1987

Characterization Of Anti-dna Autoantibody Responses From Lymphoid Cells Of Normal Human Origin

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CHARACTERIZATION OF ANTI-DNA AUTOANTIBODY RESPONSES FROM LYMPHOID CELLS OF NORMAL HUMAN ORIGIN.

by

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Submitted in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

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ISBN 0-315-36071-2

ABSTRACT

The hallmark of systemic lupus erythematosus (SLE) is a spontaneous production of autoantibodies reactive with a variety of nuclear structures. However, the origin and pathogenetic role of autoreactive immunoglobulins remains obscure. The detection in normal individuals of low levels of antibodies to self-antigens such as actin and myoglobin, suggested that anti-nuclear antibody responses might be part of the normal immune repertoire and prompted us to search for anti-DNA antibody production in normal B lymphocytes. Spontaneous and mitogen induced anti-DNA antibodies were assayed in tonsillar and peripheral blood lymphocytes (PBLs) of healthy donors as well as in PBLs from SLE patients. IgM antibodies to single stranded DNA (ssDNA) were detected frequently in pokeweed mitogen stimulated cultures of both SLE PBLs (88%) and normal tonsillar lymphocytes (78%). IgM and IgG antibodies to DNA were found at high frequencies of 73% and 59% respectively with mitogen stimulated SLE PBLs. Spontaneous production of IgM antibodies reactive with ssDNA was identical in normal tonsillar lymphocytes and PBLs of SLE patients (43% and 44% respectively). Human hybridoma cell lines were generated by fusion of normal tonsillar lymphocytes with lymphoblastoid cell line GM 4672. 11.8% of these hybridomas synthesized and secreted anti-nucleic acid antibodies, all of which were IgM. These monoclonal antibodies reacted with ssDNA, DNA, polydG.polydC, poly(dA-dT), low molecular weight supernatant DNA and RNA as well as with cardiolipin. Four of the ten hybridomas studied reacted with the HEp2 cytoskeletal

component vimentin or a vimentin-related compound. Anti-idiotypic antibody raised against one of the monoclonal anti-DNA antibodies, was utilized to examine the expression of the corresponding idiotype (ID), 4.6.3, in the collection of human hybridoma cell lines as well as in sera of normals and SLE patients. The 4.6.3 ID was expressed by a third of all DNA and non-DNA binding monoclonal antibodies and was detected in sera from 90% of SLE patients and 24% of normals. The level of 4.6.3 ID in patients was independent of total serum IgM and IgG levels and of the concentration of anti-DNA antibody. Antigen binding to the majority of the 4.6.3 ID positive monoclonal anti-DNA antibodies was not blocked by the anti-ID suggesting that 4.6.3 idiotypic determinants were localized outside the antigen binding site.

These data indicate convincingly for the first time that anti-DNA antibodies equivalent to those found in SLE patients can be derived from apparently normal individuals. As idiotypes are the serologic markers of variable immunoglobulin region genes, the existence of a common cross-reactive idiotype shared by both normal and SLE lymphocytes, implies that anti-DNA antibodies are encoded by common variable region genes present throughout the normal human population.

ACKNOWLEDGEMENTS

I would like to acknowledge the guidance offered by Dr. David A. Bell who was a "super" supervisor and an excellent teacher. The exciting atmosphere which he created and his sharing of ideas made my graduate studies a most memorable experience.

The constructive criticisms and ideas of Dr. Gil Strejan were always appreciated as was his generosity in finding time for our friendly discussions of research.

I would also like to thank Dr. Wayne Flintoff for his excellent advice during my studies, Dr. Joyce Rauch for sharing her hybridoma technology experience with me, and Ron Komar for helping with the immunofluorescence techniques.

A special thanks goes to my laboratory colleagues (Brian, Helene, John and Judy) who not only provided technical assistance to this project but cheered me up when things went wrong.

Finally, I would like to thank my family for their love, support, encouragement and patience during the studies and during writing of this thesis. I dedicate this work to my family: to my daughter, Stephanie; to my husband, Bill; to my father, Henryk; to my mother, Lusia; and to my sister, Barbara.

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LIST OF ABBREVIATIONS

AIT	Autoimmune thyroiditis
Anti-ID	Anti-idiotype
APC	Antigen presenting cell
B cell	Bone marrow cell or equivalent
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CRI	Cross-reactive idiotype
CT	Calf thymus
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenyl
EBV	Epstein Barr virus
FCS	Fetal calf serum
GM	Growth medium for hybridomas
HAT	Hypoxanthine-aminopterine-thymidine
HEp2	Human epithelial cell line
HGG	Human gammaglobulin
HSA	Human serum albumin
ID	Idiotype
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL1	Interleukin 1
I12	Interleukin 2

KLH	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
OA	Ovalbumin
PBL	Peripheral blood lymphocyte
PFC	Plaque forming cell
PFRM	Post fusion recovery medium
PLL	Poly-L-lysine
polydG.polydC	Polydeoxyguanylate-polydeoxycytidylate
poly(dA-dT)	Polydeoxyadenylate-thymidylate
PPD	Purified protein derivative of tuberculin
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RT	Room temperature
SFM	Serum free medium
SLE	Systemic lupus erythematosus
S/N	Supernatant
SRBC	Sheep red blood cell
ssDNA	Single stranded DNA
T cell	Thymus derived cell
WB	Washing buffer

INTRODUCTION

Autoantibodies of various specificities are features of many human diseases, and are particularly characteristic of the antibody mediated prototype autoimmune disease, systemic lupus erythematosus (SLE). The antibodies observed in SLE patients are directed against a broad spectrum of antigens, including nucleic acids, nuclear proteins, phospholipids and cell membranes (Kunkel et al, 1960; Hughes et al, 1971; Sharp et al, 1976; Morimoto et al, 1979; Swaak et al, 1979; Koike et al, 1982). These self-reactive antibodies have been implicated in clinical symptomatology through the formation of pathogenetic immune complexes which participate in the tissue injury observed at various sites (Landry and Sam, 1973; Koffler et al, 1974). The relationship between autoantibodies and disease pathogenicity is unclear and has emerged as one of the central issues in autoimmunity research.

A key question in examination of the origin and role of autoantibodies is whether or not autoreactive B lymphocytes are part of the normal immune repertoire. Autoantibodies are known to occur frequently outside the realm of autoimmune disease, for example, in association with chronic infections, aging and with the use of a variety of drugs (Lidman et al, 1976; Linder et al, 1979; Hirokawa, 1985). These conditions however, do not represent "normal" immune states.

At the time when this research began, anti-DNA antibodies had not been identified in vivo in normal individuals and their induction in animals experimentally had been extremely difficult (Roder et al, 1978; Madaio et al, 1984). These observations led to the speculation that the anti-DNA autoantibody response arose either from a unique immune response gene providing the genetic basis to SLE, or that the anti-DNA response arose from defective regulation of the immune response, leading to polyclonal activation of self-reactive B cells (Knight, 1982; MacKay, 1983).

In contrast to these results were reports of "natural autoantibodies", occuring in normal human sera. Guilbert et al. (1982) reported the consistent detection in normals of antibodies against self antigens such as tubulin, albumin and myoglobin. Morover, several different observations suggested that even the anti-DNA antibody response was not necessarily restricted to the disease state. Spontaneous production of anti-nucleic acid antibodies was observed with normal murine spleen cells in vitro as well as in vivo, after injection of an immunogenic low molecular weight form of DNA or of the polyclonal B cell activator LPS (Roder et al, 1978; Pancer et al, 1981; Fish and Ziff, 1982). Reports that anti-DNA antibodies generated from normal mice and from lupus-prone strains share cross-reactive idiotypes (Datta et al, 1983) provide further evidence that cells capable of making autoantibodies are present in normal mice.

The principal objective of the studies presented here was to further explore the question of whether or not autoreactive B lymphocytes are present in normal individuals and to probe the variable region immunoglobulin genes of the autoantibodies produced by B cells, including their antigen specificity and idiotype expression. It was found that the human tonsillar lymphoid cells derived from healthy donors spontaneously produced in vitro amounts of anti-DNA antibody comparable to those produced by cultured SLE PBLs (Cairns et al, 1985). This initial observation provided the basis for the experiments, in which for the first time, the successful generation of anti-DNA autoantibody producing human hybridomas was accomplished with lymphoid cells from a normal individual. Monoclonal anti-DNA antibodies secreted by these hybridomas were characterized (Cairns et al, 1984; Cairns et al, 1986a). The hybrids were shown to produce IgM anti-DNA antibodies with identical patterns of reactivity to those observed in SLE patients (Shoenfeld et al, 1983; Andre-Schwartz et al, 1984). In addition, it was demonstrated that both the monoclonal anti-DNA antibodies from a collection of hybridoma cell lines as well as serum immunoglobulins of SLE patients, expressed a cross-reactive idiotype (Cairns et al, 1986b). These results confirm that normal individuals have B lymphocytes which secrete anti-DNA antibodies and that autoantibody production is part of the normal immune repertoire. The demonstration, for the first time, of a cross-reactive idiotype shared amongst monoclonal anti-DNA autoantibodies from unrelated normal individuals, further implies that a common immunoglobulin variable region gene or closely related genes within the human

population encode anti-DNA antibodies. These results provide a first step in understanding the origins of the anti-DNA autoimmune response. Further analyses, particularly at the molecular level, should elucidate the factors involved in the genesis of these autoantibodies and their function in health and disease.

CHAPTER 1 - HISTORICAL REVIEW

1.1. Birth of the Concept of Autoimmunity.

At the turn of the twentieth century, Ehrlich and Morgenroth (1900) discovered that goats immunized with their own erythrocytes failed to elicit an autoimmune response. To explain this loss of reactivity to self, Ehrlich and Morgenroth (1900) formulated the concept of "horror autotoxicus" or autoimmunity which the organism was to avoid at all costs. Although this self tolerance became the generally accepted rule, the potential for possible deviation from it leading to autoimmune disease was realized at the same time as reflected in the following statement of Ehrlich and Morgenroth, "In the explanation of many disease phenomena, it will in the future be necessary to consider the possible failure of the internal regulation, as well as the action of directly injurious exogenous or endogenous substances".

1.2. Clonal Deletion Hypothesis - an Attempt to Explain Self Tolerance.

In 1959 Burnet proposed a clonal deletion hypothesis in parallel to his clonal selection theory. He postulated that each lymphocyte clone gives rise to progeny cells making antibody of only one specificity. On contact with antigen, lymphocytes of a given clone proliferate and differentiate into plasma cells secreting their characteristic antibodies. The diversity of antibodies is generated during embryonic

development by random somatic mutations. During that time and by the same mechanism self reactive clones (forbidden clones) would be generated. As soon as these forbidden clones emerge, they would be capable of reacting with surrounding autologous antigens and in turn they would be eliminated. The deletion of self reactive clones would result in tolerance to self.

According to Burnet, autoimmunity may arise as a consequence of the emergence of forbidden clones due to a mistake in the deletion process or due to exposure to sequestered antigens (antigens which were not exposed in embryonic life to the immunological system because of their compartmentalization or later development).

Burnet's concept of elimination of self reactive clones as a mechanism to avoid autoimmunity was challenged by different investigators who succeeded in demonstrating the presence of such clones in normal individuals. For example, Bankhurst et al (1973) and Roberts et al (1973) showed the binding of labelled thyroglobulin to the peripheral blood lymphocytes of normal individuals. Yung et al (1973) demonstrated the binding of myelin to human and guinea pig lymphocytes.

Not only could binding of autoantigens to lymphocytes from normal subjects be revealed, but also the production of autoantibodies in normal tissues. Such specific naturally occuring autoantibodies to thymus cells (Martin and Martin, 1975a), to brain antigens (Martin and Martin, 1975b), to carbohydrate components (Sela et al, 1975), to lymphocytes (Rogentine and Plocinik, 1974), to IgG (Grabar, 1975; Dresser, 1978), to spermine (Bartos et al, 1980) and to erythrocytes

(Cunningham, 1974) were reported. Guilbert et al (1982) studied immunoglobulins in the sera of healthy donors and found antibodies against tubulin, actin, thyroglobulin, fetuin, transferrin, albumin, cytochrome c and collagen. Spontaneous anti-DNA autoantibodies could be demonstrated in vitro from normal strains of mice (Roder et al, 1978).

In addition to lymphocyte binding autoantigens and the finding of autoantibodies in normal sera, it was possible to demonstrate an enhanced production of these antibodies by normal individuals under certain circumstances. Autologous proteins could induce autoantibodies when combined with adjuvants (Rose and Witebsky, 1956 a,b; Esquivel et al, 1977). Similarly, tolerance to self could be broken by injection of cross-reactive red blood cells (Playfair and Marshall-Clarke, 1973), thyroglobulin (ElRehewy et al, 1981) or virus modified self proteins (Fox and Plescia, 1973) without even a need for adjuvants. Furthermore, the polyclonal B cell activator LPS, when injected into normal animals induces autoantibody production in vivo (Izui et al, 1977a,b,c).

The discovery that antibody idiotypes are capable of immunizing an autologous host (Kluskens and Kohler, 1974; McKearn et al, 1974; Rodkey, 1974; Nisonoff and Ju, 1977) and eliciting auto-anti-idiotypic responses further undermined Burnet's hypothesis. The immunoregulatory role assigned to the auto-anti-idiotypic antibodies by Jerne (1974) in his immune network theory, provided a new perspective on autoreactivity. It became clear that certain forms of autoreactivity (auto-anti-idiotypic antibodies) may not only exist

but must exist in order to serve a regulatory function in the immune system. Whether the expression of all forbidden clones plays any role under normal physiological conditions has not yet been addressed. Burnet's clonal deletion theory not only provided a stimulus for the discovery of autoimmunity in normals but also opened entirely new lines of investigation for comparing normal versus pathological states.

1.3. B Cell Triggering and Self Tolerance.

Accepting the notion that some autoreactive cells are not eliminated during embryonic life, one can ask several questions. What prevents these autoreactive cells from being expressed at levels which may lead to autoimmune disease? How is tolerance to self induced and maintained? What are the defects in self tolerance which lead to pathological autoimmune conditions?

Studies of tolerance and immunological responsiveness are thus critical for understanding autoimmunity. The tolerant state was thought to be established only in the prenatal/neonatal life (Burnet, 1959). This idea was formed following Owen's observation (1945) that dizygotic twin cattle have erythrocytes of their own type and the type of the non-identical twin. Furthermore, Billingham et al (1953) were able to induce experimentally unresponsiveness in adult mice A to the graft tissue of strain B mice by neonatal injection of strain A mice

with the strain B cells. These investigators also showed that strain A mice which have not been neonatally injected with strain B cells have no ability to tolerate strain B grafts.

In 1962, Dresser showed that tolerance could be established in adult animals. He immunized mature CBA mice with heterologous IgG which had been deaggregated by ultracentrifugation. Subsequent injection of an immunogenic preparation (aggregated protein) of the same bovine gamma globulin failed to generate an immune response.

The discovery of B and T cell cooperation in antibody formation (Miller and Mitchell, 1968; Taylor and Wortis, 1968; Claman and Chaperon, 1969) provided new perspectives in tolerance research. It became apparent that tolerance could exist in the B and T cell compartments of in either of these compartments (Chiller and Weigle, 1972). T cell tolerance was shown to be more easily induced and longer lived than B cell tolerance (Allison, 1974).

The mechanisms by which tolerance might be induced, maintained and broken are best viewed against the background of how the normal immunological response to foreign antigens occurs. A two signal hypothesis for B cell triggering was proposed (Bretscher, 1975), one signal being provided by the antigen interacting with B cell receptors, and the other from T cell (associative antibody). This model basically remains valid, although the interactions between B and T cells are recognized as being of a more complex nature. A current mechanism for the triggering of B cells to produce antibody is described below.

Three major steps are distinguished in the process of B cell

maturation from resting cell to plasma cell: activation, proliferation and differentiation (Nakanishi et al, 1983). These steps are under influences provided by helper T cells which themselves undergo activation, proliferation and maturation into effector cells upon MHC restricted antigen presentation by antigen presenting cells, (APC) and upon the presence of two inductive signals interlukin 1 and interlukin 2 (Erb and Feldman, 1975; Kappler and Marrack, 1978; Smith et al, 1979; Durum et al, 1985). The activation of resting B cells by T cell dependent antigens is initiated by the first signal (antigen specific T helper signal) which is generated when an activated T helper cell sees the Ia determinant together with a carrier moiety of the antigen on the B cell or macrophage. This signal is either directly delivered to the B cell via antigen bridging (Mitchison, 1971) of effector T cells and B cells or may be substituted by antigen specific soluble T helper factors which were shown <u>in vitro</u> to support antibody responses (Shiozawa et al, 1977; Mozes and Haimovich, 1979; Singer and Hodes, 1983). These helper factors are antigen specific by virtue of sharing idiotypic determinants with antibodies to that antigen, but lack constant region immunoglobulin epitopes (Mozes and Haimovich, 1979). These factors express Ia determinants which constrain their action to MHC-compatible B-cells (Shiozawa et al, 1977).

The second signal for B cell activation in T cell dependent responses is generated by the binding of a hapten moiety of the antigen with the B cell surface receptors. First and second signals induce a shift of the resting B cell from G_o phase of the cell cycle

to G_1 phase during which the B cell acquires receptors for the subsequent third signal provided by T helper cells in the form of soluble lymphokines which are antigen-non-specific and Ia negative. One of these factors is B cell growth factor (BCGF) (Howard et al, 1982; Butler et al, 1983b; Okada et al, 1983) and the other is interleukin 2 (IL2) (Tsudo et al, 1984; Zubler et al, 1984). These factors (BCGF and/or IL2) were implicated in the proliferative step of B cells. There is some indication that interleukin 1 (IL1), a lymphokine of macrophage origin, may be also involved in the expansion of B cells (Hoffman, 1980; Leibson et al, 1982; Howard and Paul, 1983).

The final phase of B cell maturation, that of differentiation into a plasma cell, is under the influence of yet another factor which is also non-antigen-specific and Ia negative. This is termed the B cell differentiation factor (BCDF) (Teranishi et al, 1982; Okada et al, 1983). The activation of B cells by T independent antigens (such as anti-mu) or by mitogens differs from that of the activation step by T dependent antigens in that there is bypass of the MHC restricted signal (Mond et al, 1983). Thus the first signal is generated by the cross-linking of B cell antigen receptors by a multivalent antigen or by the direct activation of the B cell by the polyclonal B cell activators. The proliferative and differentiation stages are controlled by the same factors (BCGF, IL1, IL2, BCDF) as in the case of T cell dependent antigens.

The initiation of the immune response occurs when resting cell encounters antigen in the presence of T help. At this and even at an

earlier stage (pre-B to B transition), B cells are most susceptible to negative signalling by antigen in the absence of T cells (Raff et al, 1975; Sidman and Unanue, 1975; Pike et al, 1980). This unresponsive B cell state is established by cross-linking of membrane Ig receptors by a multivalent antigen such as the model tolerogen anti-mu Ig (Lawton and Cooper, 1974). The dose of antigen required to produce unresponsiveness varies and depends largely on the maturation stage of the B cell (Raff et al, 1975). The more mature the cell, the more antigen is required to produce B cell tolerance. An antigen concentration may be as much as a 1000 fold less for achieving tolerance in the pre-B to B cell stage than in the mature B cell as has been shown by Nossal and Pike (1978) with fluorescein conjugated human gamma globulin antigen. The modulation of membrane immunoglobbulin receptors has been shown to be responsible for the tolerogenic state. The treatment of neonatal B cell with anti-Ig sera resulted in irreversible mIg disappearance (Raff et al, 1975; Bruyns et al, 1976). Keyhole limpet hemocyanin (KLH) treatment of fetal B cells prevented development of KLH binding to mature B cells (Bruyns et al, 1976). Pike et al (1982) obtained similar results when immature B cells were cultured in the presence of monoclonal anti-mu. antibody at a concentration of 10 ug/ml. A low concentration (0.1 ug/ml) of this antibody did not influence appearance of the surface Ig and yet these cells when placed in a highly stimulative environment containing potent mitogens such as LPS and dextran sulfate did not produce immunoglobulins as measured by the protein-A reverse plaque forming cell method. Thus in this latter case, a low anti-mu antibody

concentration did not produce the disappearance of surface Ig receptors but did produce the negative signal. This finding is of importance since some of the autoreactive cells detected in normals by binding of labelled antigens (Bankhurst et al, 1973; Roberts et al, 1973; Yung et al, 1973) may still remain unresponsive to inductive signals in spite of the fact that receptors for autoantigen are present.

Since B cell surface receptors are involved in the negative antigen B cell signalling which gives rise to tolerance, it is of importance to determine whether different B cell surface markers exist on the surface of cells from normal and autoimmune subjects.

An extensive study of the B cell markers in autoimmune lupus prone strains of mice (NZB, NZB/W F_1 , MRL/lpr/lpr and BXSB) in comparison with normal strains of mice was undertaken to see whether differences could be found which would account for the differences in induction of tolerance to self. All lupus prone strains of mice with the exception of BXSB express surface Ig positive cells at similar frequencies, marker density, and isotype distribution (IgM/IgD) as normal strains of mice up to 3 months of age (Theofilopoulos et al, 1982). In the same study, modulation of the spleen B cells from these mice (1-10 days old) by anti-mu Ig revealed that BXSB and MRL/lpr/lpr mice behave similar to normal mice with respect to the regeneration of the surface Ig receptors after anti-mu treatment. NZB and NZB/NZW mice are an exception since these strains regenerate Ig receptors more rapidly. The inability of these mice to receive negative signal inducing tolerance by TNP-HGG in the B cell compartment (Goldings et al, 1980;

Goldings, 1983) may be related to this phenomenon of rapid receptor development; however, rapid receptor development did not prevent the induction of tolerance in newborn NZB and NZB/NZW mice by sDNA-poly-D-lysine (Tanpahaichitr and Hahn, 1977) or by nucleosides-coupled isogenic IgG (Borel et al, 1973).

The resistance of the immature B cells to tolerance induction even by high concentrations of antigens may be a result of low affinity of the mIg receptors for that antigen. It was shown that the antigen delivers negative signals more readily to B cells with high affinity receptors (Theis and Siskind, 1968; Nossal et al, 1979). A similar suggestion was made by Klinman and Steinberg (1986) who proposed that self antigens would tolerize only B cells with high affinity receptors.

The persistence of the membrane surface receptors on B cells beyond the proliferation stage is an indication that the mature B cells are also susceptible to modulation by an antigen. This was shown by a number of investigators, some of whom used isolated B cells, plasmacytoma cells or hybridoma cells for induction of the negative signalling by the antigen (Schrader and Nossal, 1974; Klaus, 1976; Roder et al, 1978; Boyd and Schrader, 1980). It was also demonstrated that the presence of antigen influences the secretory rate of immunoglobulins (Schrader and Nossal, 1974). This down-regulatory function of the antigen is a form of tolerance which has been called an antigen blockade effect. During this effect, the antigen was shown to concentrate at the surface of the mature B cell and the antigen clearance from the surface correlated with the

recovery of Ig secretion. The effector cell blockade is probably not operative under physiological conditions as a form of B cell tolerance since it requires a very high concentration of antigen (Nossal, 1983).

The presence of IgD on mature B cells but its absence on immature B cells cannot account for the different susceptibilities of these cell types to tolerance induction. This is evidenced by the work of Layton et al (1979) who showed that an IgD positive and an IgD negative subpopulation of mature B cells are equally responsive to negative signalling.

At very low anti-mu concentrations (10^{-4} ug/ml), but not at 10 ug/ml, membrane receptor appearance and antibody production occurs, suggesting that exposure to antigen is necessary for induction of the tolerant state (Nossal, 1983).

Exposure to antigen does not always lead to tolerance since if pre-B cells are exposed simultaneously to antigen and B-cell activator (as opposed to first antigen and then activator as described above), then proliferation and antibody secretion can occur although at a reduced level. This may suggest either a time dependency of tolerance induction; i.e., that the cell response to antigen requires a time span to implement certain chemical changes, or that there is competition of the activator and antigen for binding sites with mitogen reducing the effective concentration of antigen and the antigen reducing the effective concentration of the activator.

Perhaps not all antigens can induce a tolerogenic B response and similarly perhaps not all autoantigens can elicit a tolerogenic B cell response. If this latter situation were the case, then one might

look for mechanisms other than those described above by which the system would keep autoantibody production under control with autoantibodies being produced only at low levels. These possible control mechanisms are the topic of the next section.

Weigle (1971) proposed that if autoreactive B cells are present in normal individuals and normal animals, their potential of being expressed may not be revealed because they rely on T helper cells. This theory was born out in studies of a cytoplasmic liver antigen (Fravi and Lindenmann, 1968). This molecule can be present in two forms F1 and F2. Their concentration in serum need be only very low 10^{-8}-10^{-9} M to induce tolerance in B cells (Nossal, 1983). Mice possess either F1 or F2 protein. Their competent B cells are not reactive with the syngeneic form of the cytoplasmic liver antigen, because specific helper T cells for that antigen are tolerant. Injection of mice expressing F1 antigen with F2 antigen can break tolerance and an antibody response occurs which is not only specific for the immunizing antigen but also for the homologous F1 antigen (Iverson and Lindenmann, 1972). The help is provided by T cells which recognize the allogeneic carrier moiety of F2 and collaborate with competent B cells specific for the common syngeneic determinant of F1 and F2 proteins.

Another model in which autoreactive B cells are present while specific T cells are tolerant was demonstrated in rabbits. These animals do not respond to their own alpha fetoprotein but immunization with the altered form of this antigen induces autoantibody formation (Ruoslahti et al 1975). Similarly lactic dehydrogenase of pig origin

induces in rabbit an antibody response to the autologous enzyme (Rajewsky, 1966). These examples illustrate firstly that the autoreactivity to self components depends on the status of the specific T cells if competent B cells are present , and secondly that the tolerance to self antigens may be broken by alteration of self component or by providing a cross-reactive antigen. While F prptein, alpha fetoprotein and lactic dehydrogenase autoantibody responses may be obtained by manipulation of the immune system in providing antigens cross-reactive with self, these responses are not representative of any autoimmune disease. In other cases introduction of cross-reactive antigens to normal animals may lead to the manifestation of autoimmunity.

The characteristic autoimmune disease which can be induced experimentally in normal animals (rabbits or mice) by the injection of cross-reactive thyroglobulin, altered homologous thyroglobulin or homologous thyroglobulin emulsified in complete Freund's adjuvant is that of autoimmune thyroiditis (Rose and Witebsky, 1956a,b; Weigle, 1965; Weigle and Nakamura, 1967; Nakamura and Weigle, 1968; Clagett and Weigle, 1974). The normal animals which develop the disease after injection of the altered antigen were shown to possess immunocompetent B cells for autologous thyroglobulin, but tolerant T specific helper cells.

Bypass of specific tolerant helper T cells was achieved with the introduction of determinants onto antigens which could bind to competent T cells present in these animals, and in so doing could provide help for competent B cells. The introduction of an altered

antigen for bypassing the T cell tolerance may result in antibody production to self component; however, the specificity of these antibodies may be different from the specificities of spontaneously expressed antibodies. Knight et al (1980) studied the induced and spontaneous anti-erythrocyte antibodies in mice. NZB mice which spontaneously develop hemolytic anemia express different anti-erythrocyte antibodies than do the normal or pre-autoimmune mice injected with rat erythrocytes. Similar observations were made with anti-thyroglobulin antibodies in patients with Hashimoto thyroiditis and antibodies induced in rabbits. The latter have the ability to recognize 40-60 epitopes on the antigen, while the former antibodies have a limited recognition spectrum of only 4-6 determinants (Shulman, 1971). It was also noted that the antibodies occuring spontaneously in patients with Hashimoto thyroiditis lack cross-reactivity with mammalian thyroglobulin except for a few species (monkeys and chimpanzees) (Baur and Goodman, 1964).

