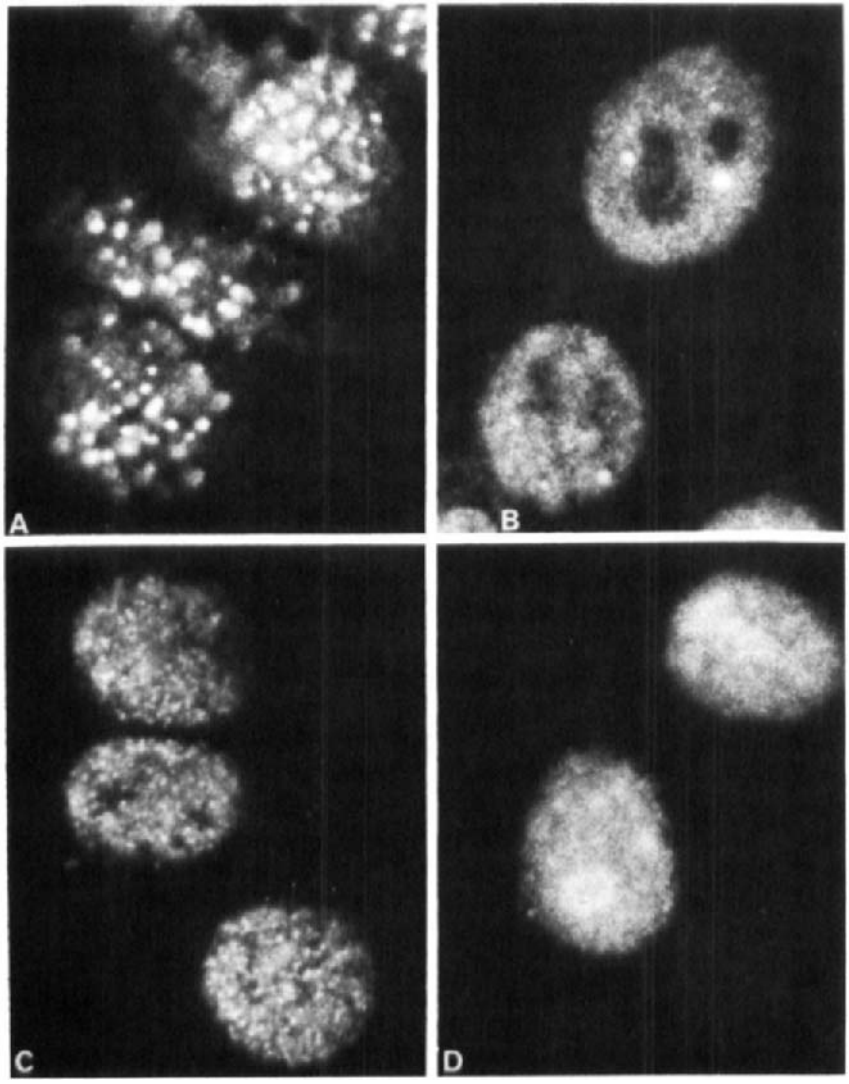

FIG. 25. Patterns of nucleoplasmic staining of scleroderma sera on HEp-2, an epithelial cell line. (A) shows centromere staining, (B) fine speckles associated with nuclear "dots." The nucleoli in (B) are negative and cytoplasm is barely visible; (C) shows coarse speckles and (D) diffusely grainy pattern. (From Bernstein *et al.*, 1982.)

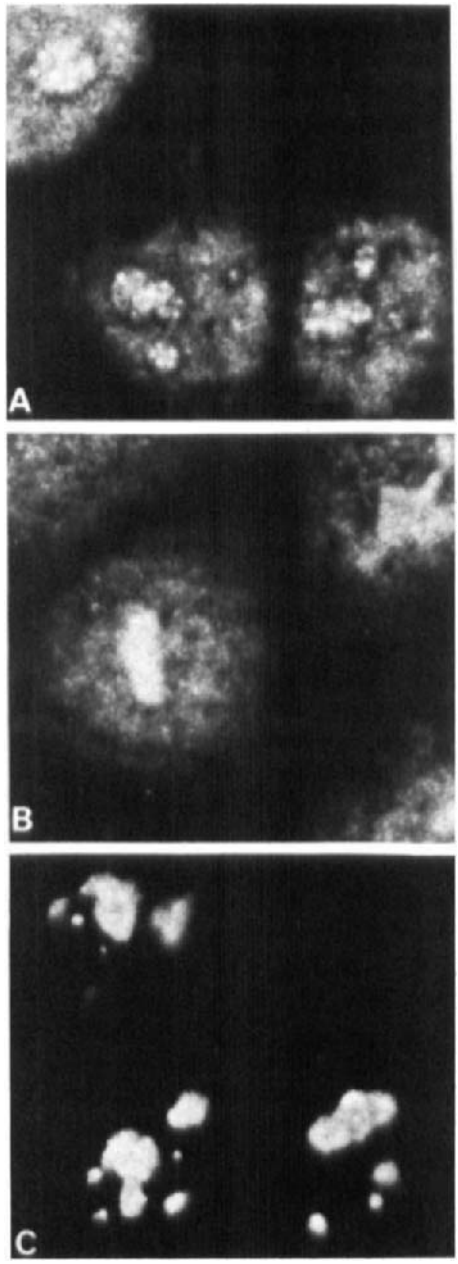

FIG. 26. Nucleolar staining of scleroderma sera. (A) shows speckled nucleolar staining associated with fine speckled nuclear stain. (B) shows homogeneous nucleolar and (C) clumpy nucleolar with associated nuclear "dot" stain. (From Bernstein *et al.*, 1982.)

Immunodiffusion analysis has been successful in identifying two types of precipitating antibody–antigen systems. One of these antibodies, anti-Scl-70, has been correlated with sera giving the diffusely grainy pattern of nucleoplasmic staining (Douvas *et al.*, 1979b). The antigen was isolated from chromatin, a property in agreement with immunofluorescence studies showing its segregation with condensing mitotic chromosomes. It is a basic protein of 70,000 molecular weight and cofractionates with histone H1. Its antigenicity was destroyed by trypsin or pronase treatment but not by deoxyribonuclease or ribonuclease. The latter phenomenon only indicates that the complete determinant of Scl-70 is protein but it could be associated *in vivo* with DNA or RNA. It is probably a highly conserved nuclear protein, since it has been identified in cells of man, rabbit, and rat (Tan *et al.*, 1980).

Other properties of nuclear and nucleolar antigens associated with scleroderma are shown in Table XII. The autoantibody to centromere/kinetochore has been elucidated by immunoelectron microscopy to be reactive with the inner and outer plates of the kinetochore and not with centromeric DNA of the chromosomes (Brenner *et al.*, 1981). This confirms earlier studies using immunocytochemistry which suggested that the centromere/kinetochore antigen was protein in nature (Moroi *et al.*, 1980). The antigen was a protein tightly bound to centromeric DNA. It also required the combination of sodium dodecyl sulfate and protease to destroy its antigenicity. Brenner *et al.* (1981) have shown that in the synchronized PtK_2

TABLE XII
ANTINUCLEAR AND ANTINUCLEOLAR ANTIBODIES IN SCLERODERMA

Immunofluorescence	Immunodiffusion	Nature of antigen
Nuclear		
Diffusely grainy	Scl-70	Chromosome-associated 70,000 MW protein
Centromere/kinetochore	—	Inner and outer plates of kinetochore
Fine speckles	—	—
Coarse speckles	—	—
Homogeneous	—	—
Dots	—	Probably centrioles
Nucleolar		
Homogeneous	4 S–6 S RNA	Probably U3 RNA
Clumpy	—	—
Speckled	—	—

cell, duplication of the centromere occurs in early G_2. Moroi *et al.* (1981) showed that in a human lymphoid cell line (Ramos) the interphase centromeres/kinetochores were associated with nuclear or nucleolar membranes in 92% of foci counted but in the Chinese Hamster ovary (CHO) cell this was observed only in 66%.

Nuclear antigens related to other patterns of nucleoplasmic staining have not been characterized although by the nature of staining, the "dot" pattern may be related to centrioles. The nature of antigens giving fine and coarse speckles remains to be identified. Certain sera giving homogeneous patterns of nucleolar staining have been shown by immunodiffusion analysis to precipitate with a low-molecular-weight 4 S–6 S nucleolus-specific RNA (Pinnas *et al.*, 1973; Miyawaki and Ritchie, 1973). In recent studies using ^{32}P-labeled cells, certain antinucleolar sera precipitate U3 RNA and it is possible that the 4 S–6 S RNA identified by immunodiffusion may be this small RNA which is localized strictly to the nucleolus (Ro-Choi and Busch, 1974). The nature of nucleolar antigens giving clumpy and speckled patterns is unknown. In a study by Alarcon-Segovia and Fishbein (1975), it was reported that almost all patients with scleroderma had precipitating antibodies to synthetic polyribonucleotides such as polyadenylic acid [poly(A)] and polyuridylic acid [poly(U)]. This was demonstrated by a counterimmunoelectrophoresis technique. It has subsequently been shown that the serum reactant was not γ-globulin but low-density lipoprotein (Garcia *et al.*, 1981). Although serum interaction with synthetic polyribonucleotides was not an immunologic phenomenon, it was nevertheless of interest that a higher percentage of scleroderma patients reacted compared to SLE and RA patients and normal controls.

Clinical Significance of ANAs in Scleroderma

Some clinicians have raised questions concerning the separation of scleroderma into diffuse scleroderma and CREST on the grounds that clinically it is sometimes difficult to differentiate them. Early in the study of anticentromere/kinetochore antibody, it became apparent this autoantibody appeared to be a serological marker for CREST (Moroi *et al.*, 1980). This has been confirmed by Fritzler *et al.* (1980) who also showed that anticentromere/kinetochore antibody could predate the appearance of a full-blown clinical syndrome of CREST. In a more recent study from this laboratory (Table XIII), clinical features of scleroderma patients with anticentromere antibody were compared to scleroderma patients with other antinuclear and antinucleolar antibodies. In those with anticentromere antibodies there were signifi-

TABLE XIII
COMPARISON BETWEEN SCLERODERMA PATIENTS WITH ANTICENTROMERE AND WITH OTHER ANTINUCLEAR ANTIBODIES

	Anti-centromere (21)	Other antibodies (55)	Significance χ^2 test
Mean age at diagnosis (years) ± SD	46 ± 13.6	43.8 ± 13.4	N.S.
Mean duration of diseases (years) ± SD	15.5 ± 9.2	7.5 ± 7.0	$p < 0.001$
Females (%)	100	67	$p < 0.003$
Calcinosis (%)	71	36	$p < 0.006$
Absence of diffuse scleroderma (%)	90	42	$p < 0.001$
Telangiectasia (%)	90	56	$p < 0.004$
Renal involvement (%)	0	18	$p < 0.04$
Mortality (1972–1980) (%)	5	23	$p < 0.06$
Raynaud's syndrome (%)	100	93	N.S.
Esophageal involvement (%)	90	96	N.S.
Small bowel involvement (%)	33	47	N.S.
Pulmonary involvement (%)	43	55	N.S.
Digital ulceration (%)	62	44	N.S.
Rheumatoid factor (titer ≥ 160) (%)	52	11	$p < 0.001$

cantly higher percentages of calcinosis, sclerodactyly, and telangiectasia and lower percentage of renal disease, confirming again the serological specificity of anticentromere for CREST. Thus, it would seem that there is strong evidence for the existence of CREST as a distinct serological entity. In preliminary studies, 20–30% of patients with Raynaud's phenomenon alone have anticentromere antibody. It would be of interest to determine in follow-up studies how many of such patients develop scleroderma as in the report of Fritzler *et al.* (1980).

Antibody to Scl-70 appears to be specific for scleroderma although it does not differentiate between diffuse scleroderma and CREST (Tan *et al.*, 1980; Bernstein *et al.*, 1982). Therefore it has the property of a specific marker antibody for scleroderma as anti-Sm is for SLE. However, with current techniques (immunodiffusion) it is detectable in only 20% of scleroderma patients. Whether the frequency will be increased with a more sensitive assay is not known. Antinucleolar antibodies of all types are present in 45% of patients with scleroderma (Berstein *et al.*, 1982). Although the earlier literature (Beck, 1963; Ritchie, 1970) describes the presence of antinucleolar antibodies in diseases other than scleroderma, it has been our experience that antinucleolar antibodies are rarely encountered in diseases such as SLE

where there is a plethora of other antinuclear antibodies. It is possible that antinucleolar antibodies may be highly specific for scleroderma or for diseases with scleroderma overlap features.

X. Autoantibody to Rheumatoid Arthritis-Associated Nuclear Antigen (RANA)

In the course of studies on the nature of autoantibodies in patients with Sjögren's syndrome, antibody to RANA was demonstrated (Alspaugh *et al.*, 1976; Alspaugh and Tan, 1976). Patients with Sjögren's syndrome (SS) can be subdivided clinically into patients with the sicca complex (xerophthalmia or dry eyes and xerostomia or dry mouth) and patients who have these symptoms in association with rheumatoid arthritis (SS-RA). A human lymphoid B cell line (WiL_2) was used as the source of whole cell extract and by double immunodiffusion, patients with SS-sicca and with SS-RA both demonstrated precipitating antibodies to antigens in cell extract. However, the antigen–antibody systems were not identical (Fig. 27). It was important to determine if the precipitating antibody in SS-RA was related to Sjögren's syndrome or to rheumatoid arthritis and the study in Fig. 27 illustrates that the relationship was with RA. As the studies developed and more information was accumulated concerning the RA antibody, it became apparent that the cell line selected for extraction of antigens was a key factor in the observations made later.

Immunofluorescence was utilized to determine whether the antigen was nuclear or cytoplasmic in location and to characterize it morphologically. The antigen proved to be highly soluble and was therefore readily eluted in washing buffers. It was also readily destroyed by commonly used tissue fixatives such as methanol, ethanol, and acetone. However, it could be fixed to cells with gentle heating at 37°C for 30 minutes and also retained antigenicity and with this method, the antigen was shown to be located in the nucleus in a finely speckled fashion (Fig. 28). The immunofluorescent speckles were not dense and tended to be readily quenched by ultraviolet light. However, Johnson and Araujo (1981) have reported a procedure which retards this phenomenon. Further studies showed that the antibody was IgG and was neither IgM nor IgG rheumatoid factor.

With the availability of two immunological techniques and especially the more sensitive immunofluorescence technique, a study was carried out on different tissues and cell lines to determine the distribution of RANA. It was not detectable in tissue sections of many organs in mouse, monkey, calf, rabbit, and man. It was also not detectable in two human T cell lines (Molt 4 and 1301) but was detected in the nuclei of

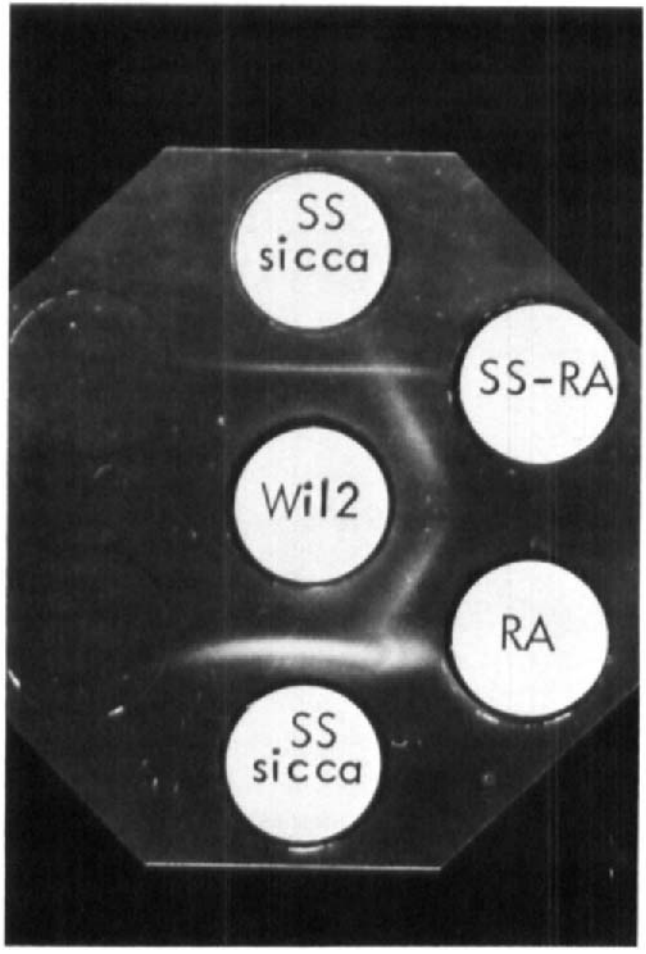

FIG. 27. Immunodiffusion study showing precipitating antibodies in Sjögren's syndrome sera reactive with antigens in extract of human B lymphoid cell line WiL_2. Of note is the immunological identity between SS-RA and RA. (From Alspaugh and Tan, 1976.)

three B cell lines (Wil_2, Raji, and Daudi). Since these B cell lines harbor Epstein–Barr (EB) virus and T cell lines do not, the question was raised concerning the possibility of RANA being an antigen related to EB virus.

Many B lymphocyte cell lines of human and simian origin are available which are infected with EB virus or herpes-related viruses. An analysis was performed using selected cell lines to look for RANA with

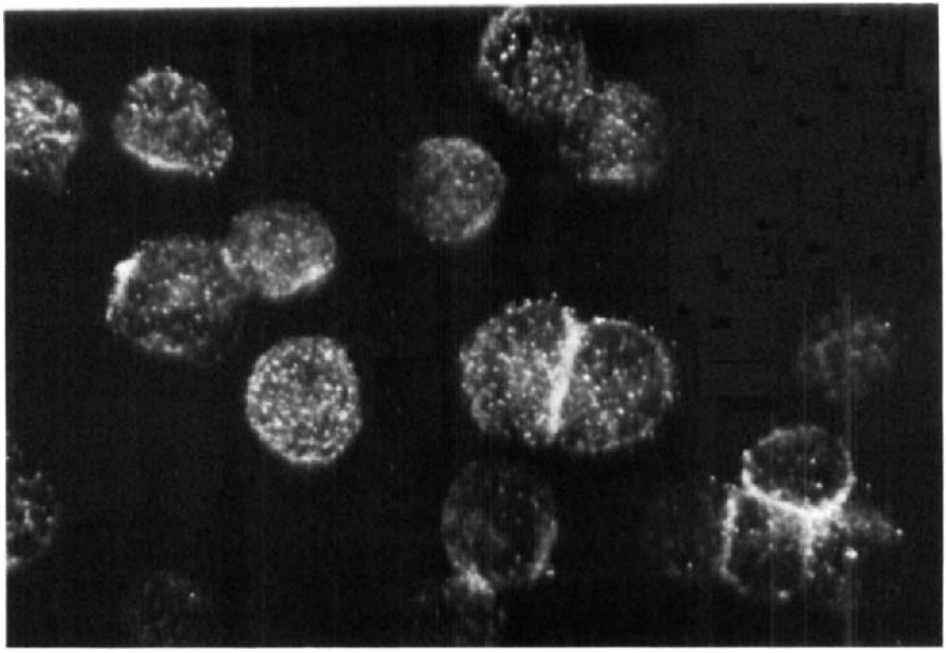

FIG. 28. Rheumatoid serum was used in immunofluorescence to demonstrate the nuclear location of rheumatoid arthritis-associated nuclear antigen (RANA). RANA is distributed as fine but distinct intranuclear speckles. (From Alspaugh and Tan, 1976.)

the immunofluorescence techniques (Alspaugh *et al.*, 1978). At the same time, the presence of EBNA (EB-associated nuclear antigen) was determined using the anticomplement immunofluorescence technique of Reedman and Klein (1973). RANA was detected in all cell lines, whether of human or simian origin which were transformed with EB virus although the intensity of RANA staining was variable. RANA was absent in all cell lines transformed with simian herpes-related viruses and in a marmoset T cell line. There was some relationship between EBNA and RANA staining although in the 531-H and B95-8 cell lines, there was strong staining for EBNA and weak staining for RANA, and in CP-81 infected with *Herpesvirus pongo*, there was weak staining for EBNA and no RANA staining. These studies tended to further support a relationship between RA antibody to RANA and EB virus. In another study, peripheral blood lymphocytes of normal controls were isolated and transformed *in vitro* with EB virus. Before transformation, the lymphocytes were negative for RANA but as transformation foci developed, RANA speckles began to appear in nuclei, initially of low

TABLE XIV
EPSTEIN–BARR NUCLEAR ANTIGEN (EBNA) AND RHEUMATOID ARTHRITIS-ASSOCIATED NUCLEAR ANTIGEN (RANA) IN HUMAN AND SIMIAN CELL LINES[a]

Cell line	Origin	Transforming virus	Lymphocyte cell type	Immunofluorescent staining for	
				EBNA	RANA
WiL_2	Human spleen	EBV	B	+++	+++
HCL-3/B95-8	Human umbilicus	EBV	B	+++	++
HCL-3/KMPG-1	Human umbilicus	*Herpesvirus papio*	B	–	–
531-H	Owl monkey with *in vitro* EBV-induced lymphoreticular hyperplasia	EBV	B	+++	+
B95-8	Cotton-topped marmoset with *in vitro* EBV-transformed lymphocytes	EBV	B	+++	+
CP-81	Orangutan with monomyelogenous leukemia	*Herpesvirus pongo*	Undifferentiated	+	–
594-S	Baboon with lymphoreticular hyperplasia	*H. papio*	B	–	–
MLC-1	Cotton-topped marmoset with *Herpesvirus saimiri*-induced lymphoma	*H. saimiri*	T	–	–

[a] From Alspaugh *et al.* (1978).

density but increasing in density with establishment of permanent cell lines (Alspaugh *et al.*, 1978).

The evidence linking EB virus and diseases such as infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma has been established in part through seroepidemiological studies. Such studies attempting to relate EB virus to RA have been carried out by several investigators (Alspaugh *et al.*, 1976, 1981; Catalano *et al.*, 1979, 1980; Ng *et al.*, 1980; Ferrell *et al.*, 1981). Table XV depicts data on the frequency of anti-RANA determined by immunodiffusion and immunofluorescence. In the earlier studies, immunodiffusion analysis

TABLE XV
ANTIBODY TO RA-ASSOCIATED NUCLEAR ANTIGEN (RANA)

Method	Reference	Serum dilution	Antibody in RA(%)	Antibody in Normal(%)
Immunodiffusion	Alspaugh *et al.* (1976)	Undil.	60/90(67)	6/71(8)
	Alspaugh *et al.* (1981)	Undil.	43/48(90)	1/16(6)
	Ferrell *et al.* (1981)	Undil.	62/87(71)	3/53(6)
	Catalano *et al.* (1979)	Undil.	44/47(94)	12/48(25)
Immunofluorescence	Ng *et al.* (1980)	1:8	116/124(94)	8/50(16)
	Catalano *et al.* (1980)	1:10		26/110(24)[a]

[a] This was from a study of 110 healthy U.S. Coast Guard Academy cadets. The 110 subjects could be subdivided into 52 positive for anti-VCA and 58 negative for anti-VCA. The 26 patients with anti-RANA all came from anti-VCA positive group.

detected anti-RANA in two-thirds of patients with RA but in some later reports, this has increased to over 90%. In normal control subjects, the frequency varies from 6 to 25%. Immunofluorescence analysis, used by two groups, have shown frequencies similar to that observed in immunodiffusion. A problem encountered in immunofluorescence is that RA sera frequently contain ANAs of other specificities and this had to be differentiated from speckled RANA staining. To help in this differentiation it was necessary to use two cell lines, one positive and the other negative for EB virus. Ng *et al.* (1980) have reported that they have experienced no difficulty differentiating RANA staining from other coexisting ANAs. Ng *et al.* (1980) have found a high frequency (95%) of anti-RANA in seronegative (rheumatoid factor negative) RA and Roitt (1981) reported that patients with early RA also have a higher frequency and titer of anti-RANA than normal individuals. Anti-RANA has been examined in other autoimmune diseases such as SLE, SS without RA, scleroderma, and MCTD and the frequencies have been close to that in normal subjects.

Seroepidemiological studies looking for the traditional antibodies to EB viral antigens such as viral capsid antigen (VCA), early antigen (EA), and EBNA have also been reported. The results from various studies are tabulated in Table XVI. In studies where titers of anti-VCA were examined, it was found that there was a higher frequency of RA patients with high titers ($\geq 1:320$). There was no difference in anti-EBNA frequency or titers. Anti-EA was also present in higher frequency in RA patients and in the one study where this was titered, there were also increased numbers of RA patients with high titers

TABLE XVI
SEROEPIDEMIOLOGY OF EBV ANTIGENS IN RA

EBV antigens	Reference	Serum dilution	Antibody in RA(%)	Antibody in Normal(%)
Viral capsid Antigen (VCA)	Ferrell *et al.* (1981)	1:10	83/86(97)	53/60(89)
		1:320	27/86(31)[a]	8/53(15)[a]
	Alspaugh *et al.* (1981)	1:320	27/48(56)[a]	11/96(11)[a]
	Catalano *et al.* (1979)	Undiluted	50/50(100)	48/48(100)
	Ng *et al.* (1980)	1:8	59/64(92)	41/50(82)
EB nuclear antigen (EBNA)	Ferrell *et al.* (1981)	1:4	78/80(97)	44/51(87)
		1:256	56/80(70)	33/51(65)
	Ng *et al.* (1980)	1:10	55/64(86)	38/50(76)
	Catalano *et al.* (1979)	1:2	49/50(98)	41/47(87)
		1:40	32/50(64)	15/47(32)
Early antigen	Ferrell *et al.* (1981)	1:10	46/86(53)[a]	10/53(19)[a]
		1:20	22/86(26)[a]	4/53(7)[a]
	Alspaugh *et al.* (1981)	1:10	21/50(42)[a]	12/96(12)[a]

[a] Percentages between RA and normals significantly different at $p < 0.01$.

($\geq$1:20). The results tend to reflect previously reported findings in infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma (Epstein and Achong, 1979; Henle *et al.*, 1979; Ernberg and Klein, 1979). However, abnormal values of anti-VCA and less frequently anti-EA have been observed in a variety of nonmalignant diseases which are deficient in immunoregulatory controls, either as a result of the disease per se or related to drug-induced immunosuppression. These include sarcoidosis, Hodgkin's disease, and ataxia telangiectasia (Henle and Henle, 1979).

Recently, it has been reported that proliferation of B cells induced by EB virus is regulated by cytotoxic T cells and that these cytotoxic T cells are present only in previously infected (seropositive) individuals (Rickinson *et al.*, 1979). Furthermore, this T lymphocyte cytotoxicity appears to be HLA restricted (Rickinson *et al.*, 1980; Misko *et al.*, 1980). Evidence of defective T cell control of EB virus-induced B-cell proliferation in RA has come from several sources. Slaughter *et al.* (1978) observed that unfractionated peripheral blood lymphocytes from RA spontaneously transformed at a much higher frequency than normal individuals. This was also observed by Bardwick *et al.* (1980). Further studies on this phenomenon (Depper *et al.*, 1981) showed that RA T cells were equally inefficient in controlling transformation of

both normal and RA B cells. Conversely, normal T cells controlled RA as well as normal B-cell proliferation, placing the defect in RA on the T lymphocyte population. The reservation about the latter findings is that the T- and B-cell populations were not HLA matched and allogeneic effects could not be ruled out. Recently, Tosato *et al.* (1981) were able to demonstrate regulatory defect of a specific subset of T cells in RA patients. Normal T cells mediated suppression of EBV-induced immunoglobulin production by B cells but RA T cells were deficient in this respect. Tests of several other T-cell functions in RA patients gave normal results suggesting a more restricted defect in suppressor–T-cell function relating specifically to EBV.

Antibody to RANA, a nuclear antigen which has not yet been characterized, led to the observations that RA might be linked in some manner to EB virus. This association has not been definitely established although there is increasingly supportive evidence. It is obvious that if there is an association, it is not a direct etiological relationship but involves other factors, including host response to the virus. The importance of T-cell regulation of EB virus-induced B-cell proliferation has been demonstrated and this might constitute a significant abnormality in the RA patient. In addition, factors related to HLA phenotype, EB virus load during infection, other deficiencies of immunoregulation, and reactivation of latently infected B cells may all play important roles in this process.

XI. Autoantibodies in Dermato/Polymositis

Dermatomyositis (DM) and polymyositis (PM) are inflammatory reactions of skin and muscle characterized by interstitial and perivascular infiltration of these tissues with lymphocytes, monocytes, and polymorphonuclear leukocytes. The disease often affects the proximal muscles (shoulder and pelvic girdle) of the extremities and is associated with skin rashes, elevated serum muscle enzymes such as creatine phosphokinase and lactic dehydrogenase, and abnormal muscle fiber contractions detectable by electromyography (Bohan *et al.*, 1977). In childhood, the disease is often always of acute onset whereas in adults, insidious and slowly progressive disease is frequently observed (Parkman and Cooke, 1980). Like many rheumatic diseases where immunological features are prominent, the etiology is unknown. In the childhood form, vasculitis of skin, muscle, and other organs can be widespread and some investigators have called this a systemic angiopathy of childhood (Banker and Victor, 1966; Whitaker and Engel, 1972).

Immunofluorescence has been used to determine the presence of ANAs in dermato/polymyositis and this has been reported to occur in frequencies varying from 16 to 60% (Weir *et al.*, 1961; Stern *et al.*, 1967; Venables *et al.*, 1981). This wide variation in frequencies may be related to sampling of different patient populations, with the higher frequencies detected in more severely ill patients but may also be related to increased sensitivity of techniques in the more recent studies. Although the occurrence of ANAs in DM/PM does not appear to be as common as in SLE, Sjögren's syndrome, and scleroderma, this may still be an open issue because of recent information that tissue substrate used in immunofluorescence is an important factor in this technique. This was most clearly demonstrated in a study of ANAs in scleroderma when tissue culture cell lines improved sensitivity of ANA detection so that the frequency in this disease was greater than 95% (Tan *et al.*, 1980). In the reported studies in DM/PM, tissue substrates which were used consisted of sections of mouse or rat liver.

Recently, three separate laboratories have employed the immunodiffusion technique to demonstrate the presence of precipitating antibodies to soluble nuclear antigens in DM/PM (Wolfe *et al.*, 1977; Nishikai and Reichlin, 1980a,b; Mimori *et al.*, 1981). All laboratories used extracts of whole calf thymus or thymus nuclei as source of antigens and showed that precipitin reactions could be observed with DM/PM sera (Table XVII). Wolfe *et al.* reported that 64% (9/14) of polymyositis and 87% (7/8) of polymyositis/scleroderma overlap patients had precipitating antibody to an antigen they called PM-1. In an interlaboratory collaborative study, it has been demonstrated that the PM-1 antigen–antibody reaction consists of multiple precipitating systems, although a major reaction line is present in high frequency in patients with polymyositis/scleroderma overlap (Reichlin, Sharp, and Tan, in preparation). This group of patients had skin changes consistent with scleroderma and in addition had symptoms of classical polymyositis.

Nishikai and Reichlin (1980a) have described an unusual nuclear antigen isolated from calf thymus which precipitated with two sera from patients with DM. The antigen, called Mi, was a dimer of 150,000 molecular weight and was reported to contain immunoglobulin determinants because precipitin lines between partially purified Mi antigen and those formed with DM serum and anti-bovine γ-globulin fused with each other. However, no demonstrable light chains were detected by polyacrylamide gel analysis of Mi antigen. It was localized to the nucleus by immunofluorescence, using antigen-affinity isolated antibody. These unusual findings remain to be further stud-

TABLE XVII
CHARACTERISTICS OF NUCLEAR ANTIGEN–ANTIBODY SYSTEMS IN DERMATOMYOSITIS (DM) AND POLYMYOSITIS (PM)

References	Antigen	Nature of antigen	Clinical relationships
Wolfe *et al.* (1977)	PM-1	Nuclear protein	PM 9/14 (64%) DM 1/6 (17%) PM/SCL 7/8 (87%)[a]
Nishikai and Reichlin (1980a)	Mi-1	150K nuclear basic protein Contains Ig determinant	DM 2/19 (11%)
Nishikai and Reichlin (1980b)	Jo-1	150K nuclear protein	PM 8/26 (31%) DM 1/22 (5%) PM/SCL 1/11 (9%)[a]
Mimori *et al.* (1981)	Ku	300K nuclear acidic protein	PM/SCL 6/11 (55%) SLE 1/150 (1%) SCL 1/45 (2%) SLE/SCL/PM 1/3 (33%)[a]

[a] Overlap diseases consisting of polymyositis and scleroderma (PM/SCL) and of SLE, scleroderma, and polymyositis (SLE/SCL/PM).

ied. Only 2 of 19 patients with DM had antibody whereas many patients with PM, SLE, scleroderma, and RA were negative.

Nishikai and Reichlin (1980b) using more concentrated extracts of whole calf thymus or calf thymus nuclei (50 mg protein/ml) have observed high frequencies of precipitin reactions with DM and PM sera. In DM, 13/22 (59%) and in PM, 17/26 (65%) sera showed precipitin reactions which were separated by them into antigen–antibody reactions of 9 different types. It is possible that in part related to the high concentration of tissue extracts used, some of the precipitin lines may not be immunological reactions. Nevertheless, they have performed further characterization of an antigen system called Jo-1 which appears to be protein in nature and of approximately 150,000 MW. Using antigen-affinity purified antibody, the antigen could be localized to the nucleus by immunofluorescence. Antibody was present in 31% of patients with PM, rarely, in DM, and not detected in SLE, scleroderma, RA, and other diseases.

The Ku antigen was reported by Mimori *et al.* (1981) to be a nuclear antigen, also isolated from calf thymus, which reacted with 55% (6/11) of patients with scleroderma/polymyositis overlap, 1/3 patients with SLE/scleroderma/polymyositis overlap, but rarely in SLE alone (1/150) or scleroderma alone (1/45). The antigen had an estimated molec-

ular weight of 300,000 and reactivity was destroyed by trypsin but not DNase or RNase. It was present in whole tissue or nuclear extracts of human and rabbit organs (thymus, liver, and spleen) but not detectable in rat tissues.

From the available data and collaborative exchange of sera from different laboratories, the precipitating antibodies described above demonstrate different immunological specificities. This testifies to the multiplicity and heterogeneity of the abnormal immune response in DM/PM. Of interest are the observations of antibodies which occur in unusually high frequency in overlap diseases consisting of DM/PM associated with scleroderma. The reasons for this at present are unknown. It is clear, however, that immunological abnormalities are a prominent feature of DM/PM and, in addition to antibodies, abnormal cellular immune reactions have been reported (Johnson *et al.*., 1972; Dawkins and Mastaglia, 1973; Lisak and Zweiman, 1975).

XII. Conclusion

Antibodies to nuclear antigens have been detected in increasing frequency in some systemic rheumatic diseases, including SLE, RA, MCTD, Sjögren's syndrome, scleroderma, and DM/PM. This is undoubtedly related to improved methods of detection in recent years. In the immunofluorescence technique, the importance of tissue substrates has become apparent. In the case of nuclear antigens present in abundance, such as DNA, histones, Sm, nRNP (U1-RNP), and SS-B/La antigens, cells from many animal species are adequate for the demonstration of the respective antibodies. However, in the case of nuclear antigens which are present in lower concentration, such as SS-A or nuclear antigens unique to cell types or animal species such as PCNA, RANA, and Ku antigen, special substrates have to be used. In addition to these features, consideration has to be given to treatment of substrate with fixatives which might destroy reactivity of certain antigens and to the extreme solubility of some antigens which might be lost from nuclei in the test procedure itself. It is difficult to designate any standard assays for the large variety of ANAs since some procedures may be superior in some aspects but inferior in others. As an approach to this problem, the use of reference sera containing assigned units of antibody reactivity may be of help since laboratories can then evaluate their own test systems in terms of type and relative quantity of antibody detected. Such a reference reagent for immunofluorescence-detected ANA is currently available from the World Health Organiza-

tion, Geneva, Switzerland but a more extensive battery of reference sera for different ANAs will soon be available from the Centers for Disease Control (CDC), Atlanta, Georgia (Tan *et al.*, 1982).

ANAs have assumed an important place in the diagnostic armamentarium of the clinician because of distinct profiles of ANAs in different diseases. Profiles of ANAs have therefore been extremely useful in differential diagnosis where the disease does not have classical or full-blown manifestations. Related to this, ANAs have been detected in many patients with undifferentiated connective tissue diseases of mild clinical activity. Whether these might constitute separate disease entities or early manifestations of classical diseases remains to be elucidated in follow-up studies. The role of certain ANAs in pathogenesis of tissue injury has been clearly demonstrated as in the case of antibodies to DNA and Sm and nRNP. Immune complexes formed in the circulation or *in situ* mediate tissue injury by activation of complement and other inflammatory mediators. Other ANAs have not been implicated but careful studies with these objectives in mind have not been reported.

More recently ANAs have been used to isolate and characterize nuclear antigens at the molecular and functional levels. Not only do these antibodies precipitate their respective antigens but also other proteins or nuclear RNAs which might be associated with them in special ways. For example, Sm antigen has been shown to be complexed to a series of nuclear RNP articles (U1, U2, U4, U5, and U6 RNPs) and the SS-B/La antigen to other small nuclear RNAs. The reasons for these special associations of protein antigens with specific sets of nuclear RNAs is unknown, but the possibility that there might be functional relationships in these complexed particles is not unreasonable. Already, as an extension of studies along these lines, U1 RNP has been proposed to perform a role in splicing of heterogeneous nuclear RNA (Lerner *et al.*, 1980). The many other nuclear and nucleolar antibodies already characterized immunologically should provide further probes for such studies.

The key question which pervades the minds of many investigators in this field is the reason for the appearance of ANAs in certain individuals. It is highly improbable that the phenomenon is a random immune response to nuclear breakdown products since the types of ANAs in different diseases are strikingly different. The explanation could be complex and dependent on multiple factors acting in concert. Those could range from environmental factors such as infectious agents, chemicals, and sunlight to host factors such as imbalance of hormones and defects in the delicate balance of the immunoregulatory

network. Some known environmental agents are drugs such as hydralazine and procainamide which together with lower levels of hepatic acetyltransferase enzyme predispose the host to the development of ANAs. Another agent may be the Epstein–Barr virus which is a ubiquitous environmental agent, but where there is a susceptable host with a specific T suppressor cell defect, uncontrolled B-cell activation may in some way lead to production of ANA to viral-induced nuclear antigen. At the present time, these are leads pointing to some directions for further investigation.

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REFERENCES

Aarden, L. A., de Groot, E. R., and Feltkamp, T. E. (1975). *Ann. N.Y. Acad. Sci.* **254,** 505.

Agelli, M., Bombardieri, S., Ioppolo, S., Siracusano, A., and Natali, P. G. (1980). *ICRS Med. Sci.* **8,** 754.

Agnello, V., Arbetter, A., Ibanez de Kasep, G., Powell, R., Tan, E. M. (1980). *J. Exp. Med.* **151,** 1514.

Alarcon-Segovia, D. (1969). *Mayo Clin. Proc.* **44,** 664.

Alarcon-Segovia, D., and Fishbein, E. (1975). *J. Immunol.* **115,** 28.

Alarcon-Segovia, D., Ruiz-Arguelles, A., and Fishbein, E. (1978). *Nature (London)* **271,** 67.

Aitcheson, C. T., Peebles, C., Joslin, F., and Tan, E. M. (1980). *Arthritis Rheum.* **23,** 528.

Akizuki, M., Powers, R., Jr., and Holman, H. R. (1977a). *J. Clin. Invest.* **59,** 264.

Akizuki, M., Boehm-Truitt, M. J., Kassan, S. S., Steinberg, A. D., and Chused, T. M. (1977b). *J. Immunol.* **119,** 932.

Alspaugh, M. A., and Tan, E. M. (1975). *J. Clin. Invest.* **55,** 1067.

Alspaugh, M. A., and Maddison, P. (1979). *Arthritis Rheum.* **22,** 796.

Alspaugh, M. A., Tan, and E. M. (1976). *Arthritis Rheum.* **19,** 711.

Alspaugh, M. A., Talal, N., and Tan, E. M. (1976). *Arthritis Rheum.* **19,** 216.

Alspaugh, M. A., Jensen, F. C., Rabin, H., and Tan, E. M. (1978). *J. Exp. Med.* **147,** 1018.

Alspaugh, M. A., Henle, G., Lennette, E., and Henle, W. (1981). *J. Clin. Invest.* **67,** 1134.

Anderson, J. R., Gray, K. G., Beck, J. S., Beechman, W. W., and McElhinney, A. J. (1962). *Ann. Rheum. Dis.* **21,** 360.

Andres, G. A., Accini, L., Beiser, S. M., Christian, C. L., Cinotti, G. A., Erlanger, B. F., Hsu, K. C., and Seegal, B. C. (1971). *J. Clin. Invest.* **49,** 2106.

Andrews, B. S., Eisenberg, R. A., Theofilopoulos, A. H., Iziu, S., Wilson, C. B., McConahey, P. J., Murphy, E. D., Roths, J. B., and Dixon, F. J. (1978). *J. Exp. Med.* **148,** 1198.

Arana, R., and Seligmann, M. *J. Clin. Invest.* **46,** 1867 (1967).

Ballou, S. P., and Kushner, I. (1979). *Clin. Exp. Immunol.* **37,** 58.

Banker, B. Q., and Victor, M. (1966). *Medicine* **45,** 261.

Bardwick, P. A., Bluestein, H., Zvaifler, N. J., Depper, J. M., and Seegmiller, J. E. (1980). *Arthritis Rheum.* **23,** 626.

Barland, P., Hamburger, M., and Hodes, S. (1975). *Arthritis Rheum.* **18,** 384.

Barque, J. P., Yeni, P., Perandeau, L., Danon, F., and Larsen, C. J. (1981). *Biochem. Biophys. Res. Commun.* **99,** 284.
Bart de la Faille-Kuyper, E. H. (1974). *Neth. J. Med.* **17,** 58.
Beck, J. S. (1963). *Scott. Med. J.* **8,** 373.
Bennett, R. M., and O'Connell, D. J. (1980). *Semin. Arthritis Rheum.* **10,** 25.
Bernstein, R. M., Steigerwald, J. C., and Tan, E. M. (1982). *Clin. Exp. Immunol.* (in press).
Beutner, E. H., Chorzelski, T. P., Bean, S. F., and Jordan, R. E. (1973). "Immunopathology of the Skin. Labeled Antibody Studies," pp. 210–212. Dowden, Hutchinson & Ross, Stroudsberg, Pennsylvania.
Blankstein, L. A., Stollar, B. D., and Levy, S. B. (1980). *Anal. Biochem.* **104,** 172.
Blomgren, S. E. (1973). *Semin. Hematol.* **10,** 345.
Blomgren, S. E., Condemi, J. J., Bignall, M. C., and Vaughan, J. H. (1969). *N. Engl. J. Med.* **281,** 64.
Blomgren, S. E., Condemi, J. J., and Vaughan, J. H. (1972). *Am. J. Med.* **52,** 338.
Bohan, A., Peter, J. B., Bohan, P. L., and Pearson, C. M. (1977). *Medicine* **56,** 255.
Brenner, S., Pepper, D., Berns, M. W., Tan, E. M., and Brinkley, B. R. (1981). *J. Cell Biol.* **91,** 95.
Bresnihan, B., Bunn, C., and Snaith, M. L. (1977). *Br. Med. J.* **1,** 610.
Brody, S., Nimmelstein, S., McShane, D., Fries, J., and Holman, H. R. (1977). *Proc. Int. Congr. Rheuma., 14th* p. 68 (Abstr).
Bruneau, C., and Benveniste, J. (1979). *J. Clin. Invest.* **64,** 191.
Burnham, T. K., Fine, G., and Neblett, T. R. (1966). *Ann. Intern. Med.* **65,** 9.
Busch, H., and Smetana, K., eds. (1970). "The Nucleolus." Academic Press, New York.
Bustin, M. (1978). *In* "The Cell Nucleus, (A. Busch, ed.), Vol 4, Part A, p. 195. Academic Press, New York.
Carpenter, J. R., McDuffie, F. C., Sheps, S. G., Spiekerman, R. E., Brumfield, H., and King, R. (1980). *Am. J. Med.* **69,** 395.
Carr, R. I. (1969). Doctorate Thesis. Rockefeller University, New York.
Carr, R. I., Koffler, D., Agnello, V., and Kunkel, H. G. (1969). *Clin. Exp. Immunol.* **4,** 527.
Catalano, M. A., Carson, D. A., Slovin, S. F., Richman, D. D., and Vaughan, J. H. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5825.
Catalano, M. A., Carson, D. A., Neiderman, J. C., Feorino, P., and Vaughan, J. H. (1980). *J. Clin. Invest.* **65,** 1238.
Cepellini, R., Polli, C., and Celado, F. (1957). *Proc. Soc. Exp. Biol. Med.* **96,** 572.
Clarke, G., Reichlin, M., and Tomasi, T. B. (1969). *J. Immunol.* **102,** 117.
Cleymart, J. E., and Nakamura, R. M. (1972). *Am. J. Clin. Pathol.* **58,** 388.
Cohen, A. S., Reynolds, W. E., Franklin, E. C., Kulka, J. P., Ropes, M. W., Shulman, L. E., and Wallace, S. L. (1971a). *Bull. Rheum. Dis.* **21,** 643.
Cohen, S. A., Hughes, G. R. V., Noel, G. L., and Christian, C. L. (1971b). *Clin. Exp. Immunol.* **8,** 551.
Davis, J. S., Godfrey, S. M., and Winfield, J. B. (1978). *Arthritis Rheum.* **21,** 17.
Dawkins, R. L., and Mastaglia, R. L. (1973). *N. Engl. J. Med.* **288,** 438.
Deegan, M. J., Waler, S. E., and Lovell, S. E. (1978). *Am. J. Clin. Pathol.* **69,** 599.
Deng, J. S., Takasaki, Y., and Tan, E. M. (1981). *J. Cell. Biol.* **91,** 654.
Depper, J. M., Bluestein, J. G., and Zvaifler, N. J. (1981). *J. Immunol.* **127,** 1899.
Dixon, F. J., Vazquez, J. J., Weigle, W. O., and Cochrane, C. G. (1958). *Arch. Pathol.* **65,** 18.
Dixon, F. J., Feldman, J. D., and Vazquez, J. J. (1961). *J. Exp. Med.* **113,** 899.
Douvas, A. S., and Tan, E. M. (1981). *In* "The Cell Nucleus" (H. Busch, ed.), Vol. 8, p. 369. Academic Press, New York.