The above findings are of interest and suggest that there may be different clones of autoreactive cells expressed in spontaneously developed autoimmune disease and intentionally provoked disease (Schwartz and Stollar, 1985).

1.4. Regulation of the Expression of Autoreactive B Cells by the T Suppressor Cell Circuit.

In 1971 Gershon and Kondo observed that lymphoid cells from mice which are tolerant to SRBC are able to confer tolerance on normal

syngeneic lymphoid cells primed with this antigen. The observed phenomenon was called "infectious tolerance" and was attributed to the suppressor T cells (Gershon and Kondo, 1971). Since then, the role of T suppressor cells (T_s cells) as elements involved in down regulation of the cellular and humoral immune responses has become much appreciated (Okumura and Tada, 1973; Elson and Taylor, 1974; Waldman et al, 1974). T suppressor cells have undergone scrutinous phenotypic characterizations. In man, these cells were originally described as Fc receptor-positive for IgG (Moretta et al, 1977) and as cells recognized by heteroantiserum TH2 (Reinherz and Schlossman, 1979). Later, OKT 5, OKT 8 and Leu 2 phenotypes were ascribed to these cells indicative of recognition by the respective anti-OKT 5 and anti-OKT 8 and anti-Leu 2 monoclonal antibodies (Reinherz and Schlossman, 1980; Reinherz et al, 1980; Gatenby et al, 1982). The T suppressor cell subset in mice was characterized by the presence of Ly 2,3 alloantigens (Cantor and Boyse, 1975; Cantor and Boyse, 1977). T cells with suppressor activity, but of Ly 1,2 or Ly 1 cell phenotypes, were also noted in mice (Sakaguchi et al, 1982; Moticka, 1983; DeMoor et al, 1985).

Suppressor effects were shown to be elicited by activated T cells in different ways: by antigens (Feldmann, 1974; Janeway et al, 1975; Kontiainen and Feldmann, 1976), by mitogens (Peavy and Pierce, 1974; Morretta et al, 1976) or by the mere pre-culturing of lymphoid cells (Burns et al, 1975). T suppressor cells and their factors were found to be either specific to the inducing antigen (Elson and Taylor, 1974; Zembala et al, 1975) or were found to inhibit immune responses to a

variety of antigens (Thomas et al, 1975; Green et al, 1983; Monroe et al, 1984). The ways by which suppression is achieved have been intensely studied, especially in animal systems (Tada et al, 1977; Beracerraf, 1980). Three levels of suppression forming suppressor cell circuits were described (Green et al, 1983). These levels of suppression include: level one suppression which prevents immune responsiveness by interference with T helper cells, contrasuppression (Gershon et al, 1981) which renders T helper cells resistant to level one suppression, and level two suppression which counters the effect of contrasuppression thereby producing the net suppression (Green et al, 1983). At each level of suppression, at least three phenotypically distinct T cells interact: T inducer, T transducer and T effector. The interaction between these cells and the target of T effector cells , i.e. T helper cells, is mediated by soluble factors (Green et al, 1983). Igh-V-linked and I-J-linked restriction elements have been shown to be involved in the communication between T suppressor cells (Bach et al, 1979; Eardley et al, 1980; Green et al, 1983). The induction of each level of suppression was proposed to depend on the antigen dose. At the antigen concentration optimal for the immune response to occur, contrasuppression is activated while sub- and supra-optimal doses of antigen will result in suppression of the immune responses regulated by level one and level two suppression respectively (Green et al, 1983).

Ever since the concept of T_s cells was introduced into the description of the immune system, the role of these cells in control of reactivity to self has been implicated (Talal, 1976). T suppressor

cell defects were shown to exist in autoimmune diseases, for example in autoimmune thyroiditis (AIT) (Rose et al, 1981), SLE (Fauci et al, 1978; Sagawa and Abdou, 1978; Sakane et al, 1978; Clough et al, 1980), and rheumatoid arthritis (RA) (Keystone et al, 1980; Sakane et al, 1982). In SLE, the defect of suppression of autoantibody production was attributed to the decreased number of suppressor T cells (Hamilton and Winfield, 1979). The decrease was shown by some investigators to be due to the lymphocytotoxic antibodies present in the disease (Sakane et al, 1979; Imai et al, 1980; Morimoto et al, 1980; Honda et al, 1982). Results contrary to the above were obtained. These latter results indicated that T suppressor cells functioned normally in pre-autoimmune and autoimmune lupus mice to inhibit production of spontaneous and LPS-induced anti-DNA antibodies (DeMoor et al, 1985). In humans, the impairment of the T suppressor function in SLE patients was reportedly undermined (Nakamura et al, 1982). These controversial results on the role of T_s cells in controlling autoantibody responses in SLE did not relate T_s defects or lack thereof to the suppressor level circuits. Others however, provided evidence and raised the possibility that SLE may be a result of defective suppression and abnormal contrasuppression (Smith et al, 1983).

That T_s cells control the autoreactivity to self thyroglobulin was also described (Okayasu et al, 1980). In these studies, mice were injected with syngeneic soluble thyroglobulin and generated T_s cells which in turn when transferred to recipients with experimentally induced thyroiditis decreased the severity of the disease.

Different studies showed that the induction of T_s cells specific for an autologous antigen (red blood cells) may follow the immunization of normal mice with rat red blood cell antigen (Naysmith et al, 1980). The expression of the autoantibodies in these mice was transient and lasted up to 3 months. Lymphoid cells from these mice taken at the decline of the autoantibody response were able to confer tolerance to autologous red blood cells in normal mice immunized with rat erythrocytes. The responses to rat erythrocytes remained unchanged or were enhanced.

This last example clearly shows firstly that competent autoreactive B and T helper cells for autologous erythrocytes exist in normal mice, secondly that these autoreactive B cells can be expressed when the animal is challenged with antigens related to their own erythrocytes, and thirdly that the expression of autoreactive cells can be terminated by antigen-specific T suppressor cells. Thus the role of suppressor T cells in preventing autoimmune disease in normal individuals and animals possessing autoreactive lymphoid cells can not be over emphasized.

1.5. Idiotype Network Interactions and Autoreactivity.

Prior to the development of the idiotype network theory formulated by Jerne (1974), several observations were made. Kunkel et al (1963), Oudin and Michel (1969) had defined idiotypic markers on murine myeloma proteins and Kluskens and Kohler (1974) described an auto-anti-idiotype antibody. Thus Jerne's theory proposing that the

immune system operates through a highly connected web of idiotypes was made on a solid experimental basis. This networking was proposed to exist between cells expressing idiotype receptors and antibodies. The evidence that B and T cells express the same idiotype in the T15 system and that T cell recognized B cell idiotypes (Julius et al, 1977) came in support soon after the network theory came to light. Jerne (1974) visualized idiotype interactions as follows.

In response to an antigen, antibody Ab1 is made. This antibody posses idiotype determinants (markers in the variable portion of Ig) which subsequently evoke an auto-anti-idiotype (Ab2) response by interaction with the cells carrying an appropriate receptor. In turn, Ab2 idiotype evokes production of Ab3.

The chain phenomenon could be repeated several times. Of interest is the fact that the anti-idiotype antibody, like the original antigen, can react with the receptor of the B1 cells. If the combining site (idiotype) is close enough to the antigen binding site, then interference with antigen binding could occur. On the other hand, if the anti-idiotype antibody were directed against the antigen binding site, then the antibody might be expected to mimic antigen and enhance antibody formation.

Support for Jerne's concept was provided in many ways. For example, the appearance of Ab2 after injection of Ab1 (Clevinger et al, 1980), the appearance of Ab1 after immunization with Ab2 (Sachs et al, 1981), and the appearance of Ab2 and Ab3 after immunization with antigen (Bona et al, 1981; Cowdery and Steinberg, 1981; Geha, 1982) are all consistent with the hypothesis. Moreover, internal antigen

image antibodies which mimic insulin (Sege and Peterson, 1978), thyrotropin (Islam et al, 1983), reovirus (Noseworthy et al, 1983) and self Ia antigens (Holmberg et al, 1984) were reported. Immune responses against phosphorylcholine (Kelsoe and Ceryn, 1979), (1,3)dextran (Bona et al, 1981) and p-azophenylarsonate (Reth et al, 1981) were either stimulated or inhibited by anti-idiotype antibodies depending on dose and isotype of the anti-idiotype antibodies used.

Klinman and Steinberg (1986) in their consideration of autoimmunity and idiotypy, point to the frequent expression of cross-reactive idiotypes (CRI) by autoantibodies. CRIs were reported on the majority of anti-DNA antibodies produced by lupus prone MRL/lpr/lpr and (New Zealand Black x New Zealand White)F_1 ((NZBxNZW)F_1) mice (Rauch et al, 1982; Tron et al,1982; Hahn and Ebling, 1984). CRIs are also present amongst Ig and anti-DNA antibodies in many humans with SLE (Nasu et al, 1982; Zouali and Eyquem, 1984), in cerebrospinal fluid of patients with multiple sclerosis (Tachovsky et al, 1982), in the sera of patients with myasthenia gravis (Lefvert et al, 1982; Wassermann et al, 1982), on the antibodies which comprise rheumatoid factor in patients with rheumatoid arthritis (Kunkel et al, 1973) and on antibodies specific for thyroid tissue, insulin receptors and other autoantigens (Zanetti and Bigazzi, 1981; Zanetti et al, 1984). The interest in CRI comes from the possibility that if the network concept is at work, and anti-idiotype antibodies are normally functional in regulation, one might not expect to see high expression of CRI. The fact that they are observed in individuals with autoimmune disease would be consistent with a breakdown in the network regulation

mechanisms. A number of mechanisms were postulated which could account for anti-idiotype antibody stimulation of autoimmunity. For example, if an environmental antigen (bacteria, exogenous or endogenous viruses, drugs or even unrelated self antigens) were to produce antibodies and the anti-idiotype antibodies to these were coincidently to be cross-reactive with the antibodies to self antigens (and hence to the B cell self-antigen receptors), then antigen binding to the B cell might be prevented. As discussed earlier, self-antigen binding is a prerequisite for maintenance of tolerance and hence an autoimmune state would be induced.

The role of bacteria and viruses has been mentioned before, but it is worth noting that in MRL/lpr/lpr mice, a particular idiotype was found which is also found on a large number of serum antibodies which do not react with DNA (Tron et al, 1982; Teitelbaum et al, 1984)).

Other mechanisms for anti-idiotype involvement in autoimmune induction could be proposed. For example, interaction with T cells and the consequences thereof could be considered. As an example, Neilson and Phillips (1982) observed that rats injected with tubular basement membrane developed autoimmune renal disease; however, if the animals were pre-treated with T cells reactive against tubular basement membrane-specific autoantibodies, the autoimmune response did not develop. In this case, the T cell receptor could mimic the antigen and through interaction with the B cell inhibit the response to the autoantigen.

There are also clinical arguments which would suggest the role of anti-idiotype antibodies in autoimmunity. Abdou et al (1981) reported

that coincident with remission in a patient with SLE, there was the appearance of autologous anti-idiotype specific for the patient's anti-DNA. Anti-idiotype antibodies specific for autoantibodies were reported in the sera of other SLE patients (Nasu et al, 1982) and in patients with myasthenia gravis (Tachovsky et al, 1982). Anti-idiotype has also been implicated as a contributor to reduced immunoresponsiveness of aging individuals (Szewczuk and Campbell, 1980).

1.6. Polyclonal Activation of Ig Synthesis and Self Reactivity.

The induction of B cell activation, proliferation and differentiation by polyclonal B cell activators is of interest since some of these substances (LPS, PPD) were implicated in autoantibody production as is the case with anti-nucleic acid antibody (Izui et al, 1977a), anti-thyroglobulin antibody (Esquivel et al, 1977) or anti-thymocyte antibody (McHugh and Bonavida, 1978). The polyclonal B cell activators comprise a long list of molecules: LPS, PPD (purified protein derivative of tuberculin), protein A from Staphylococcus aureus, bacterial polysaccharides from Klebsiella pneumoniae, Streptococcus diplococcus, polyanions (dextran sulfate, poly IC), viral components and viruses (e.g. EBV), PWM etc. (Goodman and Weigle, 1982). The mechanism by which these diverse agents interact with resting B cell is unknown; however, a specific LPS receptor on these cells or LPS intercalation into the B cell membrane was suggested as a possible means of mitogen-cell interaction (Kabir and Rosenstreich,

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1977; Forni and Cutinho, 1978). The requirement for T cells in eliciting polyclonal Ig synthesis by some of the mitogens (LPS, PWM) was demonstrated (Theofilopoulos et al, 1980; Goodman and Weigle, 1982)). The suppressive effect of T cells on mitogen driven (LPS, PWM) Ig secretion was also described (Goodman and Weigle, 1982; Bellamy et al, 1983). In the case of LPS however, suppressive T cell effects were observed only under special conditions in vitro, i.e. in cultures where the ratio of T/B cells in splenic murine lymphocyte was high and in cultures to which ConA stimulated T cells were added (Primi et al, 1979; Goodman and Weigle, 1982).

The role of polyclonal B cell activators as possible agents in breaking B cell tolerance to self antigens was investigated since many of these activators are components of ubiquitous infectious agents to which an animal may be exposed during its life time. LPS and PPD were shown to induce anti-DNA antibody production in vivo in normal strains of mice (Izui et al, 1977a,b,c). Similar influences of LPS on generation of anti-thymocyte antibodies, antibodies reactive with autologous albumin and erythrocytes were reported (Primi et al, 1977; McHugh and Bonavida, 1978).

In normal human individuals, stimulation of autoantibody production by polyclonal B cell activators was also documented, although to a lesser extent than in animal systems. Rheumatoid factor (RF) was shown (Slaughter et al, 1978) to be produced in normal peripheral blood cultures in the presence of EBV, an agent implicated in the etiology of rheumatoid arthritis (RA) (Vaughan et al, 1983). The degree of RF generation and the affinity of RF after EBV stimulation

was lower in normal peripheral blood lymphocyte cultures than in the peripheral blood lymphocyte cultures from RA patients (Slaughter et al, 1978). These higher responses to EBV of RA peripheral blood lymphocytes were ascribed to the defective suppression frequently seen in RA (Hasler et al, 1983; Tosato et al, 1983).

The above experimental observations are strongly suggestive that tolerance to self antigens can be overriden in vivo or in vitro by polyclonal B cell activators even when the immunoregulatory T cell circuits are present.

An enhanced production of anti-DNA antibody, B cell reactivity and the autoimmune state was demonstrated in normal thymectomized mice strains injected frequently with polyclonal B cell activators (LPS and/or poly rI.rC) as compared with mice treated only with mitogens or thymectomized (Smith et al, 1983). These authors further showed that this effect was attributable to the decreased suppression and enhanced contrasuppression. Thus the combined effect of polyclonal B cell activators and defective T cell regulation may be responsible for the development of autoimmunity as seen in lupus prone mice.

In contrast, in normal mice and normal humans, the stimulatory effect of polyclonal activators such as viruses or bacteria which are encountered may not contribute to the development of autoimmune disease as long as T suppressor circuits or other control mechanisms (e.g. immune network) are not defective.

In summary, several different mechanisms have been proposed for the regulation of the expression of autoreactive clones. It is not valid at the moment to expect that only one mechanism is functional, nor

that any of the discusssed mechanisms are necessarily correct at their present level of formulation. Neither is it valid to assume that the presented mechanisms are mutually exclusive. It can be expected that reality may eventually involve a synthesis or synergy of some of the above and other mechanisms.

1.7. Development of Hybridoma Technology.

The ability to produce monoclonal antibody in vitro dates to 1966 when continuous cell lines were established by Fahey et al (1966) and Tanigaki et al (1966). The immortalization of human B lymphocytes by transformation with EBV was described shortly thereafter (Henle et al, 1967).

The first human mouse-hybrids were obtained in 1973 by Schwaber and Cohen. In all the above cases, the specificity of the immunoglobulin produced was unknown. The field of monoclonal antibodies was revolutionized by Kohler and Milstein (1975) who developed the technique of somatic cell hybridization which allows for the production of a large quantity of monoclonal antibodies of pre-defined specificity. These investigators showed that the immunization of mouse with an antigen (SRBC was originally used) and subsequent fusion of splenocytes from the immune animal to mutant (azaguanine-resistant) myeloma cells (P 3) results in hybrid cell clones (hybridomas) producing antibodies, some of which recognize the immunogen. The hybridoma technique developed by Kohler and Milstein is based on the principle outlined below. Two cell types are needed. One of these

cells is a myeloma cell which confers immortality to the hybrid, but which by itself would not grow in a selective medium containing hypoxanthine, thymidine and aminopterine, an inhibitor of de novo DNA synthesis. Thus the myeloma cell line not only must be sensitive to the inhibitor but must lack the enzyme hypoxanthine phosphoribosyl transferase which enables it to proceed with DNA synthesis via the purine salvage pathway. The other cell type is B lymphocyte which when fused with the mutant myeloma cell will provide genetic information to the hybrid for the production of the hypoxanthine phosphoribosyl transferase enzyme allowing the hybrid to survive under the selective conditions. The B cell lymphocytes by themselves will grow only for a limited time. The original myeloma cell line used by Milstein and Kohler was an Ig producer. Thus two monoclonal species of antibodies were made by the hybridomas, one of lymphocyte origin and the other of myeloma fusion partner origin.

Soon after the discovery of hybridoma technology, a search began for an "ideal" fusion partner which would form stable hybrids and would not secrete its own antibody. Such an "ideal" fusion partner would form hybrids in which only immunoglobulin genes of the B lymphocytes would be expressed. Non-Ig secreting murine myeloma cell lines SP-1, NS-1, Sp2/0Ag14, P3X63-Ag8.653 were found (Kohler et al, 1976; Shulman et al, 1978; Kearney et al, 1979). These lines have been widely used to produce mouse:mouse or mouse:human hybridomas (Astaldi et al, 1982; Bundle et al, 1982; Lane et al, 1982; Butler et al, 1983a; Houghton et al, 1983; Abrams et al, 1984). The interspecies hybridomas however are unstable and preferentially lose

human chromosomes (Ruddle, 1973; Croce et al, 1980). Furthermore, heterohybridomas often failed to secrete human Ig even if the chromosomes carrying immunoglobulin genes are present (Raison et al, 1982).

The search for suitable non-Ig secreting cell lines of human origin to produce human:human hybridomas has been unsuccessful, except for one B cell lymphoma cell line RH-L4 which produces but does not secrete IgG kappa (Houghton et al, 1983) and a lymphoblastoid cell line UC 729-6 which has been shown to have cytoplasmic and surface IgM but not to secrete antibody (Glassy et al, 1983). The RH-L4 line was used for obtaining human:human hybridomas producing monoclonal antibodies specific for human leukemia cells (Olsson et al, 1984). All the other human cell lines for fusion were shown to secrete Ig and the majority of these lines are lymphoblastoid cells. A plasmacytoma cell line (SKO-007) was used to generate hybridomas specific for DNP hapten (Olson and Kaplan, 1980).

The first lymphoblastoid cell line, GM 1500-6TG-2 was established from a patient with multiple myeloma by Croce et al (1980). This line produces IgG kappa and was used to derive two sublines KR-4 and GM 4672 (Croce et al, 1980; Kozbor et al, 1982). The original GM 1500-6TG-2 line and its sublines were used successfully for human:human monoclonal antibody production in different laboratories (Croce et al, 1980; Osband et al, 1981; Shoenfeld et al, 1982; Massicotte et al, 1984). The other lymphoblastoid cell fusion partners described include LICR-LON-HMy2 (Edwards et al, 1982) and H351.1 (Chiorazzi et al, 1982).

The production of specific human:human hybridomas was shown to depend on many variables such as the source of lymphoid cells for fusion, donor's immune status, activation of lymphocytes prior to fusion, fusion procedure and post-fusion conditions (Andrzejewski et al, 1980; Astaldi et al, 1982; Denis et al, 1983; Pintus et al, 1983; Massicotte et al, 1984). Even under optimal conditions defined in the individual laboratory, the frequencies of finding hybridomas with the desired specificity were found to be low: 0.7 - 15% of all hybrids formed (Shoenfeld et al, 1982; Kozbor and Roder, 1983). Moreover, a low amount of antibody ranging between 0.1 and 20 ug/ml was secreted. The majority of human monoclonal antibodies described were of IgM class with the few exceptions where IgG was produced (Osband et al, 1981; Edwards et al, 1982). This occured regardless of the lymphoblastoid cell line used for fusion. The secretion of the antibodies by human hybrids would readily cease during the stage up procedure as reported by Andrzejewski et al (1980).

A novel approach was undertaken by Kozbor and Roder (1983) to improve upon human:human hybridoma technology. These investigators utilized EBV transformation of human peripheral blood lymphocytes before fusing them with the KR-4 lymphoblastoid cell line. This transformation increases the fusion frequency 36 fold over that obtained with resting peripheral blood lymphoctytes and 6 - 11 fold over the frequency achieved after stimulation of lymphoctyes with mitogen (Kozbor and Roder, 1983). This new technique described above has another advantage over the conventional hybridoma procedure; namely, the ability to use a continuous source of the same EBV

established specific cell line for a number of fusions. The disadvantage of the method is the presence of the virus and possible viral components in the hybridoma supernatants. This contamination can limit supernatant usages, for example, as a source of antibodies for therapeutic purpose. More recently, an in vitro immunization technique was implemented to increase the efficiency of obtaining specific human hybridomas, for example, to SRBC (Strike et al, 1984). The amount of antigen required for stimulation was very low (0.1 ug/ml).

The potential of hybridoma technology was recognized soon after Kohler and Milstein's discovery. An enormous variety of monoclonal antibodies has been generated (to list them is beyond the scope of this review) for different purposes: to study the structure of antibodies (Parhami-Seren et al, 1984; Greenspan and Davie, 1985) and antigens (Elkins and Metcalf, 1984; Reeves, 1985) to determine the genetic elements of antibodies (Siekevitz et al, 1983; McKean et al, 1984), to study the regulation of Ig synthesis (Hamano and Asofsky, 1984), to develop reagents for clinical use (vaccination, detection of tumor antigens, modulation of disease) (Miller et al, 1982; Peng et al, 1982; Briles et al, 1984) and many other applications. Perhaps one may use the phrase that the possible uses of hybridomas are as limitless as hopefully limitless are their products.

CHAPTER 2 - MATERIALS AND METHODS

2.1. Source of Human Sera.

Sera from 59 Systemic Lupus Erythematosus (SLE) patients, 12 patients with unexplained polyclonal hypergammaglobulinemia and 15 patients with monoclonal IgM, were obtained from the Clinical Immunology Laboratory or from Dr. D.A. Bell's laboratory serum bank (University Hospital, University of Western Ontario, London, Ontario). The control normal human sera (NHS) were obtained from healthy hospital personnel (16 sera) or from the Hematology Laboratory (University Hospital, University of Western Ontario) (50 sera). Sera were separated by centrifugation at 400 g for 15 min at 20° C and were stored frozen at -20° C before testing.

2.2. Source and Separation of Lymphoid Cells for Short Term Culturing.

Tonsillar tissue was obtained from individuals (age 3 to 40 yr) who had undergone routine tonsillectomy. The donors had been treated for recurrent chronic tonsillitis but had no symptoms of SLE and therefore are referred to as normal individuals. The microscopic examination of the tonsillar tissue was not performed because there were no gross abnormalities noted in these tonsils. Tissue was kept in RPMI 1640 (Gibco Laboratories, Grand Island, NY) at pH 7.4, was cut into small fragments and was homogenized with a Dounce homogenizer. Peripheral blood lymphocytes from 14 normal donors and 11 SLE patients as well as

the tonsillar lymphoid cells from 16 normal individuals were purified on Ficoll-Hypaque (specific gravity 1.081) (Ficoll 400, Pharmacia Fine Chemicals AB, Uppsala, Sweden; Hypaque, Winthrop Laboratories, Aurora, Ontario) by density gradient centrifugation according to Boyum (Boyum, 1968). Cells were washed three times in RPMI 1640 by resuspension after centrifugation at 300 g for 8 min. The centrifugation was done at 20 °C. Six different samples of the tonsillar lymphoid cells were separated on nylon wool as described elsewhere (Bellamy et al, 1983) in order to obtain adherent (B-enriched) and nonadherent (T-enriched) cell fractions. In four instances, separated B- and T-enriched fractions were also obtained from the peripheral blood of the same tonsil donor.

2.3. Source and Preparation of Donor's Lymphoid Cells for Hybridomas.

In preparation for hybridoma production, palatine tonsils were obtained from a normal female donor (age 7). Her serum exhibited normal immunoglobulin levels, and lacked antibodies to single stranded DNA (ssDNA) and to DNA as determined by enzyme linked immunosorbent assay (ELISA) (2.10). Tonsillar lymphoid cells were obtained after purification by Ficol-Hypaque density gradient centrifugation (Boyum, 1968), washed three times in RPMI 1640 by pelleting the cells by centrifugation at 300 g for 8 min following resuspension. The final pellet was resuspended in serum free medium (SFM) to the cell concentration needed for fusion.

SFM contained RPMI 1640 supplemented with 1% 200 mM L-glutamine

(Gibco Laboratories), 1% 10 mM sodium pyruvate (M.A. Bioproducts, Walkersville, MD), 1% 2.5 M N-2-hydroxyethylpiperazine-N-2-ethanosulfonic acid (Hepes) (Gibco Laboratories), 1.25% of a solution containing 10,000 U/ml penicillin, 10,000 ug/ml streptomycin (Gibco Laboratories), 0.5% 10 mM non-essential amino acids (M.A. Bioproducts), and 0.2% 50 mg/ml gentamycin (Schering Corp., Kenilworth, NJ).

2.4. Human Lymphoblastoid Cell Line for Hybridoma Production.

The hypoxanthine phosphoribosyl transferase-deficient, hypoxanthine-aminopterine-thymidine (HAT) sensitive IgG_2 kappa producer mutant cell line GM 4672 was obtained from the Institute for Medical Research (Camden, NJ). The GM 4672 cells were cultured in RPMI 1640 supplemented with 1% 200 mM L-glutamine, 10% fetal calf serum (FCS) (Gibco Laboratories) which was heat inactivated for 30 min at 56°C, and 1×10^{-4} M 6-thioguanine (Sigma Chemical Co., St. Louis, MO). Five days before fusion, cells were staged up into 75 cm^2 polystyrene flasks (Corning Glass Works, Corning Medical and Scientific, Corning, NY) without 6-thioguanine. Prior to fusion, cells were washed twice by pelleting the cells at 200 g for 10 min at 20°C and gently resuspending them in the desired volume of SFM (defined in 2.3).

2.5. Short Term Cultures.

Ficoll-Hypaque purified and washed lymphoid cells (unseparated) were cultured in 12 x 75 mm polystyrene tubes (Fisher Scientific Co., Don Mills, Ontario) at 1 x 10^6 cells/ml and 5 x 10^6 cells/ml in one ml culture media. In addition to unseparated cell cultures, cultures from six different tonsils were also set up containing a constant number of B-enriched cells (5 x 10^5 nylon adherent) and different numbers of nylon nonadherent T-enriched cells (5 x 10^5 , 2.5 x 10^5 , 1.25 x 10^5) in 1 ml of culture media. Culture media contained RPMI 1640, pH 7.4, supplemented with 10% FCS (Gibco Laboratories) that was heat inactivated at 56° C for 30 min, 0.5% gentamycin 10 mg/ml (Schering Corporation), and 0, 0.01, 0.025, 0.05, 0.1, 0.5 and 1.0% pokeweed mitogen (PWM) (Gibco Laboratories). Cells were cultured for 7 days at 37° C in a 5% CO_2 humidified atmosphere.

2.6. Cell Fusion and Post-fusion Anti-DNA Hybridoma Cultures.

Fusion was performed according to the following modifications of the procedure described by Shoenfeld et al (1982). Cells were mixed at ratios 1 : 1 and 5 : 1 (tonsillar lymphoid cells : GM 4672 cells), co-pelleted by centrifugation (200 g, 10 min, 20° C) and treated with 0.5 ml 44.4% polyethylene glycol 1440 (J.T. Baker Chemical Co. Phillipsburg, NY) in SFM (defined in 2.3). After gentle resuspension, the pelleted cells were centrifuged at 300 g for 3 min, resuspended in 10 ml SFM, and pelleted again at 200 g for 5 min. Finally, cells were

resuspended in post-fusion recovery medium (PFRM) and cultured for 24 h at 37° C in a humidified 5% CO_2 atmosphere. PFRM consisted of RPMI 1640 supplemented with 1% 200 mM L-glutamine and 1% solution containing 10,000 U/ml penicillin, 10,000 ug/ml streptomycin, 1% 100 mM sodium pyruvate, 0.5% 10 mM non-essential amino acids, 15% heat-inactivated FCS, and 0.2% 50 mg/ml gentamycin.

After 24 h of incubation in PFRM, cells were centrifuged at 200 g for 5 min and resuspended in hypoxanthine-aminopterine-thymidine (HAT) medium (Littlefield, 1964) supplemented with 5% NCTC 109 (M.A. Bioproducts). Cells were dispensed in 2 ml at 4 x 10^5 cells/well on 24-well Costar trays (Costar, Data Packaging, Cambridge, MA) and at 1 x 10^5 cells/well and 2 x 10^5 cells/well in 0.2 ml on 96-well Costar trays and cultured at 37° C in a humidified 5% CO_2 atmosphere. One half of the HAT medium was removed and replenished weekly. Positive growth was scored after 4 weeks. Twenty four-well tray supernatants were then screened for anti-ssDNA antibodies by using an ELISA (2.10) while 96-well tray hybridomas were transfered to 24-well trays for 7 days before screening for anti-ssDNA antibody. Anti-ssDNA antibody producing hybridomas were cloned by limiting dilution at 2 cells/well in 0.2 ml growth medium (GM); and the cells were left unfed for 4 weeks. Growing cells were then transfered to 24-well trays and after 10 days were tested for anti-ssDNA antibody production. GM contained RPMI 1640 supplemented with 1% 200 mM L-glutamine, 1% 100 mM sodium pyruvate, 0.5% NCTC 109, and 1% of a solution containing 10,000 U/ml penicillin, 10,000 ug/1ml streptomycin, and 12% heat- inactivated FCS. Positive clones for anti-ssDNA antibody were transfered to 25 cm^2

culture flasks and cultivated in GM. Cultures were supplied with fresh GM medium after 10 days of growth and within the next 10 days they were transfered into 75 cm^2 flasks.

The anti-ssDNA antibody producing hybridomas were cloned 3 times by limiting dilution. The culture supernatants containing anti-nucleic acid antibodies were collected by centrifugation at 400 g, 10 min, 20° C and stored at -20° C. Anti-ssDNA antibody negative hybridoma culture fluids were collected and stored in an identical manner.

2.7. Enumeration of Immunoglobulin and Anti-single Stranded DNA (ssDNA) Antibody-producing Cells.

Cultured cells were washed three times in Hanks' balanced salt solution (Gibco Laboratories) at pH 7.4. Immunoglobulin-producing cells were detected by the reverse hemolytic plaque-forming cell (PFC) assay according to the procedure described previously (Bellamy et al, 1983). Anti-ssDNA antibody producing cells were enumerated by an anti-ssDNA PFC assay (Bell et al, 1973).

2.8. Determination of the Radiolabelled DNA Binding to PLL Coated Wells.

Two commercially available preparations of double stranded DNA were used: 1) *E. coli* ^{14}CDNA (43 µCi/mg) (Amersham Canada Limited, Oakville, Ontario) and 2) polydeoxy-adenylate-thymidilate (poly dA-(Methyl- ^{3}H)) (13 µCi/umole P) (Miles, Elkhart, Indiana).

These nucleic acid preparations were prepared at 10 µg/ml, 5 µg/ml and 2.5 µg/ml in 0.1 M Tris-HCl pH 7.4, and 50 µl antigen was pipetted into round bottom wells of polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc.) which were uncoated or coated for 1 h at room temperature (RT) with 50 µl of 50 µg/ml poly-L-lysine (PLL) (Sigma Chemical Co.) in 0.1 M Tris-HCl pH 7.4. The wells were then washed with 0.1% BSA 0.1 M Tris-HCl pH 7.4 and air dried at RT. Individual wells were cut out from the plate and put into vials with 5 ml of scintillation fluid containing 3 g PPO (2,5 diphenyl-1-oxazole) (Fisher Scientific Co.) and 0.1 g POPOP (1,4-bis(2,5-phenyl-oxazolyl-benzene)) (Fisher Scientific Co.). The vials were counted in a Searle Delta 300 beta counter (Searle Analytic Inc., Toronto).

2.9. ELISA Determination of SLE Serum Binding to DNA-coated Polyvinyl Chloride Wells and to DNA-coated Polystyrene Wells.

Three SLE sera were chosen to determine binding of their anti-DNA antibodies to double stranded calf thymus (CT) DNA (Millipore Corporation, Freehold, NJ) coated onto round bottomed polyvinyl chloride or polystyrene (Dynatech Laboratories, Inc.) plates. The plates were untreated or treated with 50 µl/well of 50 µg/ml of poly-L-lysine (PLL) (Sigma Chemical Co.) in 0.1 M Tris-HCl pH 7.4 prior to coating with 50 µl/well of 10 µg/ml antigen. The plates were incubated with DNA for 3 h at RT and excess antigen was removed by washing three times with 0.1% BSA, 0.05% Tween 20 in 0.1 M Tris-HCl pH 7.4. The wells were then filled with 2% BSA in 0.1 M Tris-HCl pH 7.4

and incubated for 2 h at RT. After one wash with a solution of 0.1% BSA and 0.05% Tween 20 in 0.1 M Tris-HCl pH 7.4, 50 µl/well of serum (heat inactivated at 56°C for 30 min and diluted $1/10^2$, $1/10^3$, $1/10^4$ $1/10^5$ in 0.1% BSA and 0.1 M Tris-HCl pH 7.4) was pipetted into the wells. Further treatment of the plates was identical to that described in 2.10.

2.10. Enzyme Linked Immunosorbent Assay (ELISA) for Determination of Anti-DNA Antibody.

Polystyrene wells of 96-well round bottomed Cooke microtiter plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were coated with 50 µl of 50 µg/ml poly-L-lysine (Sigma Chemical Co.) in 0.1 M Tris-HCl pH 7.4. After 1 h of incubation at room temperature (RT) wells were washed three times with 0.1 M Tris-HCl, pH 7.4, and 50 µl of antigen was pipetted into the wells. Nucleic acid antigen was left in the wells for 1 h at RT. Alternate wells served as controls and 50 µl of 0.1 M Tris-HCl pH 7.4 was added instead of antigen.

The following antigens were used in ELISA: 10 µg/ml calf thymus (CT) DNA (Millipore Corporation, Freehold, NJ) in 0.1 M Tris-HCl pH 7.4; 10 µg/ml single stranded calf thymus DNA in 0.1 M Tris-HCl pH 7.4 prepared as described by Doty et al (1960); 10 µg/ml polydeoxyguanylate-polydeoxycitidylate (polydG.polydC) (Miles Laboratories, Inc., Kankakee, IL) in 0.1 M Tris-HCl pH 7.4; 10 µg/ml polydeoxyadenylate-thymidylate sodium salt (poly (dA-dT)) (Miles Laboratories, Inc.) in 0.1 M Tris-HCl pH 7.4; 10 µg/ml low molecular

weight supernatant DNA (S/N DNA) obtained from New Zealand Black/White (NZB/W) mice thymocytes which was prepared as previously described (Pancer et al, 1981) in 0.1 Tris-HCl pH 7.4; and 10 µg/ml RNA type III from bakers yeast (Sigma Chemical Co.) in 0.1 M Tris-HCl pH 7.4.

Antigen coated wells were washed three times with washing buffer (WB) 0.1 M Tris-HCl pH 7.4 containing 0.1% bovine serum albumin (BSA) (Gibco Laboratories) and 0.05% Tween 20 (Sigma Chemical Co.). The wells were then filled with 2% BSA in 0.1 M Tris-HCl pH 7.4 and incubated for 2 h at RT. After one wash with WB, 50 µl/well of serum (heat inactivated at 56° C for 30 min) or of supernatant (from a short term lymphocyte culture) or of hybridoma supernatant was added and the tray was incubated overnight at 4° C. After five washings with WB, 50 µl of either goat anti-human IgM alkaline phosphatase conjugate, 1 µg/ml (Cordis Laboratories, Inc., Miami, FL), or goat anti-human IgG alkaline phosphatase conjugate, 1 µg/ml (Cordis Laboratories, Inc.) diluted with 1% BSA, 2% bovine gammaglobulin (Sigma Chemical Co.) in 0.1 M Tris-HCl pH 7.4 was added to the wells and incubated for 3 h at RT. Five further washings of the wells with WB were followed by addition of 50 µl p-nitrophenyl phosphate 1 mg/ml (Sigma Chemical Co.) in diethanolamine buffer pH 9.8. After 30 min incubation (when sera or hybridoma supernatants were tested) or 45 min incubation (when short term culture supernatants were tested) with the substrate at 37° C, the enzymatic reaction was stopped with 25 µl/well of 3M NaOH. Optical densities were read at 405nm in a Titerek Multiskan (Dynatech Laboratories, Inc.).

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2.11. Inhibition of Anti-DNA Antibody in ELISA.

To determine ELISA specificity for anti-ssDNA antibody and anti-DNA antibody, 500 µl samples of two SLE sera diluted 1/100 were preincubated without or with 1, 5, or 25 µg ssDNA or DNA for 1 h or 1.5 h at RT before testing for binding in ELISA (2.10).

2.12. DNase I Digestion of DNA Bound to the Wells.

Native CT DNA-coated wells and uncoated wells (controls) were incubated for 60 and 90 min at 37°C with 50 µl of 0.1 mg/ml DNase I from bovine pancreas (Sigma Chemical Co.), dissolved in 0.1 M Tris-HCl buffer, pH 7.4 containing 0.002 M magnesium sulfate. After incubation wells were washed two times with 0.02 M EDTA in 0.1 M Tris-HCl, pH 7.4, and then three times with 0.1% BSA in 0.1 M Tris-HCl, pH 7.4. Further treatment of the wells was as described in determination of anti-DNA antibody by ELISA (2.10).

2.13. DNase I Digestion of Short Term Culture Supernatants.

All supernatants from short term lymphocyte cultures were incubated with DNase I (Sigma Chemical Co.) for 90 min at 37°C before testing for binding to CT DNA in ELISA (2.10). Final enzyme concentration was 0.1 mg/ml. Enzymatic digestion was carried out in the presence of 0.002 M magnesium sulfate. The reaction was stopped with EDTA (0.02 M final concentration).

DNase I treatment of culture supernatants tested for binding to DNA was chosen since the treatment with this enzyme resulted in:

1) enhanced detection of IgM anti-DNA and IgG anti-DNA antibodies in 2/9 and 6/9 of SLE PBL cultures respectively,
2) an increase (1.3 to 3.5 times) of IgM anti-DNA antibodies in 3/9 of the SLE cultures,
3) an increase (3 times) in IgM anti-DNA antibodies of 1/5 normal PBL cultures

Treatment with DNase I never resulted in a decreased detection of anti-DNA antibodies in normal or SLE PBL culture supernatnants.

2.14. ELISA for Determination of Hybridoma Supernatant Binding to Non-nucleic Acid Antigens.

Anti-nucleic acid positive hybridoma supernatants were tested for binding to cardiolipin, chicken egg albumin (ovalbumin, OA), human serum albumin (HSA), 2,4-dinitrophenyl (DNP), keyhole limpet hemocyanin (KLH), DNA-free human histones and human IgG. The wells of polystyrene round bottomed Cooke microtiter plates (Dynatech) were coated overnight at 4° C with 50 µl of: 20 µg/ml cardiolipin (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA), 20 µg/ml OA (Sigma Chemical Co.), 20 µg/ml HSA fraction V (Sigma Chemical Co.), 20 µg/ml DNP-OA , 20 µg/ml KLH, 20 µg/ml human histones and 20 µg/ml human IgG (Cappel Laboratories, Cochranville, PA). The control wells without antigen were coated with diluent only. All antigens were diluted in carbonate-bicarbonate buffer pH 9.6 except cardiolipin which was diluted in 0.01 M phosphate buffered saline (PBS) (Gibco Laboratories) pH 7.4. DNP-OA and KLH antigens were obtained from Dr. G. Strejan (Dept. Microbiology and Immunology, University of Western Ontario) and human histone antigen was provided by The Clinical Immunology Laboratory (University of Western Ontario). Antigen coated wells were washed three times with washing buffer (WB) 0.1 M Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin (BSA) (Gibco Laboratories) and 0.05% Tween 20 (Sigma Chemical Co.). The wells were then filled with 2% BSA in 0.1 M Tris-HCl pH 7.4 and incubated for 2 h at RT. After one wash with WB, 50 µl/well of hybridoma supernatant was added and the tray was incubated overnight at 4° C. After five

washings with WB, 50 µl of goat anti-human IgM alkaline phosphatase conjugate was added to the wells and incubated for 3 h at RT. This conjugate was purchased from Cordis Laboratories Inc., was diluted to 1 µg/ml in 0.1% BSA 0.1 M Tris-HCl pH 7.4 for all the tests excluding the test in which cardiolipin was used. The conjugate for developing the anti-cardiolipin reactivity was diluted also to 1 µg/ml in 0.1 M Tris-HCl pH 7.4 but the buffer contained 1% bovine gammaglobulin (Sigma Chemical Co.) in addition to 1% BSA.

The wells were subsequently washed five times with WB which was followed by addition of 50 µl p-nitrophenyl phosphate, 1 mg/ ml (Sigma Chemical Co.) in diethanolamine buffer pH 9.8. After 30 min incubation with the substrate at 37 °C, the enzymatic reaction was stopped with 25 µl/well of 3 M NaOH. Optical densities were read in a Titerek Multiskan (Dynatech Laboratories, Inc.) at 405nm. The mouse serum containing anti-DNP antibodies (obtained from Dr. G. Strejan, University of Western Ontario) and human serum containing anti-histone antibodies (obtained from The Clinical Immunology Laboratory, University of Western Ontario) were also tested in this assay for binding to DNP-OA and human histone antigen respectively. Reactivity of mouse anti-DNP serum with DNP-OA was revealed using 1:1000 dilution in 0.1 M Tris-HCl pH 7.4 and 0.1% BSA of a goat anti-mouse alkaline phosphatase conjugated IgG (Jackson Immuno Research Laboratories Inc.). Optical density values at 405nm of 1.5, 0.7, 0.4, 0.24, 0.1 were obtained with this mouse antiserum tested at 1/4000, 1/8000, 1/16000 and 1/32000 dilutions respectively.

The human anti-histone serum binding to the histone antigen was

detected by the use of alkaline phosphatase conjugated goat anti-human IgG and IgM (Cordis Laboratories, Inc.) which were diluted in 0.1 M Tris-HCl pH 7.4 containing 0.1% BSA to the final concentration of 1 µg/ml. The OD at 405nm for the human anti-histone serum at 1/100 dilution was 0.49. Both mouse anti-DNP serum and human anti-histone serum were diluted for testing in 0.1 M Tris-HCl pH 7.4, 0.1% BSA.

2.15. Solid Phase Radioimmunoassay (RIA) for Determination of Anti-DNA Antibody.

The solid phase radioimmunoassay was performed in an identical fashion to anti-DNA antibody ELISA described in 2.10 with the following exceptions:

1) The antigen CT DNA was coated onto PLL-treated polyvinyl chloride plates (Dynatech Laboratories).

2) The developing antibody was goat anti-human IgG (^{125}I) (5 µCi/mg) (New England Nuclear, Boston, MA) and was used at the concentration of 0.5 µg/ml.

3) The reading of the individual wells which were cut out from the plates and placed in the scintillation vials with 5 ml of scintillation fluid (described in 2.8) was done in the automatic gamma counter 1185 Series (Nuclear Chicago, A Subsidiary of G.D. Searle Co., Des Plaines, Illinois).

2.16. ELISA for Determination of Human IgM.

The round bottomed wells of polystyrene Cooke microtiter plates (Dynatech Laboratories, Inc.) were coated overnight at 4 °C with 50 µl of 10 µg/ml goat F(ab')2 anti-human IgM (µ chain specific) (Jackson Immuno Research Laboratory) in carbonate-bicarbonate buffer pH 9.6 or with buffer alone (control wells). Wells were washed three times with washing buffer (WB) composed of 0.1% BSA, 0.05% Tween 20 in 0.1 M Tris-HCl pH 7.4. Next the blocking solution (2% BSA in 0.1M Tris-HCl pH 7.4) was added to the wells and allowed to adsorb for 2 h at RT. The wells were afterwards washed once with WB and were filled with 50 µl of various dilutions of serum or hybridoma supernatant or with serially diluted human IgM (Cappel Laboratories) or with diluent alone (0.1% BSA in 0.01 M PBS pH 7.4). The IgM standard concentrations ranged from 5 µg/ml to 2 ng/ml. The trays were subsequently incubated at 4 °C overnight and washed five times with WB. Fifty µl of either goat anti-human IgM alkaline phosphatase conjugate, 1 µg/ml, or goat-anti-human IgG alkaline phosphatase conjugate, 1 µg/ml, (both from Cordis Laboratories, Inc.) diluted with 1% BSA, 2% bovine gammaglobulin in 0.1 M Tris-HCl pH 7.4 was added to the wells and left for 3 h at RT. Five further washings of the wells with WB were followed by addition of 50 µl p-nitrophenyl phosphate (Sigma Chemical Co.) in diethanolamine buffer pH 9.8. After 30 min incubation with the substrate at 37 °C, the enzymatic reaction was stopped with 25 µl/well of 3 M NaOH. Optical densities were read in a Titerek Multiskan (Dynatech Laboratories, Inc.) at 405nm. OD 405nm readings

of zero were obtained with the controls: 1) the wells coated with carbonate-bicarbonate buffer pH 9.6 alone followed by the blocking solution and test samples, 2) the wells coated with goat $F(ab')_2$ anti-human IgM followed by blocking solution and diluent of the test samples (0.1% BSA in 0.01 M PBS pH 7.4, and 3) the control and test wells developed with alkaline phosphatase conjugated goat anti-human IgG.

2.17. ELISA for Determination of Human IgG.

The round bottomed wells of polystyrene Cooke microtiter plates (Dynatech Laboratories, Inc.) were coated overnight at 4°C with 50 µl of 10 µg/ml goat $F(ab')_2$ anti-human IgG (gamma chain specific) (Jackson Immuno Research Laboratory) in carbonate-bicarbonate buffer pH 9.6, or with buffer alone (control wells). Wells were washed three times with washing buffer (WB) containing 0.1% BSA, 0.05% Tween 20 in 0.1 M Tris-HCl pH 7.4. The blocking solution of 2% BSA in 0.1 M Tris-HCl pH 7.4 was added to the wells next. After 2 h at RT incubation with blocking solution, wells were washed once with WB and were filled with 50 µl of various dilutions of sera or hybridoma supernatant, or with serially diluted human IgG (Cappel Laboratories) or with diluent alone (0.1% BSA in PBS pH 7.4). The IgG standard concentrations ranged from 5 µg/ml to 2 ng/ml. The trays were subsequently incubated overnight at 4°C and washed five times with WB. Fifty µl of either goat anti-human IgM alkaline phosphatase conjugate 1 µg/ml or goat anti-human IgG alkaline phosphatase

conjugate 1 µg/ml (both from Cordis Laboratories, Inc.) diluted with 1% BSA in 0.1 M Tris-HCl pH 7.4 was added to the wells and left for 3 h at RT. Five further washings of the wells with WB were followed by addition of 50 µl p-nitrophenyl phosphate (Sigma Chemical Co.) in diethanolamine buffer pH 9.8. After 30 min incubation with the substrate at 37°C, the reaction was stopped with 25 µl/well of 3 M NaOH. Optical densities were read in Titertek Multiskan (Dynatech Laboratories, Inc.) at 405nm. OD_{405nm} readings of zero were obtained with the controls: 1) the wells coated with carbonate-bicarbonate buffer pH 9.6 alone followed by the blocking solution and test samples, 2) the wells coated with goat $F(ab')_2$ anti-human IgG followed by blocking solution and diluent of the test samples (0.1% BSA in 0.01 M PBS pH 7.4), and 3) the control and test wells developed with alkaline phosphatase conjugated goat anti-human IgM.

2.18. Preparation of Hybridoma Supernatants for Immunofluorescence Staining of HEp2 Cells.

Ten randomly selected supernatants containing monoclonal IgM anti-DNA antibodies and 2 hybridoma supernatants containing IgM monoclonal antibodies with unknown specificities as well as supernatant from the lymphoblastoid GM 4672 cell line fusion partner were precipitated with an equal volume of saturated ammonium sulfate (J.F. Baker Chemical Co., Phillipsburg, NJ). Precipitations were carried out at 4°C overnight and were followed by 3 washes with 50% ammonium sulfate. Afterwards pellets were resuspended in an

appropriate volume of 0.01 M PBS pH 7.4 (Gibco Laboratories) to give 5X concentrates of the original supernatant, and they were dialyzed against 0.01 M PBS pH 7.4 (Gibco Laboratories) for 48 h at 4° C with 4 changes of the buffer. Concentrated supernatants prepared in the above manner were used for binding to human epithelial cells (HEp2). The IgM and IgG concentration in these preparations was measured by ELISA as described above (2.16 and 2.17).

2.19. Immunofluorescence Staining of HEp2 Cells.

HEp2 cell line substrate prepared slides were purchased from Kallestad Laboratories Inc., Austin, Texas as part of the Quantafluor kit routinely used for determination of autoantibodies to nuclear antigens (ANA). The handling of the HEp2 slides was performed according to the Kallestad protocol attached to the reagent kit. In brief, HEp2 slides which were stored at 4° C were equilibrated to RT and placed in a moist chamber before use. Twenty-five ul of undiluted hybridoma supernatant or GM 4672 cell line supernatant or normal human IgM (100 µg/ml) (Cappel Laboratories) or a 1:2 dilution of mouse anti-vimentin antibody B11.5.1 (Dales et al, 1983) (a gift from Dr. S. Dales, Department of Microbiology and Immunology, University of Western Ontario) was applied to the appropriate HEp2 containing wells on the slides. All dilutions were in 0.01 M PBS pH 7.4. The slides were then incubated for 30 min at RT in a moist chamber. Slides were then washed with 0.01 M PBS pH 7.4 and 25 µl of a 1:10 dilution of rhodamine conjugated goat $F(ab')_2$ anti-human IgM (µ chain specific)

(Jackson Immuno Research Laboratories Inc.) or fluorescein conjugated goat $F(ab')_2$ anti-mouse IgM (μ chain specific) (Jackson Immuno Research Laboratories Inc.) was applied to appropriate wells. Slides were incubated for 30 min at RT, washed with 0.01 M PBS pH 7.4 and viewed under the microscope (Zeiss, W. Germany) equipped with UV illumination and necessary filters (KP 490 blue interference filter for fluorescein, FT 580 chromatic splitter filter for rhodamine).

2.20. Absorptions of Hybridoma Supernatants with ssDNA or Cardiolipin Prior to HEp2 Staining.

Five positive and two negative hybridoma supernatants staining HEp2 cells and the GM 4672 lymphoblastoid cell line supernatant were absorbed with 10 μg/ml ssDNA prepared according to Doty et al (1960) or with 20 μg/ml cardiolipin (Calbiochem-Behring Corp.) or with 0.1% BSA all in 0.1 M Tris-HCl pH 7.4 buffer immobilized on polystyrene wells of microtitre Cooke plates (Dynatech Laboratories, Inc.). Absorptions were done overnight at 4° C until there was no binding of antibodies to cardiolipin or to ssDNA as demonstrated by ELISA (2.10). Absorbed supernatants were used for immunofluorescence staining of HEp2 cells according to the methodology outlined above.

2.21. Competitive Blocking by Anti-vimentin Antibody of Human Hybridoma Supernatant Binding to HEp2 Cells.

Mouse IgM monoclonal anti-vimentin antibody B11.5.1 was used to

block the binding of human hybridoma supernatants to HEp2 cells. The cells were pre-incubated for 30 min at RT with B11.5.1 which was diluted 1:1 in 0.01 M PBS pH 7.4 or with 1:20 diluted in PBS pH 7.4 normal CBA/J mouse serum. After extensive washing with PBS pH 7.4 HEp2 cells were treated for 30 min at RT with ammonium sulfate precipitated human hybridoma supernatants. HEp2 cells were then washed 3 times with PBS pH 7.4 and developed with a 1:10 dilution in 0.01 M PBS pH 7.4 of rhodamine conjugated goat $F(ab')_2$ anti-human IgM (μ chain specific) (Jackson Immuno Research Laboratories Inc.). The binding of B11.5.1 to HEp2 cells was confirmed using a 1:10 dilution in 0.01 M PBS pH 7.4 of fluorescein conjugated goat $F(ab')_2$ anti-mouse IgM (μ chain specific) (Jackson Immuno Research Laboratories Inc.).

2.22. Photography of Stained HEp2 Cell.

Photographs were taken with a microscope-attached camera (Zeiss, W. Germany) using EL 135-36 Kodak Ektachrome film (Kodak Canada Inc., Toronto). Automatic exposure time was used to photograph the positively stained slides and this time varied between 6-15 seconds depending on the anti-DNA hybridoma supernantant used. The negatively stained slides were exposed for 15 seconds. The brightest areas of these negatively stained slides were photographed to show the maximum background staining.

2.23. Determination of Immunoglobulin Light Chains in Hybridoma Supernatants Using Double Immunodiffusion.

The selected hybridoma supernatants and GM 4672 cell line supernatant were concentrated by precipitation at 4° C overnight with saturated ammonium sulfate (J..T. Baker Chemical Co.) to a final concentration of 50%. After 3 washes with 50% ammonium sulfate, pellets were dissolved to one fifth of the original volume in 0.01 M PBS pH 7.4 , and extensively dialyzed at 4° C against 4 changes of 0.01 M PBS pH 7.4 during 48 h. Twenty five µl of the concentrated hybridoma supernatant and GM 4672 supernatant was applied to the outer wells in 0.8% agarose (Marine Colloids Division FMC Corp., Rockland, ME) in 0.01 M PBS pH 7.4 prepared on a microscope slide. The central wells contained 25 µg of rabbit anti-human lambda or kappa light chain antibodies (obtained from the Clinical Immunology Laboratory, University of Western Ontario). The antibodies were allowed to diffuse in the gel overnight at RT. The slides were kept in a moist chamber during that time. The slides were then extensively washed with 0.01 M PBS pH 7.4, rinsed with distilled water and dried by covering them with layers of filter paper. Drying was performed at RT overnight. To visualize immunoprecipitin lines, slides were stained with Amido Black (Ouchterlony and Nilson, 1973) for 15 min at RT and destained for 30 min at RT with 2% acetic acid.