Douvas, A. S., Stumph, W. E., Reyes, P., Tan, E. M. (1979a). *J. Biol. Chem.* **254,** 3608.
Douvas, A. S., Achten, M., and Tan, E. M. (1979b). *J. Biol. Chem.* **254,** 10514.
Dubroff, L. M., and Reid, R. J. (1980). *Science* **208,** 404.
Eisenberg, R. A., Tan, E. M., and Dixon, F. J. (1978). *J. Exp. Med.* **147,** 582.
Eldredge, N. T., van Robertson, W. B., and Miller, J. J. (1974). *Clin. Immunol. Immunopathol.* **3,** 262.
Elling, P., Grandal, H., and Faber, V. (1968). *Ann. Rheum. Dis.* **27,** 225.
Epstein, M. A., and Achong, B. G. (1979). "The Epstein-Barr Virus," pp. 1–12. Springer-Verlag, Berlin and New York.
Epstein, P., Reddy, R., and Busch, H. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1562.
Ernberg, I., and Klein, G. (1979). *In* "The Epstein-Barr Virus" (M. A. Epstein and B. G. Achong, eds.), pp. 39–60. Springer-Verlag, Berlin and New York.
Farber, S. J., and Bole, G. G. (1976). *Arch. Intern. Med.* **136,** 425.
Ferrell, P. B., Aitcheson, C. T., Pearson, G. R., and Tan, E. M. (1981). *J. Clin. Invest.* **67,** 681.
Fidelus, R. K., Lightfoot, R. W., and Jones, J. V. (1981). *Arthritis Rheum.* **24,** 5111 (Abstr.).
Forstot, J., Forstot, S. L., Greer, R. O., and Tan, E. M. (1982). *Arthritis Rheum.* **25,** 156.
Franco, H. L., Weston, W. L., Peebles, C., Forstot, S. L., and Phanuphak, P. (1981). *J. Am. Acad. Dermatol.* **4,** 67.
Friou, G. J. (1958). *J. Immunol.* **80,** 476.
Fritzler, M. J., and Tan, E. M. (1978). *J. Clin. Invest.* **62,** 560.
Fritzler, M. J., Kinsella, D., and Garbutt, E. (1980). *Am. J. Med.* **69,** 520.
Garcia, I., Wolbers, R., and Tan, E. M. (1981). *J. Immunol.* **126,** 706.
Gibbons, J. J., Jr., Tsai, C. C., and Roodman, S. T. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 1023.
Gilliam, A., Lang, D., and Lo Spalluto, J. J. (1980). *J. Immunol.* **125,** 874.
Gilliam, J. N., and Prystowsky, S. D. (1977). *Arch. Dermatol.* **113,** 583.
Ginsberg, B., and Keiser, H. (1973). *Arthritis Rheum.* **16,** 199.
Goessens, G., and Lepoint, A. (1974). *Exp. Cell Res.* **87,** 63.
Gold, E. F., Ben-Efraim, S., Faivisewitz, A., Steiner, Z., and Klajman, A. (1977). *Clin. Immunol. Immunopathol.* **7,** 176.
Grossman, L., and Barland, P. (1981). *Arthritis Rheum.* **24,** 927.
Gurney, T., Jr., and Eliceiri, G. L. (1980). *J. Cell Biol.* **87,** 398.
Hahn, B. H., Sharp, G. C., Irwin, W. S., Kantor, O. S., Gardner, C. A., Bagby, M. K., Perry, H. M., and Osterland, C. K. (1972). *Ann. Intern. Med.* **76,** 365.
Hannestad, K. (1978). *Scand. J. Immunol.* **7,** 127.
Hannestad, K., and Johanessen, A. (1976). *Scand. J. Immunol.* **5,** 541.
Hannestad, K., and Stollar, B. D. (1978). *Nature (London)* **275,** 671.
Harada, S., and Kato, N. (1980). *Nucleic Acids Res.* **8,** 1273.
Harbeck, R. J., Bardana, E. J., Kohler, P. F., and Carr, R. I. (1973). *J. Clin. Invest.* **52,** 789.
Hargraves, M. M., Richmond, H., and Morton, R. (1948). *Mayo Clin. Proc.* **23,** 25.
Harmon, C., Peebles, C., and Tan, E. M. (1981). *Arthritis Rheum.* **4,** 5122.
Haserick, J. R., Lewis, L. A., and Bortz, D. W. (1950). *Am. J. Med. Sci.* **219,** 660.
Hekman, A., and Sluyser, M. (1973). *Biochim. Biophys. Acta* **295,** 613.
Hellung-Larsen, P. (1977). "Low Molecular Weight RNA Components in Eukaryotic Cells." FADL, Copenhagen.
Hendrick, J. P., Wolin, S. L., Rinke, J., Lerner, M. R., and Steitz, J. A. (1981). *Mol. Cell Biol.* (In press).
Henle, W., and Henle, G. (1979). *In* "The Epstein-Barr Virus" (M. A. Epstein and B. G. Achong, eds.), pp. 61–73. Springer-Verlag, Berlin and New York.

Henle, W., Henle, G., and Lennette, E. (1979). *Sci. Am.* **241,** 48.
Henningsen, H. C., Cederberg, A., and Hanson, A. (1975). *Acta Med. Scand.* **198,** 475.
Holborow, E. G., Weir, D. M., and Johansson, G. D. (1957). *Br. Med. J.* **2,** 732.
Holman, H. R., and Deicher, H. R. (1959). *J. Clin. Invest.* **38,** 2059.
Holman, H., and Kunkel, H. G. (1957). *Science* **126,** 162.
Inami, Y. H., Nakamura, R. M., Tan, E. M. (1973). *J. Immunol. Methods* **3,** 287.
Inami, Y. H., Nakamura, R. M., and Tan, E. M. (1975). *Am. J. Clin. Pathol.* **64,** 65.
Isenberg, I. (1979). *Annu. Rev. Biochem.* **48,** 159.
Izuno, G. T. (1978). *Br. J. Dermatol.* **98,** 391.
Johnson, G. D., and de C. Hogueira Arango, E. M. (1981). *J. Immunol. Methods* **43,** 349.
Johnson, P. M. (1979). *Scand. J. Immunol.* **9,** 464.
Johnson, R. L., Fink, C. W., and Ziff, M. (1972). *J. Clin. Invest.* **51,** 2435.
Johnston, M. F. M., and Eisen, H. N. (1976). *J. Immunol.* **117,** 1190.
Jonsson, J., and Norberg, R. (1978). *Scand. J. Rheum.* **7,** 229.
Jonsson, J., and Norberg, R. (1980). *Scand. J. Immunol.* **12,** 119.
Jonsson, J., Steiner, M., and Klein, E. (1976). *Clin. Exp. Immunol.* **25,** 144.
Jonsson, J., Schilling, W., and Norberg, R. (1981). *Scand. J. Immunol.* **13,** 175.
Kassan, S. S., Akizuki, M., Steinberg, A. D., Reddick, R. L., and Chused, T. M. (1977). *Am. J. Med.* **63,** 328.
Kephart, D. C., Hood, A. F., and Provost, T. T. (1981). *J. Invest. Derm.* (in press).
Klajman, A., Camin-Belsky, N., Kimche, A., and Ben-Efraim, S. (1970). *Clin. Exp. Immunol.* **7,** 641.
Klein, G., Steiner, M., Wiener, F., and Klein, E. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 685.
Klipple, G. L., Peebles, C., and Tan, E. M. (1981). *Arthritis Rheum.* **4,** S95.
Koffler, D., Schur, P. H., and Kunkel, H. G. (1967). *J. Exp. Med.* **126,** 607.
Koffler, D., Carr, R. I., Agnello, V., Thoburn, R., and Kunkel, H. G. (1971). *J. Exp. Med.* **134,** 294.
Koffler, D., Agnello, V., and Kunkel, H. G. (1974). *Am. J. Pathol.* **74,** 109.
Korn, J. H., and Leroy, E. C. (1979). *In* "The Science and Practice of Clinical Medicine. Rheumatology and Immunology" (A. S. Cohen, ed.), Vol. 4, p. 249. Grune & Stratton, New York.
Kornberg, R. D. (1977). *Annu. Rev. Biochem.* **40,** 931.
Kunkel, H. G. (1977). *Arthritis Rheum. (Suppl.)* **20,** S139.
Kunkel, H. G., and Tan, E. M. (1964). *Adv. Immunol.* **4,** 351.
Kunkel, H. G., Holman, H. R., and Deicher, H. R. G. (1960). *Ciba Found.* **8,** 429.
Kurata, N., and Tan, E. M. (1976). *Arthritis Rheum.* **19,** 574.
Lachmann, P. (1961). *Immunology* **4,** 142.
Lachmann, P., Muller-Eberhard, H. E., Kunkel, H. G., and Paronetto, F. (1962). *J. Exp. Med.* **115,** 63.
Lacour, F., Nahon-Merlin, E., and Michelson, M. (1973). *Curr. Top. Microbiol. Immunol.* **62,** 1.
Lahita, R., Kluger, J., Drayer, D. E., Koffler, D., and Reidenberg, M. M. (1979). *N. Engl. J. Med.* **301,** 1382.
Lambert, P. H., and Dixon, F. J. (1968). *J. Exp. Med.* **127,** 507.
Lamelin, J. P., De-The, G., Revillard, J. P., and Gabbiani, G. (1978). *IARC Sci. Publ.* **20,** 523.
Lee, S. L., and Chase, P. H. (1975). *Semin. Arthritis Rheum.* **5,** 83.
Lerner, M. R., and Steitz, J. A. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5495.
Lerner, M. R., and Steitz, J. A. (1981). *Cell* **25,** 298.

Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L., and Steitz, J. A. (1980). *Nature (London)* **283,** 220.
Lerner, M. R., Boyle, J. A., Hardin, J. A., and Steitz, J. A. (1981a). *Science* **211,** 400.
Lerner, M. R., Andrews, N. C., Miller, G., and Steitz, J. A. (1981b). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 805.
Lisak, R. P., and Zweiman, B. (1975). *J. Neurol. Neurosurg. Psychiatry* **38,** 521.
McBride, C. M., Bowen, J. M., and Dmochowski, L. (1972). *Surg. Forum* **23,** 92.
McCain, G. A., Bell, D. A., Chodirker, W. B., and Komer, R. R. (1978). *J. Rheum.* **5,** 399.
McCarty, G. A., Barada, F. A., Snyderman, R., and Fritzler, M. J. (1981). *Arthritis Rheum.* **24,** S109.
McCluskey, R. T., Benacerraf, B., Potter, J. L., and Miller, F. (1960). *J. Exp. Med.* **111,** 181.
McGhee, J. D., and Felsenfeld, G. (1980). *Annu. Rev. Biochem.* **49,** 1115.
MacGillwary, A. J., and Carroll, A. R. (1982). *In* "Biochemistry and Biology of the Cell Nucleus: Non-histone Proteins" (L. S. Huileca, ed.). CRC Press, New York (in press).
MacGillwray, A. J., Dahi, S., Naxakis, G., and Wallis, C. M. (1981). *Cell Biol. Int. Rep.* **5,** 69.
Maddison, P. J., Mogavero, H., and Reichlin, M. (1978). *J. Rheumatism* **5,** 407.
Manak, R. C., and Voss, E. W. (1978). *Immunochemistry* **15,** 643.
Martinez-Lavin, M., Vaughan, J. H., and Tan, E. M. (1979). *Ann. Intern. Med.* **91,** 185.
Masi, A. T., and Rodnan, G. F. (1981). *Bull. Rheum. Dis.* **31,** 1.
Mattioli, M., and Reichlin, M. (1971). *J. Immunol.* **107,** 1281.
Mattioli, M., and Reichlin, M. (1973). *J. Immunol.* **110,** 1318.
Mattioli, M., and Reichlin, M. (1974). *Arthritis Rheum.* **17,** 421.
Medoff, M. E., Locker, J. D., Bennett, R. M., and Sukhupunyarksa, S. (1976). *Arthritis Rheum.* **19,** 963.
Mellors, R. C., Ortega, L. G., and Holman, H. R. (1957). *J. Exp. Med.* **106,** 191.
Miescher, P., and Strassle, R. (1957). *Vox Sang.* **2,** 283.
Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S., and Homma, M. (1981). *J. Clin. Invest.* **68,** 611.
Misko, I. S., Moss, D. J., and Pope, J. H. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4247.
Miyachi, K., and Tan, E. M. (1979). *Arthritis Rheum.* **22,** 87.
Miyachi, K., Fritzler, M. J., and Tan, E. M. (1978). *J. Immunol.* **121,** 2228.
Miyawaki, S., and Ritchie, R. F. (1973). *Arthritis Rheum.* **16,** 726.
Moroi, Y., Peebles, C., Fritzler, M. J., Steigerwald, J. C., and Tan, E. M. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1627.
Moroi, Y., Hartman, A. L., Nakaae, P. K., and Tan, E. M. (1981). *J. Cell Biol.* **90,** 254.
Morton, D. L., Malmgren, R. A., Holmes, E. C., and Ketchum, A. S. (1968). *Surgery* **64,** 233.
Mulli, J. C., and Cruchand, A. (1977). *Int. Arch. Allergy Appl. Immunol.* **53,** 279.
Nakamura, R. M., and Deodhar, S. (1976). "Laboratory Tests in the Diagnosis of Autoimmune Disorders." American Society for Clinical Pathologists, Chicago, Illinois.
Nakamura, R. M., and Tan, E. M. (1977). *Hum. Pathol.* **9,** 85.
Natali, P. G., and Tan, E. M. (1971). *Radiat. Res.* **46,** 506.
Natali, P. G., and Tan, E. M. (1972). *J. Immunol.* **108,** 318.
Natali, P. G., and Tan, E. M. (1973). *Arthritis Rheum.* **16,** 579.
Ng, K. C., Perry, J. D., Brown, K. A., and Holborow, E. J. (1980). *Lancet* **1,** 447.
Nishikai, M., and Reichlin, M. (1980a). *Mol. Immunol.* **17,** 1129.
Nishikai, M., and Reichlin, M. (1980b). *Arthritis Rheum.* **23,** 881.

Northway, J. D., and Tan, E. M. (1972). *Clin. Immunol. Immunopathol.* **1,** 140.
Notman, D. D., Kurata, N., and Tan, E. M. (1975). *Ann. Intern. Med.* **83,** 464.
Nydegger, U. E., Lambert, P. H., Gerber, H., and Miescher, P. A. (1974). *J. Clin. Invest.* **54,** 297.
Ouchterlony, O. (1964). *In* "Immunological Methods" (J. F. Ackroyd, ed.), p. 68. Blackwell, Oxford.
Packman, L. M., and Cooke, N. (1980). *J. Pediatr.* **96,** 226.
Papalian, M., Lafer, E., Wong, R., and Stollar, B. D. (1980). *J. Clin. Invest.* **65,** 469.
Parker, M. D. (1973). *J. Lab. Clin. Med.* **82,** 769.
Paronetto, F., and Koffler, D. (1965). *J. Clin. Invest.* **44,** 1657.
Peltier, A. P., Aussel, C., Haim, T., and Cyna, L. (1977). *J. Immunol. Methods* **16,** 153.
Perry, H. M., Jr. (1973). *Am. J. Med.* **54,** 58.
Perry, H. M., Jr., Tan, E. M., Carmody, S., and Sakamoto, A. (1970). *J. Lab. Clin. Med.* **176,** 114.
Picazo, J. J., and Tan, E. M. (1975). *Scand. J. Rheum. (Suppl.)* **11,** 35.
Pincus, R., Schur, P. H., Rose, J. A., and Steinberg, A. D. (1969). *N. Engl. J. Med.* **281,** 701.
Pinnas, J. L., Northway, J. D., and Tan, E. M. (1973). *J. Immunol.* **111,** 996.
Portanova, J. P., Rubin, R. L., Joslin, F. G., Agnello, V. D., and Tan, E. M. (1982). (In press).
Powers, R., Jr., Akizuki, M., Boehm-Truitt, M. J., Daly, V., and Holman, H. R. (1977). *Arthritis Rheum.* **20,** 131 (Abstr.).
Provost, T. T. (1979). *J. Invest. Dermatol.* **72,** 110.
Provost, T. T., Ahmed, R. R., Madison, P., and Reichlin, M. (1977). *Arthritis Rheum.* **20,** 1457.
Puritz, E. M., Young, W. J., and Newell, M. (1973). *Clin. Immunol. Immunopathol.* **2,** 98.
Raptis, L., and Menard, H. A. (1980). *J. Clin. Invest.* **66,** 1391.
Reddy, R., and Busch, H. (1981). *In* "The Cell Nucleus" (H. Busch, ed.), Vol. 8, p. 261. Academic Press, New York.
Reedman, B. M., and Klein, G. (1973). *Int. J. Cancer* **11,** 499.
Reichlin, M., and Mattioli, M. (1972). *N. Engl. J. Med.* **286,** 908.
Reichlin, M., and Mattioli, M. (1974). *Bull. Rheum. Dis.* **5,** 756.
Reyes, P., and Tan, E. M. (1977). *J. Exp. Med.* **145,** 749.
Richards, F. F., Konigsberg, W. H., Rosenstein, R. W., and Varga, J. M. (1975). *Science* **187,** 130.
Rickinson, A. B., Moss, D. J., and Papa, J. H. (1979). *Int. J. Cancer* **23,** 610.
Rickinson, A. B., Wallace, L. E., and Epstein, M. A. (1980). *Nature (London)* **283,** 865.
Ritchie, R. F. (1970). *N. Engl. J. Med.* **282,** 1174.
Robbins, W. C., Holman, H. R., Deicher, H., and Kunkel, H. G. (1957). *Proc. Soc. Exp. Biol. Med.* **96,** 575.
Robitaille, P., and Tan, E. M. (1973). *J. Clin. Invest.* **52,** 316.
Ro-Choi, T. S., and Busch, H. (1974). *In* "The Cell Nucleus" (H. Busch, ed.), Vol. 3, pp. 152–208. Academic Press, New York.
Ro-Choi, T. S., Reddy, R., Herring, D., Takano, T., Taylor, C. W., and Busch, H. (1972). *J. Biol. Chem.* **247,** 3205.
Rodnan, G. P. (1979). *In* "Arthritis and Allied Conditions" (D. J. McCarty, ed.), p. 762. Lea & Febiger, Philadelphia, Pennsylvania.
Roitt, I. M. (1981). *In* "Rheumatoid Arthritis" (M. Ziff, ed.). Elsevier, Amsterdam (in press).
Romac, J., Bouley, J. P., and Van Regenmortel, M. H. V. (1981). *Anal. Biochem.* **113,** 366.

Romani, M., Vidali, G., Tahourdin, C. S. M., and Bustin, M. (1980). *J. Biol. Chem.* **255,** 468.
Rothfield, N. F., and Rodnan, G. P. (1968). *Arthritis Rheum.* **11,** 607.
Rubin, R. L., Joslin, F., and Tan, E. M. (1982). *Scand. J. Immunol.* (in press).
Rubin, R. L., Joslin, F. G., and Tan, E. M. (1982). *Arthritis Rheum.* (in press).
Samaha, R. J., and Irwin, W. S. (1975). *J. Clin. Invest.* **56,** 446.
Sano, H., and Morimoto, C. (1981). *J. Immunol.* **126,** 538.
Schur, P. H., and Sandson, J. (1968). *N. Engl. J. Med.* **278,** 533.
Scopelitis, E., Biundo, J. J., Jr., and Alspaugh, M. A. (1980). *Arthritis Rheum.* **23,** 287.
Sekeris, C. E., and Guialis, A. (1981). *In* "The Cell Nucleus" (H. Busch, ed.), Part A, p. 247. Academic Press, New York.
Seligmann, M. (1957). *C. R. Acad. Sci. Paris* **245,** 243.
Sharp, G. C., Irwin, W. S., La Rogue, R. L., and Holman, H. R. (1971). *J. Clin. Invest.* **50,** 350.
Sharp, G. C., Irwin, W. S., Tan, E. M., Gould, R. C., and Holman, H. R. (1972). *Am. J. Med.* **52,** 148.
Sharp, G. C., Irwin, W. S., May, C. M., Holman, H. R., McDuffie, F. C., Hess, E. V., and Schmid, F. C. (1976). *N. Engl. J. Med.* **295,** 1149.
Shu, S., Provost, T. T., Croxdale, M. B., Reichlin, M., and Beutner, E. H. (1977). *Clin. Exp. Immunol.* **27,** 238.
Singsen, B. H., Komreich, H. K., Koster-King, K., Brink, S. J., Bernstein, B. H., Hanson, V., and Tan, E. M. (1977). *Arthritis Rheum.* **20,** 355.
Slaughter, L., Carson, D. A., Jensen, F. C., Holbrook, T. L., and Vaughan, J. H. (1978). *J. Exp. Med.* **148,** 1429.
Sontheimer, R. D., Gilliam, J. N., and Ziff, M. (1978). *Ann. Intern. Med.* **89,** 186.
Stec, G. P., Lertora, J. J. L., and Atkinson, A. J., Jr. (1979). *Ann. Intern. Med.* **90,** 799.
Steinberg, A. D., Pincus, T., and Talal, N. (1969). *J. Immunol.* **102,** 788.
Steiner, M., Klein, E., and Klein, G. (1975). *Clin. Immunol. Immunopathol.* **4,** 374.
Steinman, C. R., Deesomchok, U., and Spiera, H. (1976). *J. Clin. Invest.* **57,** 1330.
Steinman, C. R., Grishman, E., Spiera, H., and Deesomchok, U. (1977). *Am. J. Med.* **62,** 319.
Stern, G. M., Rose, A. L., and Jacobs, K. (1967). *J. Neurol. Sci.* **5,** 181.
Stollar, B. D. (1970). *Science* **169,** 609.
Stollar, B. D. (1971). *Arthritis Rheum.* **14,** 485.
Stollar, B. D. (1973). *In* "The Antigens" (M. Sela, ed.), Vol 1, p. 1. Academic Press, New York.
Stollar, B. D. (1975). *CRC Crit. Rev. Biochem.* p. 45.
Stollar, B. D. (1978). *In* "Methods in Cell Biology" (G. Stein and J. Stein, eds.), Vol 18, Part 3, p. 105. Academic Press, New York.
Stollar, B. D., and Levine, L. (1963). *Arch. Biochem. Biophys.* **101,** 417.
Stollar, B. D., and Papalian, M. (1980). *J. Clin. Invest.* **66,** 210.
Stollar, B. D., Levine, L., Lohrer, H. I., and Van Vanakis, H. (1962). *Proc. Natl. Acad. Sci. U.S.A.* **48,** 874.
Takano, M., Golden, S., Sharp, G. C., and Agris, P. F. (1981). *Biochemistry* **20,** 5929.
Takasaki, Y., Deng, J. S., and Tan, E. M. (1981). *J. Exp. Med.* **154,** 1899.
Tan, E. M. (1967a). *J. Clin. Invest.* **46,** 735.
Tan, E. M. (1967b). *J. Lab. Clin. Med.* **70,** 800.
Tan, E. M. (1974). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33,** 1894.
Tan, E. M. (1976). *J. Invest. Dermatol.* **67,** 360.
Tan, E. M. (1978). *In* "The Cell Nucleus" (H. Busch, ed.), Vol. 7, p. 457. Academic Press, New York.

Tan, E. M., and Kunkel, H. G. (1966a). *J. Immunol.* **96,** 464.
Tan, E. M., and Kunkel, H. G. (1966b). *Arthritis Rheum.* **9,** 37.
Tan, E. M., and Natali, P. G. (1970). *J. Immunol.* **104,** 902.
Tan, E. M., and Peebles, C. (1980). *In* "Manual of Clinical Immunology" (N. R. Rose and H. Friedman, eds.). American Society for Microbiology Press, Washington, D.C.
Tan, E. M., Schur, P. H., Carr, R. I., and Kunkel, H. G. (1966). *J. Clin. Invest.* **45,** 1732.
Tan, E. M., Robinson, J., and Robitaille, P. (1976). *Scand. J. Immunol.* **5,** 811.
Tan, E. M., Christian, C., Holman, H. R., Homna, M., Kunkel, H. G., Reichlin, M., Sharp, G. C., Ziff, M., and Barnett, E. V. (1977). *Arthritis Rheum.* **20,** 1419.
Tan, E. M., Rodnan, G., Garcia, I., Moroi, Y., Fritzler, M. J., and Peebles, C. (1980). *Arthritis Rheum.* **23,** 617.
Tan, E. M., Fritzler, M. J., McDougal, J. S., McDuffie, F. C., Nakamura, R. M., Reichlin, M., Reimer, C. B., Sharp, G. C., Schur, P. H., Wilson, M., and Winchester, R. J. (1982). *Arthritis Rheum.* (in press).
Teppo, A. M., Gripenberg, M., Kurki, P., Baklien, K., Helve, T., and Wegelius, O. (1982). *Scand. J. Immunol.* **15,** 1.
Thoburn, R., Koffler, D., and Kunkel, H. G. (1971). *Proc. Soc. Exp. Biol. Med.* **136,** 711.
Tosato, G., Steinberg, A. D., and Blaese, M. (1981). *N. Engl. J. Med.* **305,** 1238.
Tuffanelli, D. L. (1975). *J. Invest. Dermatol.* **65,** 143.
Unanue, E. R., and Dixon, F. J. (1967). *Adv. Immunol.* **6,** 1.
Vasquez, J. J., and Dixon, F. J. (1957). *Lab. Invest.* **6,** 205.
Venables, P. J. W., Mumford, P. A., and Maini, R. N. (1981). *Ann. Rheum. Dis.* **40,** 217.
Waelti, E. R., and Hess, M. W. (1980). *J. Immunol. Methods* **32,** 177.
Weinberg, R. A., and Penman, S. (1968). *J. Mol. Biol.* **38,** 289.
Weinstein, A. (1980). *Prog. Clin. Immunol.* **4,** 1.
Weir, D. M., Holborow, E. J., and Johnson, G. D. (1961). *Br. Med. J.* **1,** 933.
Whitaker, J. N., and Engel, W. K. (1972). *N. Engl. J. Med.* **286,** 333.
White, P. J., Gardner, W. D., and Hoch, S. O. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 626.
Whittingham, S., Mackay, I. R., and Whitworth, J. A. (1972). *Am. Heart J.* **84,** 228.
Wiik, A., Jensen, E., and Friis, J. (1974). *Ann. Rheum. Dis.* **33,** 515.
Winfield, J. B., and Davis, J. S., IV. (1974). *Arthritis Rheum.* **17,** 97.
Winfield, J. B., Koffler, D., and Kunkel, H. G. (1975a). *J. Clin. Invest.* **56,** 563.
Winfield, J. B., Koffler, D., and Kunkel, H. G. (1975b). *Arthritis Rheum.* **18,** 531.
Winfield, J. B., Faiferman, F., and Koffler, D. (1977). *J. Clin. Invest.* **59,** 90.
Winn, D. M., Wolfe, J. F., Lindberg, D. A., Fristoe, F. H., Kinsland, L., and Sharp, G. C. (1979). *Arthritis Rheum.* **22,** 1334.
Wold, R. T., Young, F. E., Tan, E. M., and Farr, R. S. (1968). *Science* **161,** 806.
Wolfe, J. F., Adelstein, E., and Sharp, G. C. (1977). *J. Clin. Invest.* **59,** 176.
Woosley, R. L., Drayer, D., Reidenberg, M., and Nies, A. S. (1978). *N. Engl. J. Med.* **298,** 1157.
Yang, V. W., Lerner, M. R., Steitz, J. A., and Flint, S. J. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1371.
Yoshida, T. O., Yasuda-Yasaki, Y., and Utsumi, K. R. (1975). *IARC Sci. Publ.* **11** (2), 259.
Ziegler, G. B., Heinkel, K., Buchholz, H., and Habermann, W. (1976). *Z. Gastroenterology* **14,** 548.
Ziff, M. (1961). *Am. J. Med.* **30,** 1.

ADVANCES IN IMMUNOLOGY, VOL. 33

The Biochemistry and Pathophysiology of the Contact System of Plasma[1]

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I. Introduction

Plasma proteins contribute in a major way to the maintenance of homeostasis of living organisms. They form major transport systems as in the case of lipoproteins and iron-binding proteins, or recognition systems as in the case of immunoglobulins, and they play important roles in the defense of the organism. Aside from the immunoglobulins, four major pathways of proteins in the plasma contribute to the host's capacity to defend against foreign substances, traumatic events, and internal disorders: they are the complement, contact (Hageman factor), extrinsic clotting, and fibrinolytic systems. These four systems have the capacity to act in concert and cross-activate one another. They may function as a direct or indirect consequence of immunologic activity. While each is involved in the host's defense, each system can also contribute to the detrimental aspect of the inflammatory process which produces structural injury of tissues with a resulting loss of function.

We are already aware of considerable knowledge gained in the biochemistry and in the physiologic and pathologic functions of the

complement and extrinsic clotting systems. Although smaller in numbers of known components, the fibrinolytic system has yielded only a modest amount of sure information about the pathways of activation that occur *in vivo* or in plasma *in vitro*. Similarly, the contact system has been difficult to study. It is only recently that sufficient information on the biochemistry and technology have been advanced to begin to allow accurate probing of its role in pathologic and physiologic processes. In large measure the difficulty was the result of problems in purifying adequate amounts of the proteins since they are present in concentrations less than 100 μg/ml and activation of the proenzymes which occurs spontaneously during and after purification. The development and employment of new technology in the past 10 years has provided adequate amounts of the proteins for a considerable expansion of our knowledge.

This article is directed at a compilation of much of the information that has been assembled during the past decade. It is our hope that the information compiled on the foundations of the contact system will provide a basis upon which investigational probes can be launched into the function of the system *in vivo*. The investigations of the past 10 years have already shed considerable light on the interactions of proteins with surfaces and with other protein molecules and in the cascading conversion of proenzymes to their activated form. Inroads have also been made into the pathologic implications of the contact system when set into motion.

We have limited in great part our review to studies published since 1970. The reader is referred to earlier reviews that deal in greater detail with much of the work performed prior to this time (Schachter, 1969; Ratnoff, 1966; Nossel, 1964). We apologize in advance for omissions of important studies that have escaped either our search of the literature or our written description.

II. Description of the Contact System

In its most condensed form, the contact system consists of four proteins: Hageman factor (HF), prekallikrein, high-molecular-weight (MW) kininogen, and coagulation Factor XI. However, several other proteins may be involved: plasminogen is acted upon by kallikrein and to a lesser extent by HFa and Factor XIa; C5 of the complement system can be activated by kallikrein; and, even more indirectly, C1 which may be activated by plasmin and, to a lesser extent, the active fragment of HF; Factor IX and the remainder of the clotting pathway are activated by Factor XI, and, reportedly, by kallikrein; and Factor

VII of the extrinsic clotting system may be activated by HFa. Hence, in a broader sense, the contact system may be extensive. In particular circumstances, each of these extensions of the system may function. Nevertheless, this article will concentrate on the biochemistry and functions of the central four proteins noted above.

III. Components of the Contact System

A. Hageman Factor (HF, Coagulation Factor XII)

HF has been highly purified from human, bovine, rabbit, and guinea pig plasmas (Cochrane and Wuepper, 1971; Movat and Ozge-Anwar, 1974; Griffin and Cochrane, 1976a; Fujikawa *et al.*, 1977a,b; Claeys and Collen, 1977; Yamamoto and Cochrane, 1981). HF is a glycoprotein that exists as a single polypeptide chain of molecular weight 74,000–80,000. Human HF contains 16.8% carbohydrate, including 4.2% hexose, 4.7% hexosamine, and 7.9% *N*-acetylneuraminic acid. The protein is a serine protease zymogen, the full activation of which is associated with limited proteolysis as will be discussed below. The sedimentation coefficient of human and bovine HF is approximately 4.5 S (Donaldson and Ratnoff, 1965; Cochrane and Wuepper, 1971; Revak *et al.*, 1974; Fujikawa *et al.*, 1977b).

Amino acid composition data of Hageman Factor of several species reveal considerable similarities (Table I). Partial amino acid sequence data are available in the case of bovine and human HF (Fujikawa *et al.* 1977a, 1980a,b). The amino acid sequence that includes the active site serine residue of bovine HF and several other bovine plasma serine

Factor VII	Phe Cys Ala Gly Tyr	Thr - Asp Gly Thr Lys - -	Asp Ala Cys Lys Gly Asp SER Gly Gly Pro His
Thrombin	Phe Cys Ala Gly Tyr	Lys Pro Gly Glu Gly Lys Arg Gly	Asp Ala Cys Glu Gly Asp SER Gly Gly Pro Phe
Factor IX_a	Phe Cys Ala Gly Tyr	His - Glu Gly Gly Lys - -	Asp Ser Cys Gln Gly Asp SER Gly Gly Pro His
Factor X_a	Phe Cys Ala Gly Tyr	Asp - Thr Gln Pro Glu - -	Asp Ala Cys Gln Gly Asp SER Gly Gly Pro His
Factor XI	Val Cys Ala Gly Tyr	Arg - Glu Gly Gly Lys - -	Asp Ala Cys Lys Gly Asp SER Gly Gly Pro ?
HF	Leu Cys Ala Gly Phe	Leu - Glu Gly Gly Thr - -	Asp Ala Cys Gln Gly Asp SER Gly Gly Pro Leu

FIG. 1. Active-site sequence of HF and several other coagulation proteins. Amino acid residues in HF that are identical with other coagulation factors are shown in blocks. Dashes refer to spaces that have been inserted to bring the six proteins into alignment for better homology. The question mark refers to an amino acid not known. The active-site serine analogous to serine-195 in chymotrypsin is shown in capital letters. (From Kisiel *et al.*, 1977.)

proteases is shown in Fig. 1. Sequence homologies are noted in the figures. The data indicate that the amino terminal and the active site region of the light chain of activated HF contain amino acid sequences which are common to a number of serine proteases that participate in coagulation.

The level of HF antigen in normal plasma was reported to be 29 μg/ml (a range of 23–40 μg/ml) by Revak *et al.* (1974). Saito *et al.* (1976) determined a normal value of 40 μg/ml using a radioimmunoassay for HF antigen.

Cleavage of the single chain native HF molecule within a disulfide loop generates a two chain active enzyme designated α-HFa, consisting of disulfide-linked fragments of 52,000 and 28,000 M_r (Fig. 2). A

TABLE I
AMINO ACID COMPOSITION OF HAGEMAN FACTOR

	Guinea pig[a] HF (%)	Human HF (%)[b]	Human HF (%)[c]	Bovine[d] HF (%)	Rabbit[c] HF (%)
Lysine	3.65	6.45	3.73	4.44	3.55
Histidine	5.29	3.57	4.73	4.61	5.33
Arginine	5.65	5.52	6.91	5.76	6.54
Aspartic acid	7.06	8.22	6.56	7.67	7.09
Threonine	5.16	5.58	5.98	5.55	5.45
Serine	6.53	9.73	6.43	6.04	7.16
Glutamic acid	11.49	12.02	12.31	11.41	11.31
Proline	9.55	5.81	9.94	8.44	10.0
Glycine	8.28	9.71	9.02	8.27	8.91
Alanine	7.50	7.10	9.33	8.30	9.30
Cysteine	6.18	3.56	3.07	4.47	2.54
Valine	4.15	5.11	5.97	5.25	5.60
Methionine	0.91	0.28	0.210	0.24	0.22
Isoleucine	1.87	2.77	1.48	2.31	1.80
Leucine	9.05	8.34	9.46	8.77	9.85
Tyrosine	3.09	2.71	2.43	2.31	2.31
Phenylalanine	3.31	3.54	2.60	3.71	3.02
Tryptophan	1.36	NR[e]	NR	2.44	NR
Total	99.99	100.02	100.16	99.99	99.98

[a] From Yamamoto and Cochrane (1981).
[b] From Revak *et al.* (1974).
[c] From Griffin and Cochrane (1976a).
[d] From Fujikawa *et al.* (1977a).
[e] NR, Not reported.

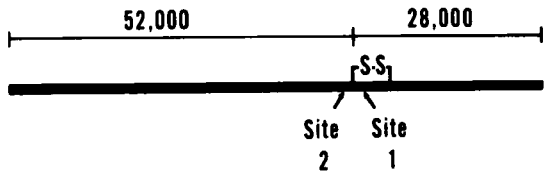

FIG. 2. Cleavage of HF by kallikrein. Cleavage occurs initially at site 1, producing two chain α-HFa. The molecule remains intact by virtue of the S–S linkage. Since the amino acids responsible for binding HF to the negatively charged surface are located on the 52,000 M_r chain, the α-HFa remains surface-bound. Cleavage at site 2 by kallikrein, which occurs after that at site 1, releases the 28,000 M_r fragment β-HFa into the supernatant fluid. The enzymatic site is located on the 28,000 M_r fragment.

second cleavage outside the disulfide bond generates a 28,000-molecular weight active enzyme derived from the carboxy terminal of the molecule (Revak *et al.*, 1974, 1977; Revak and Cochrane, 1976; Fujikawa *et al.*, 1977a). This smaller fragment has been designated β-HFa (Revak *et al.*, 1978) and was the fragment detected originally as a cleavage product of HF in plasma (Kaplan and Austen, 1970) and as an activator of prekallikrein generated by the contact of plasma with negatively charged surfaces (Wuepper *et al.*, 1970) or preparations of antigen and antibody (Soltay *et al.*, 1972). The amino terminal polypeptide of α-HFa has been shown to contain the major binding site for negatively charged surfaces while the carboxy terminal 28,000-molecular weight portion, β-HFa, contains the enzymatic active site (Revak and Cochrane, 1976). Thus, α-HFa binds to negatively charged surfaces whereas β-HFa does not. Activity of the β-HFa light chain was shown not only by its ability to activate prekallikrein (Revak *et al.*, 1977), but also by the binding of antithrombin III (Fujikawa *et al.*, 1977a), [^{3}H]DFP (Griffin, 1977; Griffin and Beretta, 1979; Fujikawa *et al.*, 1977a; Claeys and Collen, 1977; Meier *et al.*, 1977a), and C1 inh (Revak and Cochrane, 1976).

Cleavage of HF in plasma into two chains results principally from the action of kallikrein (Cochrane *et al.*, 1972a, 1973; Bagdasarian *et al.*, 1973; Revak *et al.*, 1974; Weiss *et al.*, 1974; Revak and Cochrane, 1976; Chan *et al.*, 1976; Griffin and Cochrane, 1976b; Griffin, 1978; Fujikawa *et al.*, 1980a), but also occurs through the action of plasmin (Kaplan and Austen, 1971; Burrowes *et al.*, 1972; Revak *et al.*, 1974) and Factor XIa (Revak *et al.*, 1974).

Although complete quantitative data for the enzymatic parameters of these various forms of HFa are not yet available, existing data indicate that both α- and β-HFa molecules are potent prekallikrein activators whereas α-HFa is at least 100 times more active than β-HFa in

the activation of Factor XI (Kaplan and Austen, 1971; Cochrane *et al.*, 1972a, 1973; Revak *et al.*, 1978). The differing potencies of different forms of HFa in activating its various substrates may ultimately allow a quantitative description of preferential activation of different HF-dependent pathways. Further information about the chemical changes accompanying proteolytic activation of HF come from the amino acid sequence studies of Fujikawa *et al.* (1977a, 1980a) on bovine and human α-HFa. Partial amino acid sequence data for bovine α-HFa are presented in Fig. 3 which indicate that cleavage of a specific internal peptide bond is responsible for activation of HF. The amino terminal sequence of the 52,000-MW fragment (or heavy chain) of human HF was found to be identical to that of the intact zymogen molecule starting at residue 12 (Fujikawa *et al.*, 1980a). In addition, the amino-terminal sequences of the 30,000-MW fragment (light chain) of the human and bovine fragments are nearly identical. The sequence data indicate that the 52,000-MW heavy chain derives from the amino terminal, and the 28,000- to 30,000-MW light chain derives from the carboxy terminal portion of the native molecule.

The basic mechanism responsible for the proteolytic activation of HF is most likely the same as that for trypsinogen and chymotrypsinogen (Sigler *et al.*, 1968; Stroud *et al.*, 1975), namely, the formation of an ion pair between the newly formed amino terminal residue of the light chain with the carboxyl group of the aspartic acid residue that is adjacent to the active-site serine (Fig. 3) (Fujikawa *et al.*, 1977a). Cleavage of an internal peptide bond of this type is typical of the activation of all of the coagulation zymogens. For more details, the reader is referred to Davie *et al.* (1979). The important question whether the single chain

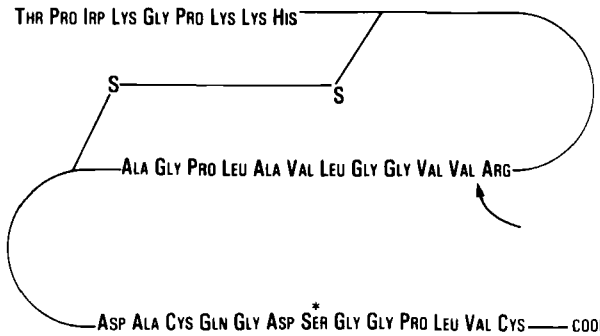

FIG. 3. Partial structure of bovine HF (Factor XII). The kallikrein-cleavage site is noted by the arrow, lying within the disulfide loop of the peptide chain (Fujikawa *et al.*, 1977a).

form of HF is active is discussed below in the section concerning the mechanisms for surface-dependent activation of HF.

Biochemical studies indicate that HFa activates a number of plasma protease zymogens, including prekallikrein (Kaplan and Austen, 1970, 1971; Cochrane and Wuepper, 1971; Takahashi *et al.*, 1972), Factor XI (Ratnoff *et al.*, 1961; Nossel, 1964; Wuepper, 1972; Heck and Kaplan, 1974), Factor VII (Radcliffe *et al.*, 1977; Kisiel *et al.*, 1977; Seligsohn *et al.*, 1979), plasminogen (Goldsmith *et al.*, 1978), HF (Wiggins and Cochrane, 1979; Silverberg *et al.*, 1980), and C1 (Gebrehewit *et al.*, 1981). Based on the reported kinetic studies of the action of HFa on its various substrates, it appears that prekallikrein is highly sensitive to low levels of HFa (Tankersley *et al.*, 1980; Hojima *et al.*, 1980c). In coagulation studies, the action of HFa on Factor XI is well appreciated as highly significant. However, the relatively weak observed action of HFa on Factor VII, plasminogen, HF, and C1 suggests that the physiologic significance of these reactions remains to be defined. In the *in vitro* activation of Factor VII, HFa can exert its influence both directly and indirectly. Seligsohn *et al.* (1979) showed that HFa could work indirectly because kallikrein, generated by HFa, can activate Factor IX and Factor IXa can then activate Factor VII.

B. Plasma Prekallikrein

Plasma prekallikrein has been highly purified from human, bovine, rabbit, and guinea pig plasmas (Wuepper and Cochrane, 1972a; Kaplan and Austen, 1972; Wendel *et al.*, 1972; Laake and Vennerod, 1974; Mandle and Kaplan, 1977; Gallimore *et al.*, 1978a; Takahashi *et al.*, 1972, 1980; Heimark and Davie, 1979; Ulevitch *et al.*, 1980; Bouma *et al.*, 1980b; Yamamoto *et al.*, 1980). Prekallikrein is a glycoprotein that exists as a single polypeptide chain of approximately 80,000–88,000 molecular weight (Table II) and sediments at 4.5 S to 5.2 S in a sucrose gradient (Wuepper and Cochrane, 1972a; Wuepper, 1972). The isoelectric point varies between species, with prekallikrein of human plasma having a p*I* of 8.5 to 9, bovine of 6.98 (Takahashi *et al.*, 1972), rabbit of 5.6 (Wuepper and Cochrane, 1972a), and guinea pig of 9.0 (Yamamoto *et al.*, 1980). Amino acid compositions of rabbit, human, and guinea pig prekallikrein are similar (Ulevitch *et al.*, 1980; Bouma *et al.*, 1980b; Yamamoto *et al.*, 1980). Sequence homologies between bovine prekallikrein and Factor XI have been observed (Heimark and Davie, 1979).