2.24. Karyotyping of Hybridomas.

Rapidly proliferating cells were obtained by seeding 1 x 10^6 cells in 5 ml of hybridoma growth medium (defined in 2.6) and culturing for 48 h. Then 50 µl of 10 µg/ml Colcemid (Gibco Laboratories) was added and cultures were incubated for 35 min. Cells were centrifuged at 200 g for 8 min and the pellet was resuspended in 4 ml of 75 mM potassium chloride. After incubation for 15 min at 37 °C, cells were recentrifuged, resuspended in 4 ml of fixative (1:3 acetic acid:methanol), and left for 30 min at 4° C.

Cells were then washed twice in fixative and resuspended in 1 ml of fixative. Two drops of the cell suspension were dispensed onto glass slides (ethanol washed and stored at minus 20 °C) and allowed to air dry. Slides were washed in saline, stained with 4% Giemsa (Fisher Scientific Co., Fair Lawn, NY) in 6 x 10^{-4} M phosphate buffer pH 6.8, destained in distilled water and mounted with Permount (Fisher Scientific Co.). Slides were examined under the light microscope and 50 karyotypes were scored.

2.25. HLA Typing of Hybridomas.

Actively proliferating cells were washed twice with hybridoma growth medium (defined in 2.6), centrifuged for 8 min at 200 g and resuspendded in hybridoma growth medium at 2 x 10^6 cells/ml.

Terasaki (T-27) and the Canadian Red Cross (NRLT 85-5) HLA typing trays were used for HLA typing by a microcytotoxicity assay as described by Terasaki et al (1978).

2.26. Purification of KIM 4.6.3 Anti-DNA Antibody.

KIM 4.6.3 anti-DNA antibody was of IgM lambda type and it displayed reactivity with various nucleic acid antigens including: ssDNA, native DNA, polydG.polydC, poly(dA-dT), a low molecular weight S/N DNA and RNA. It also bound to cardiolipin. This 4.6.3 anti-DNA antibody was chromatographically purified on silica (Boehringer Mannheim GmbH, W. Germany) coupled rabbit anti-human IgM (μ chain specific) (DAKO, Denmark). The coupling of rabbit anti-human IgM to silica was performed according to the instructions from Boehringer Mannheim GmbH attached to the silica reagent. Approximately 300 ml of KIM 4.6.3 hybridoma supernatant was passed at RT through the silica-rabbit anti-human IgM packed column (15 cm height, 1 cm diameter). The column was then extensively washed with 3% NaCl until absorbance at 280 nm was less than 0.005 OD units. The bound antibody was eluted with 2 mol/l sodium thiocyanate (J.T. Baker Chemical Co., Phillipsburg, NJ). One ml fractions were collected and those which contained protein (determined spectrophotometrically at 280 nm) were pooled. The eluted antibody solution (20-25 ml) was dialyzed for 48-72 h at 4 ^{o}C against six changes of 0.01 M PBS pH 7.4. This dialyzed antibody solution was subsequently concentrated by Amicon filtration on an XM 300 membrane (Amicon Corporation, Lexington, Mass)

and finally dialyzed at 4 °C with three changes of 0.01 M PBS pH 7.4 during 48 h. This preparation yielded 5 ml of IgM antibody solution (120 µg/ml) which had reactivity with ssDNA and which lacked detectable human IgG or rabbit IgG when tested by ELISA. The detection of rabbit immunoglobulins was done using alkaline phosphatase conjugated sheep-anti-rabbit IgG (Sigma Chemical Co.) to develop the wells directly coated with prurified KIM 4.6.3 antibody.

2.27. Production of Anti-4.6.3 Idiotype.

The anti-4.6.3 idiotype (anti-4.6.3 ID) was prepared in 6-8 week old female New Zealand rabbits. The test rabbits were injected with 100 µg/rabbit of purified KIM 4.6.3 antibody in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) and the control rabbits received saline (equivalent volume to KIM 4.6.3 antibody solution) in CFA. These injections were made intradermally into 3 different places in the neck region. Three and 5 weeks later, test rabbits were immunized again with 50 µg of KIM 4.6.3 antibody per rabbit in incomplete Freund's andjuvant (IFA) (Difco Laboratories) and control rabbits were immunized with saline in IFA. These injections were given intramuscularly in the thigh region. Two weeks after the last immunization, rabbits were bled through the ear and the control serum (normal rabbit serum, NRS) and the test serum (anti-4.6.3 serum) were collected.

The anti-4.6.3 serum as well as NRS were extensively absorbed on normal human IgM (Cappel Laboratories) coupled to silica (Boehringer

Mannheim GmbH) and on normal human IgG coupled to agarose (Sigma Chemical Co.) to remove antibodies to the constant region of human immunoglobulin light and heavy chains from the anti-4.6.3 serum. All absorptions (2 on IgG and 4 on IgM) were carried out in 15 ml screw cap tubes (Corning Glass Works, Corning, NY) containing 2 ml of packed absorbent and 3 ml of serum diluted 1:1 with 0.01 M PBS pH 7.4. Sera were incubated with each absorbent for 2 h at RT. Tubes were in a constant rocking motion (45°, 6 cycles/min) during that time. Sera were removed from the tubes after centrifugation at 400 g for 10 min. Anti-4.6.3 serum which had no reactivity in ELISA with normal human IgG and IgM (both from Cappel Laboratories), but bound to KIM 4.6.3 antibody was used and was considered as anti-4.6.3 ID.

2.28. ELISA for Binding of Rabbit Sera to IgM, IgG or KIM 4.6.3.

Fifty µl of normal human IgM (Cappel Laboratories) or normal human IgG (Cappel Laboratories) or purified KIM 4.6.3 antibody, each at 5 µg/ml in corbonate-bicarbonate buffer pH 9.6 were coated onto polystyrene round bottomed wells of Cooke Microtiter plates (Dynatech Laboratories Inc.). Wells coated with buffer served as controls. After coating, the plates were incubated at 4° C overnight and then washed 3 times with the washing buffer (WB) 0.1 M Tris-HCl, pH 7.4 containing 0.1% BSA. Subsequently all the wells were totally filled with 2% BSA in 0.1 M Tris-HCl pH 7.4 and incubated for 2 h at RT. After one wash with WB, 50 µl of unabsorbed anti-4.6.3 serum or anti-4.6.3 ID or unabsorbed NRS or absorbed NRS diluted 1:50 to $1:10^5$

in 0.1% BSA 0.1 M Tris-HCl pH 7.4 were pipetted into the antigen coated and control wells. The plates were left overnight at 4° C. The following morning they were washed 5 times with WB and 50/ul of 1:1000 dilution in 0.1% BSA 0.1 M PBS pH 7.4 of alkaline phosphatase conjugated sheep $F(ab')_2$ anti-rabbit IgG (Sigma Chemical Co.) was added to the wells. After overnight incubation at 4° C plates were washed again 5 times with WB and the wells were filled with 50/ul p-nitrophenyl phosphate at 1 mg/ml in diethanolamine buffer pH 9.8. The enzymatic reaction was developed for 30 min at 37° C and then stopped with 25/ul/well of 3 M NaOH. Optical densities were read in a Titerek Multiskan (Dynatech Laboratories Inc.) at 405nm.

2.29. Inhibition of Anti-4.6.3 ID Binding to KIM 4.6.3 by the Homologous ID.

Two hundred/ul of anti-4.6.3 ID or NRS (adjusted to a protein concentration of 5/ug/ml were mixed either with an equal volume of serially diluted affinity purified KIM 4.6.3 antibody or with KIM 4.6.3 hybridoma supernatant (both at the same IgM concentration of 0.6 /ug/ml). In addition, the following mixtures were also used: 0.6 /ug/ml normal human IgM (Cappel Laboratories) with anti-4.6.3 ID and 0.1% BSA 0.1 M Tris-HCl pH 7.4 with anti-4.6.3 ID. A solution of 0.1% BSA 0.1 M Tris-HCl pH 7.4 was used as a diluent for all above preparation. The mixtures were incubated for 2 h at RT proior to testing in ELISA. The ELISA procedure for binding of anti-4.6.3 ID to KIM 4.6.3 antibody coated on the wells using sheep $F(ab')_2$ anti-rabbit

IgG alkaline phosphatase conjugate was described in 2.28. The percent inhibition was expressed according to the formula: percent inhibition = 100 - 100 (OD_{405nm} anti-4.6.3 ID + 4.6.3 ID)/ OD_{405nm} anti-4.6.3 ID + buffer). There was no inhibition of anti-4.6.3 ID binding to KIM 4.6.3 antibody by normal human IgM. NRS did not show any reactivity with KIM 4.6.3 antibody in the absence or presence of the inhibitor, KIM 4.6.3 antibody.

2.30. ELISA for Binding of Anti-4.6.3 ID to Monoclonal DNA or Non-DNA-binding Monoclonal Antibodies or to Human Sera.

The following samples were used in the ELISA determination for the presence of 4.6.3 ID: 1) undiluted hybridoma supernatants containing DNA binding monoclonal antibodies, 2) undiluted hybridoma supernatants containing non-DNA-binding antibodies, 3) undiluted hybridoma growth medium (defined in 2.6), 4) undiluted GM 4672 lymphoblastoid cell line supernatant, 5) human sera obtained from normal individuals, SLE patients, patients with unexplained polyclonal hypergammaglobulinemia, patients with monoclonal IgM which were diluted 10^{-2}, 5×10^{-2}, 10^{-3}, 2×10^{-3}, 5×10^{-3}, 10^{-4}, 2×10^{-4} in carbonate-bicarbonate buffer pH 9.6, 6) SLE or normal sera immunoglobulin fractions obtained by 50% ammonium sulfate precipitation and after extensive dialysis diluted $1:10^{-3}$ in carbonate-bicarbonate buffer pH 9.6. Fifty µl of these samples were coated overnight at 4° C onto polystyrene wells of Cooke Microtiter plates (Dynatech Laboratores Inc.). The plates were then washed 3 times with washing buffer (WB) containing 0.1% BSA in

0.1 M Tris-HCl pH 7.4 and incubated for 2 h at RT with a blocking solution of 2% BSA in 0.1 M Tris-HCl pH 7.4. Following one wash with WB, 50 µl of anti-4.6.3 ID or NRS diluted 5 x 10^{-2} in 0.1% BSA was pipetted into the wells and left overnight at 4°C. The plates were subsequently washed 5 times with WB and 50 µl of alkaline phosphatase conjugated sheep $F(ab')_2$ anti-rabbit IgG (Sigma Chemical Co.) diluted 10^{-3} in 0.1% BSA in 0.01 M PBS pH 7.4 was added to the wells. These plates were left overnight at 4°C and were washed 5 times with WB. The enzymatic reaction was developed with p-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer pH 9.8 for 1 h at 37°C. After addition of 25 µl/well of 3 M NaOH, plates were read in a Titerek Multiskan (Dynatech Laboratories Inc.) at 405 nm.

2.31. Inhibition by Anti-4.6.3 ID of 4.6.3 ID-positive Anti-DNA Antibody Binding to ssDNA.

Five 4.6.3 ID positive IgM anti-DNA antibodies diluted in 0.1 M Tris-HCl pH 7.4 were mixed with an equal volume of anti-4.6.3 ID diluted in 0.1% BSA 0.1 M Tris-HCl pH 7.4. In these reaction mixtures, the final concentration of IgM anti-DNA antibodies ranged from 50 to 500 ng/ml and the final dilution of anti-4.6.3 ID was 1:100 which was equivalent to a protein concentration of 50 µg/ml. These mixtures were incubated for 2 h at 37°C before using them in ELISA for binding to ssDNA as described in 2.10.

2.32. Statistics.

The statistical methods used were described by Snedecor (1956).

CHAPTER 3 - RESULTS

3.1. Sensitivity and Specificity of Anti-DNA Antibody ELISA.

A prerequisite for detecting anti-DNA antibodies by ELISA is the binding of DNA to the wells of the microtiter plates. The microtiter plates used in anti-DNA ELISA are made of polystyrene, a rigid plastic. Direct evidence for the ability of this polystyrene plastic to bind DNA is difficult to produce due to difficulties in measuring radioactive DNA retained in the individual wells. An attempt was made to evaluate polystyrene DNA binding properties in comparison with polyvinyl chloride DNA binding. Polyvinyl chloride is a flexible plastic. Its DNA binding ability was directly explored with use of two radiolabelled nucleic acid antigen preparations: 1) Escherichia coli ^{14}C-DNA and 2) a synthetic nucleic acid polymer, poly ^{3}H-(dA-dT). Both radiolabelled antigens bound only to the polyvinyl chloride wells which were pre-coated with the positively charged amino acid polymer, poly-L-lysine (PLL) (Tables 1 and 2). The binding of E. coli ^{14}C-DNA and poly ^{3}H-(dA-dT) was similar (45 ng and 42 ng respectively) when both antigens were coated at 10 µg/ml (500ng/well). When lower antigen concentrations of 5 µg/ml, 2.9 µg/ml and 1.25 µg/ml were used for coating, better binding was achieved with poly ^{3}H-(dA-dT) (37 ng/well at 5 µg/ml, 34 ng/well at 2.5 µg/ml) than with E. coli ^{14}C-DNA (25 ng/well at 5 µg/ml, 12 ng/well at 2.5 µg/ml) (Tables 1 and 2).

Table 1. Binding of Poly ^{3}H(dA-dT) to Assay Wells Coated with Poly-L-Lysine (PLL).

PLL	Poly. ^{3}H(dA-dT)				
50 λg/ml	amount put into the well		total cpm bound	bound minus control[3]	amount bound
	λg/well	cpm/well	cpm/well	cpm/well	ng/well[4]
+ PLL[1]	0.500	14595	1237	1219	42
+ PLL	0.250	7555	1125	1107	37
+ PLL	0.125	3130	878	860	34
- PLL[2]	0.500	14595	33	19	0.65
- PLL	0.250	7555	33	19	0.63
- PLL	0.125	3130	31	17	0.68
+ PLL	0	0	18		
- PLL	0	0	14		

1 wells coated with 50 λl/well of 50 λg/ml PLL in 0.1 M Tris-HCl pH 7.4

2 wells coated with 50 λl/well of 0.1 M Tris-HCl pH 7.4

3 represents cpm/well difference between PLL-coated antigen-bound well and PLL-coated well or cpm/well difference between non-PLL-coated antigen-bound well and non-PLL-coated well

4 $\dfrac{\text{λg poly } ^{3}\text{H(dA-dT) put into the well x (total cpm bound-control)/well}}{\text{cpm put into the well}}$

Table 2. Binding of *E. coli* ^{14}C-DNA to Assay Wells Coated with Poly-L-Lysine (PLL).

PLL	*E. coli* ^{14}C-DNA				
50 µg/ml	amount put into the well		total cpm bound	bound minus control[3]	amount bound
	µg/well	cpm/well	cpm/well	cpm/well	ng/well[4]
+ PLL[1]	0.500	42100	3796	3779	45
+ PLL	0.250	26027	2586	2569	25
+ PLL	0.125	12295	1223	1206	12
- PLL[2]	0.500	42100	33	18	0.21
- PLL	0.250	26027	53	38	0.36
- PLL	0.125	12295	29	14	0.14
+ PLL	0	0	17		
- PLL	0	0	15		

1 wells coated with 50 µl/well of 50 µg/ml PLL in 0.1 M Tris-HCl pH 7.4

2 wells coated with 50 µl/well of 0.1 M Tris-HCl pH 7.4

3 represents cpm/well difference between PLL-coated antigen-bound well and PLL-coated well or cpm/well difference between non-PLL-coated antigen-bound well and non-PLL-coated well

4 $\frac{\text{µg } \textit{E. coli } ^{14}\text{C-DNA put into the well} \times \text{(total cpm bound-control)/well}}{\text{cpm put into the well}}$

The binding of SLE serum anti-DNA antibodies to PLL pre-coated polystyrene and polyvinyl chloride wells containing calf thymus DNA (coated at 10 ug/ml) is presented in Table 3. The serum antibodies from three different SLE patients showed similar binding at serum dilutions of $1:10^4$ and $1:10^5$ to the antigen coated polyvinyl chloride and polystyrene wells. With sera diluted $1:10^2$ and $1:10^3$ better binding was achieved with polystyrene plates (Table 3). This indicates that the DNA binding capacity of PLL-treated polystyrene plates is similar or higher than that of PLL-treated polyvinyl chloride plates. The SLE sera did not bind to DNA coated polyvinyl chloride plates and polystyrene DNA coated plates in the absence of PLL. The binding of these sera to PLL-coated wells of both plastic types was less than 10% of the binding to the PLL-antigen coated wells. The results presented above indicate the need for PLL treatment of the wells prior to coating with DNA and justify the choice of polystyrene plates over polyvinyl chloride plates in anti-DNA antibody ELISA.

The determination of specificity and sensitivity of anti-DNA measurement by ELISA was evaluated with sera of SLE patients and normal controls before employing this method for anti-DNA antibody detection in supernatants from short term lymphoid cell cultures and in hybridoma supernatants. Figure I shows the presence of IgG and IgM anti-DNA antibodies in different dilutions ($1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$) of SLE and normal sera. IgG anti-DNA antibodies (mean $\pm$ SD = 0.843 ± 0.453) were present in 92% (11/12) and IgM anti-DNA antibodies (mean $\pm$ SD = 0.726 ± 0.414) in 100% (12/12) of SLE sera diluted $1:10^2$.

Table 3. Binding of SLE Sera to PLL-CTDNA-coated Polyvinyl Chloride Wells or Polystyrene Wells.[1]

	SLE serum	Anti-DNA antibody ELISA[2]	
	dilution	polyvinyl chloride wells (OD_{405nm})[3]	polystyrene wells (OD_{405nm})[3]
Serum I	$1/10^2$	1.62	2.00
	$1/10^3$	1.10	1.30
	$1/10^4$	0.30	0.40
	$1/10^5$	0.13	0.14
Serum II	$1/10^2$	0.44	0.53
	$1/10^3$	0.23	0.40
	$1/10^4$	0.08	0.19
	$1/10^5$	0.04	0.00
Serum III	$1/10^2$	0.80	1.40
	$1/10^3$	0.51	0.73
	$1/10^4$	0.10	0.12
	$1/10^5$	0.00	0.00

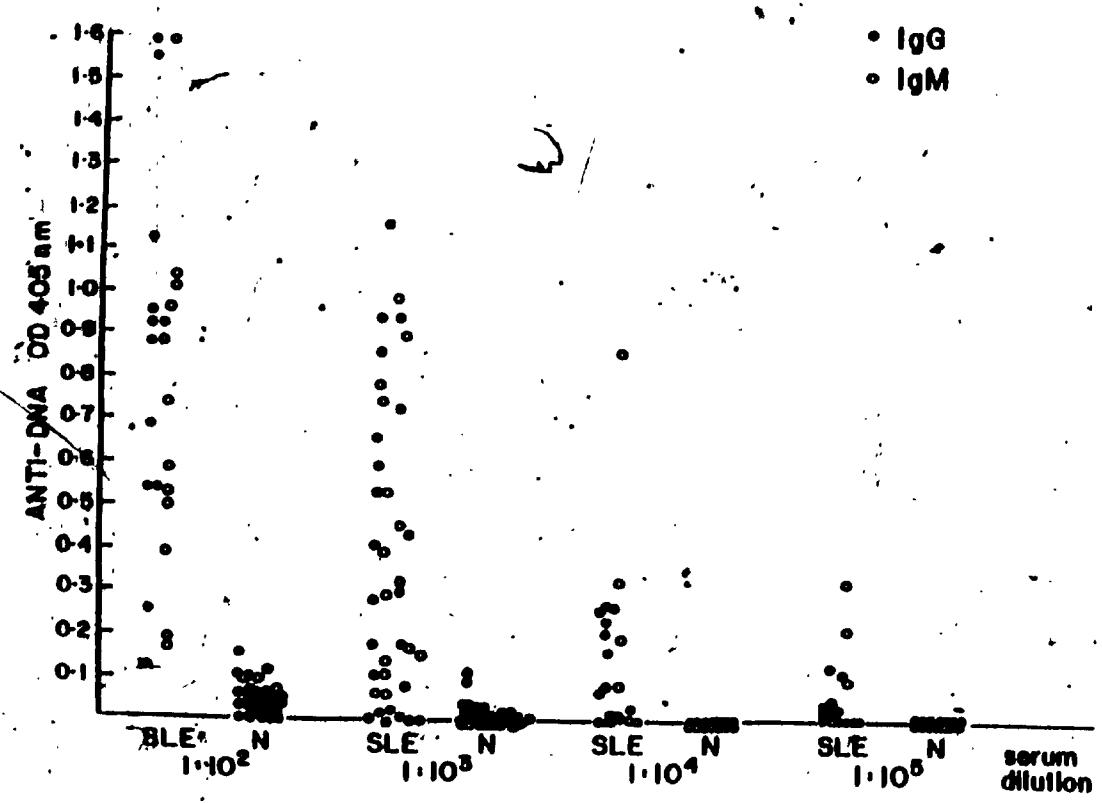
1 50 µl/well of calf thymus DNA at 10 µg/ml was used

2 developing reagent in ELISA was alkaline phosphatase-conjugated goat anti-human IgM and IgG, both at a final concentration of 1 µg/ml

3 OD_{405nm} represents differences between OD_{405nm} of PLL-antigen-coated well and OD_{405nm} of PLL-non-antigen-coated well. The OD_{405nm} of PLL-non-antigen-coated well was 10% or less of the OD_{405nm} of PLL-antigen-coated well.

Figure 1.

Presence of IgG anti-DNA antibodies (●) and IgM anti-DNA antibodies (O) in normal or SLE sera. Anti-DNA antibodies were detected by ELISA method in which CT DNA was used as the antigen.


IgG
IgM
ANTI-DNA OD 405 nm
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
SLE
N
1·10²
1·10³
1·10⁴
1·10⁵
serum dilution

An OD_{405nm} of 0.153 or less was considered negative because it corresponded to IgG anti-DNA antibody mean value + 2SD (0.061 + 2 x 0.46) of the normal controls at $1:10^2$ dilution. For IgM anti-DNA antibodies at $1:10^2$, values above OD_{405nm} = 0.117 were considered positive since they represented 2SD values above the mean of normals (mean + 2SD = 0.051 + 2 x 0.033). At $1:10^3$ dilution, 74% (14/19) SLE sera were positive for IgG anti-DNA antibodies (mean ± SD = 0.438 ± 0.364) and 79% (15/19) for IgM anti-DNA antibodies (mean ± SD = 0.348 ± 0.319). Positivity of SLE sera at $1:10^3$ dilution was determined also by elimination of all OD_{405nm} SLE sera values below mean + 2SD of normal sera. This mean + 2SD value for IgG anti-DNA antibodies was equal to 0.138 (0.038 + 2 x 0.05). Mean and standard deviation for IgM anti-DNA antibodies in normal sera diluted $1:10^3$, and further, approached zero values. The mean and standard deviation values of IgG anti-DNA antibodies in the control population was also zero in sera diluted $1:10^4$ and $1:10^5$.

In contrast, SLE sera at these high dilutions still showed binding to DNA above the control base line level. At $1:10^4$ dilution, IgG anti-DNA antibodies (mean ± SD = 0.14 ± 0.11) and IgM anti-DNA antibodies (mean ± SD = 0.147 ± 0.27) were detected respectvely in 72% (8/11) and in 40% (4/10) of the SLE sera. At $1:10^5$ dilution, 25% (2/8) of the SLE sera had IgG anti-DNA antibodies and 38% (3/8) had IgM anti-DNA antibodies. The mean values ± SD for SLE IgG and IgM antibodies to DNA at this serum dilution were respectively 0.05 ± 0.047 and 0.075 ± 0.117.

DNase I digestion of the DNA bound to the wells resulted in almost

complete loss of serum IgG and IgM binding to DNA, indicating that the reactivity with serum resulted from the binding to DNA and not to the plastic or blocking solution contained in the wells (Fig. 2). As a further indication of the specificity of antibody binding for DNA, test serum was pre-incubated for 60 and 90 min with ssDNA or DNA before its addition to the antigen (ssDNA or DNA)-coated wells (Fig.3). Dose-dependent inhibition of binding was seen with both ssDNA and DNA. Single stranded DNA was a better inhibitor for ssDNA coated wells, and DNA was a better inhibitor for DNA coated wells. There was, however, some inhibition by ssDNA of serum binding to DNA and vice versa (Fig. 3 a,b). This overlapping reactivity may reflect the presence of single stranded regions in DNA and double stranded regions in ssDNA (Tan and Natali, 1970) which are recognized by these sera. This is unlikely since the gross contamination of ssDNA in DNA and DNA in ssDNA can be excluded based on the spectrophotometric evaluation of these antigen preparations. The DNA at 20 µg/ml in 0.1 M Tris-HCl pH 7.4 produced an OD_{260nm} of 0.4 and heat denatured DNA (ssDNA) at the same concentration in 0.1 M Tris-HCl pH 7.4 produced an OD_{260nm} of 0.52. The thermal hyperchromicity value (Mahler and Cordes, 1966) was calculated to be 0.3. This hyperchromicity value indicates almost complete denaturation (Davidson, 1972) and hence minimal or lack of double stranded regions in ssDNA. The maximal thermal hyperchromicity value for DNA from the same source (calf thymus) as the one used in this experiment but measured under different conditions was observed to be 0.41 (Mahler and Cordes, 1966).

Figure 2.

Influence of DNase I treatment of CT DNA bound to the wells on anti-DNA antibody measurement by ELISA. IgG anti-DNA antibodies binding to the wells coated with DNA from normal (▲——▲) or SLE (●——●) serum, and to the wells not coated with DNA from normal (▲- - -▲) or SLE serum (●- - -●). IgM anti-DNA antibodies binding to the wells coated with DNA from normal (△——△) or SLE (○——○) serum and to the wells not coated with DNA from normal (△- - -△) or SLE (○- - -○) serum.

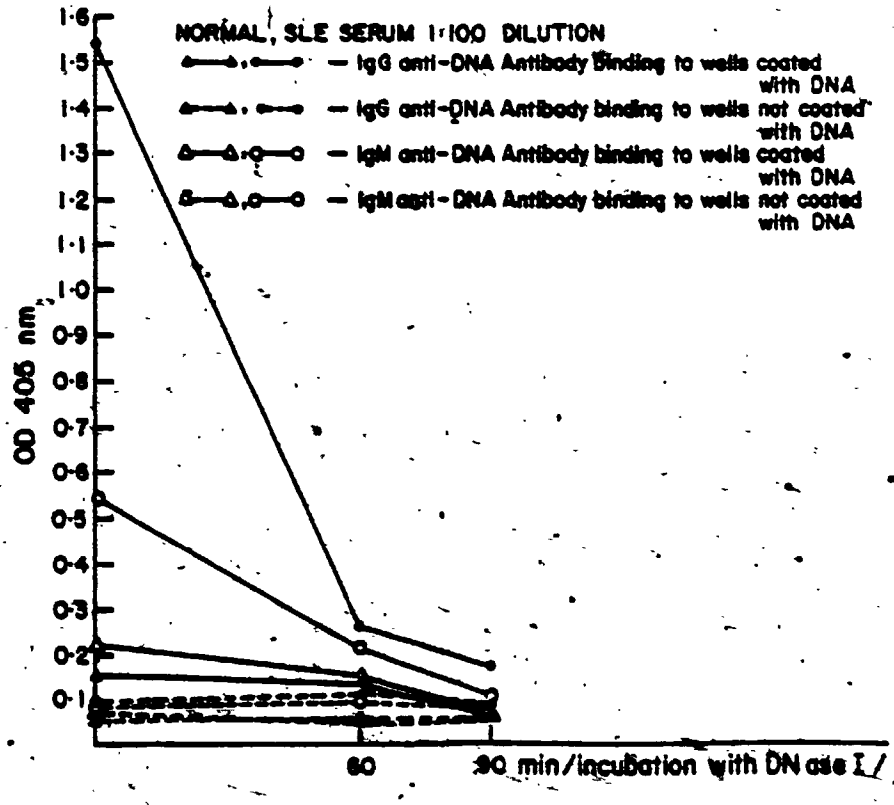
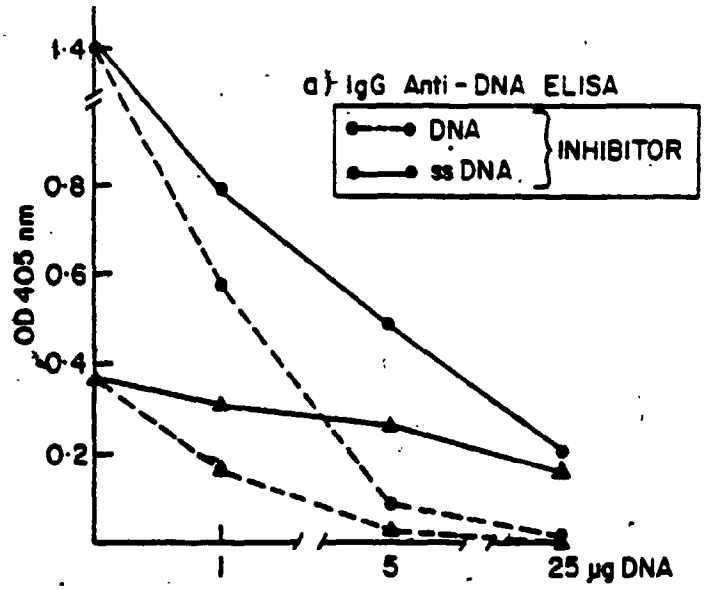
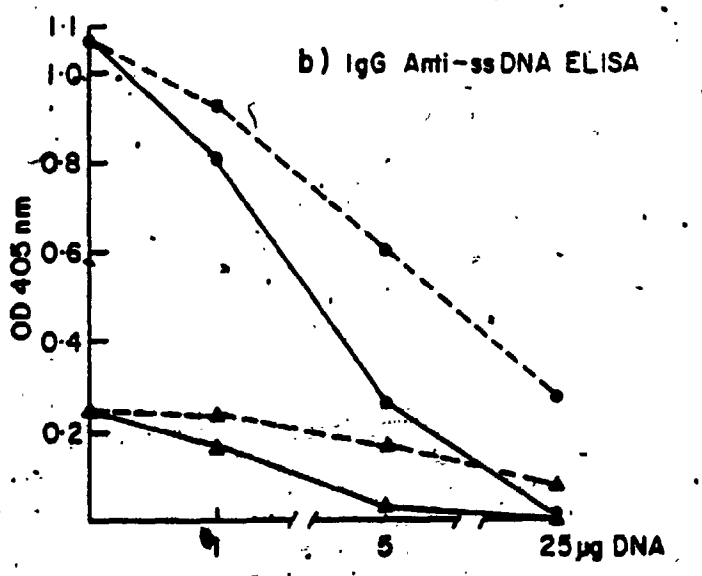

NORMAL, SLE SERUM 1:100 DILUTION
— IgG anti-DNA Antibody binding to wells coated with DNA
— IgG anti-DNA Antibody binding to wells not coated with DNA
— IgM anti-DNA Antibody binding to wells coated with DNA
— IgM anti-DNA Antibody binding to wells not coated with DNA
OD 406 nm
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
60
90 min/incubation with DNase I

Figure 3.

Inhibition of anti-DNA antibodies binding in ELISA by ssDNA or by DNA. Binding of IgG anti-DNA antibodies from two SLE sera (● , ▲) to CT DNA (a) or to single stranded CT DNA (b) after pre-incubation of the sera with CT DNA (●—● , ▲—▲) or with single stranded CT DNA (●—● , ▲—▲).


a) IgG Anti-DNA ELISA
DNA
ss DNA
INHIBITOR
OD 405 nm
1·4
0·8
0·6
0·4
0·2
1
5
25 µg DNA


b) IgG Anti-ss DNA ELISA
OD 405 nm
1·1
1·0
0·8
0·6
0·4
0·2
1
5
25 µg DNA

The most likely explanation for the inhibition of SLE sera binding to DNA by ssDNA and the inhibition of the same sera binding to ssDNA by DNA (Fig.3a,b) is that a fraction of the serum anti-DNA antibodies bound to both ssDNA and DNA and recognized common structural or conformational determinant(s) present on both antigens.

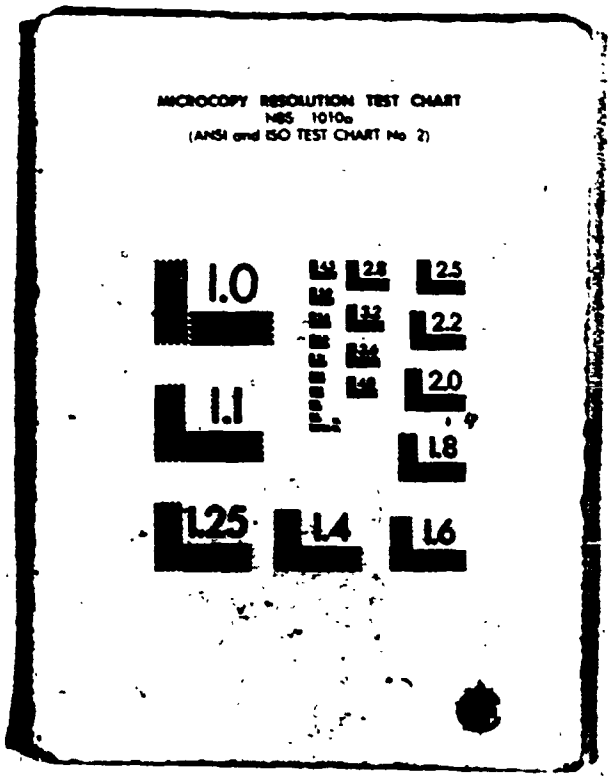
3.2. Comparison of Anti-DNA Antibody Determinations by ELISA, Farr Assay and Solid Phase Radioimmunoassay.

The anti-DNA antibody ELISA was compared with two other methods commonly employed for anti-DNA antibody measurements; namely, the Farr assay and a solid phase radioimmunoassay (RIA). Figure 4 shows ELISA IgG and IgM anti-DNA antibody levels as indicated by OD_{405nm} and Farr assay anti-DNA antibody levels as indicated by cpm in the same 25 sera. The ELISA results were obtained without prior knowledge of the anti-DNA antibody levels determined by the Farr assay. The latter assay was performed by personnel in a different laboratory (Clinical Immunology Laboratory, University of Western Ontario). The anti-DNA antibody values obtained using these two methods showed a high positive correlation. The coefficient of correlation (r) was found to be 0.821 ($p < 0.01$) (Fig.4). A similar significant cross correlation ($r = 0.810$, $p < 0.01$) was obtained in the determination of IgG serum anti-DNA antibodies by ELISA and by solid phase RIA (Fig.5).

3.3. Detection of Anti-DNA Antibodies in Supernatants from Short Term Cultures of Circulating or Tonsillar Lymphoid Cells.

Figure 4.

Comparison between serum anti-DNA antibody determination by ELISA and by Farr assay. Correlation coefficient (r)= 0.821, ($p < 0.01$). ELISA assay was developed using goat anti-human IgM or IgG antibodies. OD_{405nm} represents IgM anti-DNA plus IgG anti-DNA antibodies. Antigen in ELISA - CT DNA; antigen in Farr assay - ^{14}CDNA from E. coli.


MICROCOPY RESOLUTION TEST CHART
NBS 1010a
(ANSI and ISO TEST CHART No 2)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

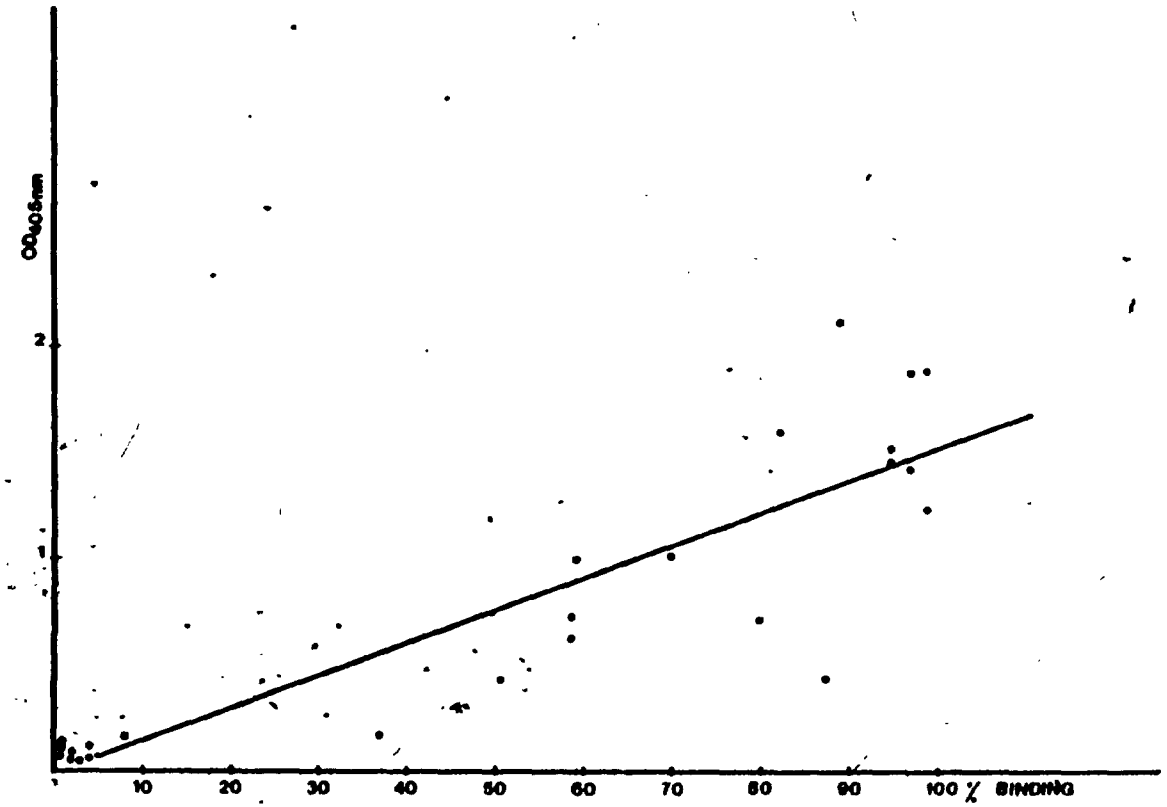
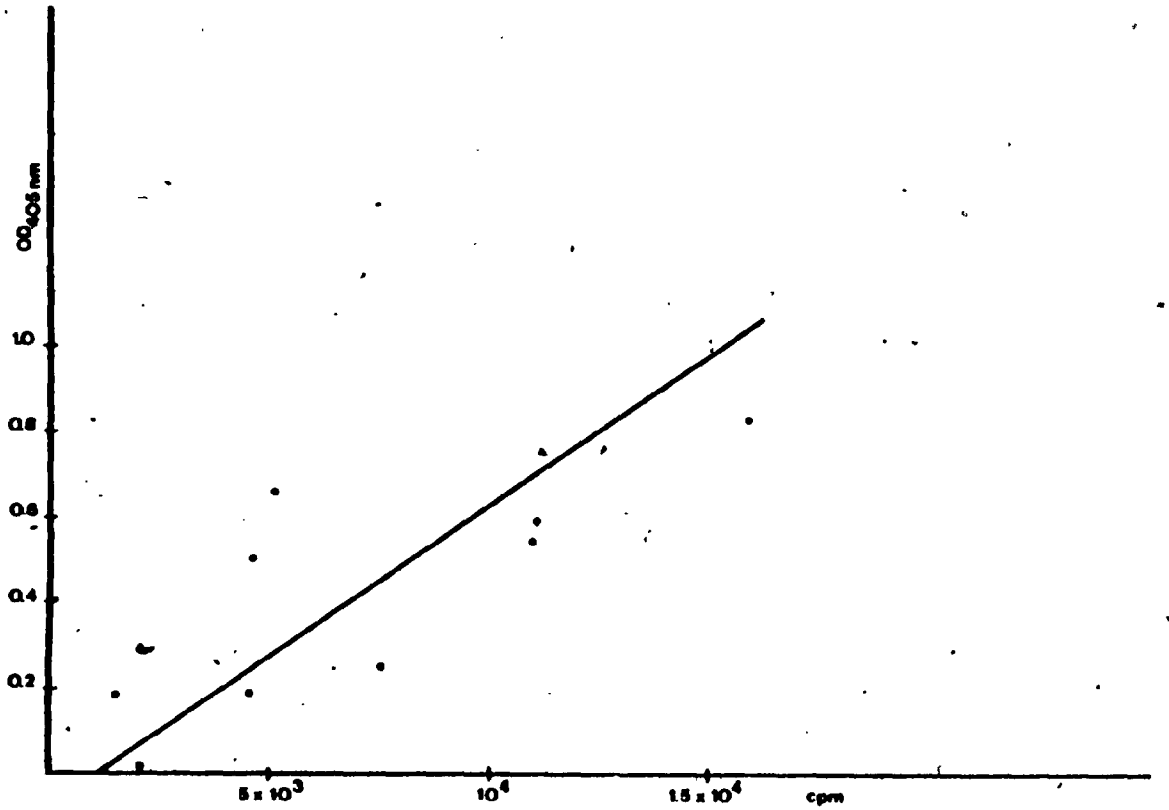

OD405nm
2
1
10
20
30
40
50
60
70
80
90
100 % BINDING

Figure 5.

Comparison between serum IgG anti-DNA antibody determination by ELISA and by solid phase radioimmunoassay. Correlation coefficient (r)= 0.810, ($p < 0.01$). Antigen used in both assays was CT DNA. Sera were diluted 1:1000 for testing.


$OD_{405\,nm}$
1.0
0.8
0.6
0.4
0.2
5×10^3
10^4
1.5×10^4
cpm

Peripheral blood lymphocytes from normals and SLE patients or tonsillar lymphoid cells from normal individuals were cultured in the presence or absence of optimal doses of pokeweed mitogen (PWM). At the end of the 7 day culture period, cell-free supernatants were removed. The level of anti-DNA antibodies in these supernatants was evaluated by using the ELISA with ssDNA or DNA as antigen. The anti-DNA antibody responses obtained with the cells cultured at 1 x 10^6 cells/ml in 1 ml are shown in Fig. 6. Anti-DNA antibodies were detected by ELISA with SLE circulating lymphocytes (88% vs ssDNA, 73% vs DNA), tonsillar lymphoid cells (78% vs ssDNA, 10% vs DNA) and normal peripheral blood lymphocytes (36% vs ssDNA, 10 % vs DNA). IgM anti-nucleic acid antibodies were obtained with all sources of lymphoid cells, but IgG antibodies were obtained only with SLE peripheral blood lymphocytes. These IgG anti-DNA antibodies were detected in one of eleven (9%) SLE peripheral blood lymphocyte cultures at 1 x 10^6 cells/ml (Fig. 6b). At the higher cell concentration of 5 x 10^6 cells/ml in 1 ml, the IgG antibody responses to DNA were more frequent. They were found in 10/17 (59%) SLE peripheral blood lymphocyte culture supernatants and in 2/12 (17%) normal peripheral blood lymphocyte culture supernatants (Fig. 7). At this high cell concentration, IgM anti-DNA antibodies were detected in 9/17 (53%), 3/12 (25%), and 2/11 (18%) culture supernatants from SLE, normal peripheral blood lymphocytes and tonsils respectively.

The spontaneous response of cultured lymphoid cells was also evaluated (Fig. 8). Although spontaneous anti-DNA antibody

Figure 6.

Anti-ssDNA (a, c, e) and anti-DNA (b, d, f) responses of PWM stimulated SLE peripheral blood lymphocytes (a, b), normal peripheral blood lymphocytes (e, f) or tonsillar lymphoid cells (c, d) measured by ELISA. Data represents peak responses of 1 x 10^6 PWM stimulated lymphoid cells cultured for 7 days (x, IgM; ●, IgG anti-ssDNA or anti-DNA antibodies).

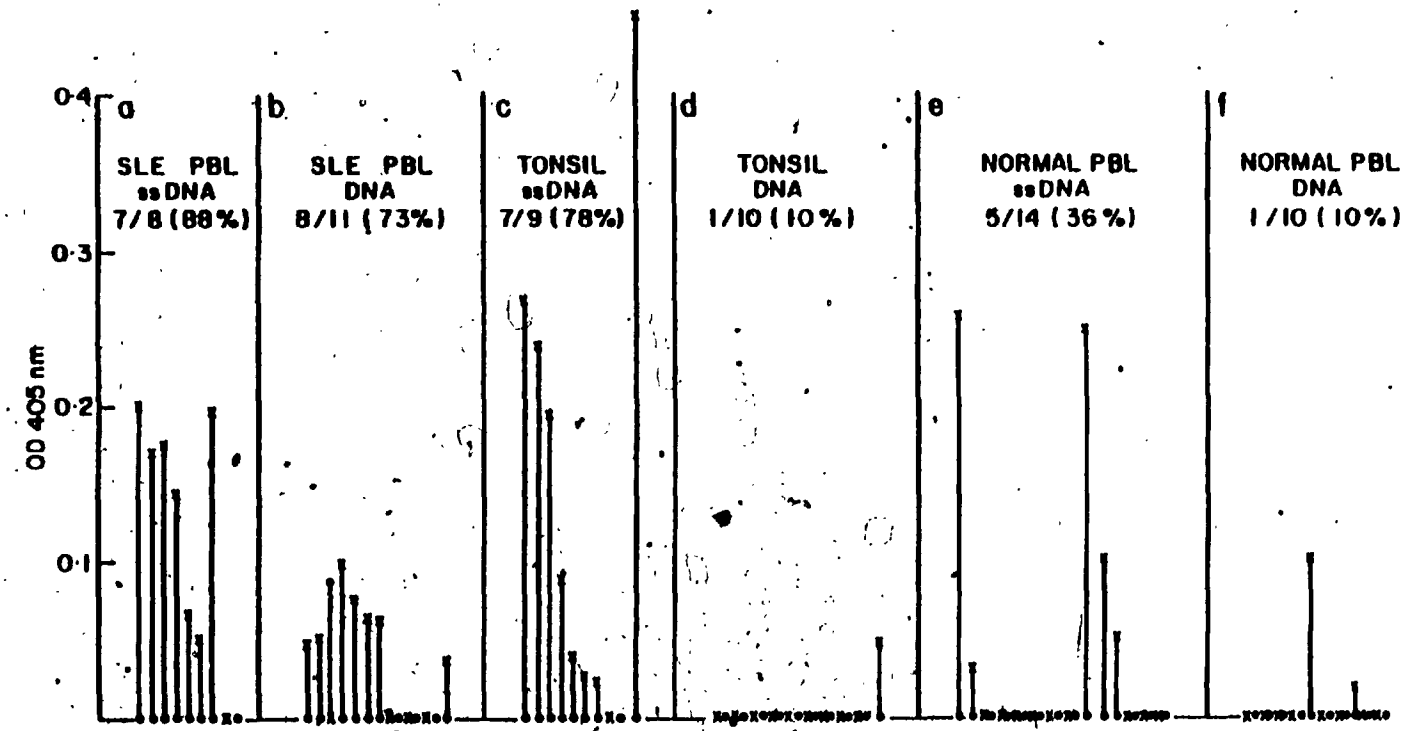

0·4
0·3
0·2
0·1
OD 405 nm
a
SLE PBL
ssDNA
7/8 (88%)
b
SLE PBL
DNA
8/11 (73%)
c
TONSIL
ssDNA
7/9 (78%)
d
TONSIL
DNA
1/10 (10%)
e
NORMAL PBL
ssDNA
5/14 (36%)
f
NORMAL PBL
DNA
1/10 (10%)

Figure 7.

Anti-DNA responses of PWM stimulated SLE peripheral blood lymphocytes, normal peripheral blood lymphocytes or tonsillar lymphoid cells measured by ELISA. Data represents peak responses of 5×10^6 PWM stimulated lymphoid cells cultured for 7 days (x, IgM; •, IgG anti-DNA antibodies).

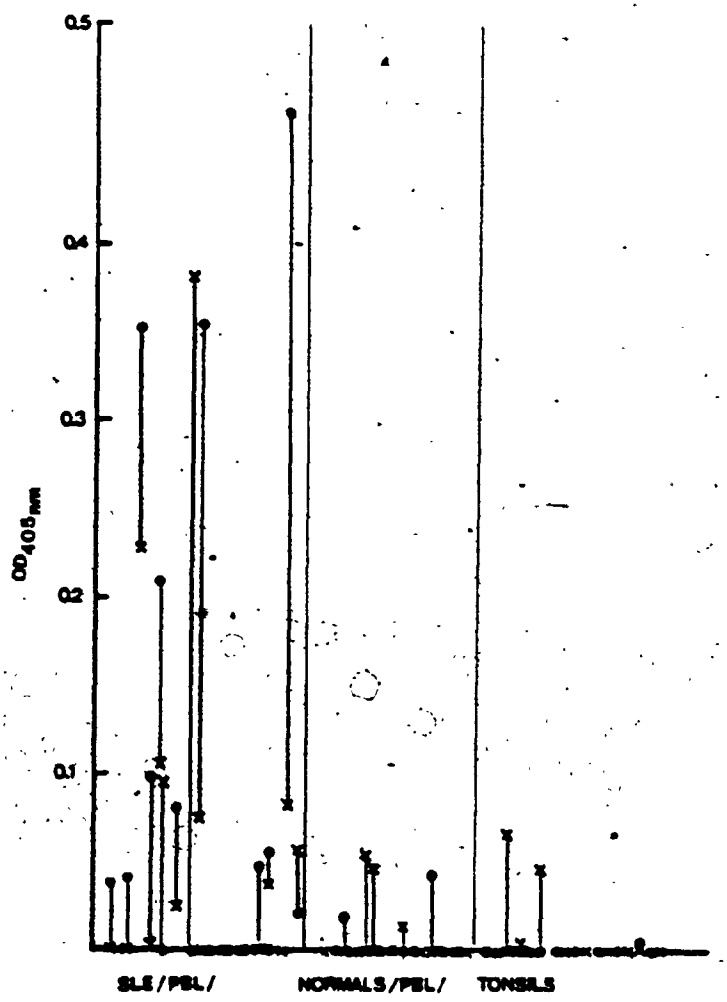
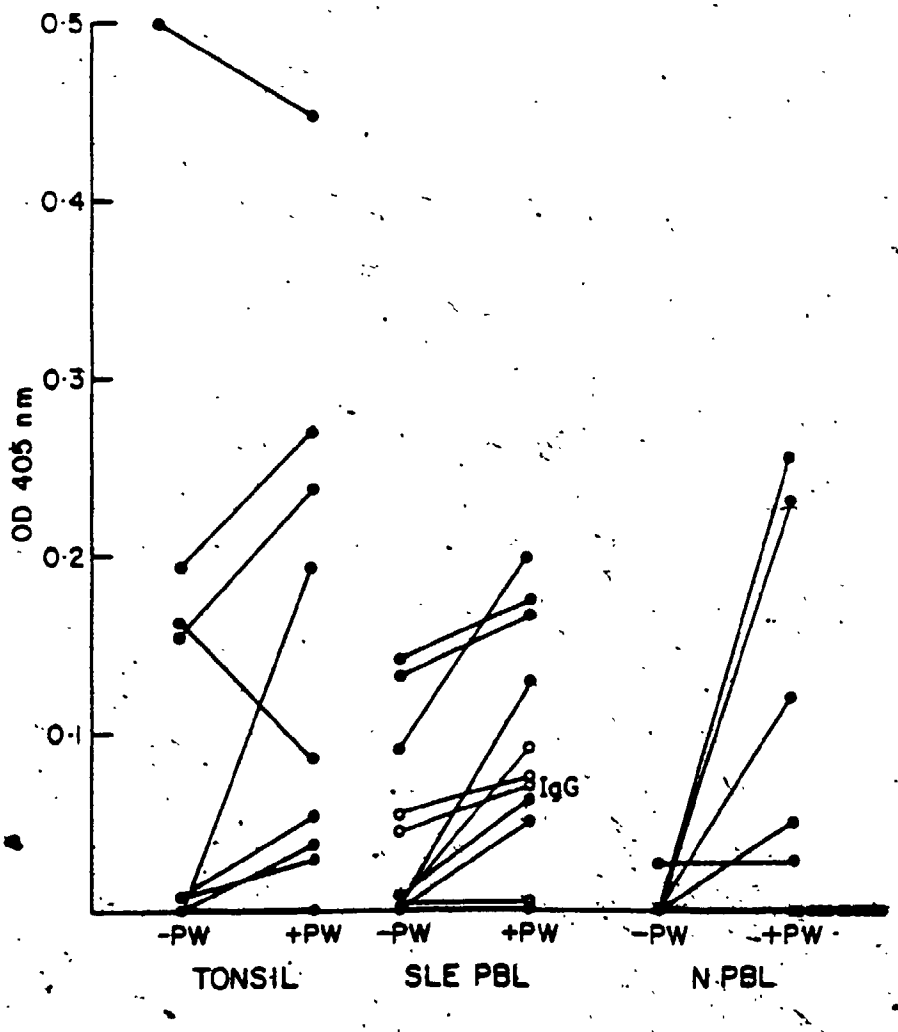

0.5
0.4
0.3
0.2
0.1
OD405nm
SLE/PBL/
NORMALS/PBL/
TONSILS

Figure 8.

Spontaneous and PWM stimulated anti-ssDNA (●) and anti-DNA (o) antibody responses from 1 x 10^6/ml cell cultures of SLE or normal peripheral blood lymphocytes or tonsillar lymphocytes. All anti-DNA and anti-ssDNA antibodies were of IgM class except one which was of IgG class as indicated.


0·5
0·4
0·3
0·2
0·1
OD 405 nm
IgG
-PW
+PW
-PW
+PW
-PW
+PW
TONSIL
SLE PBL
N PBL

responses were seen regularly with SLE peripheral blood lymphocytes (43%–3/7 vs ssDNA, 50%–2/4 vs DNA) and with tonsillar lymphoid cells (44%–4/9 vs ssDNA, 0% vs DNA), spontaneous responses were rarely seen with normal circulating lymphocytes (6%–1/16 vs ssDNA, 0% vs DNA).

3.4. Cellular Requirements for in vitro Anti-DNA Antibody Responses.

The cellular requirement for the in vitro antibody response to ssDNA was examined by culturing nylon wool-adherent (B) cells with varying concentrations of nylon passed (T) cells (Table 4). These experiments revealed in most instances a T cell dependent effect on the anti-ssDNA antibody response when tonsillar lymphoid cells were employed. These responses were optimal at 2.5×10^5 /ml T cell concentration in the majority of the cultures, and a further increase in T cell concentration had an inhibitory effect on the anti-ssDNA antibody production. In contrast, mixtures of B and varying T cell concentrations from peripheral blood lymphocytes of normal donors did not show any detectable anti-nucleic acid antibody responses. This negative result is not shown.