The plasma level of prekallikrein is approximately 50 μg/ml based on radioimmunoassay data (Saito *et al.*, 1978) and Laurell rocket im-

TABLE II
PHYSICAL PROPERTIES OF THE CENTRAL COMPONENTS OF THE CONTACT ACTIVATION SYSTEM OF HUMAN PLASMA

	Molecular weight	Concentration in citrated plasma (μg/ml)	p*I*
Hageman factor	74–80,000	24–40	6.8
Prekallikrein	80–85,000	50	8.5–9.0
Factor XI	160,000 (dimer)	4	8.5–9.0
High-molecular-weight kininogen	110,000	70	4.5

munoelectrophoresis (Bouma *et al.*, 1980a). Earlier immunologic studies overestimated the plasma level of prekallikrein because complex formation between prekallikrein and high-MW kininogen was not taken into consideration. Such complex formation, as discussed below, alters the mobility of prekallikrein in Laurell rocket determinations since it is a γ-globulin and high-MW kininogen is an α-globulin (Table II).

Prekallikrein is activated by limited proteolysis by HFa (Wuepper, 1972; Mandle and Kaplan, 1977; Ulevitch *et al.*, 1980; Bouma *et al.*, 1980b; Heimark *et al.*, 1980; Takahashi *et al.*, 1980). The first cleavage reported that was thought to be associated with activation involved the release of a fragment of 10,000 M_r from rabbit but not human prekallikrein (Wuepper and Cochrane, 1972a). This proved to be a late event, one that follows activation of rabbit but not human kallikrein (Ulevitch *et al.*, 1980). Following reduction, SDS gel analysis of plasma kallikrein of all species studied showed the presence of polypeptide fragments of approximately 55,000 and 35,000 MW (Mandle and Kaplan, 1977; Ulevitch *et al.*, 1980; Wuepper, 1972; Bouma *et al.*, 1980b; Yamamoto *et al.*, 1980). Initial studies of the cleavage and activation of human prekallikrein (Mandle and Kaplan, 1977) reported that activation preceded cleavage of ^{125}I-labeled prekallikrein. However, prekallikrein in these studies was radiolabeled by the chloramine-T method resulting in a labeled protein that is cleaved more slowly than native prekallikrein or than that labeled by the Bolton–Hunter reagent (Bouma *et al.*, 1980b). Recently, it was shown that specific peptide bond cleavage yielding 50,000- and 35,000-MW fragments is associated with acquisition of enzymatic activities attributable to kallikrein (Wuepper, 1972; Johnston *et al.*, 1976; Ulevitch *et al.*, 1980; Bouma *et*

al., 1980b). As demonstrated by the uptake of [^{3}H]DFP, the active site serine resides in the 35,000-MW chain (Johnston *et al.*, 1976; Mandle and Kaplan, 1977; Ulevitch *et al.*, 1980).

The principal actions of kallikrein are several. First, the cleavage of high-MW kininogen at two internal peptide bonds, releasing bradykinin (Nagasawa and Nakayasu, 1973). With bovine kallikrein and bovine high-MW kininogen, a third peptide bond is cleaved releasing a histidine-rich fragment from the light chain, termed fragment 1-2 (Han *et al.*, 1976). A second major function of kallikrein is the cleavage and activation of HF (Cochrane *et al.*, 1972a, 1973; Bagdasarian *et al.*, 1973; Griffin and Cochrane, 1976b; Meier *et al.*, 1977a), a reaction that is greatly potentiated with HF is bound to a surface (Griffin, 1978). The functional significance of these activities will be discussed below.

Plasma kallikrein, in euglobulin fractions derived from plasma or in purified systems, is also capable of cleaving and activating plasminogen. The major HF-dependent activator of plasminogen in early studies was thought to be a distinct protein termed plasminogen proactivator (Kaplan *et al.*, 1972, 1973) or Hageman factor cofactor (Ogston *et al.*, 1969a,b). In the former study the conclusion was based on the observation that the plasminogen proactivator was not inactivated by C1 inh and was present in nearly normal concentration in prekallikrein-deficient plasma. The latter point was underscored in the studies of Mandle and Kaplan (1977). However, more recent studies have shown an apparent absence of detectable HF-dependent plasminogen proactivator activity in the γ-globulin fraction of prekallikrein-deficient plasma (Vennerod and Laake, 1976; Bouma and Griffin, 1978), in support of the earlier finding that euglobulins of prekallikrein-deficient plasma generated only small amounts of fibrinolytic activity (Wuepper, 1973). It is now apparent that C1 inhibitor does inhibit the HF-dependent plasminogen proactivator of plasma as well as kallikrein activity (Laake and Vennerod, 1974; Mandle and Kaplan, 1977).

Bouma *et al.* (1980b) underscored the hypothesis that prekallikrein and HF-dependent plasminogen proactivator are identical. The rate of activation of each by β-HFa was identical, and monospecific antibody to prekallikrein inhibited both zymogen and active forms of prekallikrein and plasminogen proactivator in a parallel manner over a range of antibody dilutions. The data support the original conclusion (Colman, 1969; Wendel *et al.*, 1972) that kallikrein is capable of activating plasminogen and is probably the major HF-dependent plasminogen activator in plasma.

It must be emphasized that the capacity of kallikrein in whole

plasma to cleave and activate plasminogen is quite limited. Surface contact of whole plasma does not result in fibrinolytic activity in the first 15 minutes even though kallikrein has been activated. When ^{125}I-labeled plasminogen was added to plasma and subjected to contact activation so as to convert >80% of the prekallikrein to kallikrein, no detectable cleavage of plasminogen occurred (Cochrane and Revak, 1980a).

Additional activities of kallikrein will be noted in sections on the biologic functions of the Contact System.

C. High-MW Kininogen

Plasma kininogens are large proteins that contain potent vasoactive peptides, the kinins, within their primary sequences (Fujii *et al.*, 1979a,b; Erdos, 1979). Human plasma contains at least two distinct kininogens, high-MW kininogen and low-MW kininogen (Jacobsen and Kriz, 1967; Habal *et al.*, 1974). High MW-kininogen contains approximately one-fifth of the kinin content of plasma and exists as a single polypeptide chain of approximately 110,000 MW (Table II). Low-MW kininogen contains approximately four-fifths of the kinin of plasma and consists of a single polypeptide chain of approximately 60,000 MW. Kinins are released from the internal amino acid sequences of kininogens by limited proteolysis by kallikreins that may be derived from plasma or from tissue sources (Fig. 4). The tissue enzyme is equally potent in releasing kinin from both types of kininogen whereas plasma kallikrein is 40 times more active in liberating bradykinin from high-MW kininogen than from low-MW kininogen.

Although kallikreins and kininogens have been known for a number of years, the central role of high-MW kininogen in plasma contact activation reactions became apparent in 1975. This discovery has en-

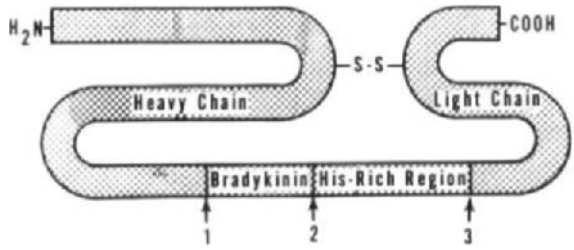

FIG. 4. Structure of high-MW kininogen. The arrows 1 and 2 delineate cleavage points induced by kallikrein in the case of human high-MW kininogen. Arrow 3 marks a further cleavage that occurs in the bovine molecule, liberating the histidine-rich peptide 1-2. (Based on data from Han *et al.*, 1978.)

abled the assembly of many pieces of the molecular puzzle of the contact activation reactions. Highly purified high-MW kininogen has been required for critical biochemical studies, and this molecule has been isolated from human (Habal *et al.*, 1974; Griffin and Cochrane, 1976b; Pierce and Guimaraes, 1976; Meier *et al.*, 1977a; Saito, 1977; Kerbiriou and Griffin, 1979; Nakayasu and Nagasawa, 1979) and bovine (Komiya *et al.*, 1974; Kato *et al.*, 1977; Han *et al.*, 1975, 1978) plasmas. High-MW kininogen is a nonenzymatic cofactor that is central to contact activation reactions (Griffin and Cochrane, 1976b, 1979; Meier *et al.*, 1977a). Low-MW kininogen plays no known role in coagulation or contact activation reactions, although it appears extensively homologous in its amino acid sequence to approximately 60% of the high-MW kininogen molecule (Han *et al.*, 1975, 1978; Kato *et al.*, 1977).

Immunologic studies of high-MW kininogen in human plasma have been presented by several laboratories. The plasma concentration of high-MW kininogen has been reported to be 70 μg/ml based on Laurell rocket immunoelectrophoresis data (Bouma *et al.*, 1980a) and 90 μg/ml based on hemagglutination inhibition assays (Kleniewski and Donaldson, 1977) and on radioimmunoassays (Proud *et al.*, 1980). High-MW kininogen antigen has not been detected in plasma from patients with high-MW kininogen deficiency whereas low-MW kininogen was reported to be present in reduced amounts of plasmas from two out of five patients with high-MW kininogen deficiency. Plasma from three of the five deficient patients that have been studied to date lack both high-MW and low-MW kininogen antigens. In patients with hepatic disease the level of high-MW kininogen is reduced implying that a major site of synthesis of this protein may be the liver.

The carboxy terminal region of high-MW kininogen that has been isolated in highly purified form is responsible for the contact activation cofactor activity of high-MW kininogen (Waldman *et al.*, 1977; Thompson *et al.*, 1978; Kerbiriou and Griffin, 1979; Sugo *et al.*, 1980). The mechanism of action of high-MW kininogen as a cofactor in contact activation reactions will be discussed below.

High-MW kininogen has been shown to contain a most unusual region of amino acid sequence that is rich in histidine, lysine, and glycine (Kato *et al.*, 1977; Han *et al.*, 1975, 1978). In a sequence of 50 amino acids, approximately 30% are histidine, 30% are glycine, and 10% are lysine. Moreover, this portion of the carboxy terminal region of the high-MW kininogen molecule has been shown to be essential for the contact activation cofactor activity (Kato *et al.*, 1977, 1979; Sugo *et al.*, 1980). It is probable that this highly positively charged region of the molecule is responsible for the critical binding of the molecule to negatively charged surfaces.

D. FACTOR XI

Factor XI has been extensively purified from human, bovine, and rabbit plasmas (Wuepper, 1972; Movat and Ozge-Anwar, 1974; Bouma and Griffin, 1977; Kurachi and Davie, 1977; Koide *et al.*, 1977b; Heck and Kaplan, 1974; Wiggins *et al.*, 1979a). Factor XI is a glycoprotein that contains two very similar or identical polypeptide chains that are held together by disulfide bonds. Each polypeptide chain is approximately 80,000 molecular weight although 60,000 is reported for bovine Factor XI (Koide *et al.*, 1977b). When activated, each molecule of Factor XI binds two molecules of antithrombin III indicating that each disulfide-linked chain bears an active site (Kurachi and Davie, 1977). Partial amino acid sequence data for Factor XI are available (Koide *et al.*, 1977a; Kurachi *et al.*, 1980) (Fig. 5). A comparison of these data to sequences for a portion of the amino terminal sequence of bovine prekallikrein indicates considerable homology (Kurachi *et al.*, 1980). It is significant that active Factor XI and kallikrein are inhibited by a common spectrum of inhibitors. The data suggest that the two proteins have evolved from a common ancestor. Some properties of human Factor XI are seen in Table II.

Immunologic studies of Factor XI antigen indicate that normal plasma contains 6 μg/ml based on radioimmunoassay data (Saito and Goldsmith, 1977). These authors also showed that Factor XI antigen was decreased in plasmas from 13 patients with congenital Factor XI deficiency in proportion to the reduction in Factor XI procoagulant activity.

Factor XI is activated to Factor XIa by limited proteolysis by HFa (Wuepper, 1972; Bouma and Griffin, 1977; Kurachi and Davie, 1977; Wiggins *et al.*, 1979b). Appearance of esterase activity of Factor XIa during activation was correlated with extent of cleavage (Kurachi *et al.*, 1980). Cleavage occurs at a single internal arginyl-isoleucine pep-

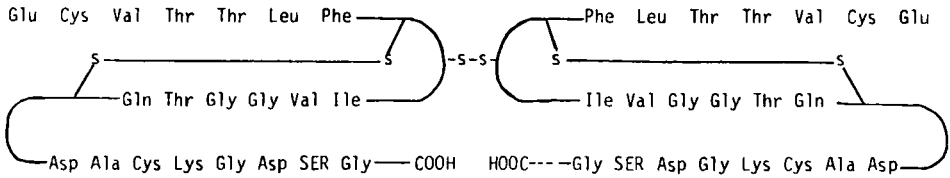

FIG. 5. Partial structure of bovine factor XI. The two nearly identical chains are held together by a disulfide bond(s). The active-site serine is shown in capital letters. The two arrows indicate the site of cleavage in the two chains when the protein is converted to factor XI_a by HFa. The resulting four chains are held together by disulfide bonds. The number and exact location of these disulfide bonds are not known. The dotted line indicates a possible difference in the carboxyl terminal region of one of the light chains of factor XI_a. (From Kurachi *et al.*, 1980.)

tide bond as depicted in Fig. 5 based on amino acid sequence studies of Kurachi and Davie (1977). Each polypeptide chain of Factor XI is cleaved by HFa to give disulfide-linked heavy and light chains. The enzymatic active site is located in the carboxy terminal 30,000-MW polypeptide chain. A detailed discussion of the molecular interactions responsible for the HFa-dependent activation of Factor XI is presented below.

The major activity of Factor XIa is the proteolytic cleavage and activation of coagulation Factor IX (Davie *et al.*, 1979). Minor activities that have been reported include the cleavage and activation of HF (Cochrane *et al.*, 1973; Griffin, 1978; Meier *et al.*, 1977b). The activation of plasminogen by Factor XIa has been reported (Mandle and Kaplan, 1977; Saito, 1980) although further studies will be required to determine the relative importance of Factor XIa.

E. Assays of Activity for Contact Activation System Proteins

The discovery of each of the contact activation system proteins was based on coagulation laboratory data of plasma with prolonged clotting times. Currently a variety of assay techniques are available to measure quantitatively HF, Factor XI, prekallikrein, and high-MW kininogen. These techniques are based either on the activity of the molecule or on the immunologic properties of the molecule. This section is concerned with functional assays for each protein and the following section is concerned with immunologic studies of the contact activation system proteins.

1. Functional Assays for Contact Activation System Proteins

The single most useful assay for identifying and roughly quantitating the activity of contact system proteins is the activated partial thromboplastin time (Proctor and Rapaport, 1961) employing plasmas deficient in the protein of interest. In this coagulation assay, a mixture of the deficient plasma and the test sample is preincubated with kaolin (or with another activating agent) in the absence of calcium ions. This allows contact activation reactions to occur to the extent permitted by the preincubation time and the available factors, and this generates Factor XIa. Calcium ions are not required for contact activation reactions but are necessary for the activation of Factor IX by Factor XIa and for subsequent steps. After a certain preincubation time, calcium ions are added and the clotting time is determined. An important variable in this assay is the preincubation time as seen in the results of Wuepper *et al.* (1975) in Fig. 6. In Fig. 6, the clotting times of plasmas deficient

in HF or in high-MW kininogen (Flaujeac trait) are extremely prolonged and remain so in spite of variation in the preincubation time. The same is also true of Factor XI-deficient plasma. However, as seen in Fig. 6, prekallikrein-deficient plasma (Fletcher trait) exhibits a retarded shortening of the clotting time that eventually becomes normal. This retarded normalization of the clotting time probably reflects a slow proteolytic activation of surface-bound HF (Revak *et al.*, 1977; Tans and Griffin, 1981).

The specific clotting activity of the purified proteins is an important parameter reflecting the purity of the protein. The absolute value varies somewhat from lab to lab and presumably reflects subtle differences in the design of the activated partial thromboplastin time assay. Purified HF varies from 59 (Saito *et al.*, 1974) to 80 units/mg (Griffin

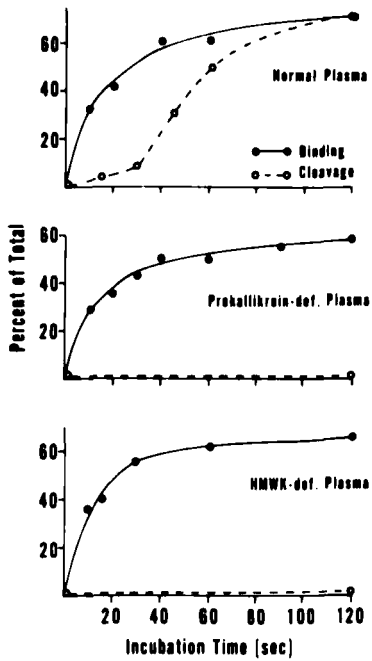

FIG. 6. Rates of binding and cleavage of ^{125}I-labeled Hageman factor in normal and prekallikrein-deficient plasmas upon exposure to glass. Note that while binding of the HF occurs in the deficient plasmas as rapidly as in the normal, cleavage of the HF failed to occur over the interval studied. Addition of purified prekallikrein and high-MW kininogen to the respective deficient plasmas reconstituted the normal rate of cleavage. It should be added that cleavage of the HF in prekallikrein-deficient plasma did occur after 120 seconds, approaching that of normal plasma in 20 minutes. (From Revak *et al.*, 1977.)

and Cochrane, 1976a) where one unit of activity is defined as that amount present in 1 ml of normal plasma. Human Factor XI has a specific clotting activity of 200 to 250 units/mg (Bouma and Griffin, 1977; Kurachi and Davie, 1977). Prekallikrein exhibits 20 to 22 units/mg (Bouma *et al.* 1980b). High-MW kininogen has a specific activity of 12 to 16 units/mg (Griffin and Cochrane, 1976b; Kerbiriou and Griffin, 1979; Thompson *et al.*, 1978).

Other useful assays for prekallikrein are based on the potent arginine esterase or amidase activity of kallikrein (Colman *et al.*, 1969; Kluft, 1978). The esterolytic activity is readily measured using tosyl-Arg-methyl ester or benzoyl-Arg-ethyl ester (Colman *et al.*, 1969; Griffin and Cochrane, 1976a). More recently, arginine oligopeptide substrates with chromophores or fluorophores on the carboxyl terminus have offered greater convenience, specificity, and sensitivity (Amundsen *et al.*, 1976; Iwanaga *et al.*, 1980).

A very useful technique for measuring prekallikrein in plasma was described by Kluft (1978). Plasma is incubated at 4°C for 8 minutes with 50 μg/ml dextran sulfate which activates the contact system and generates kallikrein. The kallikrein is measured by diluting a small aliquot of this mixture directly into a cuvette containing a substrate like B3-Pro-Phe-Arg-paranitroanilide or H-D-Pro-Phe-Arg-paranitroanilide (S-2302, Kabi Inc.). The increase in absorbance at 405 nm due to amidolysis is recorded and converted to units of kallikrein activity.

Bioassays for kinin are useful for measuring high-MW kininogen, kallikrein, prekallikrein, and prekallikrein activator (β-HFa). Bradykinin activity is most easily determined in bioassays of rat uterus contractions; however, such assays are less precise and generally less convenient than coagulation, spectrophotometric, or immunologic assays. The advantage rests in the specificity of the bioassay (for recent reviews, see articles in Fujii *et al.*, 1979a,b).

2. *Immunological Assays for Contact Activation System Proteins*

Proteins of the contact activation process were initially measured by neutralization inhibition assays. When precipitating antibodies became available, radial immunodiffusion techniques were developed to quantitate the proteins. In recent years more sensitive techniques like the Laurell rocket immunoelectrophoresis and the radioimmunoassay have become available. Qualitative analysis of the antigens in normal and patients' plasma were performed by immunoelectrophoresis and crossed immunoelectrophoresis. Immunologic studies of the contact system proteins have been reviewed recently (Bouma and Griffin, 1980).

a. Hageman Factor. Antibodies against HF have been used to study the presence of Factor XII antigen in normal and deficient plasmas. Early studies by Smink *et al.* (1965) using a semiquantitative passive hemagglutination inhibition test suggested that patients who are genetically deficient in HF procoagulant activity also lack HF antigen. The normal plasma level of HF antigen is 29 to 40 μg/ml (Revak *et al.*, 1974; Saito *et al.*, 1976). HF antigen was found to be reduced in plasmas of patients with disseminated intravascular coagulation and hepatic cirrhosis (Saito *et al.*, 1976). Although Factor XII deficiency initially was reported to be associated with a total absence of HF antigen, a more extensive study by Saito *et al.* (1979a) indicated that in the plasma of 2 out of 49 subjects with HF deficiency, nonfunctional material immunologically indistinguishable from normal HF was present.

b. Factor XI. An antibody neutralization assay was used by Forbes and Ratnoff (1972) and by Rimon *et al.* (1976) to study the presence of Factor XI antigen in plasma from patients with Factor XI clotting deficiency. Both studies indicated the absence of Factor XI antigen in plasma from these patients. The level of Factor XI antigen in normal plasma was determined by a radioimmunoassay to be approximately 6 μg/ml (Saito and Goldsmith, 1977). Factor XI antigen was decreased in plasmas from 13 patients with congenital Factor XI deficiency in proportion to the reduction in Factor XI clotting activities (Saito and Goldsmith, 1977). A reduction of Factor XI antigen was also detected in plasma from patients with hepatic cirrhosis (Saito and Goldsmith, 1977).

The use of precipitating techniques to study Factor XI antigen in plasma has been hampered by the low concentration of Factor XI in plasma and the lack of a good precipitating antiserum. Recently a specific precipitating antiserum against Factor XI was prepared (Bouma and Griffin, 1981). Laurell rocket immunoelectrophoresis performed using this antiserum indicated that Factor XI alone does not migrate at pH 8.6. However, when mixed with purified high-MW kininogen, it forms a complex which migrates on Laurell plates and forms precipitin rockets (Bouma and Griffin, 1981).

c. Prekallikrein. Immunochemical studies of plasma kallikrein were reported in 1974 by Bagdasarian *et al.* Using radial immunodiffusion they reported plasma prekallikrein antigen levels of 103 ± 13 μg/ml for normal plasma. Similar values were derived from Laurell rocket immunoelectrophoresis studies (Heber *et al.*, 1978). Later studies reported much lower levels. Based on radioimmunoassay and Laurell rocket immunoelectrophoresis techniques, values of approximately 50 μg/ml were found for normal plasma (Saito *et al.*, 1978;

Bouma *et al.*, 1980a). The reason why the earlier study (Heber *et al.*, 1978) with the Laurell rocket immunoelectrophoresis yielded higher values is probably due to the fact that the complex formation of prekallikrein with high-MW kininogen was not taken into account, as will be discussed below. The absence of prekallikrein antigen in plasma of Fletcher trait patients was reported by several authors (Wuepper, 1973; Saito *et al.*, 1978; Bouma *et al.*, 1980a). However, in several patients with a prekallikrein deficiency, low levels of prekallikrein antigen were detected using the sensitive radioimmunoassay (Saito and Ratnoff, 1977).

Preallikrein circulates in plasma complexed to high-MW kininogen (Mandle *et al.*, 1976; Donaldson *et al.*, 1977). The formation of this complex can be demonstrated by crossed immunoelectrophoresis and by Laurell rocket immunoelectrophoresis (Kerbiriou *et al.*, 1980; Bouma *et al.*, 1980a). Under conditions of the electrophoresis, prekallikrein alone does not migrate. Addition of high-MW kininogen leads to complex formation and, as a result, prekallikrein migrates with an anodal mobility. Using these immunochemical techniques it was shown that high-MW kininogen or the light chain derived from it complexes with prekallikrein or with kallikrein (Kerbiriou *et al.*, 1980; Bouma *et al.*, 1980a). These complexes contain equimolar amounts of each molecule (Kerbiriou *et al.*, 1980).

Prekallikrein antigen levels were measured in plasmas of patients with high-MW kininogen deficiency. Based on radial immunodiffusion technique, values were reported of 0.4 and 0.71 units/ml (Colman *et al.*, 1975; Donaldson *et al.*, 1977), although the latter value was reported to be 0.32 units/ml using a radioimmunoassay (Donaldson *et al.*, 1977). In studies using rocket immunoelectrophoresis no prekallikrein rocket was observed in plasmas of patients with high-MW kininogen deficiency unless the plasmas were reconstituted with normal levels of high-MW kininogen (Bouma *et al.*, 1980a). Prekallikrein antigen levels in such deficient plasmas were found to be 0.3 units/ml (Bouma *et al.*, 1980a). These levels were in agreement with the prekallikrein clotting activity levels observed for these plasmas. Increasing concentrations of high-MW kininogen above 100% of normal did not lead to a further increase in prekallikrein antigen levels. In contrast to the results of Bouma *et al.* (1980a), a normal level of prekallikrein antigen which was reported for four patients with high-MW kininogen deficiency after addition of high-MW kininogen to these plasmas (Colman and Scott, 1979). The basis for this disagreement is not clear.

For the measurement of prekallikrein antigen storage of plasma at 4°C should be avoided. Cold-promoted activation of prekallikrein was shown to take place and this leads to an erroneous increase in prekal-

likrein antigen rockets (Bouma *et al.*, 1980a). Storage for a short period at room temperature seems clearly preferable, or plasma samples should be frozen as quickly as possible and thawed just prior to analysis.

Prekallikrein antigen levels were reported to be decreased in hepatic cirrhosis, disseminated intravascular coagulation, chronic renal failure, and nephrotic syndrome (Saito *et al.*, 1978). During typhoid fever, a decrease in prekallikrein clotting activity was found (Colman *et al.*, 1978). Prekallikrein antigen levels, however, apparently remained the same, and crossed immunoelectrophoretic analysis of these plasma samples showed complexes of kallikrein with $C\bar{1}$ inh (Colman *et al.*, 1978).

d. High MW and Low MW Kininogens. Human plasma contains at least two distinct kininogens, high-MW kininogen and low-MW kininogen, that are single polypeptide chains of 110,000 and 50,000 to 78,000 MW, respectively. Human plasma kallikrein cleaves human high-MW kininogen to give a kinin-free molecule made up of a 65,000-MW heavy chain and a 44,000-MW light chain (Han *et al.*, 1978; Kato *et al.*, 1977). Following reduction and aklylation of the kinin-free molecule, the alkylated light chain can be separated from the alkylated heavy chain (Thompson *et al.*, 1978; Kerbiriou and Griffin, 1979). Antisera raised against the isolated alkylated heavy chain precipitate both high- and low-MW kininogens (Kerbiriou and Griffin, 1979). Anti-light chain antisera exclusively precipitate high-MW kininogen (Kerbiriou and Griffin, 1979). These antisera were used to measure the levels of high- and low-MW kininogens in normals and in patients with high-MW kininogen deficiency (Bouma *et al.*, 1980a). Laurell rocket immunoelectrophoresis gave a level of high-MW kininogen in 20 normal subjects of 70 μg/ml (Bouma *et al.*, 1980a). Based on hemagglutination inhibition assays (Kleniewski and Donaldson, 1977) or radioimmunoassays (Proud *et al.*, 1979), a normal level of 90 μg/ml has been reported. High-MW kininogen antigen was absent from plasma of patients with high-MW kininogen deficiency (Kleniewski and Donaldson, 1977; Donaldson *et al.*, 1977; Bouma *et al.*, 1980a; Proud *et al.*, 1979). Low-MW kininogen was present in reduced amounts in plasmas of two patients with high-MW kininogen deficiency and absent from three other plasmas (Colman *et al.*, 1975; Bouma *et al.*, 1980a; Oh-Ishi *et al.*, 1979).

IV. Surfaces That Induce Contact Activation

Contact of plasma with a variety of negatively charged surfaces leads to activation of the HF–kallikrein system. A list of inorganic and

organic substances capable of serving as an activating surface is given in Table III.

The importance of the negative charge of the surface has been shown by relating the capacity of surfaces of a variety of agents to bind basic dyes and their ability to promote activation of HF and induce clotting (Margolis, 1963). In addition, pretreatment of surfaces with molecules bearing positive charges, such as hexadimethrene bromide, cytochrome c, or silicone prevents activation of the contact system (Eisen, 1964; Haanen *et al.*, 1961; Nossel *et al.*, 1968). As binding of purified HF still occurs to the surface that is previously treated with silicone or hexadimethrine bromide (Cochrane, *et al.*, 1972a) the inhibitory effect must in part be caused by a diminution of the total negative charges on the surfaces.

A. Monosodium Urate Crystals

Crystals of monosodium urate, calcium pyrophosphate, and hypoxanthine bear a negative charge as demonstrated by migration in a charged field. These crystals, when added to normal but not HF-

TABLE III
SURFACE ACTIVATORS OF THE CONTACT SYSTEM[a]

Inorganic substances	Organic substances
Silica dioxide (2)	Microcrystalline sodium urate (4,19)
Glass (1,3)	Bacterial lipopolysaccharide (8)
Kaolin (1,3)	Ellagic acid (9)
Diatomaceous earth (1)	Carageenan (11)
Asbestos (1)	Collagen [12,13,14,15 (active), 20,21 (inactive)]
Talc (1)	Vascular basement membrane (16)
Barium carbonate (1)	Articular cartillage, chondroitin sulfate (17)
Celite (1,3)	Long chain fatty acids (5,6,7,18,20,23,24)
	Heparin (17)
Calcium pyrophosphate (4)	Dextran sulfate (22,20)
Sulfated cellulose (10)	Glycolipids (sulfatides, gangleocide 20)
	Skin (23,24)

[a] 1. Ratnoff (1966); 2. Margolis (1961); 3. Margolis (1963); 4. Ratnoff (1969); 5. Margolis (1962); 6. Botti and Ratnoff (1963); 7. Didisheim and Mibashan (1963); 8. Morrison and Cochrane (1974); 9. Ratnoff and Crum (1964); 10. Kellermeyer and Kellermeyer (1969); 11. Schwartz and Kellermeyer (1969); 12. Niewiarowski *et al.* (1964); 13. Niewiarowski *et al.* (1965); 14. Wilner *et al.* (1968); 15. Cochrane *et al.* (1972a,b); 16. Cochrane and Wuepper (1972); 17. Moskowitz *et al.* (1970); 18. James and Wheatley (1956); 19. Ginsberg *et al.* (1980); 20. Fujikawa *et al.* (1980a); 21. Griffin *et al.* (1975); 22. Kluft (1978); 23. Ogston *et al.* (1969a); 24. Nossel (1966).

deficient plasma, decreased the recalcified clotting time (Kellermeyer and Breckenridge, 1965). Addition of purified HF to urate crystals reconstituted the clotting time of HF-deficient plasma. As joint fluid contains components of the contact system, it also supports activation of HF by urate crystals (Kellermeyer and Breckenridge, 1965, 1966, 1967). Mixtures of urate crystals and plasma or joint fluid generate a factor that induces increased vascular permeability in guinea pig skin. Antibodies to HF block the generation of this activity (Kellermeyer and Breckenridge, 1967). A molecular understanding of these reactions was obtained by adding ^{125}I-labeled HF to the plasma or joint fluid prior to addition of urate crystals. Under these conditions, the ^{125}I-labeled HF was cleaved to yield α-HFa and β-HFa (Ginsberg *et al.*, 1980). The cleavage failed to occur in 20 minutes incubation in prekallikrein-deficient or high-MW kininogen-deficient plasmas. Treatment of the crystals with hexadimethrine bromide abolished their ability to induce cleavage of ^{125}I-labeled HF in whole plasma.

B. Collagen and Vascular Basement Membrane

The extravascular space, rich in negatively charged connective tissue components, must be considered a prime area for the surface activation of the contact system. Such surfaces could presumably bind HF present in the interstitial fluid. Under normal conditions it is possible that prekallikrein entrance into the interstitial space would be minimal owing to its complexing with high-MW kininogen in the plasma. However, data in these matters are insufficient for a clear understanding of the molecular relationships.

Preparations of human and bovine collagen have been shown to decrease the clotting time of normal human plasma (Wilner *et al.*, 1968). The collagen increased the clot-promoting activity of purified HF and exerted no augmentation of clotting when combined with known activators of HF. While the observation was previously made that bovine collagen (and elastin) specifically removed HF from plasma (Niewiarowski *et al.*, 1964, 1965), these results were not confirmed by Wilner *et al.* even though HF was apparently absorbed to their preparations of collagen. Esterification of carboxyl groups of glutamic and aspartic acids almost completely abolished the clot-promoting capacity of the collagen as did pretreatment with several cationic proteins to block the negative charge (Wilner *et al.*, 1968). Harpel (1972) observed that the exposure of calf skin collagen preparations to plasma generated kallikrein activity as evidenced by its ability to generate kinin in heated plasma and by the fact that this activity was inhibited by agents known to block kallikrein. Modification of the

carboxyl but not the amino groups of the collagen inhibited the capacity of the collagen to activate the kallikrein. Acid-solubilized and reprecipitated calf skin collagen bound HF and initiated contact activation (Cochrane *et al.*, 1972a).

By contrast, Griffin *et al.* (1975) were unable to demonstrate activation and cleavage of HF by a highly purified soluble human collagen. Fujikawa *et al.* (1980a) similarly found soluble calf skin collagen and purified rat skin collagen (types I and III) to be inactive in the surface activation of HF. In the latter study, small quantities (0.5 to 10 μg in 50 μl) were employed as opposed to 4 to 32 μg in the study by Wilner *et al.* (1968). The amount of surface area available and charge of the preparation are also of great importance and may account for the differences, and in cases where contact activation was observed, the presence of minor contaminants must be considered. Further studies are required to answer these questions.

Preparations of vascular basement membrane obtained from rabbit kidney by homogenization and sonication were found to bind and lead to activation of HF after addition of prekallikrein (Cochrane and Wuepper, 1972). Pretreatment of the basement membrane with hexadimethrine bromide blocked the activation.

C. Sulfatide

Bovine brain sulfatides have been observed to serve as a surface for the activation of HF by kallikrein, plasmin, and Factor XIa (Fujikawa *et al.*, 1980a). Using less than 2 μg of this sulfonated glycolipid in 60 μl volume, bovine HF was cleaved into its characteristic fragments (46,000 and 28,000 M_r) at the specific arginyl-valine peptide bond of the precursor molecule. Of interest, the presence of high-MW kininogen inhibited the action of kallikrein on the HF when sulfatide was present. Sulfatides also stimulated the activation of Factor XI by α-HFa (Kurachi *et al.*, 1980). Sulfatides are very procoagulant when added to plasma. ^{125}I-labeled HF is cleaved in plasma when sulfatides are added (Tans and Griffin, 1981). The time course of HF cleavage in plasma correlates with the appearance of procoagulant activity (Tans and Griffin, 1981).

D. Bacterial Lipopolysaccharides (LPS)

Several studies have indicated that LPS may activate HF and its related systems in plasma (Mason *et al.*, 1970; Kimball *et al.*, 1972; Rodriquez-Erdman, 1964). Employing a highly purified, chemically characterized LPS of *Escherichia coli* 0111:B4, it was possible to determine the aspect of the LPS molecule responsible for activation of

HF and the mechanism by which this is accomplished (Morrison and Cochrane, 1974). LPS consists of polysaccharide containing the O-antigen and lipid A, each linked to a heptose backbone. By employing LPS rich or poor in lipid A and genetic variants consisting only of lipid A and the 3-keto-2-deoxy-octulosonate linkage group, attention was drawn to the lipid A portion of the LPS. Removal of the lipid A by mild acid hydrolysis eliminated the capacity to activate HF in proportion to the amounts of lipid A removed. In addition, lipid A itself was found capable of activating HF. Lipid A consists of two glucosamines linked glycosidally with lipid side chains (R) and phosphate groups. The nature of the phosphate groups is not yet certain; but it appears that they give the majority, if not all, of the negative charges to the lipid A. The LPS employed was soluble, and it was possible to demonstrate binding of the ^{125}I-labeled HF to LPS by ultracentrifugation in sucrose.

E. Antigen–Antibody Complexes

It was initially suggested that antigen–antibody complexes were capable of activating HF, and thereby the kinin-forming and intrinsic clotting systems (Davies and Lowe, 1960, 1962; Davies *et al.*, 1967; Movat, 1967; Movat and DiLorenzo, 1968; Movat *et al.*, 1968, 1969; Eisen and Smith, 1970; Kaplan *et al.*, 1971, 1972; Epstein *et al.*, 1969). In our initial experiments, the capacity of antigen–antibody aggregates to activate HF was about 1/100th that of kaolin (Cochrane and Wuepper, 1972). Further studies failed to support an activating role of immune reactants. Antigen–antibody complexes prepared in different ratios and aggregated immunoglobulins (Igs) of each class and subclass of Ig failed to activate HF, as determined by both clotting and capacity to activate prekallikrein. In each case, the HF was still fully subject to activation by kaolin or trypsin in the presence of each immunologic reactant. Immune complexes and aggregated Ig failed to bind ^{125}I-labeled HF as determined by ultracentrifugation (Cochrane *et al.*, 1972b). In the few Ig preparations found capable of activating HF, bacterial contamination was present. The bacteria obtained by culture activated HF as well. Thus, in spite of many early reports to the contrary, it appears that antigen–antibody complexes do not activate the HF systems.

F. The Role of Negatively Charged Surfaces in Activation of the Contact System

Surfaces play an essential role in assembling the proteins of the contact system. As will be discussed in detail below, when a nega-

tively charged surface interacts with plasma, HF, prekallikrein, high-MW kininogen, and Factor XI assemble on the surface. Positively charged amino acid residues on the heavy chain of HF bind the HF to the negative charges of the surface, and the histidine-rich portion of the light chain of high-MW kininogen binds the kininogen to the surface together with its associated (complexed) prekallikrein or Factor XI. The immediate apposition of HF and prekallikrein then triggers the activation as discussed below.

Surface binding also alters the HF molecule providing greater susceptibility to the limited proteolytic cleavage associated with full activation of the HF. Griffin (1978) measured an increase of 500-fold in the susceptibility to cleavage by plasma kallikrein, 100-fold by plasmin, and 30-fold by Factor XIa. Cleavage of the HF resulted in the production of 52,000- and 28,000-MW fragments. Activation of only 0.04% of the prekallikrein in plasma, yielding 0.2 n*M* kallikrein, would be sufficient to trigger extensive activation of plasma levels of surface-bound HF.

Since the proposal of Margolis (1963), the theory has been considered that upon binding to a negatively charged surface, the conformation of HF is altered to expose an enzymatic site. A discussion of the data relating to this theory is offered below (Sections V and VI).

Another effect of the negatively charged surface may be its capacity to alter HF or high-MW kininogen in a manner that promotes greater binding of the other molecule (HF or high-MW kininogen) onto the surface, presumably adjacent to the first molecule bound (Wiggins *et al.*, 1977). Additional studies must be performed to verify this potentially important phenomenon.

V. Initial Events in Contact Activation

A. Does Binding to a Surface Activate Single-Chain Hageman Factor?

The triggering event in the activation of HF or prekallikrein on the surface has been studied in several laboratories. The hypothesis has been advanced that upon contact with the negatively charged surface a change occurs in the structure of the molecule exposing the active site of the enzyme (Margolis, 1963). The hypothesis was furthered by Donaldson and Ratnoff (1965) in studies in which HF in the presence of ellagic acid sedimented in a 5–20% sucrose gradient to the bottom of the tube within 24 hours at 105,000 *g*, rather than at the expected 4.5 S position in the sucrose gradient. Although it was unclear whether the

ellagic acid under the conditions of sedimentation had undergone aggregation, it seems likely. Hence, HF probably pelleted with particulate ellagic acid. McMillin *et al.* (1974) studied changes in the circular dichroism spectrum of HF after treatment with ellagic acid or application to quartz surfaces, and they observed changes compatible with alterations of the molecular conformation. Fair *et al.* (1977) using the hydrophobic fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene, which gives greater fluorescence in a hydrophobic medium, observed greater fluorescence of the probe when exposed to HF in the presence of ellagic acid than in its absence. When HF is surface bound it becomes one to two orders of magnitude more susceptible to cleavage and activation by kallikrein, plasmin, and activated Factor XI (Griffin, 1978) suggesting that cleavage sites are more readily available to several enzymes in the surface-bound HF.

Heimark *et al.* (1980) presented data that were interpreted to show that single chain bovine HF in the presence of high-MW kininogen and kaolin would initiate the cleavage and activation of prekallikrein. It was concluded that single chain HF is as active as two-chain α-HFa in this activation of prekallikrein. The conclusion was based upon experiments showing a lack of lag phase in the time course of the activation of prekallikrein by single chain HF as compared to two chain α-HFa over a period of minutes (Heimark *et al.* 1980). The initial rates of conversion of prekallikrein to this active form by single chain HF and two-chain α-HFa were comparable, with data points taken at approximately minute to 2-minute intervals initially. The possible cleavage of the single chain [^{3}H]HF was assessed and showed 35% of the HF to be cleaved in the reaction mixture by 2.5 minutes. A similar study was performed on the activation of Factor XI by HF or two chain α-HFa (Kurachi *et al.*, 1980; Heimark *et al.*, 1980). The conclusion drawn from these studies was that surface-bound, single chain HF in the presence of natural substrates, prekallikrein or Factor XI, is fully active, possibly due to substrate-induced activation of the HF zymogen. In these experiments, more than a 60-fold excess concentration of prekallikrein or Factor XI than occurs in normal plasma was used. As noted above, kallikrein at normal concentrations rapidly dissociates from the surface to cleave and activate surface-bound HF. Additional prekallikrein is able to cycle by the surface-bound α-HFa where it undergoes activation. In view of a more than 60-fold excess of prekallikrein, 35% cleavage of the HF by 2.5 minutes and a lack of time points shown in the first 60 seconds of the reaction, the conclusion that single chain HF was fully active in prekallikrein activation must be questioned.

Ratnoff and Saito (1979a,b) examined amidolytic properties of HF which was exposed to ellagic acid–Sephadex and was then eluted. About 10% of the HF appeared in the supernatant for study. Amidolytic activity was present and electrophoretic analysis of ^{125}I-labeled HF (30 μCi/μg) failed to reveal cleavage of the HF. While the data were interpreted to suggest activation of single chain HF had occurred upon exposure of the HF to the Sephadex–ellagic acid, this conclusion is in doubt because the authors did not report the specific amidolytic activity of HFa. In fact, the specific activity of HFa is much higher than the observed activity of Ratnoff and Saito (1979b) (Greengard and Griffin, 1980; Fujikawa *et al.* 1980b). Thus, the conclusion that single chain HF was active appears uncertain. A means by which highly purified HF can undergo cleavage and activation on a surface will be discussed below.

B. Evidence That Proteolytic Cleavage of HF Is Associated with the Rapid Activation of HF on a Surface

As was noted above, during a reciprocal interaction of HF and prekallikrein on a surface in the presence of high-MW kininogen, a burst of activity takes place resulting in a matter of seconds in the cleavage of HF and the activation of prekallikrein. The cleavage of the surface-bound HF into 52,000 and 28,000 M_r chains failed to occur in plasma deficient in prekallikrein or high-MW kininogen in the critical first 120 seconds of the reaction (Fig. 6), while in normal plasma, extremely rapid cleavage of the HF took place (Revak *et al.*, 1977). The HF was bound to the glass surface at the same rate in each situation. As shown in many laboratories, HF-dependent activities in prekallikrein or high-MW kininogen-deficient plasmas are only slowly expressed. This suggested that the mere binding of HF to negatively charged surfaces, in the absence of cleavage, does not result in activation in the first minutes. Data consistent with this hypothesis have been obtained in studies correlating the kaolin-dependent and sulfatide-dependent cleavage of ^{125}I-labeled HF in plasmas with the shortening of the observed clotting times (Tans and Griffin, 1981) and in studies correlating HF-coagulant activity with celite-dependent cleavage of ^{125}I-labeled HF (Walsh and Griffin, 1980). The reaction of purified HF with [^{3}H]DFP occurred about 600-fold more rapidly when HF was in the two-chain α-HFa form, and the slow uptake of DFP by single chain HF occurred at the same rate whether the HF was soluble or surface-bound (Griffin, 1977; Griffin and Beretta, 1979). HF, bound to kaolin, was found by Wiggins and Cochrane (1979) to cleave Factor XI when

present in two chain α-HFa form. The extent of the HF-induced cleavage of the Factor XI occurred in proportion to the amount of two chain α-HFa present. Similarly, the procoagulant activity of the surface-bound HF was proportional to the extent of two chain α-HFa present. Moreover, surface-bound HF does not exhibit significant enzymatic activity against small peptide or ester substrates (Fujikawa *et al.*, 1977a; Claeys and Collen, 1978). Substantial esterase and amidase activity is associated with the two chain bovine α-HFa molecule (Heimark *et al.*, 1978). Beretta and Griffin (1979), using prekallikrein treated for a prolonged period with DFP so as to saturate its active site (DIP-PK) and prevent the reciprocal activation of HF, found that surface-bound single chain HF was incapable of cleaving the DIP-PK while in controls β-HFa rapidly cleaved the DIP-PK into the heavy and light chains characteristic of its activation.

These data support the contention that α-HFa is much more active than HF and that limited proteolytic cleavage of surface-bound HF into disulfide-linked 52,000-MW and 28,000-MW chains is a prerequisite for the burst of activation of the contact system in plasma. Evidence that kallikrein is the enzyme principally involved in the cleavage of HF was presented above.

C. The Triggering Event

The question of the triggering event that initiates cleavage of surface-bound single chain HF and prekallikrein (complexed with high-MW kininogen) remains unanswered. Potentially, an extraneous enzyme, so far escaping identification, could cleave HF or prekallikrein to initiate the burst of activity. Another possibility is that both HF and prekallikrein possess a very low level of enzymatic activity which, in the case of HF, is not increased when the molecule becomes surface-bound as discussed above. Evidence of low-level activity has been obtained in two ways: first, single chain HF and prekallikrein were observed to react with [^{3}H]DFP at a slow rate, with a second order rate constant of 0.24 M^{-1} min^{-1} (compared to the binding of [^{3}H]DFP by two chain α-HFa at a second order rate constant of 150–170 M^{-1} min^{-1} (Griffin and Beretta, 1979; Griffin and Cochrane, 1979). This slow binding of DFP compares favorably with that of trypsinogen. Second, when single-chain HF was brought onto a surface in optimal proportions of protein to surface, proteolytic cleavage of the molecules slowly developed (Wiggins and Cochrane, 1979). This was observed even with prior treatment of the HF with DFP. This did not occur when the molecules were bound to the surface in insufficient concentration. This has been interpreted to be an "autoactivation" of

HF, and the cleaved, surface-bound α-HFa possessed procoagulant activity. The rate of cleavage was similar to that of the uptake of [^{3}H]DFP by single chain HF. Similar data on the "autoactivation" of HF were subsequently reported by Silverberg *et al.* (1980) who used a synthetic substrate to monitor the activation of HF bound to quartz. These latter authors interpreted their data to indicate that a very small portion of the isolated HF was in the active form, α-HFa, and that treatment with DFP failed to inactivate all of the HFa molecules present.

VI. Assembly and Activation of the Molecules of the Contact System upon Exposure to a Negatively Charged Surface

We have described in the sections above the capacity of HF and high-MW kininogen to bind to negatively charged surfaces. The question of whether the act of binding of HF, i.e., without any other molecules present, renders the molecule active was also discussed. The accumulated data suggest that surface-bound HF in the presence of plasma components undergoes rapid activation requiring, for maximal activity, limited proteolytic cleavage of HF by kallikrein. The purpose of the following section describes the molecular interactions of HF, prekallikrein, and high-MW kininogen that leads to generation of activated HF and kallikrein and cleavage of high-MW kininogen that releases bradykinin. The hypothesized assembly is illustrated in Fig. 7. Evidence suggesting this hypothesis is given in the remainder of Section VI. Factor XI activation results from the conversion of HF to its enzymatically active state, α-HFa.

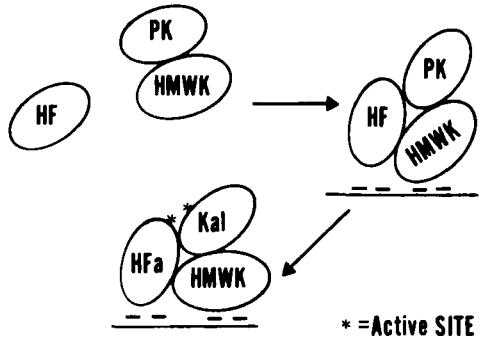

FIG. 7. Hypothetical assembly of proteins on a negatively charged surface that renders activity of the contact system.

A. A Role of Prekallikrein in the Activation of HF

When HF binds to a surface in the presence of plasma, rapid cleavage and activation of the HF takes place on the surface. A number of studies have indicated that kallikrein is the enzyme responsible for this action. This began with the rather surprising observation that when kallikrein was added to rabbit plasma in plastic containers, a dose-dependent shortening of the clotting time was observed (Wuepper and Cochrane, 1972b). α_2-Macroglobulin and soybean trypsin inhibitor both blocked the activation of the kallikrein (Wuepper and Cochrane, 1972b). The reason for the procoagulant activity became apparent when human kallikrein was observed to activate HF providing the HF with both its procoagulant and prekallikrein-activating capacities (Cochrane *et al.*, 1972a, 1973). Prekallikrein, when substituted for kallikrein, was found at higher concentrations to stimulate the activity of the HF, leading to the speculation that trace activity of either the HF or prekallikrein could lead to a reciprocal activation of the two molecules. While the above studies were performed with proteins in solution, when a surface is provided, HF is rapidly activated by PK (Griffin, 1978). Kallikrein was found to be considerably more active in cleaving surface-bound HF than other plasma proteases such as plasmin and clotting Factor XI (see below).

Strong evidence supporting the dominant role of kallikrein in the activation of HF was obtained through studies with Fletcher trait plasma. As had been described by Hathaway and his colleagues (Hathaway *et al.*, 1965; Hathaway and Alsever, 1970) and confirmed by others (Edson *et al.*, 1967; Pechet *et al.*, 1975), Fletcher trait plasma demonstrated a clotting defect in the early components of the intrinsic clotting pathway. The missing factor chromatographed independently of Factor XI and HF (Hathaway and Alsever, 1970) and plasma deficient in HF, Factor XI, and other known coagulation components corrected the Fletcher trait plasma. In 1973, Wuepper identified the missing protein as plasma prekallikrein by restoration of function of the deficient plasma and by immunoprecipitation techniques. Several functions of the contact system were found deficient in this plasma: clotting activity (kaolin-plasma thromboplastin test, kaolin-PTT), kinin, and PF/dil formation and plasmin generation (using euglobulin fractions in the latter test). Since each of these activities required activity of HF, especially PF/dil which has been found to be immunologically identical to HF (Johnston *et al.*, 1974), it appeared that the prekallikrein was important in bringing about contact activation of HF. The capacity of prekallikrein to restore fibrinolytic, clotting, and

kinin-generation activities to Fletcher trait plasma was confirmed by Weiss *et al.* (1974). In addition, these authors demonstrated that addition of activated HF, either in intact form (α-HFa), or the 28,000-M_r fragment (β-HFa) induced fibrinolytic and coagulation activity of Fletcher trait plasma but not kinin-forming activity. The latter would not be expected in the absence of prekallikrein. If prekallikrein is really the major HF-dependent plasminogen activator in plasma, the mechanism of the reconstitution of plasminogen activation by introduction of HFa to Fletcher trait plasma is unclear.

In studies of the cleavage of HF in plasma exposed to glass, as noted previously, Revak *et al.* (1977) observed that proteolytic cleavage of HF into heavy and light chains failed to occur in the absence of prekallikrein over a period of 120 seconds even though HF was bound to the surface. Restoration of prekallikrein reconstituted rapid cleavage.

The requirement of prekallikrein for full expression of HF activities in plasma was confirmed in several laboratories (Weiss *et al.*, 1974; Donaldson *et al.*, 1974; Saito *et al.*, 1974). Addition of activated HF was found to correct the coagulation and fibrinolytic defects of Fletcher trait plasma (Weiss *et al.*, 1974; and Saito *et al.*, 1974), and, as well, a chemotactic defect, purported to be mediated by kallikrein (Weiss *et al.*, 1974). Thus when the HF was previously activated, the participation of prekallikrein was not required. Removing prekallikrein with purified antibody blocked the kaolin-activated partial thromboplastin time of human plasma while not affecting the activities of other known components of the contact system (Saito and Ratnoff, 1974).

Prolonged exposure of Fletcher trait plasma to glass or kaolin, i.e., for longer than 5 minutes, resulted in a complete correction of the clotting deficiency (Hathaway and Alswer, 1970; Hattersley and Hayes, 1970) (Fig. 8). Gradual cleavage of ^{125}I-labeled HF into heavy and light chains in Fletcher trait plasma also develops over a prolonged period of exposure to kaolin (Revak *et al.*, 1977). This slow cleavage correlates with the appearance of HF-dependent procoagulant activity (Tan and Griffin, 1981). The enzyme responsible for the slow cleavage and activation of the HF has not been unequivocally identified, although plasmin and Factor XIa are possibilities.

B. High-Molecular-Weight Kininogen. A Cofactor in the Activation of the Contact System

In 1973, evidence was obtained that partially purified HFa would not adequately activate partially purified Factor XI unless diluted

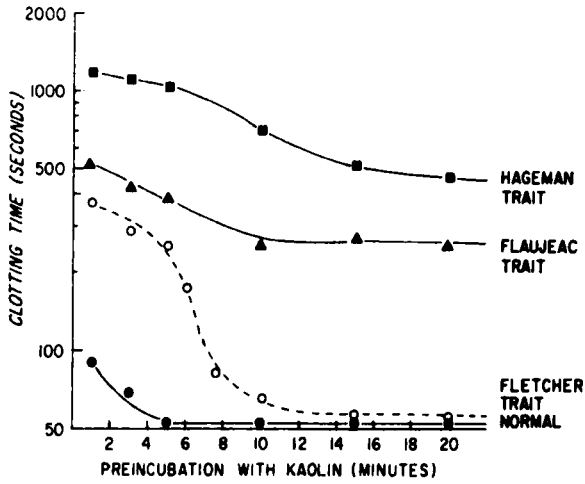

FIG. 8. Clotting times of plasmas deficient in HF, prekallikrein, and high-MW kininogen as a function of time of preincubation with a surface (kaolin) before recalcification. After exposure of prekallikrein-deficient plasma to the surface in the absence of Ca^{2+}, the plasma gradually acquires the capacity to clot when Ca^{2+} is added. This is associated with cleavage of the HF. Hageman trait, HF-deficient plasma; Flaujeac trait, high MW-kininogen-deficient plasma; Fletcher trait, prekallikrein-deficient plasma. (From Wuepper *et al.*, 1975.)

plasma was added to the mixture (Schiffman and Lee, 1974), and it was concluded that the plasma provided an essential cofactor. In a subsequent study the missing substance was partially purified by Schiffman and Lee and termed contact activation cofactor (CAC) (Schiffman and Lee, 1975). At this time, several plasmas were independently observed (Waldmann and Abraham, 1974; Lacombe, 1975; Lacombe *et al.*, 1975; Colman *et al.*, 1975) that failed to clot normally on addition of calcium and were not deficient in any known coagulation component. Antibodies to proteins in the α-globulin region of human plasma, when tested against one of these deficient plasmas (Flaujeac trait), disclosed the presence of each protein except kininogen and fractions of normal plasma rich in high-MW kininogen were found to reconstitute the deficiency (Wuepper *et al.*, 1975). The deficiency of high-MW kininogen was soon recognized in each of these deficient plasmas (Wuepper *et al.*, 1975; Colman *et al.*, 1975; Donaldson *et al.*, 1976). Biologic activities associated with the contact system, including fibrinolysis, esterase activity, and vascular permeability effects, were deficient in these plasmas (Lacombe *et al.*, 1975; Saito *et al.*, 1975; Wuepper *et al.*, 1975), and addition of high-MW kininogen corrected each of the de-

fects (Wuepper *et al.*, 1975; Matheson *et al.*, 1976; Colman *et al.*, 1975; Donaldson *et al.*, 1976).

The identity of high-MW kininogen and contact activation cofactor was subsequently determined immunologically and functionally (Schiffman *et al.*, 1975, 1977).

1. The Mechanism of Action of High-MW Kininogen

With the identification of high-MW kininogen as the agent required for full activity of the contact system in the deficient plasmas, attention was turned to the mechanism by which this molecule acted. It was reasoned that the effect of high-MW kininogen could be explained by an ability of kallikrein to activate HF more readily, that prekallikrein could be more readily activated by HFa, that both of these could be true, or that some other mechanism was responsible. Employing purified human components, Griffin and Cochrane (1976b) observed that the activation of prekallikrein by HFa on a negatively charged surface was augmented 20- to 30-fold by the presence of high-MW kininogen employing concentrations of the proteins similar to those found in normal plasma. The reciprocal reaction was also accelerated in that kallikrein was 10-fold more effective in cleaving HF on a surface in the presence of physiologic concentrations of high-MW kininogen (Griffin and Cochrane, 1976b). Meier *et al.* (1977a) observed the augmented activation of prekallikrein by α-HFa in the presence of high-MW kininogen and a surface. Liu *et al.* (1977) reported a 2- to 3-fold increase in prekallikrein activation by β-HFa in solution. The optimal concentration of high-MW kininogen required for these effects was equimolar to the concentration of HF present, suggesting a stoichiometric relationship of the two molecules on the surface. This indicated that the action of high-MW kininogen was that of a cofactor rather than as a catalytic agent. Indeed, no enzymatic activity has yet been ascribed to high-MW kininogen.

It was possible to conclude that three molecules, HF, prekallikrein, and high MW-kininogen, were found to comprise the principal proteins responsible for the generation of contact activity, since the rate of activation of Factor XI (Griffin and Cochrane, 1976b) was found to be the same in whole plasma and with mixtures of purified components at comparable concentrations (Fig. 9).

2. The Mechanism of Action of High-MW Kininogen in the Activation of Hageman Factor

Prekallikrein in whole plasma was observed by Nagasawa and Nakayasu (1973) to possess a molecular weight of approximately

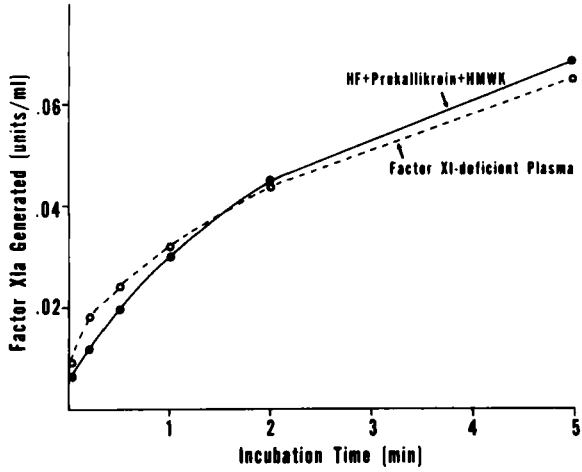

FIG. 9. Time course for the activation of Factor XI by mixtures of purified coagulation factors (HF, prekallikrein, and high-MW kininogen) or by an equivalent aliquot of Factor XI-deficient, showing that a mixture of preparations of HF, prekallikrein, and high-MW kininogen activates Factor XI as effectively as plasma does.

300,000, i.e., in excess of what would be predicted from the physical characteristics of the purified molecule. The authors suggested the existence of a physical complex between prekallikrein and another plasma protein. Wendel *et al.* (1972) reported that prekallikrein was complexed with an acidic plasma protein. These interesting observations were explained 3 years later by the finding that prekallikrein complexed with high-MW kininogen in whole plasma (Mandle *et al.*, 1976). The elution of prekallikrein from a molecular exclusion column was compared in the presence or absence of high-MW kininogen. In the presence of the high-MW kininogen, the prekallikrein eluted with an apparent molecular weight of 285,000 as opposed to 115,000 in the absence of high-MW kininogen. Comparisons were made using both plasma and purified components. Since the two molecules separate in low ionic strength buffer (as, for example, when plasma is fractionated on ionic exchange media), the molecules are not covalently coupled. Similar observations were subsequently made with clotting Factor XI and high-MW kininogen (Thompson *et al.*, 1977). Donaldson *et al.* (1977), employing immunologic techniques, were able to show physical association between prekallikrein and high-MW kininogen.

The functional role of high-MW kininogen in contact activation was then further elucidated with the observation that this molecule greatly

augmented the capacity of prekallikrein and Factor XI to bind to a negatively charged surface. Wiggins *et al.* (1977) added human ^{125}I-labeled prekallikrein or ^{125}I-labeled Factor XI to human plasmas that were normal or deficient in high-MW kininogen and exposed the plasmas to a negatively charged surface. The ^{125}I-labeled prekallikrein in normal plasma became rapidly bound to the surface and was rapidly cleaved, both on the surface and in the supernatant (Figs. 9 and 10). By contrast, the ^{125}I-labeled prekallikrein or ^{125}I-labeled Factor XI added to high-MW kininogen-deficient plasma failed to bind to the surface and did not undergo cleavage (Figs. 10 and 11). Addition of highly purified high-MW kininogen to the deficient plasma reconstituted the surface-binding and cleavage of the ^{125}I-labeled molecules. It was also

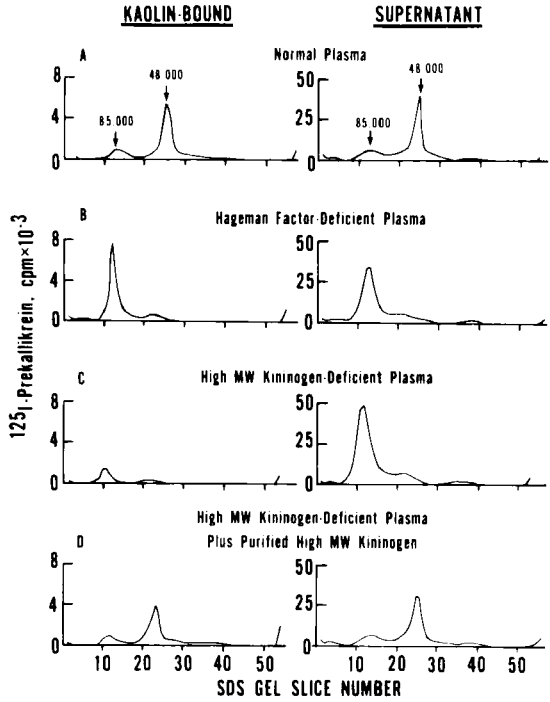

FIG. 10. The binding and cleavage of ^{125}I-labeled prekallikrein in various plasmas after addition of kaolin and incubation for 2 minutes at 20°C. The kaolin-bound material and supernatant were separated, reduced, and analyzed on 7.5% SDS–polyacrylamide gels. (A) Normal plasma; (B) Hageman-factor deficient plasma; (C) high-M_r kininogen-deficient plasma; (D) high-M_r kininogen-deficient plasma plus purified high-M_r kininogen. (From Wiggins *et al.*, 1977.)

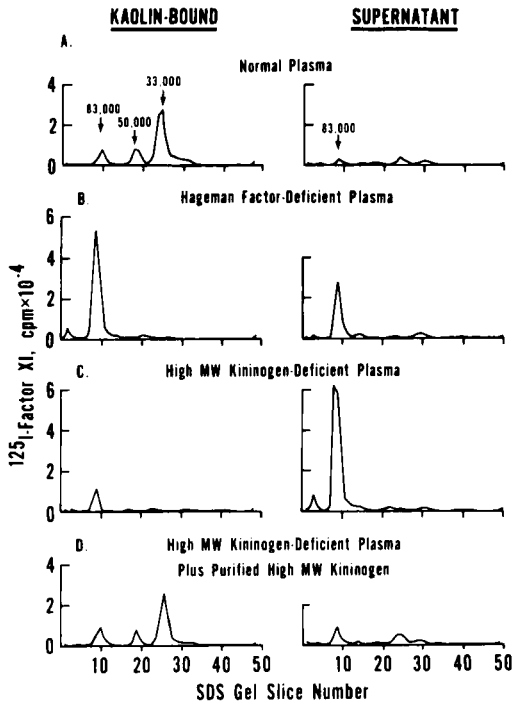

FIG. 11. The binding and cleavage of ^{125}I-labeled Factor XI in various plasmas after addition of kaolin and incubation for 2 minutes at 37°C. The kaolin-bound material and supernatant were separated, reduced, and analyzed on 7.5% SDS–polyacrylamide gels. (A) Normal plasma; (B) Hageman factor-deficient plasma; (C) high-M_r kininogen-deficient plasma; (D) high-M_r kininogen-deficient plasma plus purified high-M_r kininogen. (From Wiggins *et al.*, 1977.)

found that the surface binding of ^{125}I-labeled Factor XI also required the presence of high-MW kininogen. Similar observations have been made using rabbit proteins (Wiggins *et al.*, 1979b).

The data suggested that high-MW kininogen, through its ability to bind to a negatively charged surface and its capacity to bind prekallikrein and Factor XI in solution, promotes the association of prekallikrein (or Factor XI) with HF on the surface.

Taken together, these data suggest that an assembly of HF and the complex of prekallikrein and high-MW kininogen takes place on a negatively charged surface with HF and high-MW kininogen binding adjacent to one another. This hypothesized assembly of these molecules is shown schematically in Fig. 7.

Once the HF and prekallikrein are brought into contact, activation of one will rapidly lead to activation of the other. As discussed above, the initial triggering event remains uncertain.

C. Dissemination of Contact Activation

The question remained as to whether interaction of surface-bound molecules account for all of the activation of the contact system, or if there were means by which the activation could be propagated to other surface sites or be expanded in the fluid phase. This question has been examined in several ways. ^{125}I-labeled prekallikrein in normal plasma, when added to a surface to determine the amount bound, showed less than 20% binding even though cleavage of the prekallikrein in the supernatant plasma and on the surface had occurred (Wiggins *et al.*, 1977). By contrast nearly 80% of ^{125}I-labeled Factor XI was bound under identical conditions, and only the surface-bound Factor XI underwent cleavage. ^{125}I-labeled prekallikrein, bound to a surface, was found to be released rapidly from the surface (over 80% in five minutes) in comparison to other proteins of the contact system (Revak *et al.*, 1978). When contact activation was initiated on one set of kaolin particles, a soluble factor was rapidly released that cleaved and activated HF that was bound to a second set of kaolin particles (Cochrane and Revak, 1980a). The soluble factor was identified as kallikrein. The cleavage of HF on the second set of kaolin particles occurred as rapidly as that on the first set, indicating that the release of kallikrein from the surface, presumably bound to high-MW kininogen, was undoubtedly responsible for a great majority of activation of the surface-bound HF. Dissemination of the reaction could also result from release of the 28,000-MW fragment of HF (β-HFa) although this was found to be of lesser importance than the dissociation and dissemination of active kallikrein in fluid phase.

VII. Regulation of the Contact System

Both the activation and activity of members of the contact system are subject to regulation both by naturally occurring proteins and enzymes and by specific inhibitors that have been ascertained through knowledge of the components.

A. Inhibition of Surface Activation of the Contact System by Plasma Proteins

Activation of the system by surface contact is inhibited by the presence of protein. In 1958, Ratnoff and Rosenblum observed that HF-

deficient plasma interfered with the clot-promoting effects of glass. The findings were confirmed (Haanen *et al.*, 1961; Nossel, 1964; Nossel *et al.*, 1968; Soulier *et al.*, 1959; Hardisty and Margolis, 1959) but the mechanism of the inhibition remained obscure. Subsequent studies with partially purified HF then revealed that surface binding and activation of HF was inhibited by increasing concentrations of HF-deficient plasma (Cochrane *et al.*, 1972b). Binding to kaolin of ^{125}I-labeled HF was inhibited equally by increasing concentration of normal or HF-deficient plasma, and pretreatment of the kaolin with HF-deficient plasma also inhibited the binding. Purified human IgG at 10 mg/ml and albumin at 40 mg/ml (i.e., at plasma concentration) were about one-half as effective as whole plasma in the inhibition of binding (Cochrane *et al.*, 1973). Saito *et al.* (1974) also found that HF-deficient as well as prekallikrein-deficient plasmas inhibited binding of ^{125}I-labeled HF to glass and, in addition, that fibrinogen, along with IgG at 10 mg/ml, inhibited the uptake of HF activity from plasma. Albumin at a concentration of 10 mg/ml (i.e., one-fourth that noted above) did not appear to inhibit.

Inhibition of clot-promoting activity of surfaces, the generation of kinin, and the activation of HF result from the addition of the highly cationic, histidine-rich Fragment 1-2 of bovine high-MW kininogen (Oh-Ishi *et al.*, 1977a). This probably results from blocking of negative charges on the surface by the Fragment 1-2 (see below).

While the observations were directed at the capacity of proteins to inhibit surface-dependent activation of the contact system, information on their potential inhibitory activity on the binding of high-MW kininogen is not available.

An inhibitor of Factor XI adsorption to surfaces has been described (Margalit and Schiffmann, 1980). This, or a similar substance, was found to be of high molecular weight and its affect modified by addition of limited amounts of high-MW kininogen (Schiffmann *et al.*, 1981).

B. Inhibition of Surface Activation by Positively Charged Molecules

Neutralization of the negative charges on an activating surface results in loss of the capacity of the surface to activate the contact system. This inhibition has been observed after reacting positively charged small molecules or proteins with the surface. Silicone, hexadimethrine bromide, cytochrome c, lysozyme, polylysine, polyorinthione, and protamine (Haanen *et al.*, 1961; Eisen, 1964; Ratnoff and Miles, 1964; Nossel *et al.*, 1968) have all been employed. While hexadimethrine

bromide prevented activation of HF, it did not abrogate binding of the HF (Cochrane *et al.*, 1972a). Whether the inhibition of contact activation by neutralization of surface charges prevents changes in the molecular conformation of HF and perhaps high-MW kininogen, or whether neutralization prevents the proper assembly of HF, high-MW kininogen, and prekallikrein is unclear.

C. Specific Inhibition of Components with Heterologous Antibodies

Antibodies have been employed advantageously in studies of the function of HF and its participation in various systems. The principal cautions in such procedures lie in the necessity of employing purified specific antibody and removing the heterologous HF and other components of the contact system, i.e., HF, etc., from the heterologous serum. This is most easily accomplished by incubating the antiserum with large amounts of kaolin to activate and allow inactivation of the components in the serum, followed by isolation of the IgG fraction. Immunopurification of the antibody by affinity chromatography may also be used. We offer a few examples of the use of antibodies.

Treatment of synovial fluid or plasma with anti-HF prevented activation of HF on a surface (Kellermeyer and Breckenridge, 1967; Yamamoto and Cochrane, 1981). PF/dil was identified as activated HF by absorption of the PF/dil on a column of insolubilized anti-HF antibodies (Johnston *et al.*, 1974) as will be discussed below. Thus, either blocking the activity of the molecule or removing it from solution on an immunoabsorbent served to suggest a role of HF in biological reactions. Addition of antikallikrein antibodies to normal plasma blocked specifically, the clotting activity of prekallikrein, having no effect on HF, Factor XI, or high-MW kininogen activities (Saito and Ratnoff, 1974; Bouma *et al.*, 1980b). Antikallikrein antibodies were also employed to demonstrate the presence of kallikrein in cutaneous blister fluids of angioedema patients through the capacity of the antibodies to block the kinin-forming capacity of the fluid (Curd *et al.*, 1980).

D. Inhibition of Activated Components

Plasma Protein Inhibitors

a. Activated HF. The primary plasma protein inhibitor of activated HF is C1 inhibitor ($C\bar{1}$ inh). Using isolated $C\bar{1}$ inh, Forbes *et al.* (1970) blocked the clotting activity of HFa in the presence of kaolin particles. $C\bar{1}$ inh also inhibited PF/dil (Landerman *et al.*, 1962; Kagan and Becker, 1963; and Kagan, 1964) the activity of which is indistinguish-

able from that of HFa (Johnston *et al.*, 1974). $C\bar{1}$ inh prevented β-HFa, as designated "HF fragments," from generating kinin in plasma in a dose–response and time-dependent manner (Schreiber *et al.*, 1973a), indicating that the $C\bar{1}$ inh acted upon the β-HFa as well as kallikrein in the plasma. While mixing $C\bar{1}$ inh and β-HFa did not deplete $C\bar{1}$ inh activity, as did treatment with $C\bar{1}$s, the β-HFa antigen was lost in the reaction. Using ^{125}I-labeled HF added to plasma, $C\bar{1}$ inh bound β-HFa, forming a complex of approximately 130,000 M_r, in an apparent 1 : 1 stoichiometric combination of $C\bar{1}$ inh (104,000 M_r) and β-HFa (28,000 M_r) (Revak and Cochrane, 1976). Binding to the minor, 96,000 M_r, component of $C\bar{1}$ inh (Harpel and Cooper, 1975) was not appreciated in these studies. Complexing of the two molecules was also demonstrated by a cathodal shift in the prealbumin migratory pattern of β-HFa in immunoelectrophoresis, which was superimposed on a precipitin arc of $C\bar{1}$ inh (Revak and Cochrane, 1976). The precipitated $C\bar{1}$ inh in the immunoelectrophoresis was found to contain ^{125}I-labeled HF. A large-molecular-weight complex was also observed, although accurate measurements of size were not possible. This large M_r complex probably represented α-HFa complexed to $C\bar{1}$ inh. While β-HFa was observed to combine solely with $C\bar{1}$ inh in normal human plasma, a complex with antithrombin III was noted when ^{125}I-labeled HF was activated in $C\bar{1}$ inh-deficient plasma (Revak and Cochrane, 1976). The reaction was augmented by added heparin. In a detailed study, Stead *et al.* (1976) observed that purified α-HFa and β-HFa formed a 1 : 1 stoichiometric complex with antithrombin III, leading to inactivation of the clotting and kinin-generating activities, respectively. The addition to heparin greatly accelerated both reactions, leading to inactivation of both forms of HFa in 1–2 minutes. The inactivation of HFa by antithrombin III was also observed by Chan *et al.* (1977). It should be remembered that detectable complexing of HFa and antithrombin III in the presence or absence of heparin occurs only in $C\bar{1}$ inh-deficient plasma. The data suggest that $C\bar{1}$ inh is the major inhibitor of HFa when activation occurs in plasma.

Other plasma inhibitors, especially α_2-macroglobulin and α_1-antitrypsin (Schreiber *et al.*, 1973b; Chan *et al.*, 1977) do not appear to inactivate isolated HFa. α_2-Antiplasmin slowly inhibits purified β-HFa (Saito *et al.*, 1979a).

b. Kallikrein. $C\bar{1}$ inh and α_2-macroglobulin (α_2M) in purified form and in plasma are the principal inhibitors of plasma kallikrein. $C\bar{1}$ inh was shown in earlier studies to inactivate kallikrein along with $C\bar{1}$s, $C\bar{1}$r, plasmin, and PF/dil (Ratnoff *et al.*, 1969; Levy and Lepow, 1959). Harpel (1970) compared the inhibition of kallikrein by isolated $C\bar{1}$ inh

and α_2M and by these inhibitors in whole normal plasma and $C\bar{1}$ inh-deficient plasmas. Exposure of normal plasma to kaolin resulted in the development of esterolytic activity that was attributed to plasma kallikrein. The esterase activity reached a peak at 1–2 minutes incubation and was diminished by about 50% in 5 minutes in normal and about 30% in $C\bar{1}$ inh-deficient plasma. By 5 minutes, about 70–80% of the esterase activity remaining could not be blocked by soybean trypsin inhibitor (SBTI, see below), a plant inhibitor that rapidly inactivates the esterolytic activity of kallikrein in isolated form. The prevention of SBTI inhibition of the esterolytic activity in the plasma was attributed to the binding of α_2M to kallikrein. Using isolated components, kallikrein was observed to bind to α_2M. But while losing capacity to generate kinin activity and increase cutaneous vascular permeability, the kallikrein, complexed to α_2M, retained most of its esterolytic activity and resisted inactivation by both SBTI and $C\bar{1}$ inh. McConnell (1972) also observed the inhibition of kinin generation of kallikrein by α_2M with only a modest reduction in the kallikrein esterolytic function. The interaction of $C\bar{1}$ inh and kallikrein follows second-order kinetics and is consistent with a 1 : 1 stoichiometric complex of the two reactants (Gigli *et al.*, 1970). In these studies, the concentrations of kallikrein and $C\bar{1}$ inh were those exhibited by normal plasma.

α_1-Antitrypsin was found by McConnell (1972) to have a moderate inhibitory effect on kallikrein. Antithrombin III also inactivates kallikrein in a purified system (Vennerod and Laake, 1975; Burrowes *et al.*, 1975), the reaction being accelerated by heparin. The purity of the antithrombin III in these studies was not provided. It is important to know if this preparation was free of $C\bar{1}$ inh. $C\bar{1}$ inh activity is also accelerated by heparin (Nagaki and Inai, 1976; Revak and Cochrane, 1976). The importance of antithrombin III and α_1-antitrypsin in the inhibition of kallikrein in whole plasma appears to be less than $C\bar{1}$ inh (Harpel, 1970). Antithrombin III may be the kallikrein inhibitor in plasma studied by Haberman (1968) and Takeuchi and Movat (1972). Heating or acid treatment of plasma inactivated the inhibitors (Harpel, 1970; Jahrreiss and Habermann, 1971; McConnell, 1972).

c. Factor XI. The major inhibitor of Factor XIa in plasma is α_1-protease inhibitor (Heck and Kaplan, 1974; Scott *et al.*, 1982), accounting for 68% of the inhibitory activity of the plasma. Isolated $C\bar{1}$ inh was found to inactivate the clotting capacity of Factor XIa in a dose- and time-dependent manner (Forbes *et al.*, 1970). This differed from a second inhibitor of Factor XIa (Niemetz and Nossel, 1967) which possessed activity against Factor XIa but not HFa, in contrast to $C\bar{1}$ inh. When $C\bar{1}$s was added to normal plasma to consume $C\bar{1}$ inh, the capac-

ity of the plasma to inhibit activated Factor XIa was diminished but not entirely abolished (Forbes *et al.*, 1970).

α_2M was found not to inactivate Factor XIa (Harpel, 1971). This capacity differentiated the inhibitory patterns of Factor XIa and kallikrein.

Antithrombin III–heparin complex inhibits Factor XIa (Damus *et al.*, 1973). By removing antithrombin III immunologically from $C\bar{1}$ inh-deficient plasma, these workers concluded that antithrombin III accounts for approximately 40% of the Factor XIa inhibitory activity of normal plasma. $C\bar{1}$ inh was previously determined to account for 20% of the inhibitory activity (Gore and Larkey, 1960), an observation supported by studies of $C\bar{1}$ inh-deficient plasma (Ratnoff *et al.*, 1972). The remainder of Factor XIa inhibitory activity of plasma has been attributed to another plasma protein (Amir *et al.*, 1972; Niemetz and Nossel, 1967), although this protein of 65,000 was not clearly differentiated from antithrombin III.

Bovine antithrombin III binds to and inactivates purified bovine Factor XIa. Two molecules of antithrombin III bind to each Factor XIa molecule, indicating activity of both chains of the Factor XIa dimer (Kurachi and Davie, 1977).

E. Cold Activation of the Contact System: Blocking of Plasma Inhibitors

Regulation of the contact system activation by plasma inhibitors can be decreased by dilution and reducing temperatures. Based on the effect of cold on the spontaneous activation of Factor VII in plasma Gjonnaess (1972a–d) and Kluft (1978) observed that kallikrein was activated in plasma incubated at 0°C in the presence of dextran sulfate. Presumably cold interferes with the ability of $C\bar{1}$ inh to react with enzymes of the coagulation system although the mechanism is not well understood. The phenomenon is noted in plasmas of about 15% of normal men and women and in a higher percentage of women using estrogen-containing contraceptives or approaching term in pregnancy (Laake *et al.*, 1974). It is apparently dependent upon the presence of HF and is associated with elevated levels of prekallikrein (Laake *et al.*, 1974). During cold activation of plasma, HFa was generated and prekallikrein and kininogen levels fell.

F. Other Inhibitors

The interactions of a number of protease inhibitors derived from plants, animal tissues, or microbes with various components of the contact system have been described. In general, these inhibitors com-

bine with the enzymatic site of the activated protein, rendering it inactive. A summary of some of these inhibitors, as well as several of the more commonly used chemical inhibitors and their spectrum of interaction with the contact system enzymes, is given in Table IV.

VIII. Constituents of Cells That Activate Components of the Contact System

A. Plasma Kinin Activity Generated by Leukocytes

Following earlier studies of the pharmacologic action of peptides generated by the action of leukocytic proteases of Menkin (1938), Greenbaum and his colleagues examined the action of leukocytes on plasma protein substrates. Neutrophilic leukocytes and alveolar macrophages of rabbits were found to contain enzymes which, at an acid pH, caused release of a pharmacologically active peptide from a human plasma protein substrate (Greenbaum and Yamafujii, 1966; Greenbaum and Kim, 1967). The enzyme was located in a subcellular fraction rich in nuclear debris. The peptide released induced hypotension, contracted rat uterus, and guinea pig ileum, and caused increased vascular permeability (Freer *et al.*, 1972). However, the peptides released from the kininogen differed from bradykinin in both size (21 and 25 amino acids for the neutrophilic and macrophage enzyme induced peptides) and amino acid composition (Chang *et al.*, 1972). Thus the biochemical reactions leading to release of the kinins are unrelated, as far as can be observed, to those leading to release of bradykinin from high- or low-MW kininogen.

The interaction of leukocytes with components of the HF system was examined in a series of studies by Melmon and Cline. Leukocytes, obtained from peripheral blood of human beings, when incubated in plasma, generated kinin activity and simultaneously degraded kininogen (Melmon and Cline, 1967, 1968). The leukocyte most likely involved was the neutrophil, although eosinophils were also found to be active. Two pH optima were observed, one at neutrality and a second, at pH 5 or below. Incubation of the leukocytes in plasma deficient in HF did not generate kinin activity or lead to diminished kininogen levels. It therefore appeared that the leukocytes were acting through the mediation of the components of the HF system. This was clouded, however, by the finding that the leukocytes could act directly on an α_2-globulin fraction of human plasma rich in kininogen.

A kininase activity was also observed in the medium of cultured human neutrophils (Melmon and Cline, 1967).

TABLE IV

NONPLASMA INHIBITORS OF THE CONTACT SYSTEM[a]

Inhibitor	M_r	Active site sequence	Components inhibited				References
			HFa	XIa	Kal	Plasmin	
Plant origin							
Soybean	21,500	Pro-Ser-Tyr-Arg^{63}-Ile-Arg-Phe-Ile	−	+	+	+	1–5,13
Lima bean	8,000–10,000	Leu-{Ala/Ser}-Thy-Lys^{28}-Ser-Ile-Pro-Pro	+	−	−	+	1,2,4,6,13
Corn	11,000–14,000	Ile-Pro-Gly-Arg^{25}-Leu-Pro-Pro-Leu	+	−	−	−	7
Pumpkin	3,000–4,000		+	N.D.	−	±	8
Animal origin							
Trasylol	6,000		±	+	+	+	1–5,13
Ovomucoid	28,000	X-X-X-Arg-Ala-X-X-X	−	−	−	−	2,3,4,9,13
Microbial origin							
Leupeptin	427	Ac-Leu-Leu-Arg	+	N.D.	+	+	3,5,10
Antipain		Phe-CO-Arg-Val-Arg	N.D.	N.D.	−	±	5
Chemical							
DFP (diisopropyl phosphofluoridate)	184		+	+	+	+	2,11,12,13
PMSF (phenyl-methyl-sulfonyl fluoride)	174		+	N.D.	−	N.D.	2,11,13
TLCK (Tosyl-lysine chloromethyl ketone	336		−	−	−	+	2,12,13

[a] 1. Harpel (1970); 2. Wuepper (1972); 3. Yamamoto *et al.* (1980); 4. Wuepper (1972); 5. Umezawa (1972); 6. Burrowes *et al.* (1972); 7. Hojima *et al.* (1980a); 8. Hojima *et al.* (1980b); 9. Wuepper *et al.* (1970); 10. Yamamoto *et al.* (1980); 11. Wuepper (1972); 12. Kaplan *et al.* (1972); 13. Wuepper and Cochrane (1971).

B. Kininogenase Activity of Neutrophils

When polymorphonuclear leukocytes (neutrophils) of human blood are exposed to antigen–antibody complexes enzymes are released that cleave both low- and high-MW kininogens to release peptides bearing smooth muscle contracting and vascular permeability activities (Movat *et al.*, 1973, 1976b; Movat and Habal, 1976). The proteases involved in the reaction appear to be multiple. By molecular size, charge, and inhibition profile, the principle enzyme resembled the neutrophilic elastase (Movat *et al.*, 1975, 1976a,b; Wasi *et al.*, 1978), described by Janoff and Scherer (1968). The amount of kinin released from kininogen by the purified elastase-like enzyme was about one-tenth the amount released by trypsin (Wasi *et al.*, 1978). Treatment of the peptide with trypsin doubled the activity. While evidence suggested the enzyme was the elastase, only 60% of the kinin-generating activity was inhibited by a known inhibitor of elastase (Wasi *et al.*, 1978).

Another enzyme isolated from supernatants of neutrophils exposed to antigen–antibody complexes was found to alter the properties of the biologically active peptide cleaved from the kininogen so that its elution from CM-cellulose was identical to that of bradykinin (Pass *et al.*, 1978). The biologically active peptide released from kininogen by unfractionated neutrophilic enzymes was found to elute from carboxymethyl-cellulose in the same position as synthetic bradykinin (Movat *et al.*, 1976a) although a second peak, representing about 40% of the bradykinin peak by activity, was also observed eluting immediately after the bradykinin. Elution of the second peak was similar to, but not identical with, Met-Lys-bradykinin. The purified neutrophilic protease was found to release the second kinin only (Movat *et al.*, 1975).

That elastase is the enzyme involved in cleaving kinin from kininogen has been recently questioned. Arguing that elastase could cleave the Leu-Met bond on the N-terminal side of Met-Lys-bradykinin, it was held unlikely that the Arg-Ser bond on the C-terminal side would be cleaved by this enzyme (Fritz, 1978). In addition, purified elastase was found incapable of releasing kinin activity from the parent kininogen (Fritz, 1978; Dittmann *et al.*, 1979) and Trasylol, used in concentrations that did not inhibit elastase, was stated to block generation of kinin activity (Fritz, 1978). Unfortunately, quantitative data were not provided in the reports of Fritz and Dittmann and it is difficult to determine if 10% of the releasable kinin activity, the amount reported by Movat, had been generated by interaction of the kininogen with purified elastase.

C. Enzymes Released from Basophil-Rich Preparations of Human Leukocytes and Lung after Anaphylactic Challenge That Cleave and Activate Components of the Contact System

Human basophils have been implicated in the generation of biologic activities from components of the HF system. A kallikrein-like activity was released from human peripheral leukocytes by the addition to the leukocytes of either anti-IgE or specific antigen to which the individuals were allergic (Newball *et al.*, 1975). The leukocytes released an arginine–esterase enzyme and an enzyme that generated kinin activity from plasma or semipurified kininogen as determined by the radioimmunoassay. Release of the enzymes occurred together with histamine and was inhibited or enhanced by agents known to control the release of histamine. The presence of leukocytes enriched for basophils, but not for any other cell type, correlated with the generation of the enzymes (Newball *et al.*, 1979a,b). More recently, an enzyme has also been observed in such preparations that cleaves HF (Newball *et al.*, 1979c). Fragments of HF of 52,000 and 30,000 and 28,000 were observed. Cleavage apparently occurred outside of the disulfide loop more rapidly than within. As a result, the fragments were only partly active in cleaving prekallikrein (Newball *et al.*, 1979c, 1981). The presence of a negatively charged surface greatly augmented the reaction, but high-MW kininogen was without an enhancing effect. The enzyme also failed to cleave the synthetic peptide substrates specific to plasma kallikrein and did not activate plasminogen. Hence the leukocytic enzyme differed in several ways from plasma kallikrein.

D. HF-Activating Enzyme in Endothelial Cells

Cultured endothelial cells of both rabbit and bovine origin have recently been found to contain an enzyme which cleaves and activates HF (Wiggins *et al.*, 1980b). Cleavage of HF occurs principally inside the disulfide loop of HF, leading to activity of the molecule as revealed by an acquired capacity to cleave prekallikrein and clotting Factor XI. The heavy chain of HF is subject to continued cleavage by the endothelial enzyme so as to yield several small fragments. The enzyme has not yet been identified, although it is closely associated with the plasminogen activator and is associated with the cytoplasmic and microsomal membranes of the 100,000 *g* pellet of the disrupted cells. The enzymatic activator of HF was also inhibited by DFP (2 m*M*), but not soybean trypsin inhibitor (50 μg/ml), hirudin (8 mg/ml), or purified antibodies to prekallikrein or plasmin. Similar enzymes were found in cultured fibroblasts (Wiggins *et al.*, 1980b).

E. Reactions of the Contact System with Cells

Owing principally to the rather recent availability of purified components of the contact system, little information is at hand on the effect of components of this system on cells.

1. Chemotaxis of Neutrophils

Kaplan and Austen (1972) observed a chemotactic effect of preparations of kallikrein upon neutrophils. Eosinophils were not affected, and prekallikrein or DFP-treated kallikrein did not possess activity. These observations have been reported (Weiss *et al.*, 1974), although with kallikrein of rabbit and human plasma, we have not succeeded in inducing chemotaxis of neutrophils (unpublished observations). Recently Wiggins *et al.* (1980b) have reported that rabbit kallikrein fails to induce chemotactaxis of neutrophils. However, when a source of C5 was present, a chemotactic response of the neutrophils occurred along with release of β-glucuronidase. An anodally migrating species of C5, possibly C5a, was generated by the interaction of kallikrein and C5.