Both tonsillar and blood B and T lymphocytes from the same donor, taken at the same time, were available for comparison in some instances. In mixing experiments, peripheral blood T cells could provide some help for autologous tonsillar B cells but at much lower magnitude than did tonsillar T cells (Table 4). Similarly, tonsil T cells but not autologous peripheral blood T cells could provide some help for autologous peripheral blood B cells. These data indicate

Table 4. Cellular Requirements for the *in vitro* Anti-DNA Antibody Response.

Donor	Anti-ssDNA Antibody (OD_{405nm})[1,3]											
	$B_{T^+}T_T$ No. T cells ($\times 10^{-5}$)				$B_{B^+}T_T$ No. T cells ($\times 10^{-5}$)				$B_{T^+}T_B$ No. T cells ($\times 10^{-5}$)			
	.00	1.25	2.50	5.00	.00	1.25	2.50	5.00	.00	1.25	2.50	5.00
1	.05	.10	.28	.08								
2	.00	.33	.17	.12								
3	.31	.32	.58	.19								
4	.00	.00	.14	.13								
5	.00	.05	.08	.03								
6 [2]	.00		.42	.24					.00	.00	.00	.18
7	.06	.11	.32	.07				.11	.06	.02	.03	.06
8 [2]	.00	.25		.30	.00		.00	.00	.00	.00	.12	.16
9	.05		.13	.14					.05		.00	.13

1 Anti-ssDNA antibody level in culture supernatants detected by ELISA using anti-human IgM conjugate. $5x10^5$ (donors 1-5) or $1x10^6$ (donors 6-9) B cells from tonsil (B_T) or B cells from peripheral blood (B_B) cocultured for 7 days with PWM and varying numbers of autologous T cells from tonsil (T_T) or peripheral blood (T_B).

2 Peripheral blood B+T cell cultures from these donors tested: negative for anti-ssDNA.

3 Blanks indicate not tested.

that the more frequent antibody response to ssDNA of unseparated tonsillar lymphoid cells is not merely a reflection of an increased number of B cells in the tonsil, but in comparison with the circulation, reflects an increase in anti-nucleic acid antibody precursor B cells and/or T helper cells. On the other hand, tonsil and peripheral blood T cells appeared equally helpful for the PWM-induced immunoglobulin response as observed by others in the same laboratory (personal communication from D.A. Bell and J. St. Germain).

3.5. Hemolytic Plaque Forming Cell Response of Tonsillar Lymphoid Cells.

In addition to measuring anti-DNA responses by evaluating the cell free supernatant by ELISA, the cultured lymphocytes were harvested at 7 days and examined for anti-ssDNA plaque forming cells (PFC) (Fig. 9). In all instances, PWM was necessary to stimulate antibody responses detected by this means. The tonsil in 9/11 (81.8%) of the cases gave responses varying from 55-240 (mean $\pm$ SD = 113 $\pm$ 82) PFC/10^6 mononuclear cells (MNC) plated at day 7 of culture. In 8/11 (73%) of the supernatants from these tonsillar lymphoid cell cultures, antibodies reactive with ssDNA (mean $\pm$ SD = 0.091 $\pm$ 0.102) were detected by ELISA (Fig. 9). There was no significant positive correlation between the anti-ssDNA antibody response measured by using hemolytic PFC assay and IgM anti-ssDNA measured by ELISA in the cell free supernatant from the same tonsil on day 7. In addition, immunoglobulin PFC were enumerated by using a reverse hemolytic PFC

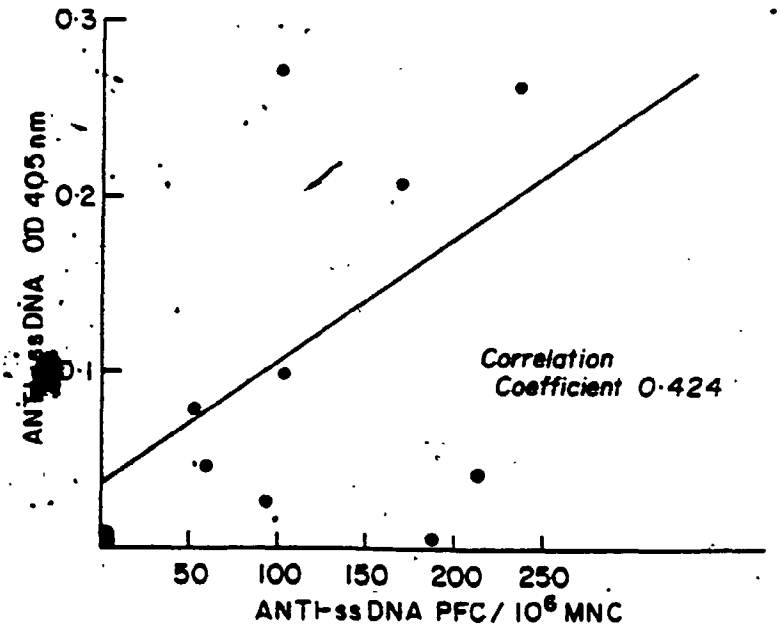


Figure 9.

Correlation of anti-ssDNA antibody PFC and anti-ssDNA antibody ELISA in cultures of PWM stimulated tonsillar lymphoid cells.

assay (Fig. 10). Immunoglobulin PFC mean ± SD was equal to 8929 ± 6705 PFC/10^6 MNC. The magnitude of this response, reflecting total immunoglobulin synthesis after PWM stimulation, correlated directly with the anti-ssDNA PFC response ($r = 0.673$, $p < 0.05$).

3.6. Production of Human:Human Hybridomas and Screening for Anti-DNA Antibody.

The observations described above on antibody production by lymphoid cells in short term cultures indicated that anti-DNA autoantibody precursor B cells exist in normal individuals and that they have the ability to be expressed in vitro.

To further study these normal anti-DNA antibody responses, including their molecular genetics, human:human hybridomas were generated. For hybridoma production, tonsillar lymphoid cells were chosen, since they were capable of frequent and often spontaneous secretion of anti-ssDNA antibodies in 7 day cultures. These lymphoid cells were fused with IgG_2 kappa producing lymphoblastoid cell line GM 4672 and after the fusion procedure, 431 wells were seeded. Growth was observed in 110 wells (25.5%). The ssDNA ELISA system was used to screen for hybridomas producing anti-nucleic acid antibody, and 13 of 110 wells (11.8%) were positive. Thus, positive wells for anti-ssDNA antibodies constituted 3% of the total 431 wells seeded.
IgM and IgG levels were also quantitated by ELISA in hybridoma supernatants which were positive or negative for anti-ssDNA antibodies. 85/110 hybridoma supernatants

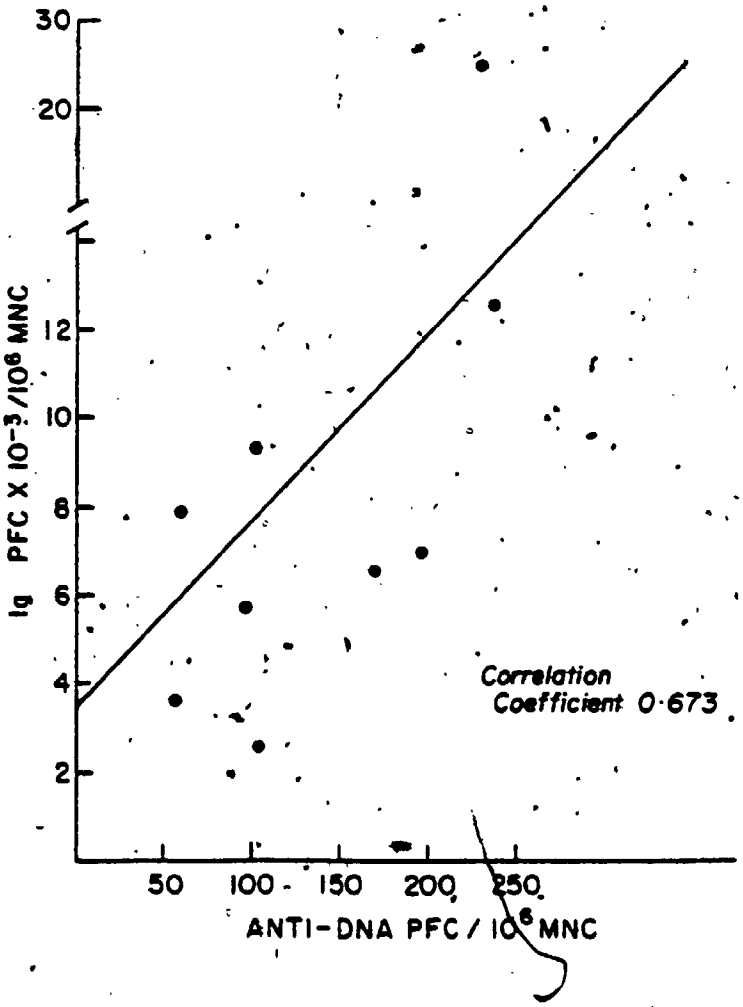


Figure 10.

Correlation between anti-ssDNA antibody PFC and immunoglobulin PFC in 7 day cultures of PWM stimulated tonsillar lymphoid cells.

were avaiable for testing, of which 13 were positive and 72 were negative for anti-ssDNA antibody. Both positive and negative hybridoma supernatants contained IgM; eight contained only IgG. None of the latter showed binding to ssDNA or DNA. The IgM levels of the positive hybridoma supernatants ranged from 0.45 to 8 /ug/ml (mean ± SD = 1.33 ± 1.4), but the anti-DNA antibody negative hybridoma supernatants contained 0.5 /ug/ml IgM or less. Despite the lower levels of IgM in the negative hybridomas, the lack of binding to ssDNA was not a reflection of lack of sensitivity of the ELISA assay, since positive hybridoma supernatants showed binding to ssDNA when these were diluted to a concentration of IgM comparable to that in the anti-ssDNA antibody negative supernatants (Fig. 11). In 60% of IgM anti-ssDNA antibody positive hybridoma supernatants, 15 ng/ml or less IgG of the lymphoblastoid cell GM 4672 origin was detected. The IgG in these supernatants did not show any reactivity with nucleic acid antigens.

The overall yield of anti-ssDNA antibody producing hybridomas is a function of both the probability of obtaining successful fusion and the probability that successful fusion will lead to anti-ssDNA antibody production. The data in Table 5 suggests that these variables may depend on both the concentration of cells used in postfusion cultures as well as the ratio of lymphocyte to GM 4672 cells used in the fusion. For any given fusion ratio of cells, the probability of finding hybridomas after 24 h postfusion recovery and 4 weeks culture period was higher when the cells were cultured at 4×10^5 cells/well than at 1×10^5 cells/well; however, at the lower cell concentration (10^5 cells/well), there was a greater probability of

Figure 11.

Binding to ssDNA of hybridoma supernatants containing high and low IgM concentration (hybridoma supernatant KIM 4.4 ●——●; KIM 7.2 ■——■; KIM 1.6 ▲——▲).

(KIM 4.4 - 0.4 µg/ml IgM; KIM 7.2 - 8 µg/ml IgM; KIM 1.6 - 0.4 µg/ml IgM)

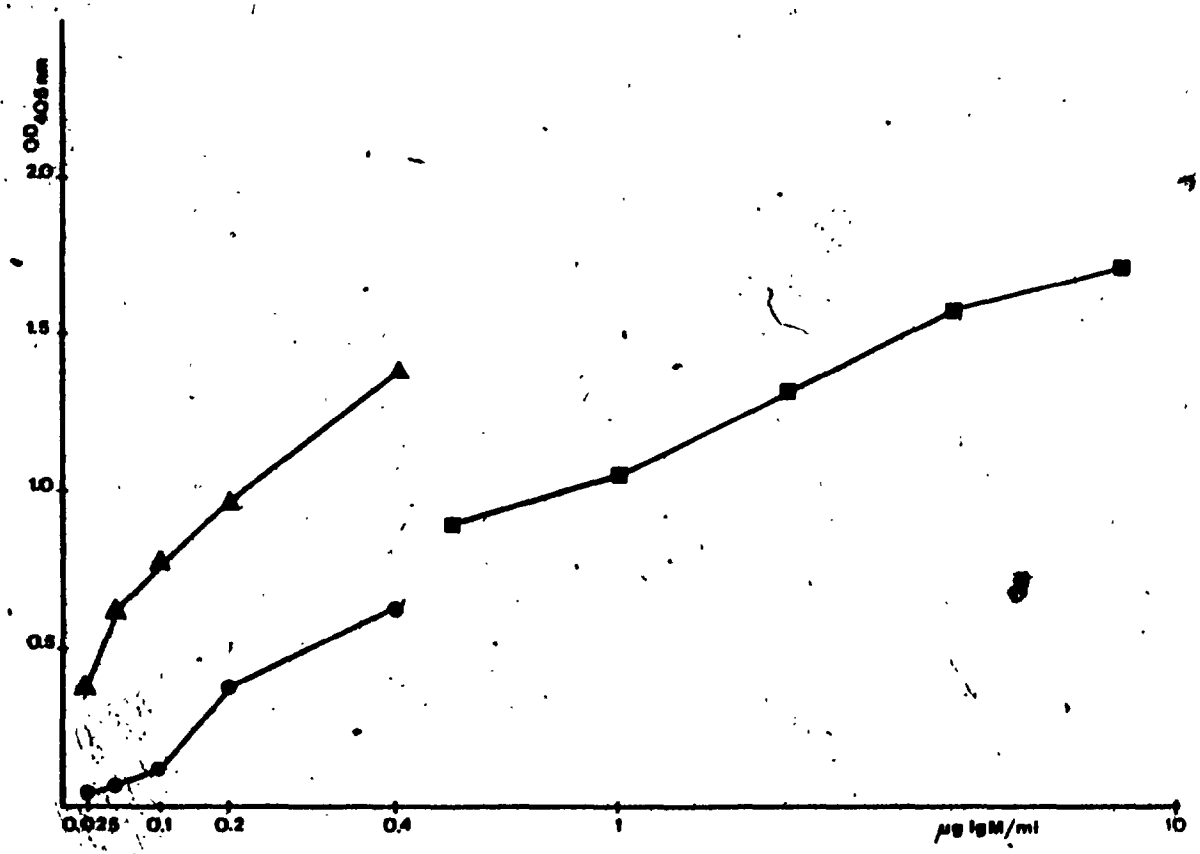

OD 405 nm
2.0
1.5
1.0
0.5
0.025
0.1
0.2
0.4
1
10
µg IgM/ml

Table 5. Growth of Hybridomas and Anti-ssDNA Antibody Production

Lymphocyte/GM4672 cell ratio	5:1			1:1		
Cells per well	4×10^5	2×10^5	1×10^5	4×10^5	2×10^5	1×10^5
Fraction and percent of wells positive for hybridoma growth	14/43 (33%)	2/13 (15%)	8/69 (12%)	39/45 (87%)	14/107 (13%)	33/154 (21%)
Fraction and percent of growth-positive wells also positive for anti-ssDNA antibody	1/14 (7%)	0/2 (0%)	1/8 (12.8%)	3/39 (7.7%)	1/14 (7%)	7/33 (21%)
Percent of all seeded wells positive for both hybridoma growth and anti-ssDNA antibody production	2.3%	0%	1.5%	6.7%	0.9%	4.4%

detecting hybridomas that produce anti-ssDNA antibodies. The probability of fusion and anti-ssDNA antibody production also appears to depend on the lymphocyte/GM 4672 cell ratio with a ratio of 1:1 producing the highest values (87% for fusion, 21% for anti-ssDNA antibody production). As indicated in Table 5 the best overall yield of anti-ssDNA antibody producing hybridomas (6.7%) was found with a 1:1 cell ratio for fusion and a culture concentration of 4×10^5 cells/well.

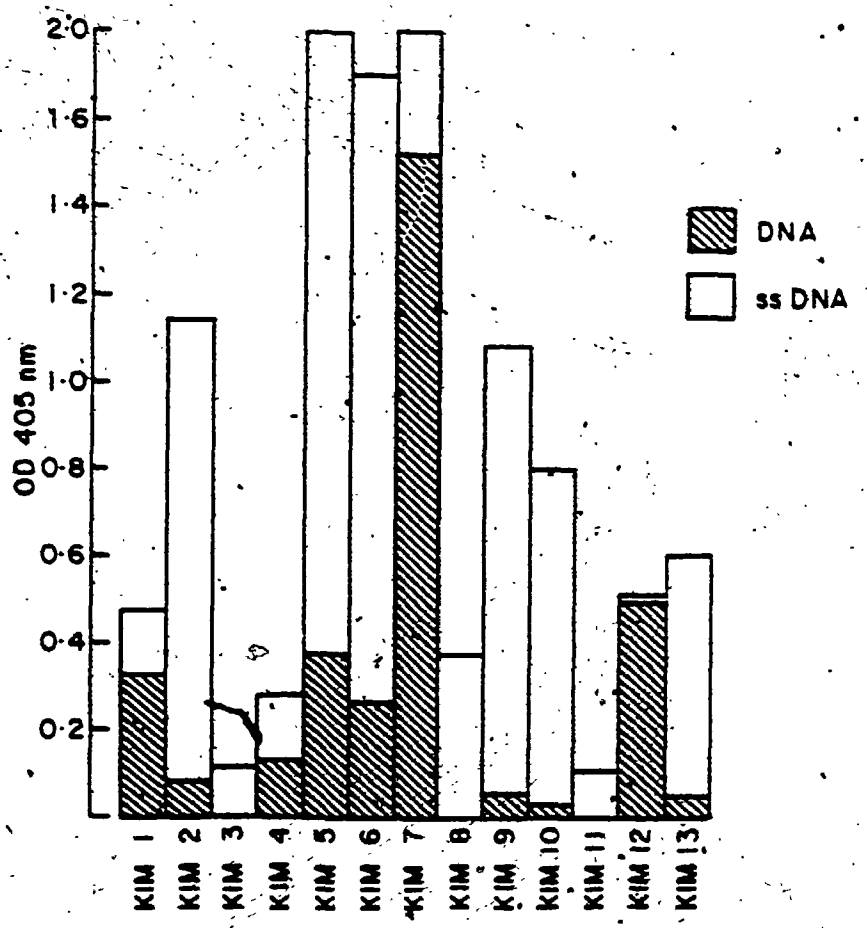
The 13 supernatants positive for anti-ssDNA antibodies were also tested for binding to undenatured DNA. All hybridomas except KIM 3, KIM 8 and KIM 11 reacted with DNA (Fig. 12). Binding of supernatahts to ssDNA was greater that to DNA except for KIM 12 which reacted equally well with both ssDNA and DNA.

3.7. Cloning of Primary Hybridomas.

Cloning was successful in 9/13 primary hybridomas and yielded 34 clones. Four of these 13 primary hybridomas (KIM 2, KIM 3, KIM 6, and KIM 8) could not be cloned. Figure 13 shows the binding of the supernatants from 34 clones to ssDNA, DNA, poly(dA-dT), polydG.poly dC, S/N DNA (150 base pairs, 80% double stranded, 20% single stranded), RNA, cardiolipin and human IgG. All antibodies which bound to these antigens were of the IgM isotype, as determined by the use of a goat anti-human IgM antibody in ELISA. Using anti-human IgG and IgM antibodies, no antibodies to any of the eight antigens used were detected in the supernatants of the fusion partner GM 4672 cell line

Figure 12.

Binding to DNA and ssDNA of antibodies from hybridoma supernatants obtained during the initial screening by ELISA.



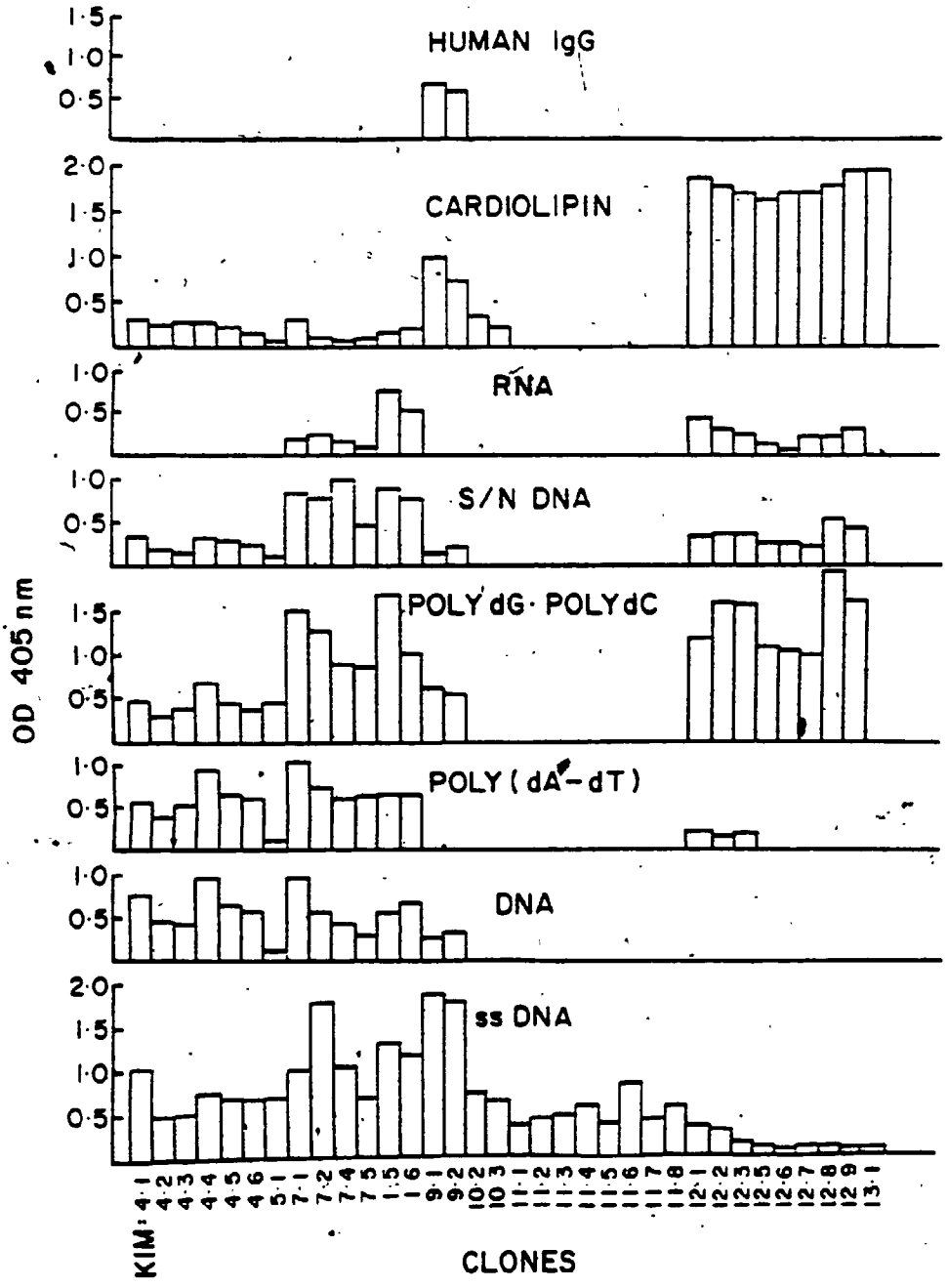
KIM 1–KIM 13 / laboratory designation of positive supernatants

cultured alone. All supernatants except KIM 11.1 - KIM 11.8 showed polyspecificity in reacting with the antigens used (Fig. 13).

There was no relationship between the IgM concentration found in the supernatants of the various clones and the number of nucleic acid antigens bound. The following examples illustrate this. KIM 11 (KIM 11.1-KIM 11.8) supernatants containing IgM in the range of 0.92-1.48 µg/ml were monospecific for ssDNA, while KIM 12 (KIM 12.5-12.9) supernatants containing similar IgM concentrations (0.6-1.3 µg/ml) bound to polydG.polydC, S/N DNA, RNA and cardiolipin in addition to ssDNA (Fig. 13). Derived from the same primary hybridoma, KIM 12 clones 12.1-12.3 and 12.5-12.9 producing similar amounts of IgM showed a similar reactivity pattern with the exception of KIM 12.1-12.3 but not KIM 12.5-12.9 binding to poly(dA-dT) (Fig. 13). KIM 7.2 containing 8 µg/ml IgM and KIM 9.1 supernatant containing 1.2 µg/ml IgM showed similar binding to ssDNA but differed in binding to other antigens. KIM 7.2 bound better to DNA, polydG.polydC, S/N DNA but less to cardiolipin than KIM 9.1 supernatant. KIM 9.1 also had reactivity with human IgG, while KIM 7.2 did not. KIM 9.1 showed no binding to RNA and poly(dA-dT) in contrast to KIM 7.2. (Fig. 13). Identical 0.4 µg/ml concentrations of IgM in KIM 1.6 and KIM 4.4 supernatants produced two fold reactivity differences of these supernatants with ssDNA (Fig. 11). In addition to better ssDNA binding, KIM 1.6 also showed higher binding to polydG.polydC and S/N DNA and less binding to DNA and poly(dA-dT) than KIM 4.4. KIM 1.6 but not KIM 4.4 reacted with RNA (Fig. 13). IgM antibody at concentrations of 0.9 µg/ml from KIM 13.1 supernatant showed almost

Figure 13.

Binding of supernatant antibodies from cloned hybridomas to ssDNA, DNA, poly(dA-dT), polydG.polydC, S/N DNA, RNA, cardiolipin and human IgG.


HUMAN IgG
CARDIOLIPIN
RNA
S/N DNA
POLY dG · POLY dC
POLY (dA-dT)
DNA
ss DNA
OD 405 nm
1·5
1·0
0·5
2·0
KIM-4-1 4-2 4-3 4-4 4-5 4-6 5-1 7-2 7-4 7-5 7-6 9-1 9-2 10-3 10-1 2-3 3-4 5-6 7-8 12-2 12-3 12-5 12-6 12-7 12-8 12-9 13-1
CLONES

undetectable binding to ssDNA but high binding to cardiolipin and no binding to other antigens. KIM 9.2 with a comparable IgM concentration of 0.7 µg/ml to KIM 13.1 was reactive with ssDNA, DNA, polydG.polydC and S/N DNA in addition to cardiolipin (Fig. 13). KIM 9.2 had half the reactivity of KIM 13.1 with cardiolipin. These examples indicate that the antigen binding differences observed in direct ELISA with different hybridoma supernatants were not related to IgM antibody concentration in these supernatants since the unique reactivity patterns were observed between hybridomas producing similar amounts of IgM.

The patterns of specificity were characterized by the ratio of antibody reactivity with each antigen to reactivity with a reference antigen . The reference antigen was chosen as ssDNA since, because of the selection method used, it was universally reactive with all the hybridoma supernatants tested. Table 6 lists for all clones the ratio of antibody reactivity with each antigen to reactivity with ssDNA. From the data in Fig. 13 and Table 6, the relative reactivity of the clones for certain nucleic acid antigens allowed these clones to be separated into eight different groups. Table 7 summarizes these groups and the range of their reactivity ratios. Groups 1-3 show no binding to double stranded antigens, while groups 4-8 do. Group 2 and 3 bind cardiolipin in addition to ssDNA. Group 4 binds human IgG in addition to ssDNA, DNA, polydG.polydC, S/N DNA and cardiolipin. Groups 7a and 7b (KIM 7 and KIM 1 clones respectively) appear very similar in the spectrum of their reactivity ratios. Group 8 reacts with RNA but not with DNA.

Table 6. Reactivity Ratios: Antibody Reactivities (OD_{405nm}) with Each Antigen, Divided by the Reactivity with ssDNA.