2. Platelets and Factor XI-Dependent Coagulation

Normal hemostasis and blood coagulation is initiated *in vivo* by the adhesion and aggregation of platelets (Walsh, 1974; Majerus and Miletich, 1978). As platelets aggregate to form a hemostatic plug, they may provide an activating surface for assembling coagulation factors that initiate and propagate the intrinsic coagulation pathway. Walsh (1972a) showed that ADP-stimulated platelets could shorten the clotting time of platelet-rich plasma in an HF-dependent reaction. Furthermore, he showed that collagen-stimulated platelets accelerate blood coagulation reactions that occur prior to the activation of Factor X (Walsh, 1972b). In this latter case of collagen-induced coagulant activity involving platelets, it was shown that coagulant activity requiring Factor XI was generated in reactions that were partially dependent and partially independent of HF. Recent studies employing washed human platelets and purified radiolabeled HF and Factor XI confirmed and extended these concepts (Walsh and Griffin, 1980). It was demonstrated that platelets can promote the proteolytic activation of HF by kallikrein as well as the activation of Factor XI by both HF-dependent and HF-independent mechanisms. Furthermore, Greengard *et al.* (1981) demonstrated the existence of specific, high-affinity binding sites on washed activated platelets for Factor XI. Approximately 500 binding sites with a dissociation constant on the order of 10^{-9} *M* appear when platelets are stimulated with low concentra-

tions of thrombin. Platelet localization of HFa and Factor XIa not only localize procoagulant reactions but also probably protect the activated clotting factors from plasma inhibitors. Platelet-dependent pathways for the activation of Factor XI in the absence of HF may explain the asymptomatic nature of HF deficiency in the face of the known bleeding tendency of Factor XI-deficient patients.

IX. Pathophysiologic Actions of the Components

A. Hageman Factor and PF/dil

1. Vascular Permeability

The relationship of a permeability factor that is generated when plasma contacts glass to the presence of HF was established many years ago. The importance of glass in the generation of a permeability factor (Spector, 1957) or a kinin-like activity (Armstrong *et al.*, 1954) pointed to the necessity of HF in the evolution of kinin activity (Margolis, 1958, 1960). Webster and Ratnoff (1961) showed that permeability failed to develop in HF-deficient plasma upon contact with surfaces and that addition of HF to the mixture reconstituted the permeability-inducing factor. Ratnoff and Miles (1964) then employed partially activated HF to induce an increase in vascular permeability of guinea pigs. They found that a minimal dose of 2 μg in 0.1 ml volume induced increased permeability in the skin. Since the HF was only partially active and partially purified, it was uncertain that this represented a true minimal dose.

When the HF was added to dilute plasma, the permeability effect was enhanced, leading the investigators to suspect that the HF had activated a second factor, thought to be the permeability factor of dilution (PF/dil). PF/dil, which was generated in plasma, diluted in the presence of glass (Miles and Wilhelm, 1955; Wilhelm *et al.*, 1958; Spector, 1957; Kagan *et al.*, 1963), was thought to act as a substrate for the activated HF. PF/dil was later found to be removed from solution by insolubilized antibodies to HF (Johnston *et al.*, 1974). PF/dil and active HF were found to chromatograph similarly (Oh-Ishi and Webster, 1975). It is probable, therefore, that PF/dil and activated HF are one and the same.

More recently, fully activated, purified guinea pig HF has been assessed for its permeability activity. Increased permeability was apparent using as little as 1 ng HFa (at a concentration of $3 \times 10^{-10} M$ in

0.1 ml), i.e., about 10–100 times more active than bradykinin in this assay (Yamamoto and Cochrane, 1981). The HFa was found to be identical to the permeability factor found in extracts of guinea pig skin (Yamamoto and Kambara, 1978; Kozono *et al.*, 1980; Yamamoto *et al.*, 1980).

2. *Hypotension*

Following earlier reports of hypotensive responses in human beings to infusions of albumin (Bland *et al.*, 1973; Harrison *et al.*, 1971a,b), a study was made of the nature of the hypotensive agent in commercial albumin preparations. These preparations had been given intravenously to patients before or during surgical procedures and were associated with a high incidence of hypotensive episodes, with a fall in arterial blood pressures ranging between 20 mm Hg and circulatory collapse. The duration of hypotension was not reported, but pressures returned to normal on cessation of administration and/or antihypotensive therapy. β-HFa (prekallikrein activator) was found in the albumin preparations as determined by physical properties, its ability to activate prekallikrein, and by its inhibition with specific antibodies to HF (Alving *et al.*, 1978). The activity was reportedly not related to bradykinin or kallikrein in the preparation.

3. *Leukocyte Accumulation in Blood Vessels and Tissues*

When exposed to ellagic acid and placed in a vascular bed of rabbit ear chambers, human HF induced margination and infiltration of leukocytes (Graham *et al.*, 1965). The reaction reached a maximum after about 90 minutes as opposed to a more transient effect of bradykinin.

B. High-MW Kininogen

Aside from being the parent molecule of bradykinin generated by the hydrolytic action of plasma kallikrein, high-MW kininogen also contains the histidine-rich residue, Fragment 1-2. In the case of bovine high MW-kininogen, Fragment 1-2 and its cleavage products, Fragment 1 and Fragment 2, are released by the action of plasma kallikrein, a property not demonstrated with the human proteins. The bovine Fragment 1-2 and Fragment 2, but not Fragment 1, produce an increase in vascular permeability when administered intradermally. Matheson *et al.* (1976), measuring the diameter of extruded blue dye in skin, observed a permeability reaction with 10^{-8} to 10^{-9} moles of Fragment 1-2, about 10^{-2} less potent than the reaction to bradykinin. An additive effect between Fragment 1-2 and bradykinin was mea-

sured. Oh-Ishi *et al.* (1977b) have noted in independent studies that Fragment 1-2 and Fragment 2, but not Fragment 1, induce vascular permeability to an extent of 1/100th that of bradykinin in rabbit skin. PGE_2, but not bradykinin, was found to enhance the permeability activity. It should be kept in mind that rabbits respond poorly to bradykinin or HFa in comparison to guinea pigs and primates.

Fragment 1-2 and Fragment 2 were also found to have $<10^{-4}$ the activity of bradykinin in the contraction of rat uterine and guinea pig ilial smooth muscle and 10^{-2} to 10^{-3} of the activity of bradykinin in the hypotensive response in rats (Oh-Ishi *et al.*, 1977a).

C. Bradykinin

The physiologic and pathologic effects of bradykinin are of extreme diversity and have received intensive study over the past 3–4 decades. Both *in vitro* and in many species of animals *in vivo*, the pharmacologic effects of this nonapeptide are now well recognized. Increase in vascular permeability, smooth muscle contraction, diminished arterial resistance and hypotension, vascular margination and infiltration of leukocytes into tissues, and pain are among the commonly studied responses to bradykinin. The actions have been subject to review and will not be specifically discussed here. Comparisons with active components of the contact system have been given above in this section. Bradykinin has been found in fluid such as plasma and joint fluid in numerous forms of experimental and clinical inflammatory disease. The reader is referred to recent compilations of studies of kinins (Pisano and Austen, 1976; Fujii *et al.*, 1979a,b; Erdos, 1979).

D. Contact Activation System and the Plasma Renin–Angiotensin System

The plasma kallikrein–kinin system and the renin–angiotensin system exhibit a number of interesting relationships. Bradykinin is hypotensive whereas angiotensin II is hypertensive. The dipeptidase-converting enzyme, found principally in pulmonary endothelium, generates angiotensin II from a less active peptide, angiotensin I, by cleaving off a dipeptide (Erdos, 1979). The same proteolytic enzyme destroys bradykinin by eliminating its C-terminal dipeptide, Phe-Arg (Erdos, 1979). Recently it has been shown that *in vitro* activation of plasma prorenin can be effected via Factor XIIa and kallikrein.

Plasma renin liberates angiotensin I from angiotensinogen. In plasma, most renin (80 to 90%) circulates in zymogen form as prorenin (Derkx *et al.*, 1976) which can be activated by trypsin, plasmin, uri-

nary kallikrein, or plasma kallikrein (Cooper *et al.*, 1977; Sealey *et al.*, 1978; Osmond and Loh, 1978; Osmond *et al.*, 1978; Derkx *et al.*, 1979a). Cryoactivation of plasma or acidification followed by neutralization of plasma also results in activation of prorenin (Sealey *et al.*, 1978; Derkx *et al.*, 1978; Atlas *et al.*, 1978; Osmond and Loh, 1978). Experiments employing plasmas deficient in HF or prekallikrein indicated that these proteins were essential for these *in vitro* activations of prorenin (Osmond *et al.*, 1978; Millar *et al.*, 1978; Derkx *et al.*, 1979c; Sealey *et al.*, 1979). Normal prorenin activation is restored by addition of kallikrein to HF-deficient plasma, but not by addition of β-HFa to prekallikrein-deficient plasma (Derkx *et al.*, 1979c; Sealey *et al.*, 1979). This shows that kallikrein is more directly required for prorenin activation than is HFa, although HF is presumably required for activation of prekallikrein.

The pathophysiologic significance of the activation of the renin–angiotensin system by HF or prekallikrein-dependent pathways is not at all clear. Nor is the significance of the observation that 10 to 20% of plasma renin exists as renin versus 80 to 90% present as prorenin apparent. However, it appears that prekallikrein influences the physiologic percentage of renin versus prorenin since the level of prorenin is abnormally high and renin is abnormally low in plasma from patients with prekallikrein deficiency (Derkx *et al.*, 1979c). In this context, one recent report that activation of the plasma kallikrein–kinin system appears unlikely to result in activation of prorenin *in vivo* based on their observations that kaolin-induced generation of plasma arginine esterase, presumably kallikrein, does not necessarily lead to prorenin activation (Blumberg *et al.*, 1981). Further work will be necessary to define the potential significance of these recent *in vitro* observations.

X. The Participation of the Contact System in Experimental and Human Disease

It is clear from the discussion in Section IX that components of the contact system may be associated with pathophysiologic processes. In this section, we address this problem in terms of diseases, reviewing the knowledge currently available. With information on the biochemistry of the contact system now available, and with sensitive techniques developed for measuring the components and their activities, it should be possible to advance our understanding of the participation of components of the contact system in the pathogenesis of disease. Already there are significant strides.

A. Criteria for Judging the Participation of Component-Proteins in the Pathogenesis of Inflammatory Disease

The pathogenic role of components of the plasma in disease states can be determined through fulfillment of various criteria. In many studies only a few of the criteria are evaluated and interpretation of the data becomes difficult. This has been true of many studies of both complement and contact system proteins. In clinical situations it is impossible to satisfy all the criteria in determining pathogenic participation of a component, and the use of animal models becomes important. We list in Table V the criteria of greatest importance in determining the pathogenic participation of components of the contact system. The criteria serve as well for proteins of the complement, fibrinolytic, and clotting systems.

Several of the points require amplification. Rather than studies of a single protein component, it is preferable to analyze several that act in sequence or together. The concentration of proteins in plasma must be related to the concentration of uninvolved proteins (often albumin), or to the total protein concentration, owing to dilutional changes that may occur *in vivo* (e.g., produced by intravenous fluid therapy). In studies of the rate of catabolism, use has frequently been made of radioisotope-labeled components, the rate of disappearance from the

TABLE V
Determination of a Pathogenic Role of Plasma Proteins in the Development of Inflammatory Lesions

1. Changes observed in component proteins
   - A. Systemic (plasma)
     - Changes in concentration of specific component(s) in plasma
     - Increased rate of catabolism
     - Evidence of activation
       - Functional assays of activity
       - Binding of activated components to inhibitor proteins
       - Specific proteolytic cleavage of components
       - Reaction of activated component with antibody specific to the active enzyme site
   - B. Local (joint fluid, lavage fluids, blister fluids, lymph, etc.)
     - Similar information to that listed above under "Evidence of activation"
2. Localization of activated components at the site of inflammation
3. Modification of the inflammatory lesion by removal or by specific inhibition of the component

circulation reflecting the catabolic rate. Comparisons are made between such rates in the presence and absence of disease. Care must be taken to account for loss unrelated to the disease, such as may occur in the urine when renal lesions are present. In studies of the activation of components, it is essential to treat the plasma or other fluids in such a way as to avoid activation *in vivo*. For example, the use of plastic containers is essential in studies of the contact activation system, and cooling of the blood can allow activation to occur as discussed above.

The data obtained in these studies must be carefully examined together with evidence of the disease state. Critical assays of clinical functions are most helpful. It is perhaps unnecessary to mention that the state of disease is the result of multiple factors and hence any single component may not play a determinant role in the degree of injury. Correlation is therefore often imprecise and changes in the plasma may be restricted to the site of the lesion, without reflection in the systemic circulation. The altered component protein may also be diluted beyond limits of detection in the systemic circulation. In fact, especially in the case of complement proteins, activation and consumption of components can occur by factors unrelated to the pathogenesis of the lesion under study.

Modification of the lesion or the disease by specific removal of a component being tested (part 3 in Table V) can be accomplished only in experimental animals, but in greater numbers, the specific inhibition of components should be possible in human disease in the future. Such capability is the consequence of careful analytic studies outlined in Table V. As a caution in these studies of inhibition, it is important to bear in mind that the lesions of inflammation are the product of many factors. Inhibition of one component may modify the lesion in a minor way, although if combined with inhibition of one other component, significant modification of the lesion may result. In studies in experimental animals, evaluation early in the development of a provoked lesion is most apt to induce a modification. It is necessary in such studies to verify that the component in question is either removed or does not function *in vivo* before interpretations can be made.

With this preface in mind, it is possible to examine various studies performed in which components of the contact system have been linked to various lesions and diseases. We will review some of these diseases and attempt to relate the data to the criteria noted in Table V. The review is not complete, as multitudes of studies of this nature have been reported in the world's literature and it is impossible to include them all in the confines of the present study. Thus the review

is meant more to point the direction of understanding in the various diseases and to delineate gaps in our knowledge.

B. ARTHRITIS

Synovial fluids have been shown to contain components of the contact system required for full activity (Armstrong *et al.*, 1957; Kellermeyer and Breckenridge, 1966; Sawai *et al.*, 1980). Addition to joint fluid of chondroitin sulfate (Eisen and Kule, 1965), articular cartilage or cartilage extracts (Moskowitz *et al.*, 1970), or urate crystals, agents known to be important in the pathogenesis of gout (Kellermeyer and Breckenridge, 1965, 1967; Ginsberg *et al.*, 1980), activate components of the contact system. Joint fluid from patients with arthritic disease contain elevated levels of kinins (Melmon and Cline, 1967; Kule and Eisen, 1970) although the correlation of elevated kinin concentration and intensity of disease was not consistent. Plasma of six patients with rheumatoid arthritis were found to contain normal levels of HF using immunologic assays (Saito and Ratnoff, 1974). The clinical state of the patients was not given. Joint fluid levels of high-MW kininogen were lowered in patients with active rheumatoid arthritis (Sawai *et al.*, 1980).

Intraarticular infusion of bradykinin in dogs induces increased warmth and pain, reaching a peak in 10 minutes, but leukocyte accumulation was not noted. Similar injections of activated components of the contact system have not been reported. However, injection of 100 mg microcrystalline sodium urate crystals in a volunteer with gout induced severe local arthritis and a rapid rise in bradykinin concentration in the joint fluid over a 24-hour period (Webster and Maling, 1970). McCarty *et al.* (1966) induced severe articular inflammation in the dog using urate crystals. Injection of 10 mg of urate crystals in the subplantar region of rats paws induced a slowly increasing edema that reached a peak in 6 hours and remained through 18 hours (Webster and Maling, 1970). Pretreatment by intraperitoneal injection of soybean trypsin inhibitor reduced the edema by 30–40%, as did separate infusions of carboxypeptidase B. Combined therapy was not employed. However Phillips *et al.* (1966), using the model of crystal-induced arthritis in dogs (McCarty *et al.*, 1966), failed to note a diminution of intraarticular pressure or leukocytic accumulation by pretreatment with an amount of carboxypeptidase B that effectively inhibited the edema caused by an intraarticular injection of bradykinin. The significance of these data has been questioned in view of the observation that dog plasma generates kinins poorly when ex-

posed to surfaces (Armstrong *et al.*, 1957; Vogt, 1966). Carboxypeptidase would also not affect the actions of previous components of the contact system and might not effectively inhibit bradykinin released in the synovial tissues.

Information on the role of the contact system in arthritis is incomplete. The turnover and rate of activation of components in the joint during developing inflammation, the presence and location of activators of the contact system in the inflammed joint, evidence of activated components of the system, and the relationship of demonstrated effective inhibitors of activated components to the disease process remain to be studied. Similar data regarding the effect of depletion of specific contact system components in the development of joint inflammation in experimental animals are unavailable. The pathophysiologic effect of intraarticularly administered active components, aside from bradykinin, is also unknown.

C. Allergic and Anaphylactic Reactions

In 1975 Pinckard and his colleagues (Pinckard *et al.*, 1975; Halonen and Pinckard, 1975) observed that rabbits, immunized to produce IgE antibodies, when challenged with antigen to induce anaphylaxis, developed shortening and then prolongation of whole blood clotting time which was associated with a diminution of HF and clotting Factors XI and IX. The three components were measured by functional activity. While an inhibitor of the clotting process was found 15 minutes after challenge, it was barely measurable at 60 minutes postchallenge at a time when levels of the clotting factors were still low. Total protein concentration of the plasma did not change sufficiently to explain the fall in clotting activities. In addition, the prothrombin time, indicative of changes in the terminal portion of the clotting sequence, was unchanged. Rabbits with nonanaphylactic IgG antibody when challenged with antigen did not demonstrate changes in functional levels of clotting components.

The above data, while not providing direct information on the proteins involved, do suggest strongly that during anaphylaxis in the rabbit, the contact system might be set in motion. The mechanism relating anaphylaxis and activation of the contact system has been derived from other studies. In studies performed with Newball as noted above (Section VIII), human lung fragments, lung mast cells, and circulating basophils, when challenged by an allergic reaction, release enzymes capable of releasing kinin from high MW-kininogen and cleaving and activating HF and prekallikrein. While the sensitivity of this reaction sequence is unclear, it represents a potential mechanism whereby the

contact system might be activated in the anaphylactic process. Support for the potential of mast cells in activation of the contact system has come from the work of Rothschild and his colleagues. Epinephrine treatment of rats induced partial depletion of total kininogen in the circulation (Castania and Rothschild, 1974). Epinephrine or 48/80 treatment of isolated peritoneal mast cells of rats resulted in generation of an enzyme bearing arginine esterolytic activity in plasma that depleted levels of trypsin-releasable kinin in plasma (Rothschild, 1981).

In addition to enzymes released from basophils and mast cells, negatively charged sulfated glycosaminoglycans and proteoglycans, contained in the cytoplasmic granules, are potentially important in the activation of the contact system. The presence of these negatively charged agents in basophils (Ornstein *et al.*, 1978) and mast cells (Yurt and Austen, 1977) has recently been demonstrated.

In summary, evidence suggests that contact system proteins may be brought into participation in the allergic and anaphylactic response. The data in rabbit anaphylaxis are indirect, showing decreased levels of circulating components by activity. Otherwise all the data come from *in vitro* assays and their significance in the allergic or anaphylactic reaction remains untested.

D. Bacteremic Shock

Bacteremic shock has been frequently considered to be a disease in which the contact system participates. During sepsis a decrease in functional levels of prekallikrein has been observed (Mason *et al.*, 1970; Robinson *et al.*, 1975; Colman *et al.*, 1978; Hirsch *et al.*, 1974; O'Donnell *et al.*, 1976; Aasen *et al.*, 1979) along with a fall in kallikrein inhibitory activity (Mason *et al.*, 1970; O'Donnell *et al.*, 1976; Aasen *et al.*, 1979). The activation of prekallikrein in the blood in patients developing typhoid fever has been postulated by virtue of a conversion in the electrophoretic migration of prekallikrein to that of a complex between kallikrein and $\overline{C1}$ inh, and by the appearance of arginine esterase activity in the plasma (Colman *et al.*, 1978). In these patients, levels of PK measured immunologically, and HF, determined by functional assay, were unchanged; and functional levels of high-MW kininogen rose as if in response to the infection. In other reports, HF (Mason *et al.*, 1970; Aasen *et al.*, 1979) and kininogen (Hirsch *et al.*, 1974; Aasen *et al.*, 1979) levels of the plasma are reportedly diminished in patients with endotoxin shock and free kinin has been detected in the circulation (Hirsch *et al.*, 1974).

In experimental hypotensive shock, a fall in kininogen level (detected as trypsin-releasable kinin) and the appearance of free kinin

have been correlated with a fall in peripheral arterial resistance in rhesus monkeys (Nies *et al.*, 1968) and in monkeys infected with *S. typhimurium* (Wing *et al.*, 1978). Similar changes have been detected in dogs (Gallimore *et al.*, 1978a; Aasen *et al.*, 1978). In rabbits, initial studies supported a fall in kininogen (Erdos and Miwa, 1968) in endotoxin shock, but subsequent studies of the turnover of human ^{125}I-labeled high MW-kininogen failed to record a significant change in its disappearance rate and the circulating ^{125}I-labeled high-MW kininogen failed to undergo cleavage despite severe shock (Cochrane and Revak, 1980b).

In vitro, mixtures of purified HF and prekallikrein are readily activated after interaction with purified bacterial lipopolysaccharides (Morrison and Cochrane, 1974).

In summary, the studies of bacterial or lipopolysaccharide-induced hypotensive shock in experimental animals and man suggest that the contact system components participate. Since edema, hypotension, and coagulation are common features of this disease, there is reason to suspect a role of the contact proteins in the pathogenesis. Nevertheless, the data to date are weak: in almost all studies of levels of components, only functional activities were obtained, and almost without exception, the levels of components were not related to levels of other, unrelated proteins. This is especially critical in shock states in which fluid balance changes rapidly and where therapeutic administration of fluids is the rule. In addition no account is given to the possible changes (or lack thereof) in circulation time of components upon activation. Information is now available on the fate *in vivo* of bacterial lipopolysaccharide (LPS), the active moiety of bacterial endotoxin. With new techniques available in the analysis of activation of the components and with greater knowledge of the movement *in vivo* of the LPS, it should be possible to obtain more definitive information on the pathogenic role of the contact system in bacteremic shock.

E. Other Diseases

In a number of other diseases, data concerning a potential role of the contact system have been accumulated. In hereditary angioedema, kallikrein in high concentration has been found in cutaneous blister fluids (Curd *et al.*, 1980). The reader is referred to a recent review of Colman and Wang (1979b) and to compilations of recent reports (Fujii *et al.*, 1978).

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REFERENCES

Aasen, A. O., Frolish, W., Saugstad, O. D., and Amundsen, E. (1978). *Eur. Surg. Res.* **10,** 50.

Aasen, A. O., Gallimore, M. J., Lyngass, K., Larsbraaten, M., Amundsen, E., and Smith, N. (1979). *Proc. Int. Congr. Thromb. Haemostasis, 7th* p. 253.

Alving, B. M., Hojima, Y., Pisano, J. J., Mason, B. M., Buckingham, R. E., Mozen, M. M., and Finlayson, J. S. (1978). *N. Engl. J. Med.* **229,** 66.

Amir, J., Ratnoff, O. D., and Pensky, J. (1972). *J. Lab. Clin. Med.* **80,** 786.

Amundsen, E., Svendsen, L., Vennerod, A. M., and Laake, K. (1976). *In* "Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease" (J. J. Pisano and K. F. Austen, eds.), p. 215. DHEW No. (NIH) 76-791.

Armstrong, D., Kech, C. A., Jipson, J. B., and Stewart, J. W. (1954). *Nature (London)* **174,** 791.

Armstrong, D., Jepson, J. B., Kule, C. A., and Stewart, J. W. (1957). *J. Physiol. (London)* **135,** 350.

Atlas, S. A., Sealy, J. E., and Lavagh, J. H. (1978). *Circ. Res.* **42** (Suppl. I), 128.

Bagdasarian, A., Talamo, R. C., and Colman, R. W. (1973). *J. Biol. Chem.* **248,** 3456.

Bagdasarian, A., Lahiri, B., Talamo, R. C., Wong, P., and Colman, R. W. (1974). *J. Clin. Invest.* **54,** 1444.

Beretta, G., and Griffin, J. H. (1979). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 811.

Bland, J. H. L., Laver, M. B., and Lowenstein, E. (1973). *J. Am. Med. Assoc.* **224,** 1721.

Blumberg, A. L., Sealy, J. E., Atlas, S. A., Lavagh, J. H., Dharingrongartama, B., and Kaplan, A. P. (1981). *J. Lab. Clin. Med.* **97,** 771.

Botti and Ratnoff (1963). *J. Clin. Invest.* **42,** 1569.

Bouma, B. N., and Griffin, J. H. (1977). *J. Biol. Chem.* **252,** 6432.

Bouma, B. N., and Griffin, J. H. (1978). *J. Lab. Clin. Med.* **91,** 148.

Bouma, B. N., and Griffin, J. H. (1980). *In* "Immunoassays: Clinical Laboratory Techniques for the 1980's" (R. M. Nakamura, W. R. Dito, and E. S. Tucker, eds), p. 325. Liss, New York.

Bouma, B. N., and Griffin, J. H. (1981). *Thrombosis Hemostasis* **46,** 257.

Bouma, B. N., Kerbiriou, D. M., Vlooswijk, R., and Griffin, J. H. (1980a). *J. Lab. Clin. Med.* **96,** 693.

Bouma, B. N., Miles, L. A., Beretta, G., and Griffin, J. H. (1980b). *Biochemistry* **19,** 1151.

Burrowes, L. E., Movat, H. Z., and Soltay, M. J. (1972). *Proc. Soc. Exp. Biol. Med.* **135,** 959.

Burrowes, C. E., Habal, F. M., and Movat, H. Z. (1975). *Thromb. Res.* **7,** 175.

Castania, A., and Rothschild, A. M. (1974). *Br. J. Pharmacol.* **50,** 375.

Chan, J. Y., Habal, F. M., Burrowes, C. E., and Movat, H. Z. (1976). *Thromb. Res.* **9,** 423.

Chan, J. Y. C., Burrowes, C. E., Habel, F. M., and Movat, H. Z. (1977). *Biochem. Biophys. Res. Commun.* **74,** 150.

Chang, J., Freir, R., Stella, R., and Greenbaum, L. M. (1972). *Biochem. Pharmacol.* **21,** 3095.

Claeys, H., and Collen, D. (1977). *Eur. J. Biochem.* **87,** 69.

Cochrane, C. G., and Revak, S. D. (1980a). *J. Exp. Med.* **152,** 608.

Cochrane, C. G., and Revak, S. D. (1980b). *Clin. Immunol. Immunopathol.* **15,** 367.

Cochrane, C. G., and Wuepper, K. D. (1971). *J. Exp. Med.* **134,** 986.

Cochrane, C. G., and Wuepper, K. D. (1972). *In* "Immunopathology VI" (P. Miescher, ed.), p. 220. Schwabe, Basel.

Cochrane, C. G., Revak, S. D., Aikin, B. S., and Wuepper, K. D. (1972a). *In* "Inflammation: Mechanisms and Control" (I. H. Lepow and P. A. Ward, eds.), p. 119. Academic Press, New York.

Cochrane, C. G., Wuepper, K. D., Aikin, B. S., Revak, S. D., and Spiegelberg, H. L. (1972b). *J. Clin. Invest.* **51,** 2736

Cochrane, C. G., Revak, S. D., and Wuepper, K. D. (1973). *J. Exp. Med.* **138,** 1564.

Colman, R. (1969). *Biochem. Biophys. Res. Commun.* **35,** 273.

Colman, R. W., and Scott, C. F. (1979). *Clin. Res.* **27,** 460A.

Colman, R. W., and Wang, P. (1977). *Thromb. Hemost.* **38,** 751.

Colman, R. W., Mattler, L., and Sherry, S. (1969). *J. Clin. Invest.* **48,** 23.

Colman, R. W., Bagdasarian, A., Talamo, R. C., Scott, C. F., Seavey, M., Guimaraes, J. A., Pierce, J. V., and Kaplan, A. P. (1975). *J. Clin. Invest.* **56,** 1650.

Colman, R. W., Edelman, R., Scott, C. F., and Gilman, R. M. (1978). *J. Clin. Invest.* **61,** 287.

Cooper, R. M., Murray, G. E., and Osmond, D. H. (1977). *Circ. Res.* **40** (Suppl. I), 171.

Curd, J., Prograis, L., and Cochrane, C. G. (1980). *J. Exp. Med.* **152,** 742.

Damus, P. S., Hicks, M., and Rosenberg, R. D. (1973). *Nature (London)* **246,** 355.

Davie, E. W., Fukikawa, K., Kurachi, K., and Kisiel, W. (1979). *Adv. Enzymol.* **48,** 227.

Davies, G. E., and Lowe, J. S. (1960). *Br. J. Exp. Pathol.* **41,** 335.

Davies, G. E., and Lowe, J. S. (1962). *Int. Arch. Allergy* **20,** 235.

Derkx, F. H. M., Wenting, G. J., Man in 't Veld, A. J., van Gool, J. M. G., Verhoeven, R. P., and Schalekamp, M. A. D. H. (1976). *Lancet* **2,** 496.

Derkx, F. H. M., Tan-Tjiong, H. L., and Schalekamp, M. A. D. H. (1978). *Lancet* **2,** 218.

Derkx, F. H. M., Tan-Tjiong, H. L., Man in 't Veld, A. J., Schalekamp, M. P. A., and Schalekamp, M. A. D. H. (1979a). *Clin. Sci.* **57,** 351.

Derkx, F. H. M., Bouma, B. N., Tan-Tjiong, H. L., and Schalekamp, M. A. D. H. (1979b). *Clin. Sci.* **57,** 89 S.

Derkx, F. H. M., Bouma, B. N., Schalekamp, M. P. A., and Schalekamp, M. A. D. H. (1979c). *Nature (London)* **280,** 315.

Didisheim, P., and Mibashan, R. S. (1963). *Thromb. Diath. Haemorrh.* **9,** 346.

Dittmann, B., Wimmer, R., Mindermann, R., and Ohlsson, K. (1979). *In* "Kinins II" (S. Fujii, H. Moriya, and T. Suzuki, eds.). Plenum, New York.

Donaldson, H., and Ratnoff, D. D. (1965). *Science* **150,** 754.

Donaldson, V. H., Saito, H., and Ratnoff, O. D. (1974). *Circ. Res.* **34,** 652.

Donaldson, V. H., Glueck, H. I., Miller, M. A., Movat, H. Z., and Habal, F. (1976). *J. Lab. Clin. Med.* **87,** 327.

Donaldson, V. H., Kleniewski, J., Saito, H., and Sayed, J. U. (1977). *J. Clin. Invest.* **60,** 571.

Edson, J. R., White, J. G., and Krivit, W. (1967). *Thromb. Diath. Haemorrh.* **18,** 342.

Eisen, V. (1964). *Br. J. Pharmacol.* **22,** 87.

Eisen, V., and Kule, C. A. (1966). *In* "Hypotensive Peptides" (E. A. Erdos, N. Back, F. Sicuteri, and A. F. Wilde, eds.), p. 551. Springer-Verlag, Berlin and New York.

Eisen, V., and Smith, H. G. (1970). *Br. J. Exp. Pathol.* **51,** 328.

Enfield, D. L., Ericsson, F. H., Fujikawa, K., Tetani, K., Walsh, K. A., and Neurath, H. (1974). *FEBS Lett.* **47,** 132.

Epstein, W. V., Tans, M., and Melmon, K. L. (1969). *Ann. N.Y. Acad. Sci.* **168,** 173.

Erdos, E., ed. (1979). "Handbook of Experimental Pharmacology," Vol. XXV, (Suppl.). Springer-Verlag, Berlin and New York.

Erdos, E. G., and Miwa, I. (1968). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **27,** 92.

Fair, B. D., Saito, H., Ratnoff, O. D., and Rippon, W. B. (1977). *Proc. Soc. Exp. Biol. Med.* **155,** 199.

Forbes, D. C., and Ratnoff, O. D. (1972). *J. Lab. Clin. Med.* **79,** 113.

Forbes, D. C., Pensky, J., and Ratnoff, O. D. (1970). *J. Lab. Clin. Med.* **76,** 809.

Freer, R., Chang, J., and Greenbaum, L. N. (1972). *Biochem. Pharmacol.* **21,** 3107.
Fritz, H. (1978). *In* "Neutral Proteases of Human Polymorphonuclear Leukocytes" (K. Havermann and A. Janoff, eds.), p. 261. Urban & Schwarzenberg, Munich.
Fujii, S., Moriya, H., and Suzuki, T. (1979a). *Adv. Exp. Med. Biol.* **120A.**
Fujii, S., Moriya, H., and Suzuki, T. (1979b). *Adv. Exp. Med. Biol.* **120B.**
Fujikawa, K., Kurachi, K., and Davie, E. W. (1977a). *Biochemistry* **16,** 4182.
Fujikawa, K., Walsh, K. A., and Davie, E. W. (1977b). *Biochemistry* **16,** 2270.
Fujikawa, K., Heimark, R. L., Kurachi, K., and Davie, E. W. (1980a). *Biochemistry* **19,** 1322.
Fujikawa, K., McMullen, B., Heimark, R. L., Kurachi, K., and Davie, E. W. (1980b). *In* "Protides of the Biological Fluids" (H. Peeters, ed.), pp. 193–196. Pergamon, Oxford.
Gallimore, M. J., Aasen, A. O., Lyngass, K. H. M., Larsbratten, M., and Amundsen, E. (1978a). *Thromb. Res.* **12,** 307.
Gallimore, M. J., Fareid, E., and Stormorken, H. (1978b). *Thromb. Res.* **12,** 409.
Ghebrehiwet, B., Silverberg, M., and Kaplan, A. P. (1981). *J. Exp. Med.* **153,** 665.
Gigli, I., Mason, J. W., Colman, R. W., and Anston, J. F. (1970). *J. Immunol.* **104,** 574.
Ginsberg, M. H., Jaques, B., Cochrane, C. G., and Griffin, J. H. (1980). *J. Lab. Clin. Med.* **95,** 497.
Gjonnaess, H. (1972a). *Thromb. Diath. Haemorrh.* **28,** 155.
Gjonnaess, H. (1972b). *Thromb. Diath. Haemorrh.* **28,** 169.
Gjonnaess, H. (1972c). *Thromb. Diath. Haemorrh.* **28,** 182.
Gjonnaess, H. (1972d). *Thromb. Diath. Haemorrh.* **28,** 194.
Goldsmith, G. H., Saito, H., and Ratnoff, O. D. (1978). *J. Clin. Invest.* **62,** 54.
Gore, I., and Larkey, B. J. (1960). *J. Lab. Clin. Med.* **56,** 839.
Graham, R., Ebert, R. H., Ratnoff, O. D., and Moses, J. M. (1965). *J. Exp. Med.* **121,** 807.
Greenbaum, L. M., and Kim, K. S. (1967). *Br. J. Pharmacol. Chemother.* **29,** 238.
Greenbaum, L. M., and Yamafuji, K. (1966). *In* "Hypotensive Peptides" (E. G. Erdos, N. Bach, and F. Scientieri, eds.), p. 252. Springer, New York.
Greengard, J. S., and Griffin, J. H. (1980). *Circulation* **62,** 56.
Greengard, J. S., Walsh, P. N., and Griffin, J. H. (1981). *Blood* **58,** 194a.
Griffin, J. H. (1977). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36,** 329.
Griffin, J. H. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 1998.
Griffin, J. H., and Beretta, G. (1979). *In* "Kinins II" (S. Fujii, H. Moriya, and T. Suzuki, eds.), pp. 39–51. Plenum, New York.
Griffin, J. H., and Cochrane, C. G. (1976a). *Methods Enzymol.* **45,** 56.
Griffin, J. H., and Cochrane, C. G. (1976b). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2554.
Griffin, J. H., and Cochrane, C. G. (1979). *Sem. Thromb. Hemost.* **5,** 254.
Griffin, J. H., Harper, A., and Cochrane, C. G. (1975). *Proc. Fed. Am. Soc. Exp. Biol.* **34,** 860.
Haanen, C., Hommes, F., and Morselt, G. (1961). *Thromb. Diath. Haemorrh.* 6, 261.
Habal, F. M., Movat, H. Z., and Burrowes, C. E. (1974). *Biochem. Pharmacol.* **23,** 2291.
Habermann, E. (1968). *Ann. N.Y. Acad. Sci.* **146,** 479.
Halonen, M., and Pinckard, R. N. (1975). *J. Immunol.* **115,** 519.
Han, Y. N., Komiya, M., Iwanaga, S., and Suzuki, T. (1975). *J. Biochem.* **77,** 55.
Han, Y. N., Kato, H., Iwanaga, S., and Suzuki, T. (1976). *J. Biochem.* **79,** 1201.
Han, Y. N., Kato, H., Iwanaga, S., and Komiya, S. (1978). *J. Biochem.* **83,** 223.
Hardisty, R. M., and Margolis, J. (1959). *Br. J. Haematol.* **5,** 203.
Harpel, P. C. (1970). *J. Exp. Med.* **132,** 329.
Harpel, P. C. (1971). *J. Clin. Invest.* **50,** 2084.
Harpel, P. C. (1972). *J. Clin. Invest.* **51,** 1813.

Harpel, P. C., and Cooper, N. R. (1975). *J. Clin. Invest.* **55,** 593.
Harrison, G. A., Robinson, M., and Stacey, R. V. (1971a). *Med. J. Anst.* **2,** 1040.
Harrison, G. A., Torda, J. A., and Schiff, P. (1971b). *Med. J. Aust.* **2,** 1308.
Hathaway, W. E., and Alsever, J. (1970). *Br. J. Haematol.* **18,** 161.
Hathaway, W. E., Belhasen, L. P., and Hathaway, H. S. (1965). *Blood* **26,** 521.
Hattersley, P. G., and Hayes, D. (1970). *Br. J. Haematol.* **18,** 411.
Heber, H., Geiger, R., and Heimburger, N. (1978). *Hoppe Seyler's Z. Physiol. Chem.* **359,** 659.
Heck, L. W., and Kaplan, A. P. (1974). *J. Exp. Med.* **140,** 1615.
Heimark, R. L., and Davie, E. W. (1979). *Biochemistry* **18,** 5743.
Heimark, R. L., Fujikawa, K., and Davie, E. W. (1978). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 1587.
Heimark, R. L., Kurachi, K., Fujikawa, K., and Davie, E. W. (1980). *Nature (London)* **286,** 456.
Hirsch, E. F., Nakayima, T., Oshima, G., Erdos, E. G., and Herman, C. M. (1974). *J. Surg. Res.* **17,** 147.
Hojima, Y., Pierce, J., and Pisano, J. (1980a). *Thromb. Res.* **20,** 149.
Hojima, Y., Pierce, J., and Pisano, J. (1980b). *Thromb. Res.* **20,** 163.
Hojima, Y., Tankersley, D. L., Miller-Andersson, M., Pierce, J. V., and Pisano, J. J. (1980c). *Thromb. Res.* **18,** 417.
Iatrides, S. G., and Ferguson, J. H. (1961). *Thromb. Diath. Haemorrh.* **6,** 411.
Iwanaga, S., Morita, T., Kato, H., Harada, T., Adachi, N., Sugo, T., Muruyama, I., Takada, K., Kimura, T., and Sakakibara, S. (1979). *Adv. Exp. Med. Biol.* 147.
Jacobsen, S., and Kriz, M. (1967). *Br. J. Pharmacol.* **29,** 25.
Jahrreiss, R., and Habermann, E. (1971). *Naunyn-Schmiedebergs Arch. Pharmakol.* **269,** 85.
James, A. T., and Wheatley, V. R. (1956). *Biochem. J.* **63,** 219.
Janoff, A., and Scherer, J. (1968). *J. Exp. Med.* **12,** 1137.
Johnston, A. R., Cochrane, C. G., and Revak, S. D. (1974). *J. Immunol.* **113,** 103.
Johnston, A. R., Ulevitch, R. J., and Ryan, K. (1976). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35,** 693.
Kagen, J. L. (1964). *Br. J. Exp. Pathol.* **45,** 604.
Kagen, J. L., and Becker, E. L. (1963). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **20,** 613.
Kagen, J. L., Liddy, J. P., and Becker, E. L. (1963). *J. Clin. Invest.* **42,** 1353.
Kaplan, A. P., and Austen, K. F. (1970). *J. Immunol.* **105,** 802.
Kaplan, A. P., and Austen, K. F. (1971). *J. Exp. Med.* **133,** 696.
Kaplan, A. P., and Austen, K. F. (1972). *J. Exp. Med.* **136,** 1378.
Kaplan, A. P., Spragg, J., and Austen, K. F. (1971). *Int. Symp. Biochem. Acute Allergic React., 2nd* p. 280.
Kaplan, A. P., Kay, A. B., and Austen, K. F. (1972). *J. Exp. Med.* **135,** 81.
Kaplan, A. P., Goetzl, E. J., and Austen, K. F. (1973). *J. Clin. Invest.* **52,** 2591.
Kato, H., Han, Y. N., Iwanaga, S., Hashimoto, N., Sugo, T., Fukii, S., and Suzuki, T. (1977). *In* "Kininogenases" (G. L. Haberland, J. W. Rohen, and T. Suzuki, eds.), p. 63. Schattauer, Stuttgart.
Kato, H., Sugo, T., Ikari, N., Hashimoto, N., Iwanaga, S., and Fukii, S. (1979). *Thromb. Haemostasis* **42,** 262.
Kellermeyer, R. W. (1962). *J. Lab. Clin. Med.* **70,** 372.
Kellermeyer, R. W., and Breckenridge, R. T. (1965). *J. Lab. Clin. Med.* **65,** 307.
Kellermeyer, R. W., and Breckenridge, R. T. (1966). *J. Lab. Clin. Med.* **67,** 455.
Kellermeyer, R. W., and Breckenridge, R. T. (1967). *J. Lab. Clin. Med.* **70,** 732.

Kellermeyer, W. F., Jr., and Kellermeyer, R. W. (1969). *Proc. Soc. Exp. Biol. Med.* **130,** 1310.
Kerbiriou, D. M., and Griffin, J. H. (1979). *J. Biol. Chem.* **254,** 12020.
Kerbiriou, D. M., Bouma, B. N., and Griffin, J. H. (1980). *J. Biol. Chem.* **255,** 3952.
Kimball, H. R., Melmon, K. L., and Wolff, S. M. (1972). *Proc. Soc. Exp. Biol. Med.* **139,** 1078.
Kisiel, W., Fujikawa, K., and Davie, E. W. (1977). *Biochemistry* **16,** 4189.
Kleniewski, J., and Donaldson, V. H. (1977). *Proc. Soc. Exp. Biol. Med.* **156,** 113.
Kluft, C. (1978). *J. Lab. Clin. Med.* **91,** 83.
Kluft, C., Trumpi-Kalshoven, N. M., Jei, A. F. H., and Veldhuyzen-Stolk, E. C. (1979). *Thromb. Diath. Haemorrh.* **4,** 285.
Koide, T., Hermodson, M. A., and Davie, E. W. (1977a). *Nature (London)* **266,** 729.
Koide, T., Kato, H., and Davie, E. W. (1977b). *Biochemistry* **16,** 2279.
Komiya, M., Kato, H., and Suzuki, T. (1974). *J. Biochem. (Tokyo)* **76,** 811.
Kozono, K., Yamamoto, T., and Kambara, T. (1980). *Am. J. Pathol.* **100,** 619.
Kule, C. A., and Eisen, V. (1970). *In* "Bradykinin and Related Kinins" (F. Sicuteri, M. Rocha e Silva, and N. Bach, eds.), p. 471. Plenum, New York.
Kurachi, K., and Davie, E. W. (1977). *Biochemistry* **16,** 5831.
Kurachi, K., Fujikawa, K., and Davie, E. W. (1980). *Biochemistry* **19,** 1330.
Laake, K., and Vennerod, A. M. (1974). *Thromb. Res.* **4,** 285.
Laake, K., Vennerod, A. M., Hangen, G., and Gjonnaess, H. (1974). *Thromb. Res.* **4,** 769.
Lacombe, M. J. (1975). *C.R. Hebd. Seances Acad. Sci. Ser. D. Sci. Nat.* **280,** 1039.
Lacombe, M. J., Varet, B., and Levy, J. P. (1975). *Blood* **46,** 761.
Landerman, N. S., Webster, M. E., Becker, E. L., and Ratcliffe, H. E. (1962). *J. Allergy* **33,** 330.
Levy, L. R., and Lepow, I. H. (1959). *Proc. Soc. Exp. Biol. Med.* **101,** 608.
Liu, C. Y., Scott, C. F., Bagdasarian, A., Pierce, J. V., Kaplan, A. P., and Colman, R. W. (1977). *J. Clin. Invest.* **60,** 7.
McCarty, D. J., Philips, P., and Pyenson, J. (1966). *J. Exp. Med.* **124,** 99.
McConnell, D. J. (1972). *J. Clin. Invest.* **51,** 1611.
McMillin, C. R., Saito, H., Ratnoff, O. D., and Walton, A. G. (1974). *J. Clin. Invest.* **54,** 1312.
Magnusson, S., Peterson, T. E., Sottrup-Jensen, L., and Ures, H. (1975). *Cold Spring Harbor Conf. Cell Prolif.* **2,** 123.
Majerus, P. W., and Miletich, J. P. (1978). *Annu. Rev. Med.* **29,** 41.
Mandle, R., and Kaplan, A. P. (1977). *J. Biol. Chem.* **252,** 6097.
Mandle, R. J., and Kaplan, A. P. (1979). *Blood* **54,** 850.
Mandle, R. J., Colman, R. W., and Kaplan, A. P. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4179.
Margalit, R., and Schiffmann, S. (1980). *Blood* **56,** 168.
Margolis, J. (1958). *Nature (London)* **181,** 635.
Margolis, J. (1960). *Aust. J. Biol. Med. Sci.* **37,** 239.
Margolis, J. (1961). *Aust. J. Exp. Biol. Med. Sci.* **39,** 249.
Margolis, J. (1962). *Aust. J. Exp. Biol. Med. Sci.* **40,** 505.
Margolis, J. (1963). *Ann. N.Y. Acad. Sci.* **104,** 133.
Mason, J. M., Kleeberg, V., Dolan, P., and Colman, R. W. (1970). *Ann. Intern. Med.* **73,** 545.
Matheson, R. T., Miller, D. R., Lacombe, M. J., Han, Y. N., Iwanaga, S., Kato, H., and Wuepper, K. D. (1976). *J. Clin. Invest.* **58,** 1395.

Meier, H. L., Pierce, J. V., Colman, R. W., and Kaplan, A. P. (1977a). *J. Clin. Invest.* **60,** 18.
Meier, H. L., Thompson, R. E., and Kaplan, A. P. (1977b). *Thromb. Haemostasis* **38,** 14.
Melmon, K. L., and Cline, M. J. (1967). *Nature (London)* **213,** 90.
Menkin, V. (1938). *J. Exp. Med.* **67,** 129.
Miles, A. A., and Wilhelm, D. L. (1955). *Br. J. Exp. Pathol.* **36,** 71.
Miles, L. A., Rothschild, Z., and Griffin, J. H. (1981). *Thromb. Hemostasis,* in press.
Miletich, J. P., Jackson, C. M., and Majerus, P. W. (1978). *J. Biol. Chem.* **253,** 6908.
Millar, J. A., Clappison, B. H., and Johnston, C. I. (1978). *Lancet* **2,** 1376.
Mori, K., and Nagasawa, S. (1981). *J. Biochem. (Japan)* **89,** 1465.
Morrison, D. C., and Cochrane, C. G. (1974). *J. Exp. Med.* **140,** 797.
Moskowitz, R. W., Schwartz, H. J., Michel, B., Ratnoff, O. D., and Astrup, T. (1970). *J. Lab. Clin. Med.* **76,** 790.
Movat, H. Z. (1967). *In* "International Symposium on Vaso-active Polypeptides," p. 177. Edart Livraria Editora, São Paulo.
Movat, H. Z., and DiLorenzo, N. L. (1968). *Lab. Invest.* **19,** 187.
Movat, H. Z., and Habal, F. M. (1976). *In* "Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease" (J. J. Pisano and K. F. Austen, eds.). Fogarty International Center Proc. 27, U.S. Govt. Printing Office, Washington, D.C.
Movat, H. Z., and Ozge-Anwar, A. H. (1974). *J. Lab. Clin. Med.* **84,** 861.
Movat, H. Z., DiLorenzo, N. L., and Treloar, M. P. (1968). *Lab. Invest.* **19,** 201.
Movat, H. Z., Trevlar, M. P., and Takeuchi, Y. (1969). *J. Immunol.* **103,** 875.
Movat, H. Z., Steinberg, S. G., Habal, F. M., and Ranadive, N. S. (1973). *Lab. Invest.* **6,** 669.
Movat, H. Z., Habal, F. M., and MacMorine, D. R. L. (1975). *In* "Kinins. Pharmacodynamics and Biologic Roles" (F. Sicuteri, N. Back, and G. L. Haberland, eds.), p. 345. Plenum, New York.
Movat, H. Z., Habal, F. M., and MacMorine, D. R. L. (1976a). *Int. Arch. Allergy Appl. Immunol.* **50,** 257.
Movat, H. Z., Minta, J. O., Saya, M. J., Habal, F. M., Burrowes, C. E., Wasi, S., and Pass, E. (1976b). *In* "Molecular and Biological Aspects of the Acute Allergic Reaction" (S. G. O. Johansson, K. Strandberg, and B. Uvnas, eds.), p. 391. Plenum, New York.
Nagaki, K., and Inai, S. (1976). *Int. Arch. Allergy Appl. Immunol.* **50,** 172.
Nagasawa, S., and Nakayasu, T. (1973). *J. Biochem.* **74,** 401.
Nakayasu, T., and Nagasawa, S. (1979). *J. Biochem.* **85,** 249.
Newball, H. H., Talamo, R., and Lichtenstein, L. (1975). *Nature (London)* **25,** 635.
Newball, H. H., Talamo, R., and Lichtenstein, L. (1975). *Nature (London)* **25,** 635.
Newball, H. H., Berninger, R. W., Talamo, R. C., and Lichtenstein, L. M. (1979a). *J. Clin. Invest.* **64,** 457.
Newball, H. H., Talamo, R., and Lichtenstein, L. (1979b). *J. Clin. Invest.* **64,** 466.
Newball, H. H., Revak, S. D., Cochrane, C. G., Griffin, J. H., and Lichtenstein, L. M. (1979c). *In* "Kinins II" (T. Suzuki and H. Moriya, eds.), p. 139. Plenum, New York.
Newball, H. H., Meier, H. L., Kaplan, A. P., Cochrane, C. G., Revak, S. D., and Lichtenstein, L. (1981). *Trans. Am. Assoc. Phys.* **94,** 126.
Niemetz, J., and Nossel, H. L. (1967). *Thromb. Diath. Haemorrh.* **17,** 335.
Nies, A. S., Forsyth, R. P., Williams, H. E., and Melmon, K. L. (1968). *Circ. Res.* **22,** 155.
Niewiarowski, S., and Prou-Wartell, O. (1959). *Thromb. Diath. Haemorrh.* **3,** 593.
Niewiarowski, S., Bankowski, E., and Fiedoreck, T. (1964). *Experientia* **20,** 367.
Niewiarowski, S., Bankowski, E., and Rogowicka, I. (1965). *Thromb. Diath. Haemorrh.* **14,** 387.
Nossel, H. L. (1964). *In* "Contact Phase of Blood Coagulation." Blackwell, Oxford.

Nossel, H. L. (1966). *Proc. Soc. Exp. Biol. Med.* **122,** 16.
Nossel, H. L., Rubin, H., Drillings, H., and Haich, R. (1968). *J. Clin. Invest.* **47,** 1172.
O'Donnell, T. F., Clowes, G. H., Jr., and Talamo, R. C. (1976). *Surg. Gynecol. Obstet.* **143,** 539.
Ogston, D., Ogston, C. M., and Ratnoff, O. D. (1969a). *J. Lab. Clin. Med.* **73,** 70.
Ogston, D., Ogston, M., Ratnoff, O. D., and Forbes, C. B. (1969b). *J. Clin. Invest.* **48,** 1786.
Oh-Ishi, S., and Webster, M. E. (1975). *Biochem. Pharmacol.* **24,** 591.
Oh-Ishi, S., Katori, M., Han, Y. N., Kanagawa, S., Kato, H., and Suzuki, T. (1977a). *Biochem. Pharmacol.* **26,** 115.
Oh-Ishi, S., Tanaka, K., Katori, M., Han, Y. M., Kato, H., and Iwanaga, S. (1977b). *Life Sci.* **20,** 695.
Oh-Ishi, S., Ueno, A., Uchida, Y., Katori, M., Hayashi, H., Koya, H., Kitajima, K., and Kimura, I. (1979). *In* "Kinins II" (S. Fujii, H. Moriya, and T. Susuki, eds.), p. 93. Plenum, New York.
Ornstein, N. S., Galli, S. J., Dvorak, A. M., Silbert, J. E., and Dvorak, H. F. (1978). *J. Immunol.* **121,** 586.
Osmond, D. H., and Loh, A. Y. (1978). *Lancet* **1,** 102.
Osmond, D. H., Lo, E. K., Loh, A. Y., Zingg, E. A., and Hedlin, A. H. (1978). *Lancet* **2,** 1375.
Pass, E., Movat, H. Z., and Wasi, S. (1978). *Agents Actions* **8,** 91.
Pechet, L., Cochios, F., and Deykin, D. (1975). *Thromb. Diath. Haemorrh.* **17,** 365.
Philips, P., Prockop, D. J., and McCarty, D. J. (1966). *J. Lab. Clin. Med.* **68,** 433.
Pierce, J. V., and Guimaraes, J. A. (1976). *In* "The Kallikrein System in Health and Disease" (J. J. Pisano and K. F. Austen, eds.), p. 121. Fogarty International Center Symp. No. 27, Washington, D.C.
Pinckard, R. N., Tanigawa, C., and Halonen, M. (1975). *J. Immunol.* **115,** 525.
Pisano, J. J., and Austen, K. F., eds. (1976). "Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease." DHEW No. (NIH) 76-791.
Proctor, R. R., and Rapaport, S. I. (1961). *Am. J. Clin. Pathol.* **35,** 212.
Proud, D., Pierce, J. V., and Pisano, J. J. (1980). *J. Lab. Clin. Med.* **95,** 563.
Radcliffe, R., Bagdasarian, A., Colman, R. W., and Nemerson, Y. (1977). *Blood* **50,** 611.
Ratnoff, O. D. (1965). *J. Exp. Med.* **122,** 905.
Ratnoff, O. D. (1966). *Prog. Hematol.* **5,** 204.
Ratnoff, O. D. (1969). *Adv. Immunol.* **10,** 155.
Ratnoff, O. D., and Crum, J. D. (1964). *J. Lab. Clin. Med.* **63,** 359.
Ratnoff, O. D., and Miles, A. A. (1964). *Br. J. Exp. Pathol.* **45,** 328.
Ratnoff, O. D., and Rosenblum, J. M. (1958). *Am. J. Med.* **25,** 160.
Ratnoff, O. D., and Saito, H. (1979a). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 958.
Ratnoff, O. D., and Saito, H. (1979b). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1461.
Ratnoff, O. D., Davie, E. W., and Mallett, D. L. (1961). *J. Clin. Invest.* **40,** 803.
Ratnoff, O. D., Pensky, J., Donaldson, V. H., and Amir, J. (1972). *J. Lab. Clin. Med.* **80,** 803.
Ratnoff, O. D., Pensky, J., Ogston, D., and Naff, G. B. (1969). *J. Exp. Med.* **129,** 315.
Revak, S. D., and Cochrane, C. G. (1976). *J. Clin. Invest.* **57,** 852.
Revak, S. D., Cochrane, C. G., Johnston, A. R., and Hugli, T. H. (1974). *J. Clin. Invest.* **54,** 619.
Revak, S. D., Cochrane, C. G., and Griffin, J. H. (1977). *J. Clin. Invest.* **59,** 1167.
Revak, S. D., Cochrane, C. G., Bouma, B. N., and Griffin, J. H. (1978). *J. Exp. Med.* **147,** 719.
Rimon, A., Schiffman, S., Feinstein, D. I., and Rapaport, S. I. (1976). *Blood* **48,** 165.

Robinson, J. A., Kloduycky, M. L., Lock, H. H., Racic, M. R., and Gunner, R. M. (1975). *Am. J. Med.* **59,** 61.
Rodriquez-Erdman (1964). *Thrombosis Diath. Haemorrh.* **12,** 471.
Rothschild, A. M. (1981). *Biochem. Pharmacol.* **30,** 481.
Saito, H. (1977). *J. Clin. Invest.* **60,** 584.
Saito, H. (1980). *In* "The Regulation of Coagulation" (K. G. Hann and F. B. Taylor, eds.), p. 555. Elsevier, Amsterdam.
Saito, H., and Goldsmith, G. (1977). *Blood* **50,** 377.
Saito, H., and Ratnoff, O. D. (1974). *Nature (London)* **248,** 597.
Saito, H., and Ratnoff, O. D. (1977). *Clin. Res.* **25,** 347A.
Saito, H., Ratnoff, O. D., Marshall, J. S., and Pensky, J. (1973). *J. Clin. Invest.* **52,** 850.
Saito, H., Ratnoff, O. D., and Donaldson, V. H. (1974). *Circ. Res.* **34,** 641.
Saito, H., Ratnoff, O. D., Waldmann, R., and Abraham, J. P. (1975). *J. Clin. Invest.* **55,** 1082.
Saito, H., Ratnoff, O. D., and Pensky, J. (1976). *J. Lab. Clin. Med.* **88,** 506.
Saito, H., Poon, M. G., Vicic, W., Goldsmith, G. H., and Menitove, J. E. (1978). *J. Lab. Clin. Med.* **92,** 84.
Saito, H., Goldsmith, G., Moroi, M., and Aoko, N. (1979a). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 2103.
Saito, H., Scott, J. G., Movat, H. Z., and Scialla, S. J. (1979b). *J. Lab. Clin. Med.* **94,** 256.
Sawai, K., Niwa, S., and Katori, M. (1980). *Proc. Int. Conf. Kinins* p. 195.
Schachter, M. (1969). *Physiol. Rev.* **49,** 509.
Schiffman, S., and Lee, P. (1974). *Br. J. Haematol.* **27,** 101.
Schiffman, S., and Lee, P. (1975). *J. Clin. Invest.* **56,** 1082.
Schiffman, S., Lee, P., and Wladmann, R. (1975). *Thromb. Res.* **6,** 451.
Schiffman, S., Lee, P., Feinstein, D. I., and Pecci, R. (1977). *Blood* **49,** 935.
Schiffman, S., Mannhalter, C., and Tyner, K. D. (1980). *J. Biol. Chem.* **255,** 6433.
Schiffmann, S., Margalit, R., Rosove, M., and Feinstein, D. (1981). *Blood* **57,** 437.
Schreiber, A. D., Kaplan, A. P., and Austen, F. F. (1973a). *J. Clin. Invest.* **52,** 1402.
Schreiber, A. D., Kaplan, A. P., and Austen, F. F. (1973b). *J. Clin. Invest.* **52.**
Schwartz, H. J., and Kellermeyer, R. W. (1969). *Proc. Soc. Exp. Biol. Med.* **132,** 1021.
Scott, C. F., Shapira, M., James, H. L., Cohen, A. B., and Colman, R. W. (1982). *J. Clin. Invest.* **69,** 844.
Sealey, J., Atlas, S. A., Laragh, J. H., Oza, N. B., and Ryan, J. W. (1978). *Nature (London)* **275,** 144.
Sealey, J., Atlas, S. A., Laragh, J. H., Silverberg, M. J., and Kaplan, A. P. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5914.
Seligsohn, U., Osterud, B., Brown, S. F., Griffin, J. H., and Rapaport, S. (1979). *J. Clin. Invest.* **64,** 1056.
Sigler, J. P., and Prou-Wartelle, D. (1960). *Br. J. Haematol.* **6,** 88.
Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968). *J. Mol. Biol.* **35,** 143.
Silverberg, M., Dunn, J. T., Garen, L., and Kaplan, A. P. (1980). *J. Biol. Chem.* **255,** 7281.
Smink, M. M., Daniel, T. M., Ratnoff, O. D., and Stavitsky, A. B. (1965). *J. Lab. Clin. Med.* **69,** 819.
Soltay, M. J., Movat, H. Z., and Ozge-Anwar, A. H. (1972). *Proc. Soc. Exp. Biol.* **138,** 952.
Soulier, J. P., Wartelle, O., and Menache, D. (1959). *Br. J. Haematol.* **5,** 121.
Spector, W. G. (1957). *J. Pathol. Bacteriol.* **74,** 67.
Stead, N., Kaplan, A. P., and Rosenberg, R. D. (1976). *J. Biol. Chem.* **251,** 6481.
Stroud, R. M., Krieger, M., Koeppe, R. E., Kossiakoff, A. A., and Chambers, J. L. (1975). *Cold Spring Harbor Conf. Cell Prolif.* **2,** 13.

Sugo, T., Ikari, N., Kato, H., Iwanaga, S., and Fujii, S. (1980). *Biochemistry* **19,** 3215.
Takahashi, H., Nagasawa, S., and Suzuki, T. (1972). *J. Biochem.* **71,** 471.
Takahashi, H., Nagasawa, S., and Suzuki, T. (1980). *J. Biochem.* **87,** 23.
Takeuchi, Y., and Movat, H. Z. (1972). *Eur. J. Immunol.* **2,** 345.
Tankersley, D. L., Fournel, M. A., and Schroeder, D. D. (1980). *Biochemistry* **19,** 3121.
Tans, G., and Griffin, J. H. (1981). *Blood* **59,** 69.
Thompson, R. E., Mandle, R., and Kaplan, A. P. (1977). *J. Clin. Invest.* **60,** 1376.
Thompson, R. E., Mandle, R., Jr., and Kaplan, A. P. (1978). *J. Exp. Med.* **147,** 488.
Titani, K., Fujikawa, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3082.
Ulevitch, R. J., Cochrane, C. G., and Johnston, A. R. (1980). *Inflammation* **4,** 9.
Umezawa, H., ed. (1972). "Enzyme Inhibitors of Microbial Origin." Univ. Park Press, Baltimore, Maryland.
Vennerod, A. M., and Laake, K. (1975). *Thromb. Res.* **7,** 223.
Vennerod, A. M., and Laake, K. (1976). *Thromb. Res.* **8,** 519.
Vogt, W. (1966). *In* "Hypotensive Peptides" (E. G. Erdos, N. Back, F. Sicuteri, and A. F. Wilde, eds.), p. 185. Springer-Verlag, Berlin and New York.
Waldman, R., and Abraham, J. P. (1974). *Blood* **44,** 934.
Waldman, R., Scicli, A. G., Scicli, G. M., Guimaraes, J. A., Carretero, O. A., Kato, H., Han, Y. N., and Iwanaga, S. (1977). *Thromb. Haemostasis* **38,** 14.
Walsh, P. N. (1972a). *Br. J. Haematol.* **22,** 237.
Walsh, P. N. (1972b). *Br. J. Haematol.* **22,** 393.
Walsh, P. N. (1974). *Blood* **43,** 597.
Walsh, P. N., and Griffin, J. H. (1980). *Blood* **57,** 106.
Wasi, S., Movat, H. Z., Pass, E., and Chan, J. Y. C. (1978). *In* "Neutral Proteases of Human Polymorphonuclear Leukocytes" (K. Havermann and A. Janoff, eds.), p. 245. Urban & Schwartzenback, Baltimore, Maryland.
Webster, M. E., and Maling, H. M. (1970). *In* "Bradykinin and Related Kinins: Cardiovascular, Biochemical and Neural Actions" (F. Sicuteri, M. Rocha e Silva, and N. Bach, eds.), p. 493. Plenum, New York.
Webster, M., and Ratnoff, O. D. (1961). *Nature (London)* **192,** 180.
Weiss, A. S., Gallin, J. I., and Kaplan, A. P. (1974). *J. Clin. Invest.* **53,** 622.
Wendel, V., Vogt, W., and Seidel, G. (1972). *Hoppe Seylers Z. Physiol. Chem.* **353,** 1591.
Wiggins, R. C., and Cochrane, C. G. (1979). *J. Exp. Med.* **150,** 1122.
Wiggins, R. C., Bouma, B. N., Cochrane, C. G., and Griffin, J. H. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4636.
Wiggins, R. C., Cochrane, C. G., and Griffin, J. H. (1979a). *Thromb. Res.* **15,** 475.
Wiggins, R. C., Cochrane, C. G., and Griffin, J. H. (1979b). *Thromb. Res.* **15,** 487.
Wiggins, R. C., Giclas, P. C., and Henson, P. M. (1980a). *J. Exp. Med.* **153,** 1391.
Wiggins, R. C., Loskutoff, D., Cochrane, C. G., Griffin, J. H., and Edgington, T. E. (1980b). *J. Clin. Invest.* **65,** 197.
Wilhelm, D. L., Mill, P. J., Sparrow, E. M., Mackey, M. E., and Miles, A. A. (1958). *Br. J. Exp. Pathol.* **39,** 228.
Wilner, G. D., Nossel, H. L., and Leroy E. C. (1968). *J. Clin. Invest.* **47,** 2608.
Wing, D. A., Yamada, T., Hayley, H. B., and Pettit, G. W. (1978). *J. Lab. Clin. Med.* **92,** 239.
Wuepper, K. D. (1972). *In* "Inflammation: Mechanisms and Control" (I. Lepow, and P. A. Ward, eds.), p. 93. Academic Press, New York.
Wuepper, K. D. (1973). *J. Exp. Med.* **138,** 1345.
Wuepper, K. D., and Cochrane, C. G. (1971). *In* "Biochemistry of the Acute Allergic Reaction" (K. F. Austen and E. L. Becker, eds.), p. 303. Blackwell, Oxford.

Wuepper, K. D., and Cochrane, C. G. (1972a). *J. Exp. Med.* **135,** 1.
Wuepper, K. D., and Cochrane, C. G. (1972b). *Proc. Soc. Exp. Biol. Med.* **141,** 271.
Wuepper, K. D., Tucker, E. S., and Cochrane, C. G. (1970). *J. Immunol.* **105,** 1307.
Wuepper, K. D., Miller, D. R., and Lacombe, M. J. (1975). *J. Clin. Invest.* **56,** 1663.
Yamamoto, T., and Cochrane, C. G. (1981). *Am. J. Pathol.* **105,** 164.
Yamamoto, T., and Kambara, T. (1978). *Biochim. Biophys. Acta* **540,** 55.
Yamamoto, T., Kozono, K., Okamoto, T., Kato, H., and Kambara, T. (1980). *Biochim. Biophys. Acta* **614,** 511.
Yurt, R., and Austen, K. F. (1977). *J. Exp. Med.* **146,** 1405.

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Binding of Bacteria to Lymphocyte Subpopulations

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I. Introduction

Lymphocyte populations are composed of subpopulations which are functionally different but morphologically indistinguishable. A variety of cell surface markers have been used to identify and separate these subpopulations: T and B cells bind different heterologous erythrocytes (Wybran and Fudenberg, 1973; Pellegrino *et al.*, 1975; Forbes and Zalewski, 1976), some B cells bind erythrocytes coated with C3b (Ross *et al.*, 1973), and B cells and subpopulations of T cells have receptors for the Fc portion of immunoglobulins (Basten *et al.*, 1972; Cline *et al.*, 1972; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972a,b; Grey *et al.*, 1972; Moretta *et al.*, 1976). Also, surface antigens have been studied using alloantibodies (Cantor, 1972; Cantor *et al.*, 1976; Cantor and Boyse, 1975; Ahmed *et al.*, 1977) or xenoantibodies (Brown and Greaves, 1974; Strelkauskas *et al.*, 1975; Wilson *et al.*, 1976; Chess and Schlossman, 1977) and these antibodies have been used to identify lymphocytes subpopulations.

Functional differences between lymphocyte subpopulations with different surface antigens and receptors have been demonstrated in numerous reports. Since B and T cells have different functions and different surface antigens it was possible that their subpopulations with specialized functions also had different antigens. This has been well documented now. For example, mouse Ly alloantibodies can distinguish between three functionally different T-cell subpopulations (Huber *et al.*, 1976; Kisielow *et al.*, 1975; Shiku *et al.*, 1975) and monoclonal mouse anti-human T-cell antibodies distinguish at least two functionally different subpopulations (Reinherz *et al.*, 1979, 1980a,b; Reinherz and Schlossman, 1980). Some of the antigens found on lymphocyte surface such as Ig or Ia antigens on B cells are clearly related to their functions, namely, the interaction with antigens or with other cells. However, most other antigens identified on either B cells, T cells, or their subpopulation have no apparent reason for being present on one subpopulation but not on the other. Speculations have been made regarding the role of receptors for the Fc portion of different immunoglobulins (Moretta *et al.*, 1977), but no evidence has yet been obtained to support them. Moreover, the presence of Fc receptors for IgG, or IgM on functionally distinct human T cells has been contradicted by other reports (Lum *et al.*, 1980).

One important role played by the surface structures of lymphocytes is the interaction with other cells through membrane contacts. They

attach and interact with macrophages or other lymphocyte subpopulations, or are recognized by the endothelial cells of the postcapillary venule and allowed to pass from the blood stream into the lymph node. Alterations of surface structures by proteolytic enzymes (Woodruff, 1974; Freitas and de Sousa, 1976a), phospholipases (Freitas and de Sousa, 1976b), neuraminidase (Freitas and de Sousa, 1976a), or plant lectins (Gillette *et al.*, 1973; Freitas and de Sousa, 1975) result in a substantial alteration in lymphocyte distribution and homing. Close contact between autologous or syngeneic T cells and Ia-bearing non-T cells results in the activation of T cells as manifested by blast transformation (Howe, 1973; Ponzio *et al.*, 1975; Kunz *et al.*, 1976; Smith, 1978; Teodorescu, 1981), production of polyclonal B-cell activators (Buchholz *et al.*, 1979; Yu *et al.*, 1980; Chang *et al.*, 1981), or development of cytotoxic cells (Tomonari, 1980). Thus, through surface contacts, lymphocytes are involved in a multitude of cellular interactions with functional consequences.

The ability of lymphocyte subpopulations to interact with other cell surfaces, particularly erythrocytes, has been used for their identification. For example, the selective binding of sheep erythrocytes to human T cells (Jondal *et al.*, 1972) or of mouse erythrocytes to human B cells (Pellegrino *et al.*, 1975) showed that functionally different lymphocytes can bind to different types of cell surfaces. Therefore, looking for a variety of cells, with highly variable surface components that can interact with lymphocytes, appeared to be a useful approach for studying lymphocyte receptors and functions.

Bacteria represent a practically unlimited supply of cells that can be used as probes for lymphocyte surfaces. In the process of using bacteria as inert carriers for antibodies directed against antigens of the lymphocyte membrane (Teodorescu *et al.*, 1976) it has been found that some bacteria bind naturally or spontaneously to lymphocytes (Teodorescu *et al.*, 1977a). In what follows we shall review the work done in our laboratories as well as in others on the natural binding of bacteria to lymphocyte subpopulations. We shall review evidence that demonstrates or suggests that (a) this binding is highly reproducible; (b) the lymphocytes identified are functionally different; (c) the binding of bacteria may be related to lymphocyte functions; (d) one mechanism of binding may involve lectins on the lymphocyte surface interacting with carbohydrates on the bacteria; and (e) the use of bacteria as lymphocyte markers is clinically useful.

II. Mapping and Separation of Lymphocyte Subpopulations by Bacterial Adherence

A. Methods for Labeling Lymphocytes by Bacterial Adherence

1. General Principle

Bacteria have been used for labeling the lymphocytes in a "rosette" assay. In principle, an excess number of bacteria is centrifuged for a few minutes with lymphocytes; the mixture is resuspended and examined by phase contrast microscopy (Fig. 1). When whole blood is used the excess bacteria is removed by low-speed centrifugation, the cells are smeared, stained, and counted as for any other differential counting of leukocytes (Fig. 2). Thus, the procedures of labeling the lymphocytes with bacteria can be subdivided into (a) those involving lymphocyte suspensions (Teodorescu *et al.*, 1977a,b; Rasanen, 1981; Niemetz and Mayr, 1981a; Gaudernack *et al.*, 1981; Mayer *et al.*, 1978b) and (b) those involving stained smears of whole blood cells (Teodorescu *et al.*, 1976, 1979a; Mayer *et al.* 1978a; Bratescu *et al.*, 1980).

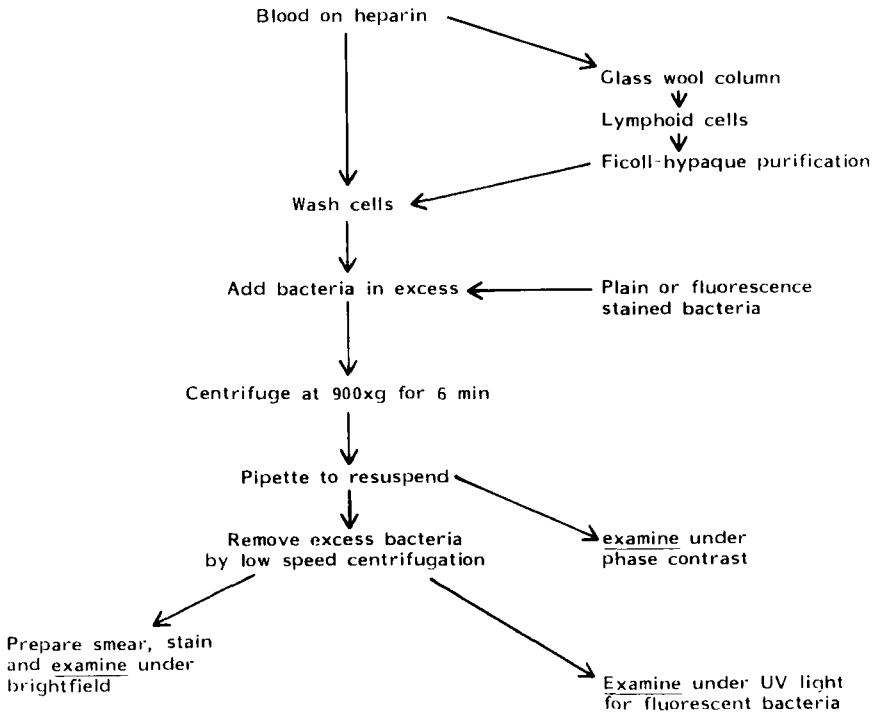

FIG. 1. Summary of the protocal for labeling lymphocytes with bacteria.

2. *Preparation of Lymphocytes or Blood Samples*

To label lymphocytes from human peripheral blood in cell suspension the lymphocytes are first purified, and then rosetted with bacteria. The monocytes, which also bind bacteria (Gaudernack *et al.*, 1981), are removed by applying the heparinized whole blood to a glass wool column and incubating at 37°C to allow the monocytes and platelets to adhere to glass. The blood is washed out of the column and centrifuged on a Ficoll–Hypaque medium to obtain the lymphocytes. This method yields populations with less than 1% phagocytic mononuclear cells (Kleinman and Teodorescu, 1978) and <3% polymorphonuclear cells. However, the possibility that some subpopulations of lymphocytes may be lost preferentially by the process of purification cannot be ruled out (Brown and Greaves, 1974). To prepare lymphoid organ suspensions (Mayer *et al.*, 1978b) the cells are centrifuged on Ficoll–Hypaque medium to remove contaminating blood cells as well as dead cells, which have the potential of forming artifactual rosettes.

Bacteria are prepared in the following manner. They are grown in suspensions, collected in late log phase, and killed either with merthiolate or by heating at 121°C. To make them stable shelf reagents, bacteria are fixed with formaldehyde, the clumps dispersed by sonication, and, after careful washing, they are finally stored at 4 or at −20°C (Teodorescu *et al.*, 1977a; Bratescu *et al.*, 1980). Not all bacteria can be heated at 121°C without altering their binding properties for lymphocytes or without causing substantial agglutination. For example, *Brucella melitensis* and *abortus* are not altered by heating at 121°C while *Arizona hinshawii* and *Escherichia coli* form large aggregates that cannot be dispersed.

For regular bright field or phase contrast microscopy bacteria can be prepared as indicated above (Teodorescu *et al.*, 1977a,b; Mayer *et al.*, 1978a). For fluorescence microscopy or for fluorescence flow cytometry bacteria are stained with xantheno-phenolrhodamine isothiocyanate (XRITC) (Gaudernack *et al.*, 1981), tetramethyl-rhodamine isothiocyanate, or fluorescein isothiocyanate (Niemetz and Mayr, 1981a,b; Spear and Teodorescu, unpublished). The conditions for labeling should be relatively mild to avoid alterations in the binding properties of the bacteria or the formation of aggregates (Spear *et al.*, unpublished). Fluorescent bacteria are useful when attempts are made to compare the binding of fluorescent antibody and of bacteria to a particular subpopulation of lymphocytes (Gaudernack *et al.*, 1981; Niemetz and Mayr, 1981a,b).

3. *Labeling Lymphocytes with Bacteria*

To demonstrate the binding of bacteria to lymphocytes, samples of the cell suspensions are mixed with a large excess of bacteria (about 100 bacteria/lymphocyte) in a medium containing bovine serum albumin (BSA), and the mixture is centrifuged at 900 *g* for 5–6 minutes. The cells are resuspended by vigorous pipetting and examined by phase contrast microscopy. The excess bacteria can be removed by centrifuging the cells at low speed. This last step is required for fluorescence (Gaudernack *et al.*, 1981; Niemetz and Mayr, 1981a) but not for phase contrast microscopy (Teodorescu *et al.*, 1977a; Mayer *et al.*, 1978b) (Fig. 2a).

To identify lymphocyte subpopulation in stained blood smears a method was also developed that takes advantage of the small size of the bacteria. This method was first described for bacteria that did not bind naturally to lymphocytyes but which could be made to bind by coating them with anti-Ig antibodies (Teodorescu *et al.*, 1976; Mayer *et al.*, 1978a). It was then adopted for bacteria that bind spontaneously to lymphocytes (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979a; Bratescu *et al.*, 1980). To remove plasma proteins the whole blood cell population, including the red cells, is washed with medium supplemented with 6% bovine serum albumin. Maintaining this protein concentration appears to be important to obtain good results (Bratescu *et al.*, 1980). Bacteria are added at a ratio of about 10 organisms for each red cell and the mixture is centrifuged at 900 *g* for 6 minutes. During the centrifugation the red cells sediment first, followed by the leukocytes which form the buffy coat, and, finally, bacteria. Under these conditions bacteria interact with the leukocytes at a ratio of about 10,000/1 and a good rosette formation is promoted. To remove the excess bacteria the cells are washed twice by low-speed centrifugation. Finally, a smear is prepared and stained and the percentage of lymphocytes that have bacteria attached is determined (Fig. 2).

When cell suspensions are used the investigator decides how many bacteria per lymphocyte will represent a rosette. This is true both for phase contrast or fluorescent microscopy. Generally, the decision is easy to make since lymphocytes either have many bacteria attached or none at all (Fig. 2). However, for an objective and accurate determination that can be standardized, blood smears offer a clear advantage, due to the fewer variables involved in sample preparation. For those lymphocytes that have only a few bacteria, the nonrandom associations (rosetting) have to be distinguished from random associations. Since none of the bacteria studied so far binds to erythrocytes, the random

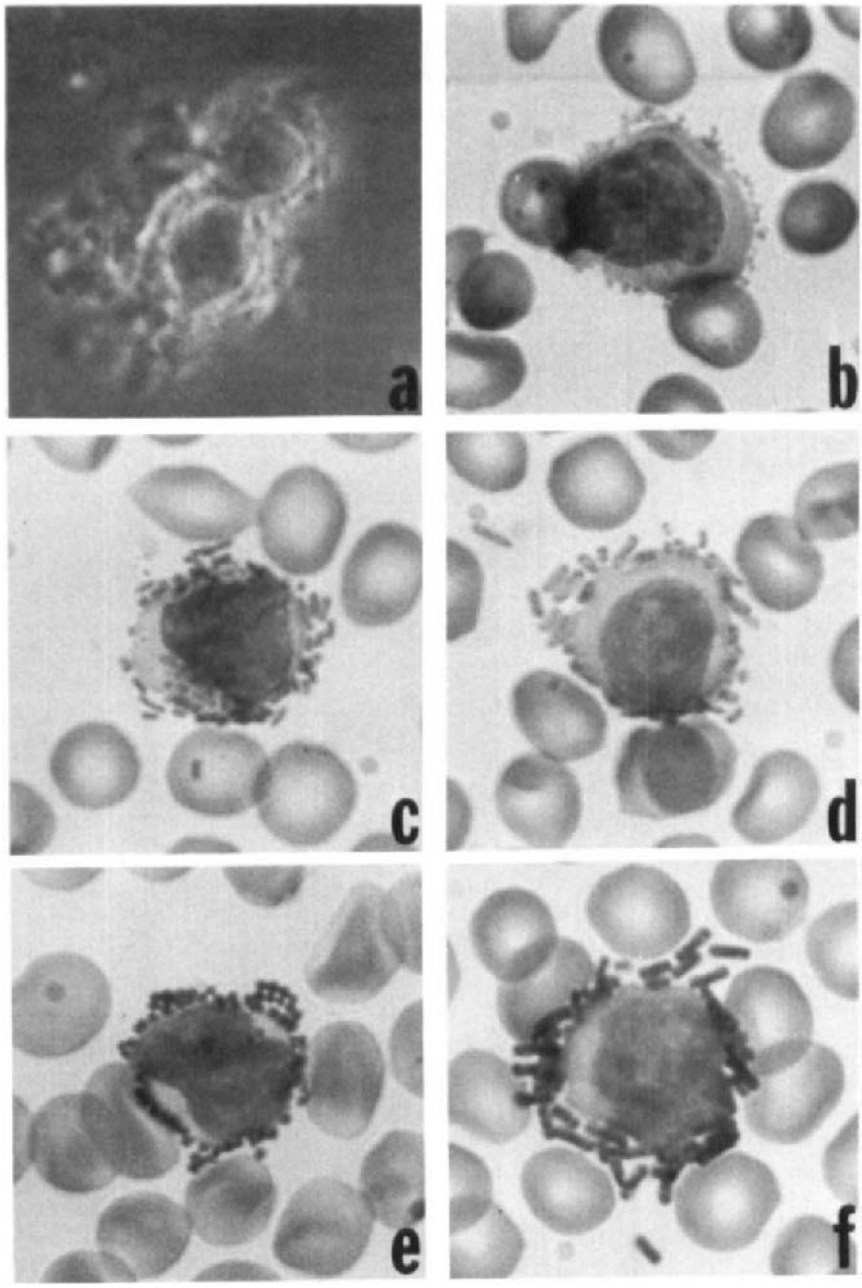

FIG. 2. Lymphocytes with bacteria attached. (a) Lymphocytes with *E. coli* attached examined under a phase contrast microscope. Lymphocytes in blood smears with bacteria attached. (b) *B. melitensis*; (c) *E. coli-2*; (d) *A. hinshawii*; (e) *S. aureus-1*; (f) *B. globigii*.

associations are considered those formed between erythrocytes and bacteria. For example, if 1% of the erythrocytes (about two microscopic fields) has two bacteria attached, any lymphocyte with three or more bacteria is considered labeled and the error will be less than 1% (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979a).

Another advantage of this method is the ability to directly observe the cellular morphology. In addition, staining for monocytes can be performed, particularly when they are in an unusually high proportion, as in some primary immunodeficiencies. However, for most practical purposes, the lymphocytes can be distinguished morphologically from monocytes. Computerized instruments for smear reading can also be used for automatic determination of the number of lymphocytes with bacteria attached (e.g., a Hematrak by Geometric Data was programmed for this purpose). Another advantage of stained smears is the use of the bacterial morphology in double-labeling experiments. For example, when an *E. coli* is used together with *B. melitensis* the two bacteria, which are morphologically different, can be seen on the same cells or on different cells (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979b).

B. Reproducibility of Bacterial Binding

From 53 strains of bacteria of different genera and species 13 have been found to bind to human lymphocytes, i.e., 10 to 70% of the lymphocytes bound these strains of bacteria. In the mouse 8 strains bound to over 10% of the spleen cell population. To determine whether this binding was reproducible three aspects have been investigated: (a) the percentages of the lymphocytes that bind each of the bacteria in different normal donors or in different strains of mice, (b) the percentages of cells that bind bacteria when the same individuals are tested repeatedly, and (c) the binding of different batches of bacteria to the lymphocytes from the same individual.

The percentages of lymphocytes that bind bacteria or antibody-coated *E. coli*-0 (Ab-*E. coli*-0) were similar in different individuals. In one study (Teodorescu *et al.*, 1977a) five normal donors were tested for the ability of their separated lymphocytes to bind several bacteria. For example, the cells that bound Ab-*E. coli*-0, i.e., practically all B cells, ranged from 11 to 24%, *B. melitensis* from 11 to 22%, and *E. coli*-2 from 56 to 63%. In a larger study on 112 normal donors in Peking, China, on the average 19.1% of the cells bound *B. melitensis* with a range of 11 to 26% (Ching-hua Wang, personal communication). Similar values were obtained by the same author for Ig-bearing cells determined by immunofluorescence. Also, in samples of purified blood

lymphocytes of 10–12 normal donors *B. melitensis* bound to 23% of the cells with a standard error of the mean of 1.3 and a strain of *E. coli* bound to 66% with a standard error of 3.0 (Rasanen, 1981). In normal adult blood the percentage Ig-bearing cells (Ig^+) and of cells that bound *B. melitensis* (Bm^+) was 19.6% (range 17 to 28%) and 20% (range 15 to 31%), respectively, in one study on blood smears (Mayer *et al.*, 1978a) and 16% for both Ig^+ and Bm^+ (range 13 to 20%) in another (Teodorescu *et al.*, 1979a). The higher range in the former study was due to one donor that had a relatively high percentage of Ig-bearing cells, probably due to stress (see Section V,A). The binding of bacteria is reproducible not only in humans but also in mice. For example, in 10 individual BALB/c mice the values were as follows: *B. melitensis*, 50% (range 43–55); *B. abortus*, 14% (range 9–18); *E. coli*-2, 47% (range 43–52); *C. xerosis*, 45% (range 40–50), etc. Also there was practically no strain difference in the binding of bacteria to lymphocytes from BALB/c, C57BL/10J, DBA/2, and AKR/J (Mayer *et al.*, 1978b).

The second aspect investigated was the reproducibility of the test on repeated samples taken from the same individual. With the exception of slight seasonal variations (see Section V,A), the percentages of lymphocytes that bind a certain bacteria remain virtually unchanged. For example, a normal donor tested four times during a 24-hour period had percentages of Bm^+ lymphocytes in blood smears of 12 ± 1, 12 ± 3.5, 12 ± 1.5, and 12 ± 3.21, percentages of Ah^+ lymphocytes of 52 ± 5.2, 50 ± 4.6, 49 ± 2.3, and 52 ± 0.6, and percentages of Ss^+ cells of 63 ± 1.5, 62 ± 7.0, 63 ± 4, and 50 ± 1.5 (Bratescu and Teodorescu, 1981).

To have reliable bacterial markers the binding properties of different batches of bacteria to lymphocytes have to remain constant. Two main factors may cause changes in these properties: (a) the culture conditions and the preparation of bacterial suspensions, which may affect the surface molecules involved in lymphocyte binding and (b) the inadvertent selection of mutants with different binding properties. Although there are no systematic studies on the culture conditions and the preparation procedures a few observations have been made. For example, harvesting *B. melitensis* at 48 hours, probably in late log phase (Bratescu and Teodorescu, unpublished), and sonication of fixed bacterial suspensions (Gaudernack *et al.*, 1981) appear to be essential steps to obtain a heavy labeling of lymphocytes.

Repeated passages of bacteria on nutrient agar or storage at 4 or −20°C, as shown by the following experiment, does not affect the binding of bacteria to lymphocytes (Bratescu *et al.*, 1980). *B. melitensis*, *B. abortus*, *A. hinshawii*, *E. coli*-2, *B. globigii*, *S. aureus*-1, and

Ab-*E. coli*-0 were stored at 4 or −20°C for 6 months. During the same period these bacteria were passed on nutrient agar every 2 weeks. New batches were then prepared and compared with the bacteria that had been stored for their binding to lymphocytes from the same individuals. No significant differences were observed, demonstrating the stability of the binding properties of bacteria. In fact, the above mentioned strains of bacteria have been used now for about 5 years without changes in their binding properties, with two exceptions. On two occasions *B. globigii* maintained by continuous passages lost its binding ability. It was likely that a nonbinding mutant was preferentially growing since by recloning the binding strain was recovered (J. Katzman, personal communication). Also, some of the bacteria showed too much variability between batches and, although used in the first studies (Teodorescu *et al.*, 1977a), they were later abandoned (e.g., *Corynebacterium xerosis* and *Staphylococcus epidermidis*).

C. Mapping Lymphocyte Subpopulations in Cell Suspensions and Blood Smears

1. The Principles of Mapping

The consistent and reproducible binding of bacteria to certain proportions of the lymphocyte population suggested that bacteria identified lymphocyte subpopulations. To determine whether different strains of bacteria bind to B cells, T cells, or to subpopulations of B or T cells double-labeling experiments were performed. In principle, Ab-*E. coli*-0 was used as a marker for B cells and each bacteria was tested alone or in combination with Ab-*E. coli*-0. Subsequently, combinations of different bacteria were tested.

Before performing these experiments the validity of Ab-*E. coli* as a B cell marker had to be demonstrated. First, to be specific for surface Ig, *Ab*-E. *coli*-0 should bind only as a result of interactions between the anti-Ig antibody on the *E. coli* and Ig on the lymphocytes. Indeed, *E. coli*-0 coated with normal IgG bound only to <3% whereas Ab-*E coli*-0 bound to about 19% of the lymphocytes (Mayer *et al.*, 1978a). Second, the specificity for B cells had to be shown. It is accepted that human B cells can make only one type of light chain κ or λ but not both. If some lymphocytes acquire their surface Ig by passive attachment from the serum, as suggested by others (Lobo *et al.*, 1975; Winchester *et al.*, 1975), they would be expected to have both light chains on the same cell. To clarify this issue the lymphocytes from eight normal donors were labeled in whole blood with either anti-κ, anti-λ, a mixture of anti-κ and anti-λ, or normal IgG-coated *E. coli*-0 (Mayer *et*

al., 1978a). After subtracting the small percentage of lymphocytes that bound *E. coli*-0 coated with normal IgG (0–3%) on the average 9.5% of the cells bound anti-κ, 7.5% bound anti-λ, and 17.6% bound the mixture of anti-λ and anti-λ antibody-coated *E. coli*-0. Since 17% were expected if B cells had only one type of light chain (9.5 + 7.5) and 17.6% were found, it was concluded that virtually no cell has passively absorbed surface Ig. Thus, Ab-*E. coli*-0 is a valid marker for B lymphocytes.