Clone	Cardiolipin/ ssDNA	RNA/ ssDNA	S/N DNA/ ssDNA	poly dG.poly dC/ ssDNA	poly(dA-dT)/ ssDNA	DNA/ ssDNA	IgG/ ssDNA
KIM 4.1	0.3	0	0.3	0.4	0.5	0.7	0
4.2	0.4	0	0.4	0.5	0.7	0.8	0
4.3	0.5	0	0.3	0.5	0.9	0.7	0
4.4	0.3	0	0.5	0.8	1.3	1.3	0
4.5	0.3	0	0.5	0.6	1.0	0.9	0
4.6	0.2	0	0.4	0.5	0.9	0.8	0
5.1	0.1	0	0.2	0.5	0.2	0.2	0
7.1	0.3	0.2	0.8	2.2	1.1	1.0	0
7.2	0.1	0.1	0.4	0.7	0.4	0.3	0
7.4	0.05	0.1	0.9	0.8	0.6	0.4	0
7.5	0.1	0.1	0.6	1.1	0.9	0.4	0
1.5	0.1	0.6	0.6	1.3	0.5	0.4	0
1.6	0.2	0.5	0.6	0.8	0.6	0.6	0
9.1	0.5	0	0.05	0.3	0	0.1	0.4
9.2	0.4	0	0.1	0.3	0	0.1	0.3
10.2	0.5	0	0	0	0	0	0
10.3	0.3	0	0	0	0	0	0
11	0	0	0	0	0	0	0
12.1	5.4	1.1	0.9	3.3	0.4	0	0
12.2	6.0	1.0	1.2	5.2	0.3	0	0
12.3	11.7	1.7	2.3	10.3	1.0	0	0
12.5	16.5	1.0	2.5	11.0	0	0	0
12.6	17.5	0.5	1.5	10.5	0	0	0
12.7	17.5	2.0	2.0	9.5	0	0	0
12.8	18.0	2.0	5.5	19.0	0	0	0
12.9	40.0	6.0	8.0	32.0	0	0	0
13.1	40.0	0	0	0	0	0	0

Table 7. Grouping of Clones According to Reactivity Ratios.

Group	Ratio of reactivity for single-stranded nucleic acid or analogue		Ratio of reactivity for double-stranded nucleic acid					Clones in each group
	Cardiolipin/ ssDNA	RNA/ ssDNA	S/N DNA ssDNA	Poly dG.Poly dC/ ssDNA	Poly (dA-dT)/ ssDNA	DNA/ ssDNA	IgG/ ssDNA	
1	0	0	0	0	0	0	0	KIM 11
2	0.3-0.5	0	0	0	0	0	0	KIM 10
3	40	0	0	0	0	0	0	KIM 13
4	0.4-0.5	0	0.0-0.1	0.3	0	0.1	0.3-0.4	KIM 9
5	0.2-0.5	0	0.3-0.4	0.4-0.8	0.5-1.3	0.7-1.3	0	KIM 4
6	0.1	0	0.2	0.5	0.2	0.2	0	KIM 5
7a	0.05-0.3	0.1-0.2	0.4-0.9	0.7-2.2	0.4-1.1	0.3-1	0	KIM 7
7b	0.1-0.2	0.5-0.6	0.6	0.8	0.6	0.4	0	KIM 1
8	5.4-40	1-6	0.9-8	3.3-32	0-1.0	0	0	KIM 12

None of the hybridoma supernatants positive for binding to nucleic antigens and/or cardiolipin and/or human IgG showed binding to DNP-OA, KLH, human histones or humar serum albumin. These negative results are not shown.

3.8. Binding of the Hybridoma Anti-DNA Antibodies to Human Epithelial Cells.

Ten hybridoma IgM anti-nucleic acid antibodies were used in immunofluorescence staining of human epithelial (HEp2) cells. The antigen specificity of these anti-DNA antibodies and their staining of the HEp2 cell components is presented in Table 8. As indicated in Table 8, 8/10 monoclonal anti-DNA antibodies were reactive with HEp2 cells and three immunofluorescence staining patterns were observed: a) a strong fibrillar cytoplasmic pattern resembling the cytoskeleton (KIM: 7.2, 7.2.4, 12.3.9, 13.1), or b) a strong homogeneous nuclear staining (KIM 1.6), or c) a weak nucleolar staining (KIM: 4.6.3, 4.6.1, 11.4.6). There was no simultaneous immunofluorescence of the HEp2 cytoskeleton and intranuclear structure by any of these HEp2 reactive monoclonal anti-DNA antibodies. Cytoskeletal, nuclear and nucleolar staining could not be correlated with any nucleic acid antigen specificity of these monoclonal anti-DNA antibodies. Two out of 10 monoclonal anti-DNA antibodies (KIM: 9.1, 10.3) did not bind to the HEp2 cells. This lack of reactivity was not a result of low IgM concentration of anti-DNA antibodies in the preparations, since the relative IgM concentration as indicated by OD_{405nm} was comparable to

Table 8. Characteristics of monoclonal anti-DNA antibodies reactive with HEp2 cell components

Hybridoma	ANTIGEN SPECIFICITY[1] ssDNA	DNA	Poly dA-dT	Poly dG Poly dC	S/N DNA	RNA	Cardio-lipin	HUMAN IgG	IgM[2] OD	IgG[2] OD	HEp2 Component Stained
KIM 9.1	+	+	--	+	+	--	+	+	0.75	0	--
KIM 7.2	+	+	+	+	+	-/+	--	--	0.93	0.06	+ cytoskel
KIM 7.2.4	+	+	+	+	+	-/+	+	--	1.01	0.09	+ cytoskel
KIM 12.3.9	+	--	-/+	+	+	+	+	--	0.77	0	+ cytoskel
KIM 1.6	+	+	+	+	+	+	+	--	0.76	0	+ nucleus
KIM 4.6.3	+	+	+	+	+	--	+	--	0.91	0.05	-/+ nucleo
KIM 4.6.4	+	+	+	+	+	--	+	--	0.92	0	-/+ nucleo
KIM 10.3	+	--	--	--	--	--	+	--	0.85	0.05	--
KIM 11.4.6	+	--	--	--	--	--	--	--	1.03	0.03	-/+ nucleo
KIM 13.1	-/+	--	--	--	--	--	+	--	1,09	0.06	+ cytoskel
KIM B.S.	--	--	--	--	--	--	--	--	0.72	0.02	--
KIM B.F.	--	--	--	--	--	--	--	--	0.79	0	--
GM 4672	--	--	--	--	--	--	--	--	0	0.36	--

(--)Negative; (-/+) Weakly Positive; (+) Positive
(Cytoskel) Cytoskeleton; (Nucleo) Nucleolus

1 determined by ELISA using neat hybridoma S/N

2 determined by ELISA using ammonium sulfate precipitated hybridoma S/N. For IgM and IgG, these S/N were used at 1/20 and 1/2 dilution respectively. OD was measured at 405nm.

that of HEp2 reactive monoclonal anti-DNA antibodies (Table 8).

No HEp2 staining was observed with DNA and/or cardiolipin negative antibodies produced by KIM B.S. (Fig. 14d), KIM B.F. (Table 8), GM 4672 hybridoma fusion partner (Fig. 14e) or the pool of human IgM (Fig. 14f). The immunofluorescence staining of HEp2 nucleus by anti-DNA antibodies is presented in Fig. 14b and staining of the cytoskeleton is presented in Fig. 14a and c. No picture was taken of nucleolar HEp2 staining since the staining was very weak.

The cytoskeletal staining of HEp2 cells by other monoclonal antibodies (KIM: 7.2, 7.2.4, 12.3.9) was identical to that of KIM 13.1 and KIM 7.2 (Fig. 14a,c) as judged by microscopic analysis. An identical HEp2 cytoskeletal staining pattern was also produced by the IgM mouse monoclonal anti-vimentin antibody B.1.5.1 (Fig. 15).

3.9. Absorptions of Monoclonal Anti-DNA Antibodies by ssDNA or by Cardiolipin.

Absorption of monoclonal anti-nucleic acid antibodies by passage over plastic immobilized ssDNA or cardiolopin was performed to demonstrate that HEp2 reactivity was indeed associated with anti-ssDNA and/or cardiolipin antibodies. Single stranded DNA and cardiolipin were chosen for absorption since all the anti-nucleic acid antibodies showed specificity for both or either of these antigens. Multiple (5 or 6) passes (Fig. 16) were required to remove all anti-ssDNA or anti-cardiolpin antibody reactivity. During the sixth absorption, there was no further binding of these antibodies to ssDNA or to

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Figure 14.

Immunofluorescence staining of HEp2 cells by: a) hybridoma supernatant KIM 13.1; b) hybridoma supernatant KIM 1.6; c) hybridoma supernatant KIM 7.2; d) hybridoma supernatant B.S. e) GM 4672 cell line supernatant; f) pooled human IgM.

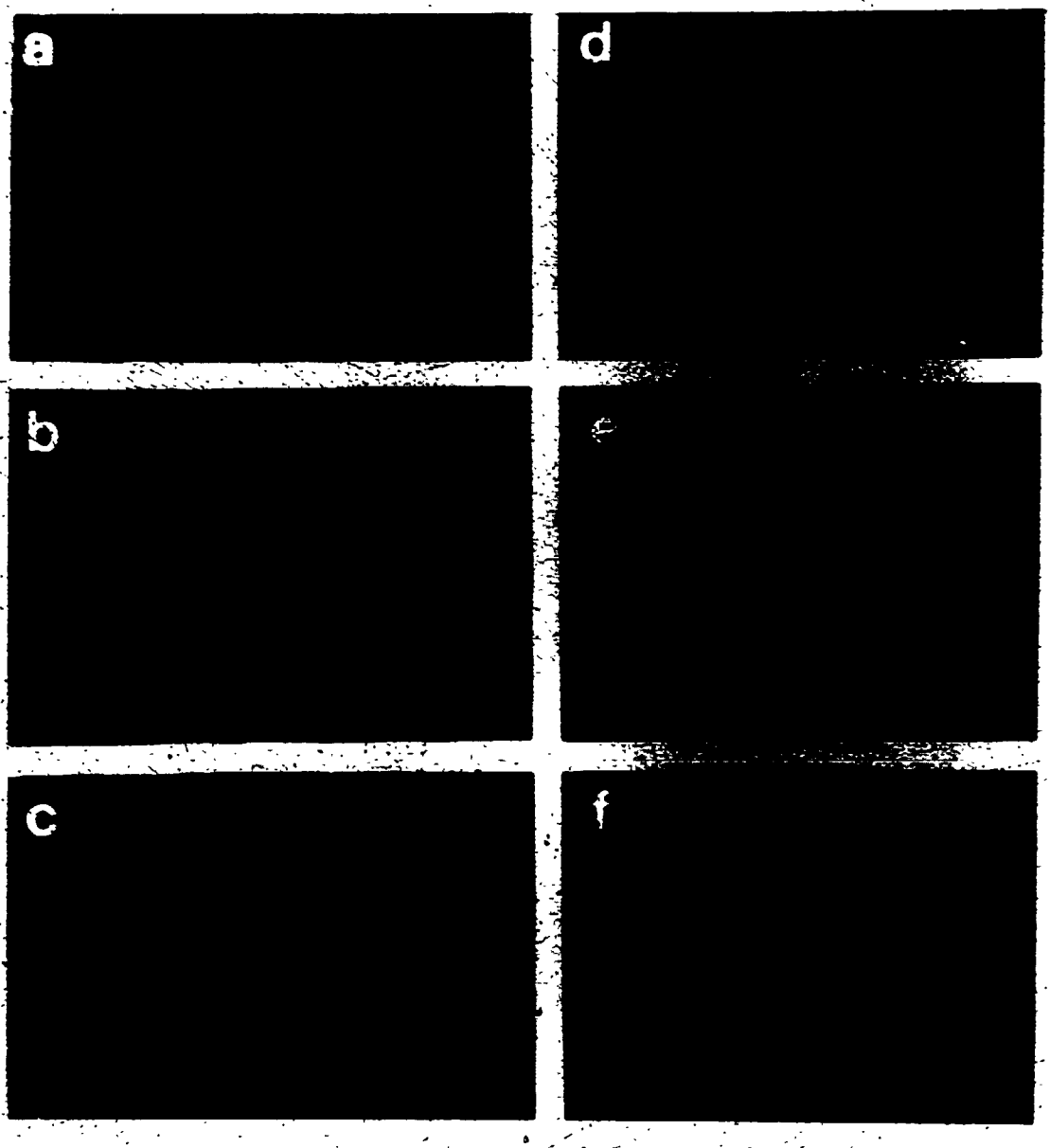

a
d
b
e
c
f

Figure 15.

Immunofluorescence staining of HEp2 cells by mouse monoclonal B11.5.1 anti-vimentin antibody.

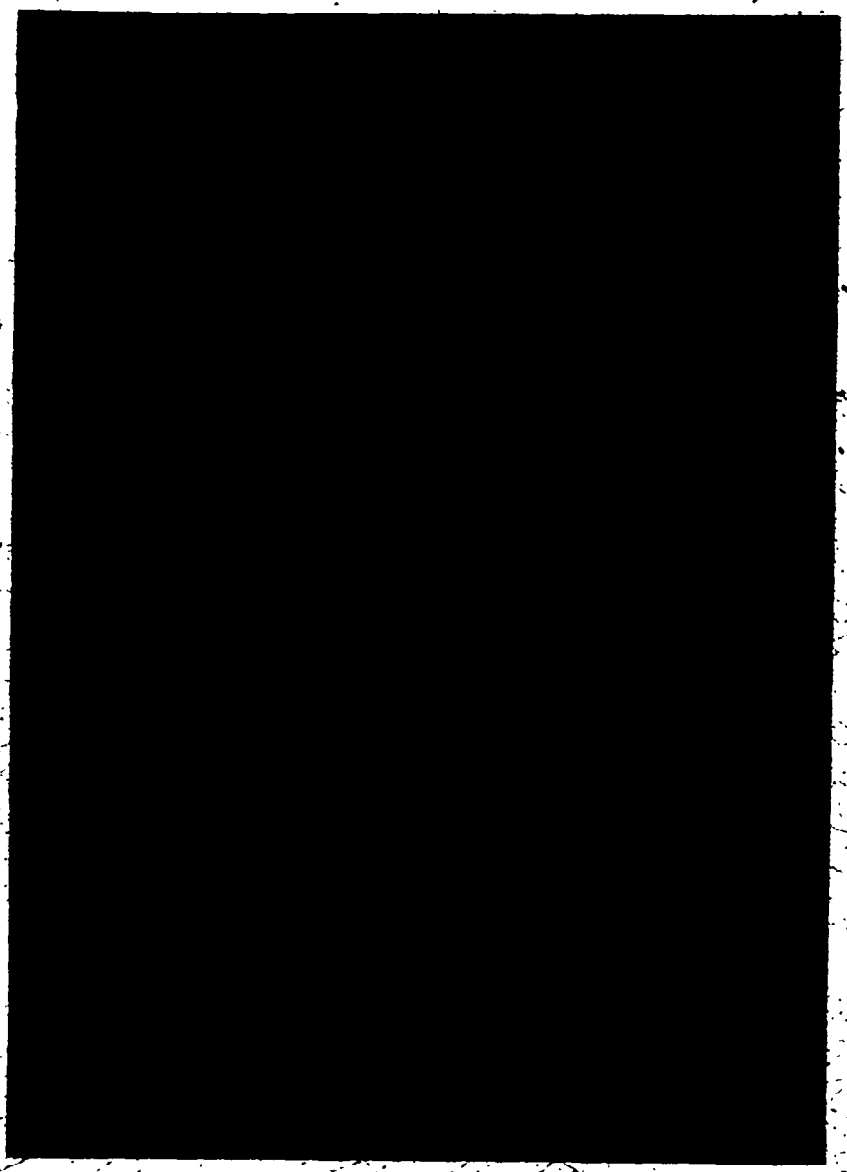
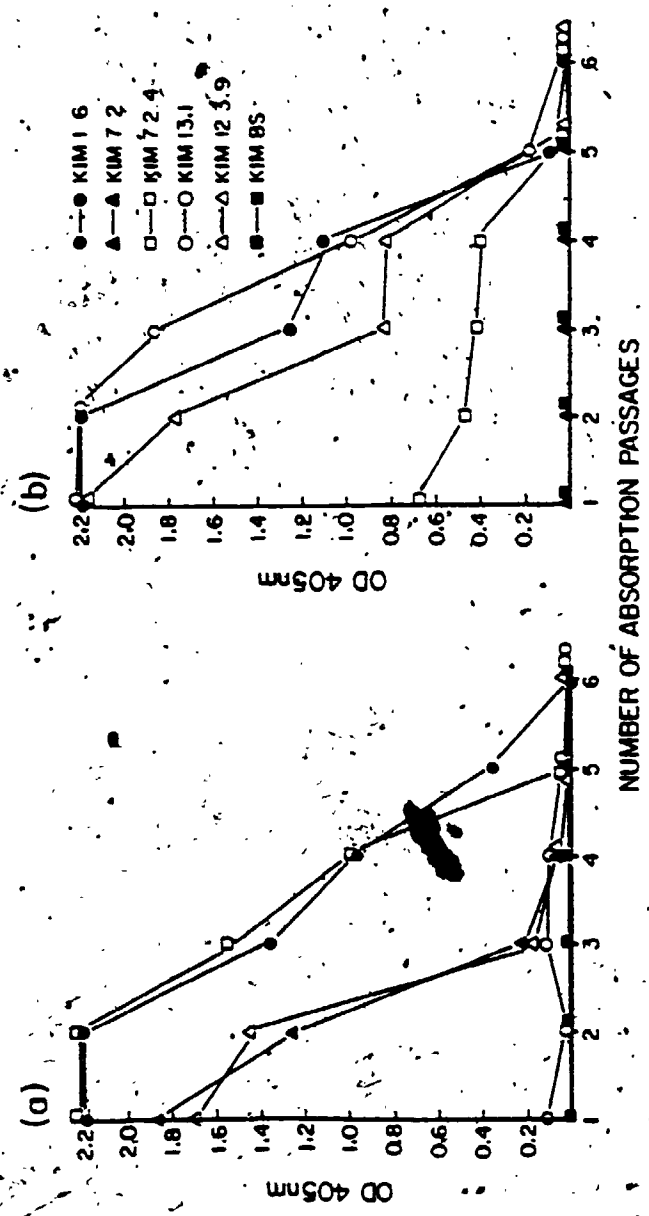


Figure 16.

The binding of hybridoma supernatants to ssDNA (a) or to cardiolipin (b) at each absorption passage: (●——● KIM 1.6; ▲——▲ KIM 7.2; □——□ KIM 7.2.4; ○——○ KIM 13.1; ■——■ KIM B.S.; △——△ KIM 12.3.9)


(a)
(b)
OD 405nm
NUMBER OF ABSORPTION PASSAGES
KIM I 6
KIM 7 2
KIM 72 4
KIM 13.1
KIM 12 3.9
KIM 85

cardiolipin as determined by ELISA and therefore absorption was considered complete. Anti-nucleic acid and /or cardiolipin negative monoclonal antibody KIM B.S. did not bind to these antigens nor to the BSA blocking solution during these procedures. In addition, there was also no binding of anti-nucleic acid and/or cardiolipin antibodies to the BSA (data not shown). These results indicate specificity of absorptions for the chosen antigens.

115a

3.10. Reactivity of Absorbed Monoclonal Anti-DNA Antibodies with HEp2 Cells.

Absorptions of all human monoclonal anti-nucleic acid antibodies except KIM 7.2.4 by ssDNA or by cardiolipin removed nuclear or cytoskeletal staining of HEp2 cells (Table 9) indicating that HEp2 reactivity was associated with anti-ssDNA and/or cardiolipin antibodies. Clear support for this is the lack of HEp2 staining by antibodies KIM 7.2 (ssDNA positive) and KIM 13.1 (cardiolipin positive) after absorptions with their respective antigens. Absorption of KIM 12.3.9 and KIM 1.6 (reactive with cardiolipin and ssDNA) with cardiolipin was successful in abolishing HEp2 binding. KIM 1.6 antibodies (Fig.16) differed from KIM 1.6 hybridoma supernatant antibodies (Fig.13) in their cardiolipin/ssDNA reactivity ratio. The higher binding to cardiolipin of KIM 1.6 antibodies used here in the absorption study resulted perhaps from selection of higher affinity antibodies to cardiolipin during ammonium sulfate fractionation. Absorptions with ssDNA or cardiolipin of KIM 7.2.4 (reactive with cardiolipin and ssDNA) did not affect HEp2 staining. Similar results were obtained with ssDNA or cardiolipin absorbed mouse monoclonal B11.5.1 anti-vimentin antibody. Pre-absorbed B11.5.1 did however show by ELISA some anti-ssDNA (OD_{405nm} = 0.265) and anti-cardiolipin (OD_{405nm} = 0.411) activity. The residual HEp2 staining in these instances may be due to a higher affinity of these antibodies for cytoskeletal antigen than for ssDNA or cardiolipin.

Table 9. Reactivity of Absorbed Monoclonal Anti-DNA Antibodies with HEp2 Cells.

Hybridoma	Staining of HEp2 cells after absorption with: BSA	ssDNA	Cardiolipin
KIM 1.6	+ (nucleus)	+	--
KIM 7.2	+ (cytoskeleton)	--	+
KIM 7.2.4	+ (cytoskeleton)	+	+
KIM 13.1	+ (cytoskeleton)	+	--
KIM 12.3.9	+ (cytoskeleton)	-/+	--
B.S.	-- (--)	--	--
B11.5.1	+ (cytoskeleton)	+	+

(--) Negative; (-/+) Weakly Positive; (+) Positive

3.11. Competitive Blocking of HEp2 Human Anti-DNA Antibody Reactivity by Mouse B11.5.1 Anti-vimentin Antibody.

Competitive blocking experiments using mouse monoclonal B11.5.1 anti-vimentin antibody were performed to determine whether human anti-nucleic acid antibodies recognize vimentin or a vimentin related component in the HEp2 cytoskeleton. Pre-incubation of HEp2 cells with B11.5.1 resulted in inhibition of the subsequent binding to HEp2 cells of all HEp2 reactive monoclonal anti-nucleic acid and/or cardiolipin antibodies including one monoclonal anti-nucleic acid antibody which showed nuclear reactivity (Table 10). The observed competition appeared to be specific since normal mouse serum did not interfere with binding to the HEp2 cells. The above results suggest that mouse monoclonal B11.5.1 and human monoclonal anti-nucleic acid antibodies recognize common epitopes expressed by vimentin or vimentin related structures in the HEp2 cytoskeleton.

3.12. HLA Typing and Karyotype Analysis.

The anti-nucleic acid antibody producing clones KIM 4.3 and KIM 4.5 were subjected to HLA typing. These cloned hybridomas expressed A-2, 3, 23/B-12, 15, 7, 22 human leukocyte antigens (HLA); however, only

Table 10. The Competitive Blocking of HEp2 Human Anti-DNA Antibody Reactivity by Mouse B11.5.1 Anti-Vimentin Antibody.

Hybridoma	Staining of HEp2 cells after blocking with: PBS	B11.5.1	Normal mouse serum
KIM 1.6	+ (nucleus)	--	+
KIM 7.2	+ (cytoskeleton)	--	+
KIM 7.2.4	+ (cytoskeleton)	--	+
KIM 13.1	+ (cytoskeleton)	--	+
KIM 12.3.9	+ (cytoskeleton)	--	+
KIM B.S.	-- (--)	--	--
GM 4672	-- (--)	--	--

(--) Negative; (+) Positive

HLA A-2,3/B-12,15 were detected on the GM 4672 lymphoblastoid line cells.

Karyotyping of KIM 4 clones was also performed. Heterogeneity of the cells within the individual clones was observed with respect to the chromosomal number (Table 11). Hyperdiploid, diploid, and hypodiploid cells were found within each clone. Figure 17 shows typical and representative karyotype spreads of KIM 4.3 hybridoma cells containing 92, 46 and 30 chromosomes.

As presented in Table 11, five of the clones, KIM 4.2-4.6 contained a large percentage of hypodiploid cells. The most frequent hypodiploid chromosomal number in all clones was 44. The lowest number of chromosomes was found to be 32, 30, 28, 31 and 36 for KIM 4.1-KIM 4.6 respectvively. In each clone, cells expressing a hyperdiploid chromosome number were observed. The percentage of hyperdiploid cells varied between 8-20% depending on the clone analyzed. The highest number of chromosomes in KIM 4.2, KIM 4.3, KIM 4.5 and KIM 4.6 was 92. KIM 4.1 and KIM 4.4 had the highest chromosomal counts of 90 and 86 respectively.

3.13. Light Chains of Antibodies Produced by the Hybridomas.

Nine hybridoma supernatants containing anti-DNA and/or anti-cardiolipin antibodies and GM 4672 cell line supernatant were concentrated five times by ammonium sulfate precipitations and tested by double immunodiffusion in agar for light chain type. Using this method, all but two supernatants (KIM 7.2 and KIM 1.5) gave precipitin

Table 11. Karyotyping of Six Hybridomas.

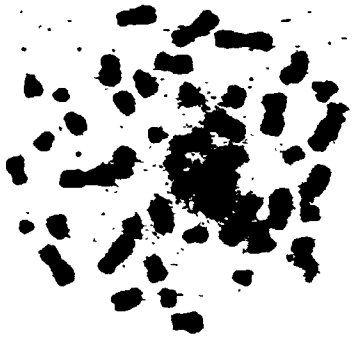
Clone	Hyperdiploid cells > 46 chromosomes	Diploid cells 46 chromosomes	Hypodiploid cells < 46 chromosomes
KIM 4.1	10 (20%)	32 (64%)	8 (16%)
KIM 4.2	4 (8%)	19 (38%)	27 (54%)
KIM 4.3	6 (12%)	21 (42%)	23 (46%)
KIM 4.4	5 (10%)	13 (26%)	32 (64%)
KIM 4.5	10 (20%)	15 (30%)	25 (50%)
KIM 4.6	10 (20%)	14 (28%)	26 (52%)

Figure 17.

Karyotypes of KIM 4.3.