To map the lymphocyte population by bacterial adherence double-labeling experiments were performed and the results were interpreted according to the following rationale (Mayer *et al.*, 1978b). Consider two bacteria—*a*, which binds to X% of the lymphocyte population and *b*, which binds to Y%. When *a* and *b* are both used in the same test, the total percentage of the population with bacteria attached may be:

1. equal to both X and Y in which case *a* and *b* identify the same subset of cells, i.e., they are interchangeable markers;
2. equal to X + Y in which case *a* and *b* must identify different, nonoverlapping, subsets of cells;
3. equal to X (where X > Y) in which case all of the cells identified by *b* must also be identified by *a*;
4. equal to Y (where Y > X), in which case all of the cells identified by *a* must also be identified by *b*;
5. greater than X (where X > Y), in which case only some of the cells identified by *b* must also be identified by *a*, i.e., there are three subsets of cells, one identified only *a*; one by *a* and *b*; and one only by *b*;
6. greater than Y (where Y > X), in which case only some of the cells identified by *a* must also be identified by *b*;
7. less than X (where Y > X), in which case one bacteria must have interfered with the binding of the other;
8. less than Y (where Y > X), in which case one bacteria must have interfered with the binding of the other.

The possibility of underestimating the percentage of labeled lymphocytes (possibilities 7 and 8 above) when a mixture of two strains of bacteria was used was unlikely for the following reasons. First, the percentage of labeled lymphocytes did not change after the removal of unbound bacteria. Second, a strain of bacteria which does not bind to the lymphocytes had no significant effect on the number of cells labeled with *B. melitensis*, which makes unlikely the possibility that one bacterium could displace another without taking its place. Third,

it was immaterial which one of the bacteria was used first, e.g., prelabeling, with *B. melitensis* and testing with *E. coli*-3 yielded similar values to those obtained when their order was reversed (Teodorescu *et al.*, 1977a). However, it appears that under certain conditions in cell suspensions some bacteria can interact with each other preventing a correct mapping (Niemetz and Mayr, 1981b).

To obtain a reliable mapping it was essential to determine whether the binding of bacteria to lymphocytes was affected by the way the "rosetted" cells were handled. It is generally known that rosettes formed by lymphocytes with heterologous erythrocytes are relatively unstable. Therefore, the effect of handling the bacterial rosettes was determined for Ab-*E. coli*-0, *B. melitensis*, *B. globigii*, and *S. aureus*-1. After the blood cells were centrifuged with bacteria, some samples were resuspended gently after each washing and some were pipetted vigorously. No significant differences were observed for the binding of bacteria to normal or leukemic lymphocytes between the samples treated so differently (Bratescu *et al.*, 1980). The slightly higher percentage of cells binding *B. melitensis* and *E. coli*-2 observed by Rasanen (1981) than by Teodorescu *et al.* (1977a) was more likely due to different seasonal or climatic factors affecting B-cell traffic than to handling of the rosettes (see Section V,A). Thus, an analogy can be made between the subpopulations of lymphocytes identified by natural bacterial adherence and those identified by Ab-*E. coli*-0. A lymphocyte subpopulation may or may not have a receptor for a certain bacteria in a fashion analogous to the presence or absence of surface of Ig which distinguishes B cells from T cells.

Based on the rationale described above it was possible to generate maps of lymphocyte subpopulations either in cell suspensions (Teodorescu *et al.*, 1977a; Mayer *et al.*, 1978b; Niemetz and Mayr, 1981b) or in blood smears (Teodorescu *et al.*, 1979a). Performing the mapping in blood smears has the advantage that the morphology of bacteria can be recorded. For example, *Brucella*, *Escherichia*, and *Staphylococcus* are easily distinguishable from each other on stained preparations (Fig. 2) (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979a) and the presence of two different bacteria on the same cell can be used as additional evidence of double labeling.

2. *Identification of B Cells*

Human peripheral blood lymphocytes have been mapped either in cell suspensions (Teodorescu *et al.*, 1977a; Niemetz and Mayr, 1981b) or in blood smears (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979a) and mouse splenic cells have been mapped in cell suspensions (Mayer *et*

al., 1978b). When human lymphoid cell suspensions were treated with either anti-Ig Ab-*E. coli*-0, *B. melitensis*, or with both bacteria, the same percentages of cells were labeled. For example, Ab-*E. coli*-0 labeled 17% of the lymphocytes, *B. melitensis* 16%, and together 16% (Teodorescu *et al.*, 1977a; Mayer *et al.*, 1978b). Thus, this situation is compatible with condition 1 described above, i.e., the two bacteria bind to essentially the same subpopulation of cells. The same principle was applied to mouse spleen cells. First, the percentages of spleen cells that bound *B. melitensis* and those that bound anti-Ig Ab-*E. coli*-0 were practically equal (50%, range 43–55 and 49%, range 43–55) (Mayer *et al.*, 1978b). When the two reagents were used together the percentage of labeled cells did not change, again showing that the same cells were identified. Moreover, in smears of human blood (Mayer *et al.*, 1978a; Teodorescu *et al.*, 1979a) or mouse spleen cells (Mayer, unpublished) it was possible to see directly that the lymphocytes that had *B. melitensis* attached (Bm^+) also had Ab-*E. coli*-0 (Ig^+). Thus, *B. melitensis* is a marker for both human and mouse B cells.

The validity of this concept was confirmed in experiments of separation of B from T cells. For example, when the stable E rosette-forming cells were removed the percentage Bm^+ cells and Ig^+ cells in the human lymphocyte populations increased in parallel (Rasanen, 1981). Also, when all Ig-bearing cells were removed by negative selection, i.e., B cells were rosetted with anti-Ig antibody-coated human erythrocytes and the rosetted cells removed by centrifugation on Ficoll–Hypaque, the remaining Ig^- population did not bind *B. melitensis* (Teodorescu *et al.*, 1979a).

Another series of experiments which proved that *B. melitensis* binds exclusively to B cells was based on fluorescence staining. When *B. melitensis* was made to fluoresce in red and the Ig-bearing in green, all green cells were also red (Gaudernack *et al.*, 1981). A small percentage of cells was only red, i.e., had only *B. melitensis* attached, suggesting that some Bm^+ cells were Ig^-. However, since essentially all Bm^+ cells were also Ig^+ when Ab-*E. coli*-0 was used (Mayer *et al.*, 1978a) it is likely that B cells that have a relatively small amount of surface Ig do not appear fluorescent. This is supported by the observation that fewer lymphocytes are found with surface Ig than those bearing Ia-like antigen (Hoffman *et al.*, 1977). Therefore, when *B. melitensis* was used together with a monoclonal anti-human Ia (DR) antibody in a double fluorescence assay all B lymphocytes were shown to be Ia^+Bm^+ (Gaudernack *et al.*, 1981). From these experiments it may be concluded that *B. melitensis* is indeed a marker for B lymphocytes and

that the rationale for identifying lymphocyte subpopulations by double-labeling experiments is valid.

3. *Identification of B- and T-Cell Subpopulations*

No bacteria tested was found that exclusively identified the entire T-cell population either in humans or in mice. Thus, the existence of subpopulations of B and T cells was investigated based on the rationale described above. One strain of bacteria was used to prelabel the lymphocytes and, on this "background," each of the other bacteria was tested. As a result, maps were generated for human lymphocyte populations from peripheral blood (Teodorescu *et al.*, 1977a) (Fig. 3) and for mouse spleen cells (Mayer *et al.*, 1978) (Fig. 4). Human lymphocytes were mapped again in blood smears (Teodorescu *et al.*, 1979a) and it was found that the number of lymphocyte subpopulations and their characteristic bacterial markers was not significantly different from that obtained in cell suspensions, with one exception. Namely, in blood smears B cells could be divided into two subpopulations B_1 (Bm^+Bg^-) and B_2 (Bm^+Bg^+ or Bm^+Sa^+). The existence of two human B-cell subpopulations was also shown by labeling enriched B-cell populations with bacteria (Rasanen, 1981). In the mouse spleen B cells can be subdivided into three subpopulations B_1 which is $Bm^+Ba^+Ah^-$

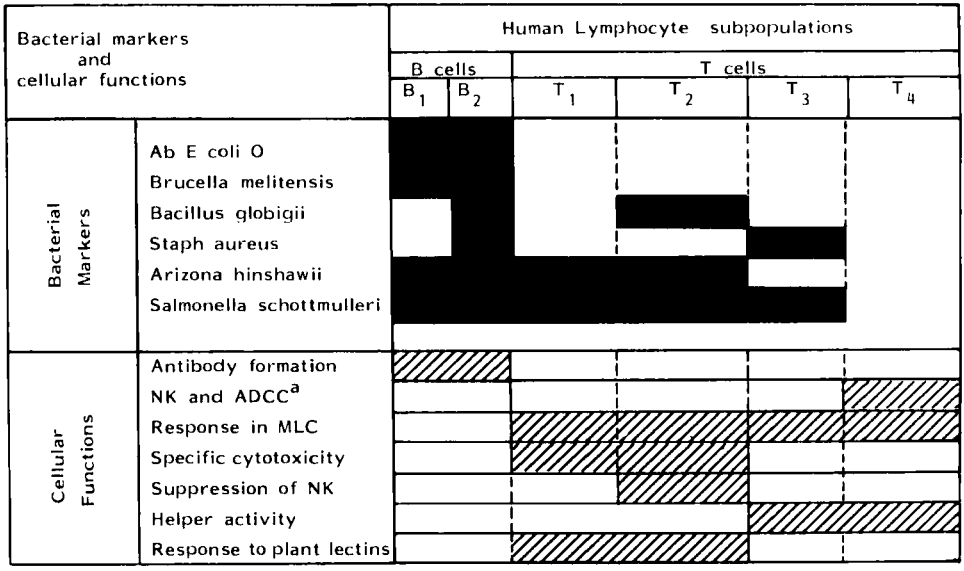

FIG. 3. The map of human lymphocyte subpopulations identified by bacterial adherence.

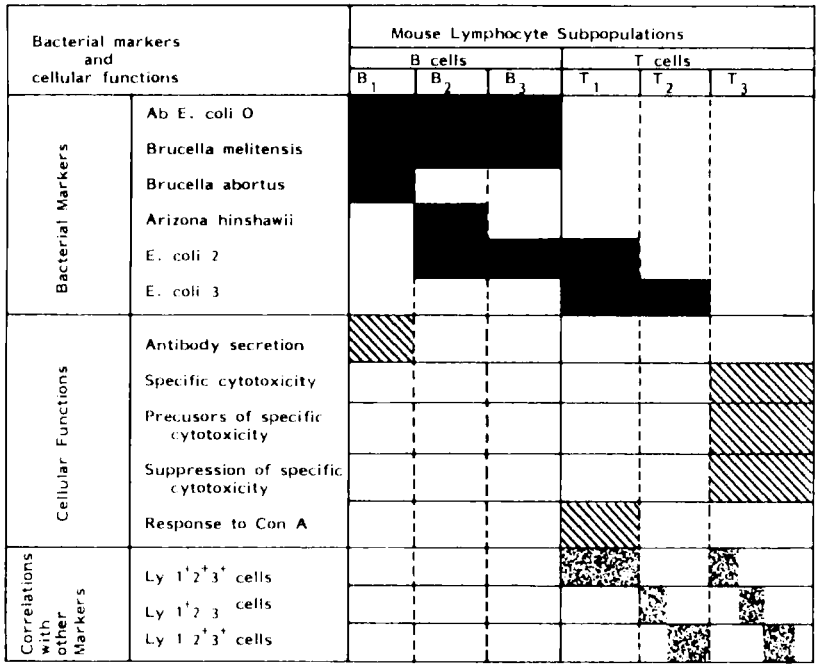

FIG. 4. The map of mouse lymphocyte subpopulations identified by bacterial adherence.

(Ba = *Brucella abortus*), B_2 which is $Bm^+Ah^+Ba^-$ (Ah = *A. hinshawii*), and B_3 which is Bm^+Pp^+ (Pp = *Pasteurella pestis*) (Fig. 4). Additional markers were found for these subsets, which allow for a more elaborate designation of the subpopulations. For example a more complete characterization of mouse B_1 cells would be $Bm^+Ba^+Cx^+Ah^-Pp^-Ec_2^-Ec_4^-$ (Mayer *et al.*, 1978b).

By bacterial adherence four T-cell subpopulations were identified in humans and three in mice and were given arbitrary numbers for convenient reference. Whereas human T_1–T_3 cells and mouse T_1–T_2 bound bacteria, human T_4 cells and mouse T_3 cells, did not bind any bacteria that have been tested so far (Teodorescu *et al.*, 1977a; Mayer *et al.*, 1978b). Thus, human T_4 and mouse T_3 are identified by difference, i.e., the difference between all T cells and those found to bind bacteria.

In humans *S. schottmulleri* binds to the highest proportion of peripheral blood lymphocytes (PBL) (De Boer *et al.*, 1981b), i.e., it includes those that bind *A. hinshawii* and in addition binds to an exclusive subset, called T_3 (Fig. 3). *A. hinshawii* binds to two subsets, T_1

which does not bind *B. globigii* and T_2 which does (Teodorescu *et al.*, 1977a, 1979a). Thus, briefly, human T-cell subpopulations have been designated as T_1 ($Bm^-Ss^+Ah^+Bg^-$), T_2 ($Bm^-Ss^+Ah^+Bg^+$), T_3 ($Bm^-Ss^+Ah^-Bg^-$), and T_4 ($Bm^-Ss^-Ah^-Bg^-$) (Fig. 3). Four bacteria were sufficient to distinguish B cells from T cells and to identify two B- and four T-cell subpopulations. Together with *S. aureus* they have been used more often in clinical laboratory investigations to identify lymphocyte subpopulations in blood smears (Nelson *et al.*, 1979; Felix *et al.*, 1981; Bratescu and Teodorescu, 1981).

In the mouse three T cells subpopulations can be identified. The T_1 subset which binds both *E. coli*-2 and *E. coli*-3 (i.e., it is $Ec_2^+Ec_3^+$), the T_2 subset which binds *E. coli*-3 but not *E. coli*-2 (i.e., it is $Ec_2^-Ec_3^+$), and the T_3 subset which does not bind any bacteria. The relative sizes of these subsets are about 20, 24, and 56% of the T cells for the T_1, T_2, and T_3 subsets, respectively (Mayer *et al.*, 1978b).

D. Separation of Lymphocyte Subpopulations by Bacterial Adherence

To determine further the validity of mapping by bacterial adherence and to study the functions of the lymphocyte subpopulations identified by bacteria, a "panning" method was developed to separate these subpopulations (Fig. 5) (Kleinman and Teodorescu, 1978). Monolayers of bacteria are created on top of a glutaraldehyde-fixed gelatin layer, and these monolayers are used to "pan" for lymphocytes (Kleinman

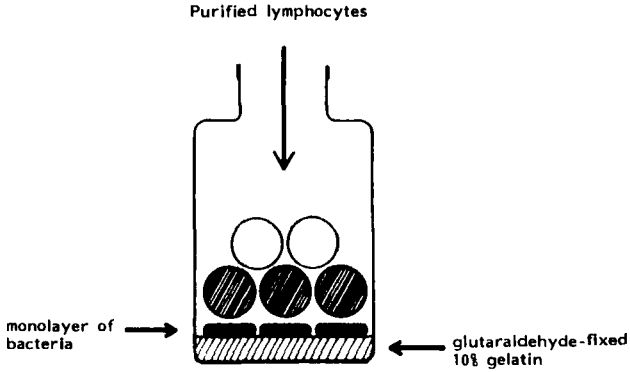

FIG. 5. Summary of the protocol for the separation of lymphocyte subpopulations by bacterial adherence.

and Teodorescu, 1978; 1979; Kleinman *et al.*, 1980; De Boer *et al.*, 1981a; Chen *et al.*, 1979, 1981). The lymphocytes are centrifuged against the monolayers to promote their binding to bacteria and those that do not bind are easily removed. The cells that bind are also removed by vigorous pipetting except B cells which bind irreversibly (Kleinman and Teodorescu, 1978, unpublished).

The nonadherent cells obtained by this method are relatively pure (>95%), i.e., the unbound lymphocytes contain few cells that bind bacteria, whereas the adherent cells are either contaminated with many nonadherent cells or they undergo membrane changes resulting in a loss of receptors for bacteria. We favor the latter possibility for the following reason. For example, if the yield in the nonadherent T-cell population is equal to that expected based on the percentage of nonlabeled lymphocytes and if practically all the cells are recovered good purity and yields are also expected for the cells that are positively selected. However, since only an enrichment is seen (Kleinman *et al.*, 1980) it is likely that the cell surface is damaged. It may be speculated that the firm attachment to the bacterial monolayer, followed by mechanical disruption of this attachment results in a "stripping" effect of the lymphocyte surface. The cells are not damaged, however, since they are fully functional (see Section III).

E. Relationship between Bacterial Markers and Other Lymphocyte Markers

The relationship between the bacterial markers and other lymphocyte markers, such as anti-Ig and anti-Ia antibodies, E and EA rosettes, OKT monoclonal antibodies, and anti-Ly antibodies, has also been investigated. In humans *B. melitensis*, *B. abortus*, *B. canis*, and *B. suis* bound only to Ig^+ and/or $Ia(DR)^+$ cells (Bratescu *et al.*, 1981; Gaudernack *et al.*, 1981) but not to those that bind sheep erythrocytes (Teodorescu *et al.*, 1979a) or those identified by monoclonal anti-T cell antibody (Spear and Teodorescu, 1982, unpublished). This confirmed the hypothesis that *B. melitensis* binds to B but not to T lymphocytes. Of the T_4 cells about 75% are lymphocytes that form rosettes with erythrocytes coated with IgG anti-ox red blood cell antibody (T_γ) (Kleinman *et al.*, 1980; DeBoer *et al.*, 1981a,b) while the other T cell subsets had few T_γ cells. When fluorescent bacteria were used along with monoclonal antibodies against T-cell subpopulations (OKT_4^+ and OKT_8^+) there were OKT_4^+ cells in all four T-cell subpopulations identified by bacterial adherence (see below, Section III on lymphocyte function).

In the mouse, an almost complete overlapping was observed be-

tween Ig-bearing cells and those that bound *B. melitensis*. However, although in man *B. abortus* bound also to all B cells, in the mouse it bound only to a subset of B cells, namely, B_1 (Mayer *et al.*, 1978b; Chen *et al.*, 1979). Mouse T_1 cells contained most of the Ly $1^+2^+3^+$ cells whereas the Ly 1^+2^- and the Ly $1^-2^+3^+$ cells were distributed between the T_2 and T_3 cells (Fig. 4) (Chen *et al.*, 1981) (see also Section III on lymphocyte functions).

III. Functional Characterization of Lymphocytes Identified by Bacterial Adherence

A. B-Cell Subpopulations: Ig Secretion

The fact that *B. melitensis* binds to B cells which have the unique function of producing and displaying Ig suggested that the binding of bacteria might be a useful way of identifying functionally different lymphocyte subpopulations. This assumption is supported by the fact that human (Teodorescu *et al.*, 1977a,b; Mayer *et al.*, 1978a; Rasanen, 1981; Gaudernack *et al.*, 1981), mouse (Mayer *et al.*, 1978b; Chen *et al.*, 1979), and rabbit (Teodorescu, unpublished) lymphocytes have a common bacterial marker, i.e., *B. melitensis*. Thus, it was possible that the three mouse B-cell subpopulations identified by bacterial adherence had different functions.

Ig secretion is obviously the most important function of B cells and the easiest to investigate. Two parameters were followed: the secretion of specific antibody by B cells from mice inoculated with sheep erythrocytes and the secretion of Ig by normal mouse spleen cells as determined by the reverse hemolytic plaque assay (Molinaro and Dray, 1974). Since B_1 cells do not bind to *E. coli*-2, mouse spleen cells were centrifuged against a monolayer of these bacteria and the cells that bound were separated from those that did not bind. Over 90% of the antibody-forming or Ig-secreting cells were found in the nonadherent population, i.e., they were B_1 cells. To obtain more direct evidence, the cells in the middle of the plaque were directly examined for bacterial binding. Essentially all the antibody-forming cells could be labeled with *Corynebacterium xerosis* which binds to B_1 and B_2 cells whereas very few were labeled with *A. hinshawii* or *E. coli*-2 which binds to B_2 and to B_2 and B_3 cells, respectively. Thus, since B_2 and B_3 subpopulations contained few if any Ig-secreting cells, it was concluded that most if not all of the Ig-secreting cells were in the B_1 subpopulation.

Although Ig-secreting cells were all contained in the B_1 subpopulation, not all B_1 cells were shown to secrete Ig. This may be due to two factors: (a) the inability of the reverse hemolytic plaque assay to detect

all Ig-secreting cells and (b) the presence of a large number of nonsecreting small lymphocytes in the B_1 subpopulation. The nonsecreting B_1 cells may correspond to the Y cells in the theoretical X–Y–Z model of B-cell differentiation (Sterzl and Silverstein, 1967). According to this model, the Y cells have had previous experience with the antigens, the Z cells secrete antibody, and the X cells are "virgin" B cells. It has been speculated that B-cell maturation is accompanied by the acquisition of a new surface structure that is recognized by *B. abortus* (Chen *et al.*, 1979). According to this hypothesis B_2 and/or B_3 may contain the cells that are immature and can differentiate into Ig-secreting B cells. In addition to *B. abortus*-binding site, surface antigens such as Ly 4 (Snell *et al.*, 1973), MBLA (Raff, 1971), and PCa-1 (Takahashi *et al.*, 1971) have been found on fully differentiated Ig-secreting mouse B cells.

In conclusion, bacteria bind to functionally different B-cell subpopulations. So far, it is not known whether the two human B-cell subpopulations, B_1 and B_2, are also functionally different.

B. T-Cell Subpopulations

1. Response to Mitogens

As shown above, four T-cell subpopulations were identified in human PBL and three in the mouse spleen. The existence of functional differences between these subpopulations has also been investigated. Human T_1T_2 cells, which adhere, to *E. coli*-2 monolayers, were compared with T_3T_4 cells, which do not adhere and with unseparated peripheral blood mononuclear cells (PBMC), for their response to mitogens: concanavalin A (Con A), pokeweed mitogen (PWM), phytohemagglutinin (PHA), and streptolysin O-associated mitogen (SLO) (Kleinman and Teodorescu, 1978). First, peripheral blood mononuclear cells (PBMC), were separated into monocytes + T_1T_2 cells and monocytes + T_3T_4 cells and their response to mitogens determined by [^{3}H]TdR uptake in 3 days cultures. The T_3T_4 cells responded much less than PBMC to all mitogens. Second, monocytes and B cells were removed, the purified T cells were separated into T_1T_2 and T_3T_4 cells and their response to variable concentrations of Con A was determined. The response of T_3T_4 cells was much lower than that of T cells or T_1T_2 cells at all concentrations of Con A, demonstrating that the T_1T_2 cells were primarily involved in mitogen responses (Kleinman and Teodorescu, 1978, 1979). As a control, the PBMC were also centrifuged against a monolayer of *E. coli*-0, a strain that does not bind to human lymphocytes; their response was not dif-

ferent from that of unprocessed cells. In addition, when various amounts of *E. coli*-2 were added to PBMC (up to 10 bacilli per lymphocyte) the response to Con A was not changed, demonstrating that free bacteria was not responsible for the difference observed. Thus, T_1T_2 cells respond well to mitogens while T_3T_4 cells respond poorly if at all.

In the mouse, a good response to Con A was almost exclusively given by the T_1 subpopulation, i.e., those cells that adhere to *E. coli*-2 and to *E. coli*-3. Sham-separated cells had the same response to Con A as the unseparated cells (Chen *et al.*, 1981). As mentioned above, T_1 cells contained most of the Ly $1^+2^+3^+$, which have been shown to be the cells that respond to Con A (Jacobs and Byrd, 1975; Cantor *et al.*, 1976). Since the response of T_1 cells to Con A could have been predicted based on their Ly markers, processing the cells on the bacterial monolayer was most probably not responsible for the responsiveness of T_1 cells. Thus, mouse T_1 cells are functionally different from T_2 and T_3 in that they account for the mitogenic response of mouse T cells.

2. *Response in Mixed Lymphocyte Cultures and Development of Cytotoxic Cells*

Human T cells were separated into T_1T_2 and T_3T_4 cells and their response in one way mixed lymphocyte cultures was determined. Both subpopulations responded by [^{3}H]TdR incorporation in 6 days cultures almost as well as the unseparated PBMC (Kleinman and Teodorescu, 1978, 1979). However, when they were tested for cytotoxic activity against ^{51}Cr-labeled target cells only T_1T_2 cells were capable of becoming significantly cytotoxic at a fixed ratio (Kleinman and Teodorescu, 1979) or at various effector/target cell ratios (Spear and Teodorescu, 1982). Thus, the cells needed for the development of specific cytotoxic cells were all contained in the T_1T_2 subpopulations.

It is likely that T_3T_4 cells are helper cells since they respond by blast transformation and proliferation but do not kill. If this is indeed the case an explanation is needed for the ability of T_1T_2 cells to generate cytotoxic cells. Several possibilities may be considered. (1) The positively selected T_1T_2 cells, which should bind *A. hinshawii* or *E. coli*-2, contain 15–35% cells that do not bind these two bacteria, which can be T_3T_4 cells. (2) The T_1T_2 cells use "foster" help from the stimulating PBMC population. (3) The T_1T_2 cells contain both precursors for cytotoxic cells and for helper cells. (4) Under these experimental conditions the helper cells for cytotoxicity are not required. These possibilities are amenable to future experimental approaches.

Since the T_1T_2 cells could be further subdivided into T_1 and T_2 cells

they were separated by adherence to monolayers of *B. globigii* which binds only to T_2 cells. Unseparated and separated T-cell subpopulations, i.e., T, T_1T_2, T_1 and the T_2 cells were activated by allogeneic cells *in vitro*. In three individual experiments all these populations except T_2 cells developed into cytotoxic cells (DeBoer and Teodorescu, 1980, unpublished) suggesting that T_1 cells contain all of the cells needed for the development of cytotoxic cells. However, as pointed out above, they might have been contaminated with enough helper cells to manifest their function.

After the precursors of cytotoxic T cells are activated into effector cells they may retain some of the former surface structures, lose some, or acquire new ones. To test for these possibilities total T cells were activated in mixed lymphocyte cultures (MLC) and were depleted of cells that bound *E. coli*-2 or *B. globigii*. If the receptors for these two bacteria remained unchanged it would be expected that *E. coli*-2 would remove all effector cells and *B. globigii* would not. In fact it was found that the effector cells were removed by both *E. coli*-2 and *B. globigii* monolayers (De Boer and Teodorescu, 1980, unpublished) suggesting that the cytotoxic cells switched from the T_1 (Ah^+Bg^-) to the T_2 (Ah^+Bg^+) subpopulation. The acquisition of new receptors by activated T cells has also been demonstrated in other systems. For example, a new antigen was found on activated mouse T cells (Kimura and Wigzell, 1978; Gately and Martz, 1981) and a binding site for a mutant of *Salmonella* developed on activated T cells, a site which was not present on resting T cells (Lehmann *et al.*, 1980).

Mouse cytotoxic T cells induced by intraperitoneal inoculation of allogeneic cells (Chen *et al.*, 1981), or in mixed lymphocyte reaction (Chen and Mayer, unpublished) did not bind to *E. coli*-2 or *E. coli*-3, i.e., they were contained in the T_3 subpopulation (Fig. 4). The precursors of cytotoxic cells were also found in the T_3 subpopulations, i.e., unlike the human lymphocyte populations, the cells remained in the T_3 subset. It is worth noting that only a small fraction of the Ly 2^+3^+ cells (1/4–1/3) contained the entire specific cytotoxic activity (Chen *et al.*, 1981). Therefore, the fact that the T_3 cells contained all of the cytotoxic cells and their precursors showed that only a small fraction of the Ly 1^-,2,3^+ subpopulation was involved in cytotoxic reactions. The MLC-activated mouse T cells develop a new binding site for a mutant of *Salmonella* (Lehmann *et al.*, 1980).

3. *Antigen Response*

Previous work by others using monoclonal antibodies (Reinherz *et al.*, 1979) showed that cells that respond to antigens are different from

those that do not respond. Therefore, we compared T_1T_2 cells with T_3T_4 cells for the response to tetanus toxoid. Since most normal individuals have memory cells for this antigen a good response was obtained with PBL from several normal donors; the T_3T_4 cells responded slightly better to tetanus toxoid than did T_1T_2 cells. Since T_1T_2 cells are mitogen responders and T_3T_4 antigen but not mitogen responders (Kleinman and Teodorescu, 1978; Spear and Teodorescu, unpublished) it appears that the two pairs of subpopulations have different functions.

4. Helper Activity in Ig Secretion

Based on the good proliferative response of T_3T_4 cells to tetanus toxoid or to allogeneic cells it was possible that these cells were also responsible for helper activity in antibody formation. Thus, PWM stimulation was used to induce Ig secretion *in vitro*. In this system B cells appear to be activated by PWM-activated helper T cells (Fauci *et al.*, 1976). When B cells were supplemented with PWM and with either T cells, T_1T_2, or T_3T_4 cells, the T_3T_4 were more efficient in inducing Ig secretion than T_1T_2 cells (Spear and Teodorescu, 1981, unpublished). However, the T_1T_2 cells were also capable of providing some help, which may be due to their contamination with T_3T_4 cells. However, it is also possible that the helper function is not an exclusive property of a single subset of human T cells. Nevertheless, it has been reported for lymphocytes separated based on surface antigens identified by monoclonal antibodies, that the helper cells belong to a clearly defined subset of T cells (Morimoto *et al.*, 1981). However, this observation requires additional independent confirmation.

C. Natural Killer Cells and Their Suppressors

Natural killer cells have been described as medium and large granular lymphocytes (Timonen *et al.*, 1979). These cells have receptors for the Fc portion of IgG and are active primarily against cell lines of hematopoietic origin such as T cell lines (Callawaert *et al.*, 1977) or the erythroblastic cell line K562. B cells lines are much less sensitive to NK cells than T cell lines or K562 cells (Callawaert *et al.*, 1977). To determine whether the NK cells are located in a particular T-cell subpopulation the T_3T_4 cells separated by removing the lymphocytes that adhere to *E. coli*-2 monolayers, and were compared with PBL for their NK activity against lymphoblastoid cell lines of B or T cell origin. When tested in a 4 hour ^{51}Cr release assay on lymphoblastoid cell lines of B cell origin, T_3T_4 cells were able to kill 29% of the cells while PBL killed only 16%. When tested on T cell lines T_3T_4 cells killed, on the

average, 44% of the cells while the PBL killed only 27%. Both PBL and T_3T_4 cells killed very well the K562 cells which are known to be very sensitive to natural killing (West *et al.*, 1977), the T_3T_4 cells being only slightly more active (DeBoer and Teodorescu, unpublished).

When T_1T_2 cells were separated from T_3T_4 cells most of the NK activity was recovered in the T_3T_4 population (Kleinman *et al.*, 1980; DeBoer *et al.*, 1981a). Moreover, when $T_1T_2T_3$ cells were removed from the T cells (DeBoer *et al.*, 1981a) or when T_3 cells were removed from T_3T_4 cells (Kleinman *et al.*, 1980) the NK activity was recovered in the T_4 cell population. This population contained about 70–75% cells with Fc receptors for IgG (Kleinman *et al.*, 1980) and about 25% cells with characteristic granular pattern (DeBoer *et al.*, 1981b). They also formed rosettes with sheep erythrocytes which would qualify them as T cells (Kleinman *et al.*, 1980). Thus, since the entire NK activity could be recovered in the T_4 subpopulation it was concluded that the human lymphocytes that do not bind any bacteria contain the NK cells. This property proved useful for their separation by negative selection (DeBoer *et al.*, 1981b).

The increase in NK activity when T_4 cells are separated from the rest of the lymphocytes could have been the result of simple enrichment in effector cells. If this was the case an increase in the number of PBL should compensate for their relatively low content in NK cells. However, at any effector/target cell ratio the T_4 cells were much more efficient than PBL in killing the CEM cells. Two possible explanations were offered for this observation: (a) irrelevant cells in the PBL population interfered with the NK cells and prevented them from binding efficiently to the target cells and (b) a particular subset of lymphocytes suppressed the function of NK cells (DeBoer *et al.*, 1981a).

The interference by irrelevant cells could have been by their simple presence or by active competition for space on target cells. The simple presence of irrelevant cells was excluded since only living T_2 cells, but not T_1T_3 cells, or dead T_2 cells, were capable of blocking the NK activity of T_4 cells in a dose-dependent manner. The competition for target cells was also excluded as follows. First, the NK activity was decreased to the same extent when T_4 cells where added first to the target cells, followed shortly by T_2 cells or when the order was reversed. Second, T_4 cells bound faster to an immobilized population of CEM cells than T_2 cells. Third, the binding of T_4 but not of T_2 cells to CEM cells was directly visualized by the following experiment. PBL were allowed to interact with CEM cells and the entire mixture was "rosetted" with *B. globigii*, a T_2 cell marker; the cells were stained and examined under the microscope. Practically no lymphocytes attached

to target cells bound bacteria while many free cells bound *B. globigii* (DeBoer *et al.*, 1981a). Thus, since there was no interference with the T_4 cell action, it was likely that T_2 cells were suppressing their cytotoxic activity. However, no soluble suppressor factor was found (DeBoer and Teodorescu, unpublished).

Along with the NK activity of the cells bearing receptors for Fc portion of IgG their ability to kill IgG antibody-coated target cells was also examined. Chang hepatoma cells, which are resistant to NK cells, were used after treatment with rabbit anti-Chang cells IgG antibody (DeBoer and Teodorescu, unpublished). When various T-cell subpopulations were tested, the activity was again found in the T_4 subpopulation. However, their activity was not inhibited by T_2 cells. Therefore, the possibility was considered that the NK cells and the cells active in antibody-dependent cellular cytotoxicity (ADCC) are different, the former sensitive and the latter insensitive to suppressor cells. This was not the case as shown by the following experiments. First, when both NK cells and ADCC active cells were rosetted with ox red blood cells coated with IgG anti-ox red cell antibody (Kleinman *et al.*, 1980), and the rosetted cells removed by centrifugation on Ficoll–Hypaque, both NK and ADCC activities were lost. Second, when PBL were allowed to bind to K562 cell monolayers in a way analogous to the preparation of bacterial monolayers (see Section II,A) the unbound cells lost both NK and ADCC activities and the cells bearing receptors for Fc of IgG (DeBoer and Teodorescu, unpublished). Thus, it is very likely that the same cells performed both functions under different conditions. Since a ligand, the antibody, is necessary for the killing of Chang cells which are resistant to NK activity, it is likely that CEM cells, K562 cells, or other hematopoietic cell lines have their own ligand for cytotoxic cells (see Section IV,D).

Although the NK cells were placed among T cells (Kleiman *et al.*, 1980; DeBoer *et al.*, 1981a,b) the nature of NK cells as part of the lymphocyte population is in doubt for several reasons. In fact, arguments can be made to place them as part of a separate granulocyte series. (1) Like other granulocytes, but unlike the lymphocytes, NK cells have granules. (2) NK cells are practically absent in the first samples of thoracic duct lymphocytes (Forber *et al.*, 1981); if they were a part of the recirculating lymphocyte pool it would be expected that the postcapillary venule of the lymphynodes would recognize them. (3) Patients with a variety of immunodeficiencies have normal levels of NK cells (Bundy and Nelson, 1980). (4) Neutrophils are capable of NK activity. (5) Common antigens between T cells monocytes and neutrophils have been described on NK cells (Orlando *et al.*, 1981). How-

ever, unlike most granulocytes and monocytes which bind bacteria, the NK cells do not bind bacteria. This characteristic, as well as their morphology, still sets them apart as a specialized population of cells probably a special line of differentiation (Orlando *et al.*, 1981).

IV. The Mechanism of Bacterial Adherence of Lymphocytes

A. Nonimmune Receptors for Bacteria on Lymphocytes

Bacteria are complex structures with numerous determinants; they are an important part of the individual's environment. Therefore, the binding of bacteria to lymphocytes might have been due to the presence of specific immune receptors. However, several arguments can be made against such a possibility. (1) The proportion of the lymphocyte population that binds bacteria is much higher than anyone would expect if immune-specific receptors were involved. (2) Normal individuals with variable immunologic history have similar percentages of lymphocytes that bind bacteria; in fact, two normal individuals examined before infection and after recovery from an infection with *B. melitensis* had the same percentages of lymphocytes that bound this microorganism (Bratescu, unpublished). (3) Lymphocytes from cord blood bind different bacteria to an extent which falls practically within adult ranges (Teodorescu *et al.*, 1981). (4) When the spleens of mice bred under germ-free conditions were examined, the percentages of cells binding bacteria was not different from those observed in conventionally bred mice (Mayer, unpublished). Thus, the receptors for bacteria were most probably nonimmune in nature.

S. aureus Cowan I strain binds to the Fc portion of IgG (Ghetie *et al.*, 1974). Therefore, the possibility that *B. melitensis* binds to surface Ig (which is mostly of IgM and IgD class) has been examined and was found to be unlikely for the following reasons. First, *B. melitensis* bound to B cells in the absence as well as in the presence of an excess of human immunoglobulins or whole serum (Mayer *et al.*, 1978a). Second, capping the surface Ig with anti-light chain antibodies had no effect on the binding of *B. melitensis* (Nelson *et al.*, 1979; Teodorescu *et al.*, 1979b; Lee *et al.*, 1982). Third, *B. melitensis* was found to bind to leukemic B cells (Teodorescu *et al.*, 1977b; Nelson *et al.*, 1979) or to "null" cells that had no detectable surface Ig (Teodorescu *et al.*, 1981).

Another possible surface component common to all B cells is the Ia-like antigen (Hoffman *et al.*, 1977). A monoclonal anti-human Ia antibody used to cap Ia antigens on human lymphocytes did not affect the binding of *B. melitensis* (Spear and Teodorescu, in preparation).

Nevertheless, by using two color fluorescence with bacteria and monoclonal anti-Ia antibody, it has been shown that all the Ia^+ cells were also Bm^+ (Gaudernack *et al.*, 1981; Spear and Teodorescu, unpublished). Thus, "immune" receptors, Ia or surface Ig were not involved in the binding of bacteria.

B. Lectins in Cellular Recognition

Another category of agents capable of specific recognition are the lectins. By definition, a lectin is a nonimmunoglobulin protein or glycoprotein, capable of specifically recognizing oligosaccharide residues (see Simpson *et al.*, 1978; Barondes, 1981). Most lectins, known as phytoagglutinins, that immunologists deal with have been extracted from plants. The methods used to detect these lectins have been based on their polyvalent character which make them capable of agglutinating red cells. The carbohydrate specificity is determined by inhibiting the agglutination of erythrocytes or trypsin-treated glutaraldehyde-fixed erythrocytes with certain free monosaccharides (Simpson *et al.*, 1977; Rosen *et al.*, 1973). This method has been applied both to plant lectins and to animal cell lectins. For example, a lectin which agglutinates red cells was extracted from the developing rat brain; this agglutination is specifically blocked by lactose but not by other sugars (Simpson *et al.*, 1977). Similar lectins have been extracted from other animal cells: liver, muscle, heart, fibroblasts etc. (see Monsigny *et al.*, 1979).

The role of lectins and carbohydrate-lectin binding in cellular recognition and interaction has been best studied in cellular slime molds. These amoebae feed on bacteria and exist as free unicellular organisms, dividing about every 3 hours. When their food supply is exhausted they stop proliferating and form large pseudoplasmodia or aggregates. The extracts of *Dictyostelium discoideum* contain a lectin called discoidin which is responsible for the formation of aggregates (Simpson *et al.*, 1974). The agglutination by this lectin can be blocked by the specific carbohydrate or by Fab antibody directed against it.

Leguminous plants recognize through their lectins different species of *Rhizobia*, by binding the carbohydrates the bacteria display in the lipopolysaccharide. This lectin recognition confers symbiont–host selectivity between the plants and bacteria (Wolpert and Albersheim, 1976) and represents an example of a recognition between a eukaryote and bacteria in which the lectin is on the eukaryotic cells. This is significant since the opposite possibility was considered as the major mechanism of bacterial binding. For example, it has been shown that an *E. coli* binds to mucosal cells as a result of a lectin specific for

D-mannose and α-D-mannopyranoside on its pilus (Ofek *et al.*, 1978; Eshdat *et al.*, 1978).

In animal cells the lectin carbohydrate interactions have been shown to have important functions. For example, the liver lectin recognizes, binds, and removes from circulation desialiated serum glycoproteins (Ashwell and Morell, 1977). This lectin is specific for D-galactose and has been shown to stimulate blast transformation in lymphocyte cultures (Novogradsky and Ashwell, 1977).

C. Lymphocyte Binding Mutants and Lectin Agglutination of Bacteria

The possible involvement of lectin–carbohydrate interactions in the binding of bacteria to lymphocytes was very appealing. Some plant lectins have been shown to bind to different subpopulations of lymphocytes suggesting that these cells may display different carbohydrates on their membranes (Reisner *et al.*, 1976; Reisner and Sharon, 1980). Thus, it is possible, as in other intercellular recognition systems, that lectins are present on some lymphocytes and their corresponding carbohydrates on other lymphocytes or other cells that interact with lymphocytes. Thus, what bacteria may identify is either a lectin or a carbohydrate that has some other important role for the lymphocyte subpopulation.

To obtain some indication regarding the nature of the binding site on the bacteria it was desirable to have both a strain of bacteria that binds to lymphocytes and a strain from which it was derived that does not bind. To accomplish this the tryptophan-dependent YS_{57} strain of *E. coli*, which does not bind to either human or mouse lymphocytes, was used. This strain of bacteria was chemically mutagenized and the mutants that bound to mouse lymphocytes were selected. In principle, when the mutated bacteria were mixed with the lymphocytes and were plated in tryptophan-free agar medium, only those mutants that bound to lymphocytes could grow by utilizing the tryptophan derived from the lymphocytes (Mayer and Teodorescu, 1978, 1980). The mutants obtained by this method had two properties: they bound to mouse lymphocytes, primarily to B cells, and were agglutinated by Con A, whereas the original strain did not bind and was not agglutinated. Moreover, these mutants also bound to human lymphocytes. Thus, this mutant selection suggested that to adhere to lymphocytes bacteria have to display carbohydrates available for binding by plant lectins, and that similar lectins may be present on the lymphocyte membrane.

To further explore this hypothesis the following experiment was performed (Teodorescu *et al.*, 1979b; Lee *et al.*, 1982). Seven strains of

bacteria that bind to human lymphocytes (binders), including those mentioned above (Section II) were compared with nine randomly selected nonbinders. Various dilutions of 14 different plant lectins were prepared in the wells of microtiter plates and agglutination titers determined for each lectin and each bacteria. The binders were agglutinated by more plant lectins and gave higher titers than did the nonbinders, the difference between the two groups being highly significant ($p < 0.001$). Thus, these experiments suggested that bacteria that bind to lymphocytes have on their surface carbohydrates available for interaction with lectins.

Evidence that bacterial carbohydrates are involved in the binding of bacteria to lymphocytes was also obtained by Lehmann *et al.* (1980). These authors have used strains of *Salmonella* that do not bind to mouse T cells activated in mixed lymphocyte cultures (MLC). Chemically induced bacterial mutants were selected for the ability to bind to these activated T cells and differences in the carbohydrate structure of their LPS demonstrated. The LPS from the mutants was extractable by a procedure which is specific for rough mutants, suggesting that they are blocked in the synthesis of the complete LPS. The heteropolysaccharides extracted from these mutants were capable of blocking the development of prekiller to killer cells in MLC without affecting the proliferative activity and without blocking the interaction between effector cells and their targets. Also, lipoteichoic acid from *B. globigii*, which binds to T_2 cells, blocks the development of cytotoxic T cells in MLC without affecting cellular viability (Whigham and Kleinman, 1982).

D. The Effect of Monosaccharides on the Binding of Bacteria to Lymphocytes

Lectins bind to the surface carbohydrates of red cells and agglutinates them. Therefore, most of the methods for the identification of lectins are based on the competition of agglutination of red cells by monosaccharides (See Simpson *et al.*, 1978). Thus, 16 different monosaccharides which are known to be constituents of LPS were tested for their ability to prevent the binding of bacteria to lymphocytes. In these experiments the binding was determined in the presence of a 5% (w/v) solution of sugar. At this concentration the cell viability and the binding of anti-Ig antibody coated *E. coli*-0 to B cells was not affected. Of all the sugars tested only four inhibited the binding of some of the bacteria: α-methyl mannoside (αMM), D-glucose, D-mannose, and D-galactose. For example, a solution of αMM blocked over 40% of the binding of the following bacteria to lymphocytes: *B.*

melitensis, *E. coli*-2, *A. hinshawii*, and the UI_{2023} strain of *E. coli*, suggesting that a Con A-like lectin was involved in the binding of these bacteria to lymphocytes. Also, a solution of 5% D-mannose or D-galactose inhibited over 40% of the binding of *E. coli*-2 or *A. hinshawii*.

The inhibition of binding by αMM only suggested the presence of a lectin–carbohydrate interaction but did not give any indication as to whether the lectin was on the lymphocytes or on the bacteria. Therefore, the lymphocytes were pretreated with αMM, washed, and immediately "rosetted" with bacteria; over 50% of the B cells did not bind *B. melitensis*. Incubation of lymphocytes at 4°C for about 2 hours made them recover the binding ability suggesting that the αMM was released. Pretreatment of bacteria with αMM had no effect on its binding to lymphocytes and preformed rosettes were not affected by the sugar (Lee *et al.*, 1982). Thus, these experiments suggested that a Con A-like lectin is present on the B lymphocyte membrane and that the corresponding carbohydrate may be on the bacteria.

Additional evidence that carbohydrates rather than lectins are on the bacteria was obtained. First, pretreatment of the bacteria with pronase had no effect on their binding to lymphocytes (Teodorescu *et al.*, 1979a; Lee *et al.*, 1978). Second, the fact that bacteria, such as *B. melitensis*, can be autoclaved and formaldehyde fixed without alteration of the binding property (Bratescu *et al.*, 1980) suggests that the protein (lectin) is not on the bacteria. Third, inhibition experiments with LPS from bacteria showed that pretreatment of lymphocytes prevented the binding of the corresponding bacteria. For example, lipid A freed LPS from *Salmonella* with chemotype Rb which is characteristic to the mutant that binds to activated T cells, inhibited the binding of *Salmonella* mutant to these cells. However, *Ra* polysaccharides were poorly inhibitory and smooth or mutant polysaccharides such as *Re*, *Rd2*, *Rd1*, or *Rc* fragments were not inhibitory (Lehmann *et al.*, 1980). Fourth, when human lymphocytes were pretreated with LPS from *B. melitensis* the binding of this bacteria was blocked (Lee *et al.*, unpublished) and when they were treated with lipoteichoic acid from *B. globigii* they did not bind *B. globigii* anymore, but continued to bind *E. coli*-2 (Shockley and Kleinman, 1981). Finally, pili, which have been shown to have lectins (Eshdat *et al.*, 1978), could not be found on *E. coli*-2 or *B. globigii* (Shockley and Kleinman, 1981).

Evidence that lectins may be on the lymphocytes has also been obtained. *B. melitensis* treated with NP40 solubilized proteins and glycoproteins from human tonsil cells lost its ability to bind to lymphocytes. Such a solubilized material was capable of agglutinating

E. coli strain UI_{2023}, a binder, but not its parental strain YS57, a nonbinder, in the wells of microtiter plates (Ralapati and Teodorescu, unpublished). Moreover, pretreatment of lymphocytes with pronase (Teodorescu *et al.*, 1979b; Lee *et al.*, 1982) or trypsin (Lehmann *et al.*, 1980) prevented the binding of bacteria.

Other enzyme treatments of lymphocytes or bacteria have also been used to determine the nature of bacterial binding. For example, neuraminidase treatment of human lymphocytes (Rasanen, 1981) or activated mouse T cells (Lehmann *et al.*, 1980) increased the percentages of cells that bound bacteria, suggesting a possible "uncovering" mechanism. The possibility that the structure on the lymphocyte membrane is a carbohydrate was also investigated using Con A or leukoagglutinin to cover up the receptors on lymphocytes. Either inhibition or enhancement of bacterial binding was observed depending on the bacteria used (Rasanen, 1981). However, no conclusion regarding the nature of the receptor on the lymphocytes can be drawn from this type of experiment for two reasons: (a) the plant lectins are large molecules that can cause steric hindrance by binding to the carbohydrate portion of a surface glycoprotein and (b) they are unusually polyvalent and create artifactual rosettes. Nevertheless, the possibility still remains that in some cases the binding of bacteria is due lectins on their surface.

E. The Binding of Phage-Resistant Mutants of *E. coli* to Lymphocytes

Evidence suggesting the possibility that some bacteria have surface lectins for lymphocyte carbohydrates was obtained from experiments with phage-resistant mutants of *E. coli*. In these experiments mutants of a nonbinding strain of bacteria that were resistant to the lytic bacteriophage T_4 were selected. The rationale for this approach was that since phage-resistant mutants have altered outer membranes they may also have altered lymphocyte binding properties. Spontaneous phage-resistant mutants (T_4 phage) of the nonbinding YS57 strain of *E. coli* were obtained and tested for their ability to bind to mouse splenic lymphocytes. All of the phage-resistant mutants tested bound to lymphocytes (Mayer, unpublished results). Spontaneous phage-resistant mutants of other nonbinding strains of *E. coli* have been obtained and they also bound to mouse lymphocytes. In addition to binding to mouse lymphocytes these phage-resistant mutants also bound to human peripheral blood lymphocytes (Bratescu *et al.*, 1982). In fact, by using a combination of the phage-resistant mutants and the mutants obtained by the selection procedure described above it has been pos-

sible to identify the same subsets of human peripheral blood lymphocytes that had been identified by using a variety of different bacteria (see Fig. 3).

In as much as carbohydrates have been implicated in the binding of bacteria to lymphocytes (see above) the ability of various sugars to inhibit the binding of the phage-resistant mutants to mouse lymphocytes was examined. The mutants fell into three categories: (1) those that were inhibited by D-mannose or α-methyl-D-mannoside; (2) those that were inhibited by phosphorylated sugars (e.g., D-mannose-6-PO_4) but not by neutral sugars (e.g., D-mannose); and (3) those that were not inhibited by any of the sugars tested. These data suggest that the binding of phage-resistant mutants may also be mediated by carbohydrates and carbohydrate binding proteins. Since the phage-resistant mutants lack one of the major outer membrane proteins (Mayer, unpublished results) which is a part of the phage receptor, the fact that the mutants bind suggests that either a new molecule is made to replace the missing protein or that preexisting binding determinants are now exposed in the mutants.

Although the data presented above suggest that the binding of various bacteria may be mediated by lymphocyte lectins, two indirect lines of evidence suggest that in the case of the mannose-inhibitable phage-resistant mutants, the "lectin" may be on the bacteria. First, the mannose-inhibitable mutants bind to yeast cells, which have mannose on their surfaces, whereas the parent strain and the noninhibitable mutants do not; this binding is inhibited by mannose. Second, the mannose-inhibitable mutants bind to formaldehyde-fixed lymphocytes whereas the binding of most other bacteria is abrogated by formaldehyde fixation of lymphocytes. If indeed the phage-resistant mutants that are mannose inhibitable bind via a mannose binding lectin on the bacteria, the binding of these mutants to the lymphocytes may be analogous to the binding of various pathogenic bacteria to epithelial cell surfaces (Ofek *et al.*, 1978) and may identify lymphocyte subsets similar to those identified by plant lectins.

F. Possible Functional Significance of the Presence of Binding Sites for Bacteria on Lymphocytes

Lectin–carbohydrate interactions play an important role in cellular recognition. The carbohydrates isolated from the cell membrane are composed of nine basic subunits: four 6-carbon sugars (glucose, galactose, mannose, and fucose), two 5-carbon sugars (arabinose and xylose), two amino sugars (*N*-acetylglucosamine and *N*-acetyl-

galatosamine), and sialic acid. These carbohydrates are joined together in different arrangements to form chains. Most glycoproteins and glycolipids are inserted in the membrane with the carbohydrate on the outside (see reviews Simpson *et al.*, 1978; Monsigny *et al.*, 1979; Weir, 1981). The number of different possible arrangements is very high, with numerous spatial configurations. For example, it has been estimated that for a 12 residue oligosaccharide of three mannose, three *N*-acetylgalatosamine, three galactose, and three sialic acid, there are 10^{24} possible structures. Thus, the number of possible cellular recognition systems that can develop between lectins and carbohydrates is very high.

One consequence of the lectin–carbohydrate interactions that may develop between bacteria and animal cells may be the initiation of the infectious process which is known in many cases to begin with the binding of bacteria to mucosal cells. When this type of binding was analyzed it has been found that bacteria have lectins that can interact with carbohydrates on the epithelial cells. The demonstration was based on monosaccharide inhibition of the binding of *E. coli* (Ellen and Gibbons, 1972; Duguid, 1968; Ofek *et al.*, 1977; Selinger *et al.*, 1978) and on the isolation of a lectin from a subunit of the bacterial pilus (Eshdat *et al.*, 1978).

The development of lectins on bacteria, however, may be only one of many mechanisms involved in the binding of pathogens to mucosal cells. It is also possible that bacteria had a selective advantage in displaying carbohydrate structures that bound to lectins of the epithelial cells. If some of these lectins are shared by macrophages and lymphocytes, bacteria would also bind to them. For example, macrophages from the mouse peritoneal exudate bind *S. typhimurium*, and this binding is inhibited by some monosaccharides, depending on the bacterial strain and their LPS chemotype (Freimer *et al.*, 1978). The presence of the lectins on macrophages and lymphocyte subpopulations is probably related to other functions in the immune system. It is possible that different lymphocyte subpopulation express different assortments of lectins, in relationship to their homing destiny and their functions. Also, during differentiation toward a functional cell or blast transformation lymphocytes may acquire new lectins or lose some of those present in a previous stage (Lehman *et al.*, 1980). Thus, the presence of lectins on either lymphocytes or bacteria may explain why some bacteria bind to lymphocyte subpopulations.

The likelihood of the presence of lectins on lymphocytes is also supported by other experimental evidence. For example, by coupling monosaccharides to an albumin backbone it was possible to trace the

presence of lectins on various subpopulations of mouse lymphocytes (Kieda *et al.*, 1979). Also, in the extracts of membrane vesicle preparations from lymphocytes a lectin capable of agglutinating trypsin-treated rabbit erythrocytes was found. These lectins appeared capable of binding complex glycopeptides such as those with terminal γ-galactosides and rich in mannose (Kieda *et al.*, 1978).

The possibility that lectins are present on lymphocytes and macrophages may have functional consequences. Lectins extracted from plants are usually potent stimulators when added to lymphocytes. The lectin extracted from liver was also shown to be mitogenic for lymphocytes (Novogradsky and Ashwell, 1977). Therefore, it is possible that some of the cellular interactions between the elements of the immune system that result in lymphocyte activation are due to lectin–carbohydrate binding. For example, we may speculate that antigen brings together B and T cells or macrophages and T cells and allows for a specific lectin–carbohydrate interaction to develop resulting in lymphocyte activation (Teodorescu, 1981). Thus, it is possible that the major stimulus is not the antigen but the specific lectins present on macrophages and lymphocytes. This hypothesis may explain, at least in part, the autologous mixed lymphocyte reaction in which T cells are polyclonally activated by non-T cells (Kunz *et al.*, 1976), macrophages (Dos Reis and Shevach, 1981), or B cells (Teodorescu, 1981; Chang *et al.*, 1981). It may also explain the selective effect of some monosaccharides on the immune response *in vitro* (Muchmore *et al.*, 1980).

Another possible role of lectins is in the function of NK cells. Specific inhibition of NK cells by some monosaccharides such as mannose and αMM was demonstrated (Stutman *et al.*, 1980). In fact, it is possible that the sensitivity of some lymphoblastoid cell lines to NK activity is due to the expression of lectins on lymphoblastoid cells, i.e., the target cells commit suicide by expressing a variety of lectins that bind to carbohydrates on NK cells and initiate the killing mechanism. If this were the case, it was expected that cells of the lymphoblastoid lines would bind bacteria while the NK cells would not. Indeed it has been found that lymphoblastoid cells lines bind bacteria well (De Boer and Teodorescu, unpublished) to the extent that T cell lines also bind *B. melitensis* (Gaudernack *et al.*, 1981), although this bacteria is a marker for freshly isolated B cells (Teodorescu *et al.*, 1979a; Mayer *et al.*, 1978a). Hence, on the one hand it appears that lymphoblastoid cells express lectins abnormally and on the other the NK cells are in a population that does not bind any bacteria (Fig. 3), i.e., they may express the carbohydrates available for the lectins on lymphoblastoid cells. It may be speculated that the physiologic role of NK cells is to

eliminate those hematopoietic cells that express abnormal lectins during their differentiation in the bone marrow. Other consequences of this concept for malignant lymphocytes *in vivo* have been explored in patients with CLL (see Section V,C).

Much more is needed to elucidate the physiologic role of the binding sites for bacteria on lymphocytes. The possibility that bacteria identify functional "arms" of lymphocyte subpopulations is suggested by at least two observations: (a) bacteria can be used as markers of functionally distinct lymphocyte subpopulations and (b) the binding site for *B. melitensis* is present on B cells from different species. Thus, elucidating the nature of these binding sites may lead to a better understanding of lymphocyte functions.

V. Clinical Use of Bacteria for the Identification of Lymphocyte Subpopulations

A. Discrete Changes in Lymphocyte Subpopulations: Stress, Solid Tumors, Aging

Counting lymphocytes and their subpopulations in peripheral blood has been done for many years, usually, we feel, for the wrong reasons. Increases and decreases in the number of cells in a subset of circulating lymphocytes have been interpreted as meaning that there are changes in the total number of lymphocytes of a certain subpopulation in the lymphoid system of the patient (Morimoto, 1978; Strelkauskas *et al.*, 1978). However this may not be the case since only less than 1% of the lymphocytes are in traffic, i.e., the change may only reflect lymphocyte redistribution. For example, it is well known that the apparent increase in the number of polymorphonuclear cells following treatment with steroid hormones is due to inhibition of their margination in capillary vessels.

Two changes in the lymphocyte subpopulations in the peripheral blood are possible: (1) discrete changes, most probably due to alterations in lymphocyte homing and traffic, and (2) dramatic changes due to primary immunologic deficiencies or malignant proliferation of lymphocytes. As shown above (Section II,B) the percentages of lymphocytes that bind bacteria are very similar in different individuals, when the blood is collected at approximately the same time. Therefore, it was of interest to follow longitudinally the changes in the proportion of different lymphocyte subpopulations in the peripheral blood. Every other month B cells were counted in the blood of six normal donors by using three markers: anti-Ig Ab-*E. coli*-0, *B. melitensis*, and *B. abortus*. Consistent seasonal changes in the relative as well

as absolute number of peripheral blood B cells have been observed with all three markers. In winter the number of circulating B cells was about double that found in summer; at the same time the absolute number of leukocytes and lymphocytes did not show any significant variation. However, short-time exposure under winter conditions in a freezer room did not affect the level of B cells (Bratescu and Teodorescu, 1981). The ratio between $T_1(Ah^+Bg^-)$ cells and $T_2(Ah^+Bg^+)$ cells also showed changes the spring–summer season. It is likely that very wide ranges, from 2 to 15% given by some authors for normal percentages of Ig-bearing cells in human peripheral blood (Hoffman *et al.*, 1977), were due, at least in part, to these seasonal variations.

The effect of stress hormones may offer one possible explanation for the changes in lymphocytes subpopulations in the peripheral blood. It is possible that summation of "ministresses" produced by exposure to cold causes a slight increase in the steroid hormones, resulting in more B cells being released in the circulation. This can occur either by release from storage compartments in lymph nodes or spleen or by inhibition of margination; this assumes that B cells marginate like the polymorphonuclear cells. The effect of stress hormones such as steroids may also explain the increase in the relative and absolute number of B cells, to three to four times the normal level in patients with burns (Kagen *et al.*, 1982). When burn patients recovered the number of B cells decreased to normal levels. The role of steroids in the increase in the number of circulating B cells was investigated in patients with rheumatoid arthritis treated with prednisone. A significantly higher number of B cells was found in the peripheral blood of patients treated than in those not treated with steroids. Also, acute inoculation of hydrocortisone resulted in an increase in B cells level (Beilke *et al.*, 1981).

The factors that determine discrete changes in the number of circulating lymphocytes of different subpopulations are largely unknown. Nevertheless, it was of interest to determine whether by using bacteria as markers for lymphocyte subsets it was possible to detect such changes in cancer patients. A double blind study was performed on patients with breast cancer to determine whether the level of lymphocytes or their subpopulations can be correlated with the clinical status (Felix *et al.*, 1981). The patients were subdivided into four groups: I (18 patients) with benign breast disease, II (13 patients) with breast cancer but free of disease, III (7 patients) with breast cancer under treatment but free of disease, and IV (19 patients) with breast cancer with metastases under treatment. The absolute numbers in the periph-

eral blood of B cells, T_1T_2 cells, T_2 and T_3 cells were determined and the data analyzed statistically.

First, the following differences have been observed in the absolute number of lymphocytes: (a) the total number of lymphocytes was significantly increased in patients free of disease that had been treated only surgically; (b) it was decreased in patients with metastatic disease undergoing treatment; and (c) it was not changed by treatment alone (chemotherapy and/or radiotherapy). Second, the identification of the lymphocyte subpopulations in blood smears by bacterial adherence showed the following: (a) The T_1T_2 cells (Bm^-Ah^+) were at a lower level in patients with metastatic disease (see Section IV) than any other group, suggesting that the change might have been due to the tumor load and not to the treatment (chemotherapy and/or radiotherapy). In some patients values as low as 7 or 12% T_1T_2 cells were observed compared with a normal average of 49%. In absolute numbers the T_1T_2 level was even lower. (b) The mean level of lymphocytes in the T_2 subset was significantly lower in group IV than in group I but not significantly different than the other two groups (II and III) suggesting that both therapy and tumor load were important. (c) The level of B cells or T_3 cells was not significantly changed in any of the patients.

The low level of T_1T_2 cells may be explained in different ways. One possibility is that individual lymphocytes lost some essential maturation receptor. This is suggested by the lack of binding of bacteria by human thymocytes (DeBoer and Teodorescu, unpublished) with the exception of about 20% of the cells binding *S. schottmulleri*. The other possibility was a change in lymphocyte traffic and homing: homing at the tumor sites or in the lymphoid organs. Since lymph nodes are generally involved, and since the egress of lymphocytes from the lymph nodes is slowed by antigenic stimulation, it is possible that some subpopulations are more affected than others. Finally, it is possible that a selective destruction of T_1T_2 cells occurred. Only direct and simultaneous examination of the lymphoid tissues and peripheral blood lymphocytes can determine the correct explanation.

The T_1T_2 cells are primarily responsible for *in vitro* responses to plant lectins (Kleinman and Teodorescu, 1978). The decrease in the relative proportion of these cells is expected to be reflected in the mitogen response of PBL in patients with high tumor load. Indeed, Dean *et al.* (1980) showed that PBL from patients with metastatic lung cancer have a depressed response to plant lectins. Other reports on the makeup of lymphocyte subpopulations in the peripheral blood in cancer patients have been rather controversial (Stjernsward *et al.*,

1972; Whitehead *et al.*, 1976; Evans *et al.*, 1977). One possible explanation may be found in the method used to separate the lymphocytes by buoyant density centrifugation on Ficoll–Hypaque. It appears that with advancing disease mononuclear cells purified from the blood of cancer patients are contaminated with an increasing number of other white blood cells (Check and Hunter, 1979). This contamination can be as high as 50% of the cell population.

Aging is accompanied by multiple changes in the immunologic potential. With advancing age autoimmune diseases became more prevalent (Teague *et al.*, 1970) and the incidence of neoplasia increases (Teller, 1972). Lymphocyte number also change resulting in depressed mitogen (Hori *et al.*, 1973) or antigen (Heindrick and Makinodan, 1973) responses. The animal model offers a good possibility of determining changes in the subpopulations of lymphocyte if the spleen, a major lymphoid organ is examined. Gilette (1982) examined the lymphocyte subpopulations identified by bacterial adherence in the spleen of aging mice. The T_1 cells ($Ec3^+$ cells) responsible for mitogen reactivity are virtually undetectable in very young animals and are also at very low level in old animals. The T_2 cells were also at a very low level both in very young and very old animals and represent the dominant class in adult animals. The T_3 subpopulation which contains most of the cells active in immune response decreased continuously with age. Similar observations were made by Lewis and Kleinman (1982).

Changes in the B-cell subpopulations have also been observed in aging animals. The B_1 subpopulation (Ba^+) which contains the Ig-secreting cells, i.e., the mature B-cell population, increased continuously throughout life, in agreement with the hypothesis that B_1 cells contain the memory lymphocytes (Chen *et al.*, 1979). The B_3 subpopulation was the largest in newborn animals and decreased with age while the B_2 cells remained relatively constant throughout life. All these changes in the B- and T-cell subpopulations with age are somehow expected based on the functions of these subpopulations in mice (Chen *et al.*, 1979, 1981). Thus, bacterial markers can detect changes related to functional potential in aging animals.

B. Dramatic Changes in Lymphocyte Subpopulations: Leukemia and Primary Immunodeficiencies

Malignant proliferation of a clone that belongs to a certain lymphocyte subpopulation may result in a dramatic change in the make-up of the lymphocyte population in the peripheral blood. This change is reflected in the imbalance between different lymphocyte subpopula-

tions and allows for direct observations of the malignant clone. In chronic lymphocytic leukemia (CLL), which is mostly of B cell origin, a large increase in the Ig^+ cells causes changes the B/T cell ratio. Since the malignant clone usually expresses only one type of Ig light chain, κ or λ, a change in their normal 6/4 ratio will also occur. *E. coli*-0 coated with anti-κ or anti-λ light chain antibody was used to count the Ig-bearing cells and *B. melitensis* was used to count B cells (Teodorescu *et al.*, 1977b; Mayer *et al.*, 1978a; Nelson *et al.*, 1979). Compared with about 15% Ig^+ or Bm^+ lymphocytes in the peripheral blood of normal donors, 52–77% of the CLL cells were Ig^+ and 80–93% Bm^+. Sometimes small alterations in the percentage of Bm^+ cells or in the ratio between the cells bearing κ or λ chains can be suggestive of CLL (Teodorescu and Mayer, 1978). In some cases the percentages of Ig^+ cells were equal to the percentage of Bm^+ cells, in others there were more Bm^+ cells than Ig^+ cells (Teodorescu *et al.*, 1977b; Nelson *et al.*, 1979) suggesting that some leukemic cells did not express detectable amounts of surface Ig.

The lymphocyte population of the same leukemic clone displayed a relatively large heterogeneity of binding ability for various bacteria (Teodorescu *et al.*, 1977b). Thus, to determine whether the percentages of lymphocytes that bound different bacteria can be correlated with disease activity a double-blind study on 24 CLL patients was set up (Nelson *et al.*, 1979). Samples of peripheral blood from patients with high counts of leukemic cells were labeled with 10 different bacteria in blood smears and a binding index was determined as the mean of percentages of lymphocytes that bound different bacteria. The data were plotted on scattergrams in which the patients were preclassified according to symptoms (symptomatic and asymptomatic) or stage (I–IV). A significant correlation was found between the binding indices and the symptoms. Also, a good correlation appeared to exist between these indices and the stage of the disease.

As shown in Section IV, one likely mechanism of binding of bacteria to lymphocytes is the presence of lectins on the lymphocyte membrane. These lectins, as in other biological systems, may be involved in cell–cell recognition and possibly in the control of cellular proliferation. Thus, it may be speculated that one of the mechanisms of escape from control of malignant cells is their paucity in surface lectins, i.e., translated here as the binding sites for bacteria (Nelson *et al.*, 1979). If this were true it was expected that the patients with low binding index will also have a poor prognosis. To test this hypothesis, the patients were tabulated according to their binding indices and were followed for 2 years. Seven out of seven patients with indices higher than 45%

were alive after this period and four out of five patients with indices of less than 36% were dead (Teodorescu *et al.*, 1981). Thus, it is possible that the loss of lectins on malignant lymphocytes contributes to their escape from control.

Patients with acute lymphocytic leukemias (ALL) have also been investigated. The blood and bone and bone marrow lymphocytes from 12 patients with ALL were examined for natural binding of bacteria and for the presence of surface Ig and E rosette-forming cells (Teodorescu *et al.*, 1981). The leukemic cells of five patients bound *B. melitensis* although they were classified as "null cells" suggesting that they may be precursors of B cells, i.e., the cells were E rosette-negative and Ig-negative. The B-cell population in these patients had a normal κ/λ ratio. If *B. melitensis* bound to pre-B cells it is likely that the B cells were not capable of further differentiation to Ig^+ cells. If this occurred the κ/λ ratio would be expected to change as it does in CLL (Teodorescu and Mayer, 1979).

Another catastrophic change occurs in primary immunodeficiencies. Peripheral blood lymphocyte of 12 patients with various primary immunodeficiencies have been examined: severe combined immunodeficiency (SCID), hypogammaglobulinemia, common variable and transient hypogammaglobulinemia. In all patients the mapping of the lymphocyte population by bacterial adherence showed severe abnormalities (Bernales and Teodorescu, 1980, and unpublished observations). The four patients with SCID had values of Ah^+ cells much lower than normal, and, at the same time, the cells responded very poorly to plant lectins. By contrast, the lymphocytes from the patients with hypogammaglobulinemia had normal–high levels of Ah^+ cells and responded well to plant lectins. No patient with thymus aplasia has been investigated so far. However, the spleen of nude mice contain over 90% cells that bind *B. melitensis* much more than the 50% found in other mouse strains (Mayer *et al.*, 1978b).

VI. Summary and Conclusions

Lymphocytes bind certain strains of bacteria in a highly reproducible manner. Both in humans and in mice it has been possible to map the lymphocyte subpopulations using bacteria as specific membrane markers. All human B cells, normal or leukemic, bind *Brucella melitensis* as well as other human pathogens from the genus *Brucella*, while T cells do not bind these bacteria. By using four strains of bacteria, *B. melitensis*, *A. hinshawii*, *B. globigii*, and *S. schottmulleri*, besides B cells (Bm^+), two B- and four T-cell subpopulations have

been identified which were given arbitrary numbers, B_1, B_2, T_1–T_4. Mouse spleen cells were also subdivided into B cells (Bm^+) and T cells (Bm^-), *B. melitensis* being the only known common marker for all human and mouse B cells. Also, three B cell subpopulations, B_1–B_3, and three T cell subpopulations, T_1–T_3, were identified in the mouse spleen using a minimum of four bacteria: *B. melitensis, A. hinshawii, E. coli*-2, and *E. coli*-3. Since the function of B cells is definitely different from that of T cells, the existence of *B. melitensis* as a B cell marker suggested that the lymphocyte subpopulations identified by bacterial adherence are functionally different. Mouse B_1 cells contain almost exclusively the Ig-secreting cells and is probably a more mature lymphocyte population increasing in size with advancing age. Mouse T_1 cells coincide largely with the Ly 123^+ cells and respond well to plant lectins while the other T-cell subpopulations respond poorly to plant lectins; the T_3 cells contained most cells active in specific cytotoxicity. Human T_1T_2 contain the precursors for specifically cytotoxic cells and are mitogen responders. The T_3T_4 cells appear to contain helper cells: they respond well in mixed lymphocyte cultures, do not become cytotoxic, respond well to soluble antigens, and help in Ig secretion induced by PWM. The T_4 cells population is defined as those lymphocytes that do not bind any bacteria. They contain the cells with receptors for Fc of IgG capable of NK and ADCC activity. The T_2 cells are capable of suppressing the activity of NK cells.

The mechanism of binding of bacteria to lymphocytes has only been partially investigated. Experiments with *B. melitensis* and with a mutant of *E. coli* suggested that there may be lectins on the lymphocyte membrane that interact with carbohydrates on the bacteria, an interaction that can be inhibited by certain monosaccharides, or by the specific LPS. The availability of carbohydrates for lectin binding appears to differentiate bacteria that bind from those that do not bind to lymphocytes. Nevertheless, some of the bacteria may bind due to the presence on their surface of lectins that interact with carbohydrates on lymphocyte subpopulations. The existence of certain lectins or carbohydrates on lymphocyte subpopulations may be related to their function in cellular interactions, such as the development of cytotoxic cells.

Bacteria bind consistently to lymphocytes, are much smaller, and their binding properties are not affected by heating or formaldehyde fixation. These properties have allowed their use as shelf reagents in stained smears prepared from peripheral blood or from bone marrow. Accurate determinations of lymphocyte subpopulations in normal individuals and patients have been performed and several observations

made. (1) The relative and absolute number of B cells and T_1T_2 cells in the peripheral blood changes with the season. (2) The number of circulating B cells increases in stressful conditions or as a result of treatment with steroid hormones. (3) Leukemic B cells and probably pre-B cells bind *B. melitensis*. (4) CLL patients with lymphocytes that have a low binding index for bacteria have a poor prognosis. (5) Abnormalities in the lymphocyte subpopulation identified by bacteria are easily detected in patients with primary immunodeficiencies. (6) An increase in tumor load and metastases is associated with a decrease in the number of circulating T_1T_2 cells. Some of the changes observed in the peripheral blood are discrete in nature and are most likely due to changes in the lymphocyte traffic. Any extrapolation to the entire immune system is likely to be misleading whatever markers for lymphocytes are used. In the case of dramatic changes such as leukemias or primary immunodeficiencies the changes in the peripheral blood are probably more representative of the entire system.

The use of bacteria as markers of lymphocyte subpopulations has several advantages. (1) Bacteria are stable shelf reagents, easy to prepare and store. (2) The extreme variety of bacteria and the selection of desired mutants provide the opportunity to identify many subpopulations of cells. (3) The use of bacteria in blood smears offers the possibility of morphologic examination of the lymphocytes and of obtaining a permanent record.

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REFERENCES

Ahmed, A., Scher, I., Sharrow, S. O., Smith, A. H., Paul, W. E., Sachs, D. H., and Sell, K. W. (1977). *J. Exp. Med.* **145**, 101.

Ashwell, G., and Morell, A. G. (1977). *Trend Biochem. Sci.* **2**, 76.

Barondes, S. M. (1981). *Annu. Rev. Biochem.* **50**, 207.

Basten, A., Sprent, J., and Miller, J. F. A. P. (1972). *Nature (London) New Biol.* **235**, 178.

Beilke, M., Bratescu, A., Skosey, F. L., and Teodorescu, M. (1982). In preparation.

Bernales, R., and Teodorescu, M. (1980). *Int. Congr. Immunol., 4th, Paris* 14.2.02. (Abstr.)

Bratescu, A., and Teodorescu, M. (1981). *J. Allergy Clin. Immunol.* **68**, 273.

Bratescu, A., Mayer, E. P., and Teodorescu, M. (1980). *Clin. Exp. Immunol.* **41**, 547.

Bratescu, A., Mayer, E. P., and Teodorescu, M. (1981). *Infect. Immun.* **31**, 816.

Bratescu, A., Mayer, E. P., and Teodorescu, M. (1982). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 838.

Brown, G., and Greaves, M. F. (1974). *Scand. J. Immunol.* **3,** 161.
Buchholz, D., Dray, S., and Teodorescu, M. (1979). *Immunology* **37,** 731.
Bundy, B., and Nelson, D. (1980). *In* "Neoplasm Immunity Exp. Clin." (R. G. Crispen, ed.), p. 443. Elsevier, Amsterdam.
Callawaert, D. M., Kaplan, J., Peterson, W. D., Jr., and Lightbody, J. J. (1977). *Cell. Immunol.* **33,** 11.
Cantor, H. (1972). *Cell. Immunol.* **3,** 461.
Cantor, H., and Boyse, E. A. (1975). *J. Exp. Med.* **141,** 1376.
Cantor, H., Shen, F. W., and Boyse, E. A. (1976). *J. Exp. Med.* **143,** 1391.
Chang, J.-L., Ganea, D., Dray, S., and Teodorescu, M. (1981). *Immunology* **44,** 745.
Check, I. J., and Hunter, R. L. (1979). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 1224.
Chen, W.-Y., Teodorescu, M., and Mayer, E. P. (1979). *J. Immunol.* **123,** 2817.
Chen, W.-Y., Teodorescu, M., McKenzie, I. F. C., and Mayer, E. P. (1981). *Immunology* **42,** 285.
Chess, L., and Schlossman, S. F. (1977). *Adv. Immunol.* **25,** 213.
Cline, M. J., Sprent, J., Warner, N. L., and Harris, A. W. (1972). *J. Immunol.* **108,** 1126.
Dean, J. H., Cannon, G., Jerrels, J., McCoy, R., and Herberman, R. (1980). *In* "Neoplasm Immunity" (G. Crispen, ed.), p. 357. Elsevier, Amsterdam.
DeBoer, K. P., Kleinman, R., and Teodorescu, M. (1981a). *J. Immunol.* **126,** 276.
DeBoer, K. P., Bratescu, A., and Teodorescu, M. (1981b). *Immunobiology* **160,** 173.
Dickler, H. B., and Kunkel, H. G. (1972). *J. Exp. Med.* **136,** 191.
Dos Reis, G. A., and Shevach, E. M. (1981). *J. Immunol.* **127,** 2456.
Duguid, J. P. (1968). *Arch. Immunol. Ther. Exp.* **16,** 173.
Ellen, R. P., and Gibbons, R. J. (1972). *Infect. Immun.* **5,** 826.
Eshdat, Y., Ofek, I., Yashouv-Gan, Y., Sharon, N., and Mirelman, D. (1978). *Biochem. Biophys. Res. Commun.* **85,** 1551.
Evans, J. T., Goldrosen, M. H., Han, T., Minowada, J., Howell, J. H., Mittelman, A., and Holyoke, E. D. (1977). *Cancer* **40,** 318.
Fauci, A. S., Pratt, K. R. K., and Whalen, G. (1976). *J. Immunol.* **117,** 2100.
Felix, E., Katz, S., Teodorescu, A., and Teodorescu, M. (1981). *J. Surg. Oncol.* **18,** 323.
Forber, J. T., Niblack, G. D., Fuchs, R., Richie, R. E., Johnson, H. K., and Oldham, R. K. (1981). *Cancer Immunol. Immunother.* **11,** 139.
Forbes, I. J., and Zalewski, P. D. (1976). *Clin. Exp. Immunol.* **26,** 99.
Freimer, N. B., Ogmundsdottir, H. M., Blackwell, C. C., Sutherland, I. W., Grahame, L., and Weir, D. M. (1978). *Acta Pathol. Microbiol. Scand. Sect. B* **86,** 53.
Freitas, A. A., and deSousa, M. A. B. (1975). *Eur. J. Immunol.* **5,** 831.
Freitas, A. A., and deSousa, M. A. B. (1976a). *Cell. Immunol.* **22,** 345.
Freitas, A. A., and deSousa, M. A. B. (1976b). *Eur. J. Immunol.* **6,** 703.
Gately, M. K., and Martz, E. (1981). *J. Immunol.* **126,** 709.
Gaudernack, G., Curry, R., and Messner, R. (1981). *J. Immunol. Methods* **43,** 251.
Ghetie, V., Nilsson, K., and Sjoquist, J. (1974). *Eur. J. Immunol.* **4,** 500.
Gillette, R. W. (1982). *Clin. Exp. Immunol.* **111,** 1502.
Gillette, R. W., McKenzie, G. O., and Swanson, M. H. (1973). *J. Immunol.* **111,** 1902.
Grey, H. M., Kubo, R. T., and Cerottini, J.-C. (1972). *J. Exp. Med.* **136,** 1323.
Heindrick, M. L., and Makinodan, T. (1973). *J. Immunol.*
Hoffman, T., Wang, C. Y., Winchester, R. J., Ferrarini, M., and Kunkel, H. G. (1977). *J. Immunol.* **119,** 1520.
Hori, Y., Perkins, E. H., and Halsall, N. K. (1973). *Proc. Soc. Exp. Biol. Med.* **144,** 48.
Howe, M. L. (1973). *J. Immunol.* **110,** 1090.
Huber, B., Devinsky, O., Gershon, R. K., and Cantor, H. (1976). *J. Exp. Med.* **143,** 1534.

Jacobs, D. M., and Byrd, W. (1975). *Nature (London)* **255,** 113.
Jondal, M., Holman, G., and Wigzell, H. (1972). *J. Exp. Med.* **136,** 207.
Kagen, R., Bratescu, A., Jonasson, O., and Teodorescu, M. (1982). In preparation.
Kieda, C. M. T., Bowles, D. J., Raviel, A., and Sharon, N. (1978). *FEBS Lett.* **94,** 391.
Kieda, C., Roche, A.-C., Delmotte, F., and Monsigny, M. (1979). *FEBS Lett.* **99,** 329.
Kimura, A. K., and Wigzell, H. (1978). *J. Exp. Med.* **147,** 1418.
Kisielow, P., Hirst, J. A., Shiku, H., Beverley, P. C. L., Hoffman, M. K., Boyse, E. A., and Oettgen, H. F. (1975). *Nature (London)* **253,** 219.
Kleinman, R., and Teodorescu, M. (1978). *J. Immunol.* **120,** 2020.
Kleinman, R., and Teodorescu, M. (1979). *Cell. Immunol.* **48,** 43.
Kleinman, R., DeBoer, K. P., and Teodorescu, M. (1980). *Clin. Exp. Immunol.* **39,** 510.
Kondracki, E., and Milgrom, F. (1977). *J. Immunol.* **118,** 381.
Kunz, M. M., Innes, J. B., and Weksler, M. E. (1976). *J. Exp. Med.* **143,** 1042.
Lee, C.M.-J., Mayer, E. P., Molnar, J., and Teodorescu, M. (1982). *Immunobiology,* in press.
Lehmann, V., Streck, H., Minner, I., Krammer, P. H., and Ruschmann, E. (1980). *Eur. J. Immunol.* **10,** 685.
Lewis, J. A., and Kleinman, R. (1982). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 384.
Lobo, P. I., Westervelt, F. B., and Horwitz, D. A. (1975). *J. Immunol.* **114,** 116.
Lum, L. G., Benveniste, E., and Blaese, R. M. (1980). *J. Immunol.* **124,** 702.
McKenzie, I. F. C., Clarke, A., and Parish, C. R. (1977). *J. Exp. Med.* **145,** 1039.
Mayer, E. P., and Teodorescu, M. (1978). *Infect. Immun.* **29,** 66.
Mayer, E. P., and Teodorescu, M. (1980). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 1654.
Mayer, E. P., Bratescu, A., Dray, S., and Teodorescu, M. (1978a). *Clin. Immunol. Immunopathol.* **9,** 37.
Mayer, E. P., Chen, W.-Y., Dray, S., and Teodorescu, M. (1978b). *J. Immunol.* **120,** 167.
Molinaro, G., and Dray, S. (1974). *Nature (London)* **248,** 515.
Monsigny, M., Kieda, C., and Roche, A. C. (1979). *Biol. Cell.* **36,** 289.
Moretta, L., Ferrarini, M., Mingari, M. C., Moretta, A., and Webb, S. R. (1976). *J. Immunol.* **117,** 2171.
Moretta, L., Webb, S. R., Grossi, C. E., Lydyard, P. M., and Cooper, M. D. (1977). *J. Exp. Med.* **146,** 184.
Morimoto, C. (1978). *Clin. Exp. Immunol.* **32,** 125.
Morimoto, C., Reinherz, E. L., and Schlossman, S. (1981). *J. Immunol.* **127,** 514.
Muchmore, A. V., Decker, J. M., and Blaese, R. M. (1980). *J. Immunol.* **125,** 1306.
Mukherji, B., Flowers, A., Rothman, L., and Nathanson, L. (1975). *J. Immunol.* **124,** 412.
Nelson, R., Bratescu, A., and Teodorescu, M. (1979). *Cancer* **44,** 1665.
Niemetz, A. H., and Mayr, W. R. (1981a). *Vox Sang.* **489.**
Niemetz, A. H., and Mayr, W. R. (1981b). *Vox Sang.* **490.**
Novogradsky, A., and Ashwell, G. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 676.
Ofek, I., Mirelman, D., and Sharon, N. (1977). *Nature (London)* **265,** 623.
Ofek, I., Heachey, E. H., and Sharon, N. (1978). *Trends Biochem. Sci.* **3,** 159.
Orlando, J. R., Sharrow, S. O., Timonen, T., and Herberman, R. H. (1981). *J. Immunol.* **127,** 2401.
Paraskevas, I., Lee, S.-T., Orr, K. B., and Israels, L. G. (1972a). *J. Immunol.* **108,** 1319.
Paraskevas, F., Orr, K. B., Anderson, E. D., Lee, S. T., and Israels, L. G. (1972b). *J. Immunol.* **108,** 1729.
Pellegrino, M. A., Ferone, S., and Theofilopoulos, N. F. (1975). *J. Immunol.* **115,** 1065.
Ponzio, N. M., Finke, J. H., and Batisto, J. R. (1975). *J. Immunol.* **114,** 971.
Raff, M. C. (1971). *Transplant. Rev.* **6,** 52.

Rasanen, L. (1981). *Cell. Immunol.* **58,** 19.
Reinherz, E. L., and Schlossman, S. F. (1980). *New Engl. J. Med.* **303,** 370.
Reinherz, E. L., Kung, P. C., Goldstein, G., and Schlossman, S. F. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4061.
Reinherz, E. L., Kung, P. C., Goldstein, G., and Schlossman, S. F. (1980a). *J. Immunol.* **124,** 1301.
Reinherz, E. L., Morimoto, C., Penta, A. C., and Schlossman, S. F. (1980b). *Eur. J. Immunol.* **10,** 570.
Reisner, Y., and Sharon, N. (1980). *Trends Biochem. Sci.* **5,** 29.
Reisner, Y., Linker-Israeli, M., and Sharon, N. (1976). *Cell. Immunol.* **25,** 129.
Rosen, S. D., Kafka, J. A., Simpson, D. L., and Barnes, S. M. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2554.
Ross, G. D., Rabellino, E. M., Polley, M. J., and Grey, H. M. (1973). *J. Clin. Invest.* **52,** 377.
Selinger, D. S., Julie, N., Reed, W. P., and Williams, R. C. (1978). *Science* **201,** 455.
Shiku, H., Kisielow, P., Bean, M. A., Takahashi, T., Boyse, E. A., Oettgen, H. F., and Old, L. J. (1975). *J. Exp. Med.* **141,** 227.
Shockley, R., and Kleinman, R. (1981). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40,** 1155.
Simpson, D. L., Rosen, S. D., and Borondes, S. H. (1974). *Biochemistry* **13,** 3487.
Simpson, D. L., Thorne, D. R., and Loh, H. H. (1977). *Nature (London)* **266,** 367.
Simpson, D. L., Thorne, D. R., and Loh, H. H. (1978). *Life Sci.* **22,** 727.
Smith, J. B. (1978). *Cell. Immunol.* **36,** 203.
Smith, R. T., Norcross, M., Maino, V., and Konaka, Y. (1980). *Immunol. Rev.* **51,** 193.
Snell, G. D., Cherry, M., McKenzie, I. F. C., and Bailey, D. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1108.
Spear, G., and Teodorescu, M. (1982). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 812.
Strelkauskas, A. J., Teodorescu, M., and Dray, S. (1975). *Clin. Exp. Immunol.* **22,** 62.
Strelkauskas, A. J., Callery, R. T., McDowell, J., Borel, Y., and Schlossman, S. F. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5150.
Sterzl, J., and Silverstein, A. M. (1967). *Adv. Immunol.* **6,** 337.
Stjernsward, J., Sandal, J., Vanky, F., Wigzell, H., and Sealy, R. (1972). *Lancet* **1,** 1352.
Stutman, O., Dien, P., Wilson, R. E., and Lattime, E. C. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2895.
Takahashi, T., Old, L. J., Hsu, C. J., and Boyse, E. A. (1971). *Eur. J. Immunol.* **1,** 478.
Teague, P. O., Yunis, E. J., Fish, A. J., Stutman, O., and Good, R. A. (1970). *Lab. Invest.* **22,** 212.
Teller, M. N. (1972). *Adv. Gerontol. Res.* **4,** 25.
Teodorescu, M. (1981). *Immunol. Rev.* **55,** 155.
Teodorescu, M., and Mayer, E. P. (1978). *Ann. Clin. Lab. Sci.* **8,** 353.
Teodorescu, M., Mayer, E. P., and Dray, S. (1976). *Cell. Immunol.* **24,** 90.
Teodorescu, M., Mayer, E. P., and Dray, S. (1977a). *Cell. Immunol.* **29,** 353.
Teodorescu, M., Mayer, E. P., and Dray, S. (1977b). *Cancer Res.* **37,** 1715.
Teodorescu, M., Bratescu, A., and Mayer, E. P. (1979a). *Clin. Immunol. Immunopathol.* **13,** 194.
Teodorescu, M., Wen, C.M.-J., and Mayer, E. P. (1979b). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 1461.
Teodorescu, M., Hsu, C., Bratescu, A., DeBoer, K. P., Nelson, R., Kleinman, R., Wen, C.-M.-J., Mayer, E. P., and Pang, E.J.-M. (1981). *Mod. Trends Hum. Leuk.* **26,** 355.
Timonen, T., Saksela, I., Ranki, A. M., and Hayry, P. (1979). *Cell. Immunol.* **48,** 133.
Tomonari, K. (1980). *J. Immunol.* **124,** 1111.

Weir, D. M. (1981). *Immunol. Today* **Aug.,** 45.
West, W., Cannon, G., Kay, H. D., Bonard, G., and Herberman, R. (1977). *J. Immunol.* **118,** 355.
Whigham, E., and Kleinman, R. (1982). *Annu. ASM Meeting,* Abstr. p. 5.
Whitehead, R. H., Thatcher, J., and Teasdale, C. (1976). *Lancet* **1,** 330.
Wilson, B. S., Teodorescu, M., and Dray, S. (1976). *J. Immunol.* **116,** 1312.
Winchester, R. J., Fu, S. M., Hoffman, T., and Kunkel, H. G. (1975). *J. Immunol.* **114,** 1210.
Wolpert, J. S., and Albertsheim, P. (1976). *Biochem. Biophys. Res. Commun.* **70,** 729.
Woodruff, J. (1974). *Cell. Immunol.* **13,** 378.
Wybran, J., and Fudenberg, H. H. (1973). *J. Clin. Invest.* **52,** 1026.
Yu, D. T. Y., Chiorazzi, N., and Kunkel, H. G. (1980). *Cell. Immunol.* **50,** 305.

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