HYPERDIPLOID
92 chromosomes

DIPLOID
46 chromosomes



HYPODIPLOID
30 chromosomes

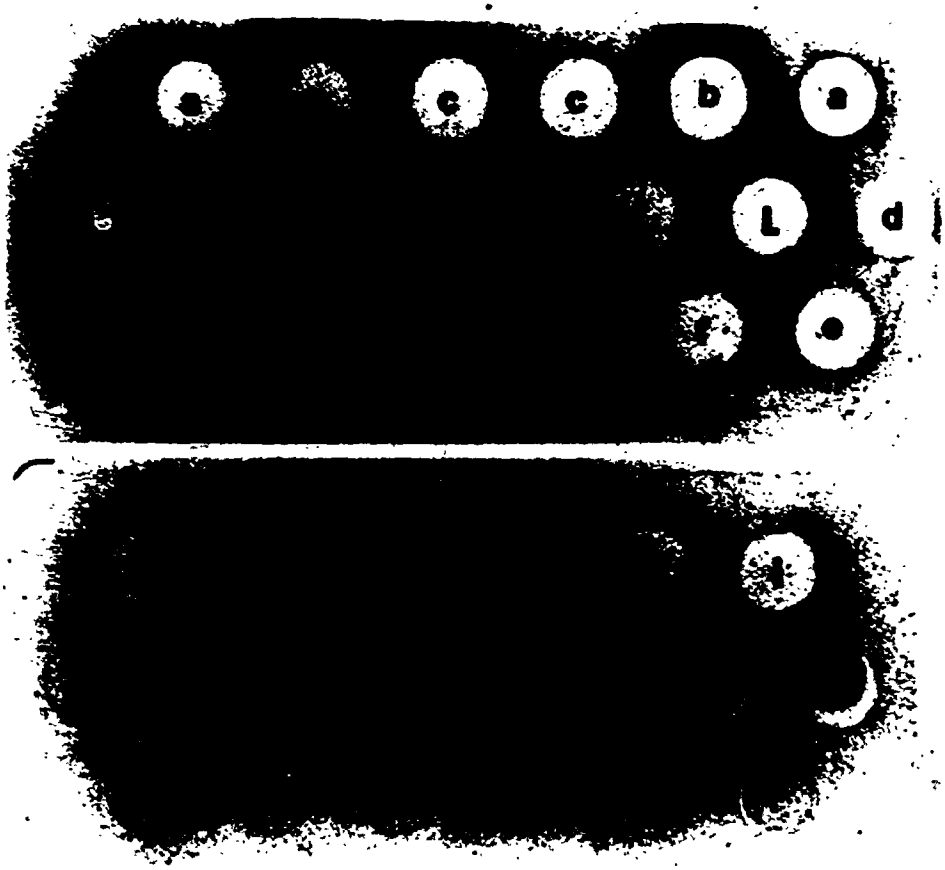
lines with anti-human lambda or kappa antibodies (Fig. 18). The lambda producers were: KIM 10.2, 11.6, 4.1 and 5.1. The kappa producers were KIM 13.1, 9.2 and 12.1. None of the supernatants positive for lambda light chains also expressed the kappa light chain derived from the GM 4672 fusion partner, an IgG kappa producer. The supernatant from the GM 4672 cell line did however give a precipitin line with anti-human kappa antibody (Fig. 18). The presence of lambda chains only in some hybridoma supernatants may be due to very low IgG kappa secretion by GM 4672 after the fusion as previously referred to in Table 8. The inability to detect anti-Light chains in two of the hybridoma supernatants tested (KIM 7.2 and KIM 1.5) may be related to the levels of light chains in these supernatants; i.e., too low to result in a visible precipitin line.

3.14. Characteristics of Monoclonal Anti-DNA Antibody KIM 4.6.3 Used for Anti-idiotypic Serum Production.

Human hybridoma KIM 4.6.3, a subclone of KIM 4.6 (Fig. 13) was continuously maintained in culture for a period of one year and was a stable IgM lambda anti-DNA antibody producer. KIM 4.6.3 anti-DNA antibody exhibited a broad spectrum of reactivity similar to the parent KIM 4.6 in binding to a variety of nucleic acid antigens (ssDNA, DNA, poly(dA-dT), polydG.polydC, low molecular weight S/N DNA)

Figure 18.

Light chains produced by the hybridomas: KIM 5.1, a; KIM 9.2, b; KIM 10.2, c; KIM 12.1, d; KIM 7.2, e; KIM 13.1, f; KIM 11.6, g; KIM 1.5, h; KIM 4.1, j and by the GM 4672 cell line (IgG kappa producer), i. Central wells contain anti-human lambda (L) antibodies or anti-human kappa (k) antibodies.


c
c
b
a
L
d
1

as well as to cardiolipin. The reactivities of the parent KIM 4.6 anti-DNA antibody and subclone KIM 4.6.3 antibody tested in July/83 and in April/84 respectively are shown in Fig. 19. This figure also shows the reactivity of KIM 4.6.3 antibody with ssDNA, poly(dA-dT).polydG.polydC and cardiolipin after its purification by an affinity column consisting of rabbit anti-human IgM. The similarities in reactivity of unpurified (supernatant) KIM 4.6.3 anti-DNA antibody and purified KIM 4.6.3 anti-DNA antibody adjusted to the concentration of the original KIM 4.6.3 supernatant can also be noted. The results suggest that the purification procedure did not affect KIM 4.6.3 anti-DNA antibody reactivity.

The polyspecific nature of KIM 4.6.3 antibody was also observed with 55% of the other monoclonal anti-DNA antibodies produced by different clones obtained in the same fusion (Fig. 13). For this reason, the KIM 4.6.3 antibody was considered a prototype for polyspecific anti-DNA antibody and was chosen for anti-idiotypic antibody production.

3.15. Specificity of Anti-4.6.3 Idiotype for 4.6.3 Idiotype.

The repeated immunizations of rabbits with adjuvant emulsified, affinity purified human IgM monoclonal KIM 4.6.3 anti-DNA antibody resulted in the production of an antiserum which bound in ELISA to normal human IgM, IgG (both without anti-DNA antibody reactivity) (Fig. 20, 21) and to KIM 4.6.3 monoclonal anti-DNA antibody (Fig. 22). This indicated that the immunization schedule and the chosen dosages

Figure 19.

Binding of KIM 4.6 (□), KIM 4.6.3 (▨) supernatants and affinity purified KIM 4.6.3 (■) antibody to ssDNA, DNA, poly(dA-dT), polydG.polydC, S/N DNA and cardiolipin. Binding of purified KIM 4.6.3 antibody to DNA and S/N DNA was not tested.

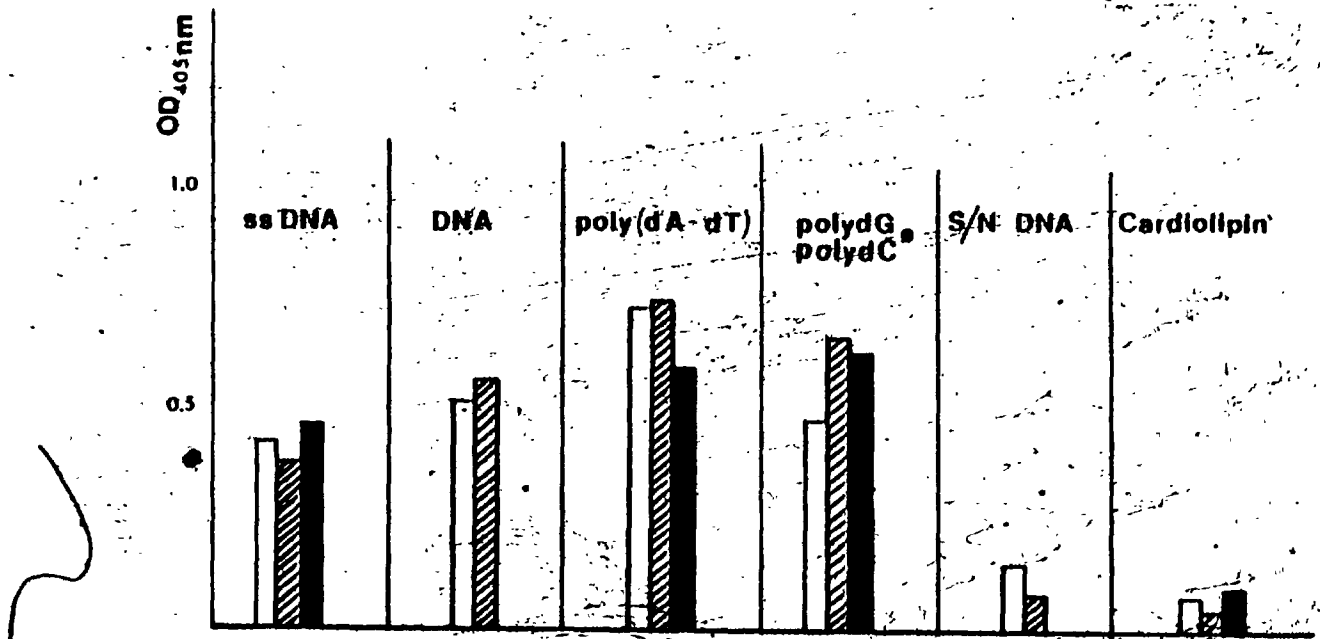

OD405nm
1.0
0.5
ss DNA
DNA
poly(dA-dT)
polydG
polydC
S/N DNA
Cardiolipin

Figure 20.

Binding of rabbit sera before and after absorptions on human IgG and IgM to human IgM coated on the wells. (Anti-4.6.3 serum: before (▲——▲) and after (△——△) absorptions; NRS: before (●——●) and after (○——○) absorptions)

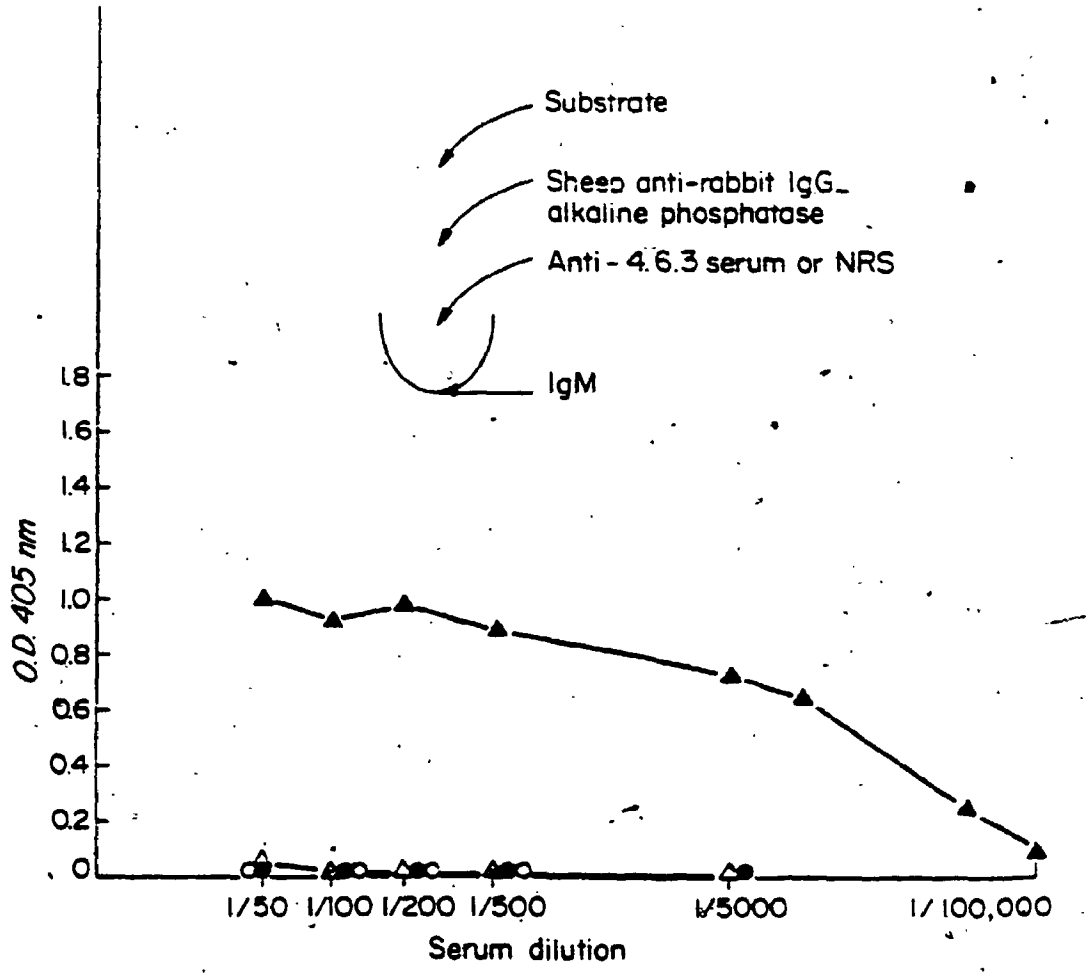

Substrate
Sheep anti-rabbit IgG-alkaline phosphatase
Anti-4.6.3 serum or NRS
IgM
O.D. 405 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1/50
1/100
1/200
1/500
1/5000
1/100,000
Serum dilution

Figure 21.

Binding of rabbit sera before and after absorptions on human IgG and IgM to human IgG coated on the wells. (Anti-4.6.3 serum: before (▲——▲) and after (△——△) absorptions; NRS: before (●——●) and after (○——○) absorptions)

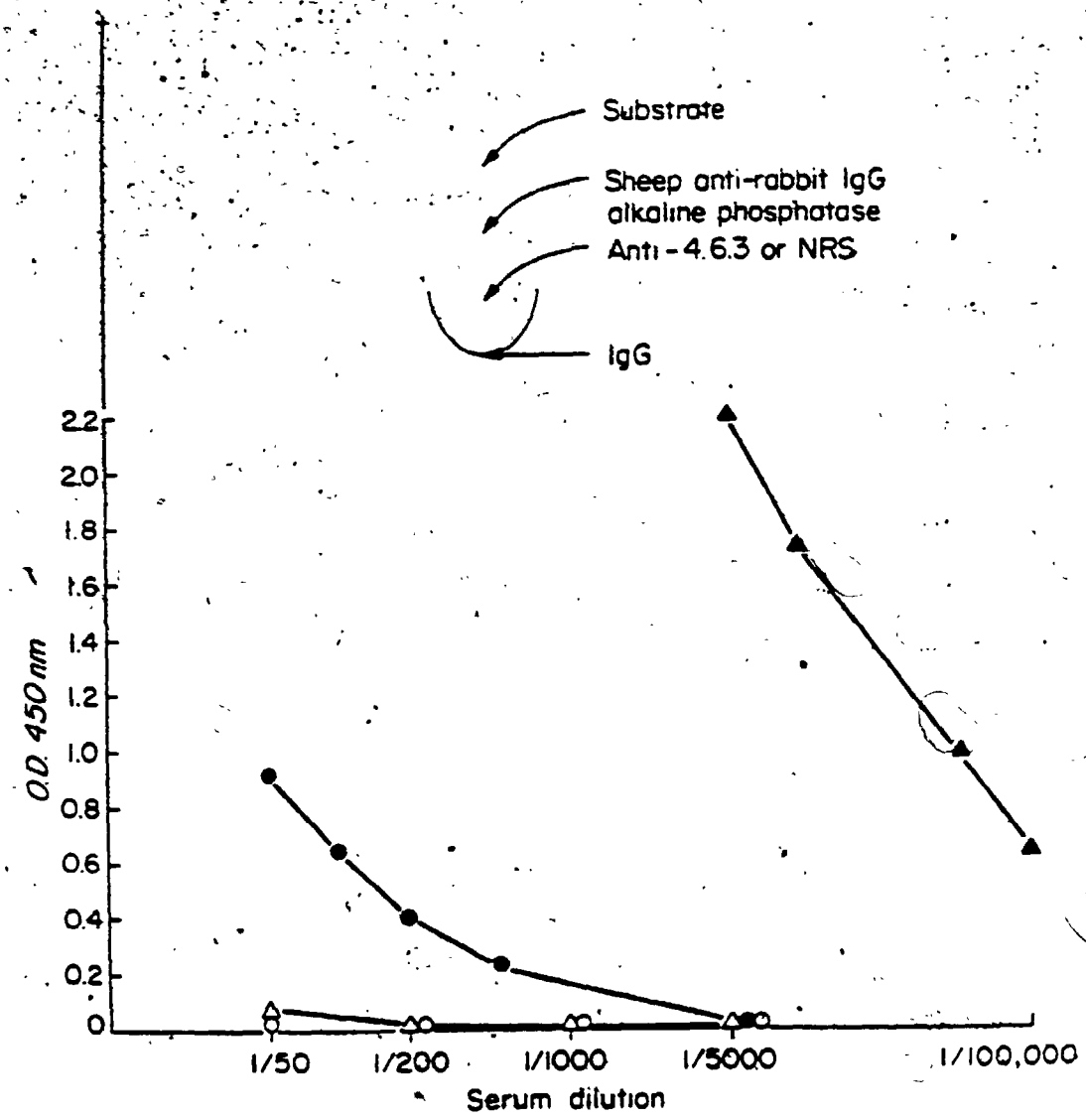
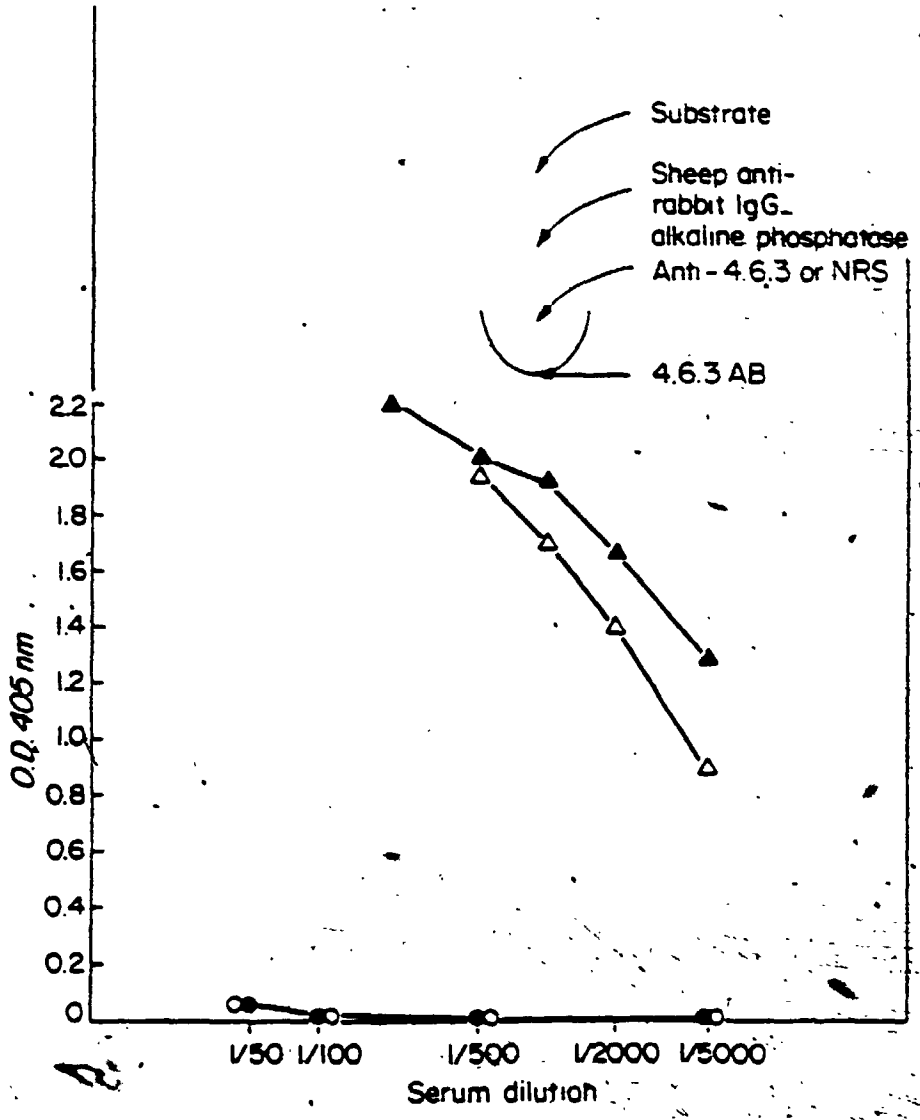

Substrate
Sheep anti-rabbit IgG
alkaline phosphatase
Anti-4.6.3 or NRS
IgG
O.D. 450nm
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1/50
1/200
1/1000
1/5000
1/100,000
Serum dilution

Figure 22.

Binding of rabbit sera before and after absorptions on human IgG and IgM to KIM 4.6.3 antibody coated on the wells.

Anti-4.6.3 serum: before (▲——▲) and after (△——△) absorptions; NRS before (●——●) and after (○——○) absorptions.

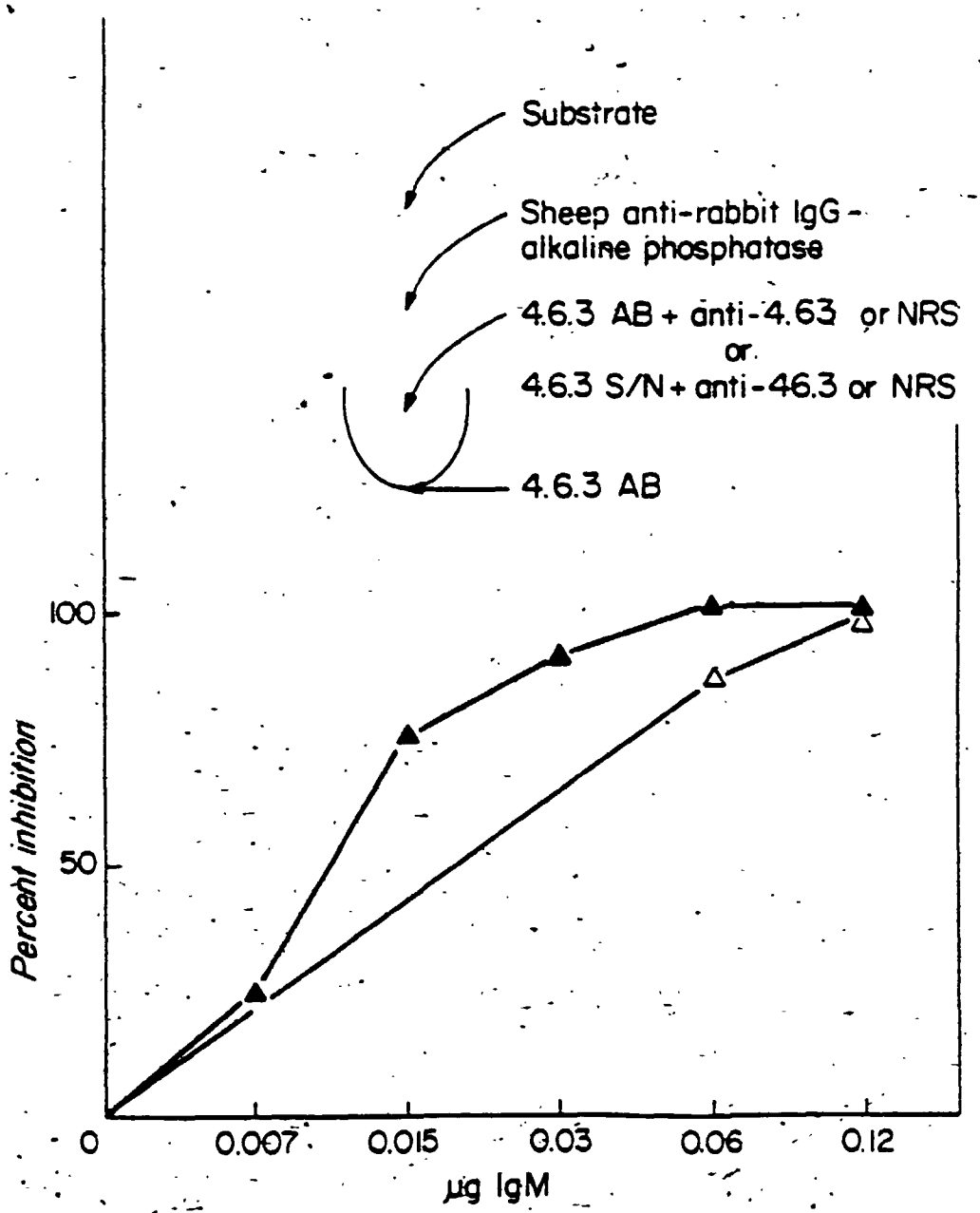

Substrate
Sheep anti-rabbit IgG-alkaline phosphatase
Anti-4.6.3 or NRS
4.6.3 AB
O.D. 405 nm
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1/50
1/100
1/500
1/2000
1/5000
Serum dilution

of antigen (100 µg/rabbit in primary immunization, 50 µg/rabbit in secondary immunizations) were sufficient to elicit in the rabbit an antibody response of IgG isotype directed towards human immunoglobulins. The antiserum obtained from the control rabbit injected with adjuvant only and adjusted to the same protein concentration of 5 mg/ml as anti-4.6.3 antiserum showed no reactivity with normal human IgM or with KIM 4.6.3 anti-DNA antibody (Fig. 20, 22). This control normal rabbit serum (NRS) however bound at low dilutions (1:50 to 1:1000) to normal human IgG (Fig. 21). The absorption of anti-4.6.3 serum with normal human IgM and IgG removed its reactivity with the constant portion of immunoglobulin light and heavy chains, and rendered this serum specific for the variable portion of KIM 4.6.3 antibody. Therefore, this serum was considered as an anti-4.6.3 idiotype (anti-4.6.3 ID). The specificity of anti-4.6.3 ID for KIM 4.6.3 idiotype (ID) is indicated in Fig. 22 by the binding of absorbed anti-4.6.3 serum to KIM 4.6.3 antibody and in Fig. 20 and 21 by its lack of binding to IgM and IgG respectively. The normal rabbit serum had undergone an identical absorption procedure in order to remove its reactivity with normal human IgG and to produce a true control reagent for further study. NRS and anti-4.6.3 ID were always used at identical starting protein concentrations of 5 mg/ml.

The specificity of anti-4.6.3 ID for the variable portion of KIM 4.6.3 anti-DNA antibody was subsequently explored in inhibition studies. As shown in Fig. 23, the ability of anti-4.6.3 ID to bind to KIM 4.6.3 antibody was inhibited by affinity purified homologous

Figure 23.

Inhibition of anti-4.6.3 serum binding to KIM 4.6.3 purified antibody by KIM 4.6.3 purified antibody or KIM 4.6.3 hybridoma supernatant (S/N). Inhibitor: KIM 4.6.3 purified antibody (▲——▲); KIM 4.6.3 S/N (△——△).


Substrate
Sheep anti-rabbit IgG - alkaline phosphatase
4.6.3 AB + anti-4.63 or NRS
or
4.6.3 S/N + anti-46.3 or NRS
4.6.3 AB
100
50
0
Percent inhibition
0.007
0.015
0.03
0.06
0.12
µg IgM

antibody. The homologous hybridoma supernatant used at an IgM concentration equivalent to the purified KIM 4.6.3 antibody showed similar inhibition. This suggests that during the purification procedure, the variable portion of KIM 4.6.3 antibody was not altered. The anti-4.6.3 ID also inhibited KIM 4.6.3 antibody reactivity with ssDNA antigen (Fig. 24). This inhibition was dependent on the dose of the anti-4.6.3 ID. At 1:50 and 1:100 dilutions of anti-4.6.3 ID, there was 100% inhibition of KIM 4.6.3 antibody binding to ssDNA as compared to the binding of KIM 4.6.3 antibody to ssDNA in the presence of 0.1% BSA in 0.1 M Tris-HCl pH 7.4 buffer control. At 1:100 to 1:5000 dilutions, NRS did not interfere with KIM 4.6.3 antibody binding to the nucleic acid antigen. These results indicate that the rabbit anti-4.6.3 ID recognized an epitope (idiotope) on KIM 4.6.3 antibody that is at, or close to, the antigen binding site.

3.16. Reactivity of Anti-4.6.3 ID with Other DNA-Binding and Non-DNA-Binding Monoclonal Antibodies.

The expression of the 4.6.3 ID on monoclonal anti-DNA antibodies other than KIM 4.6.3 and on non-DNA-binding antibodies which were derived from the same fusion as KIM 4.6.3 is shown in Figures 25a and 25b respectively. The 4.6.3 ID was detected in 7/22 (32%) of the hybridoma supernatants (KIM: 4.3, 4.5, 5.1, 10.1, 9.1, 9.2, 12.5) containing anti-DNA antibodies as well as in KIM 4.6.3 (Fig. 25a) and in 7/22 (32%) of the hybridoma supernatants (KIM: J, A1, C, AD, T, AK, BN) with antibodies of unknown specificity (Fig. 25b).

Figure 24.

Inhibition of KIM 4.6.3 antibody binding to ssDNA by anti-4.6.3 ID or NRS. Inhibitor: anti-4.6.3 ID (▲——▲); NRS (●——●).

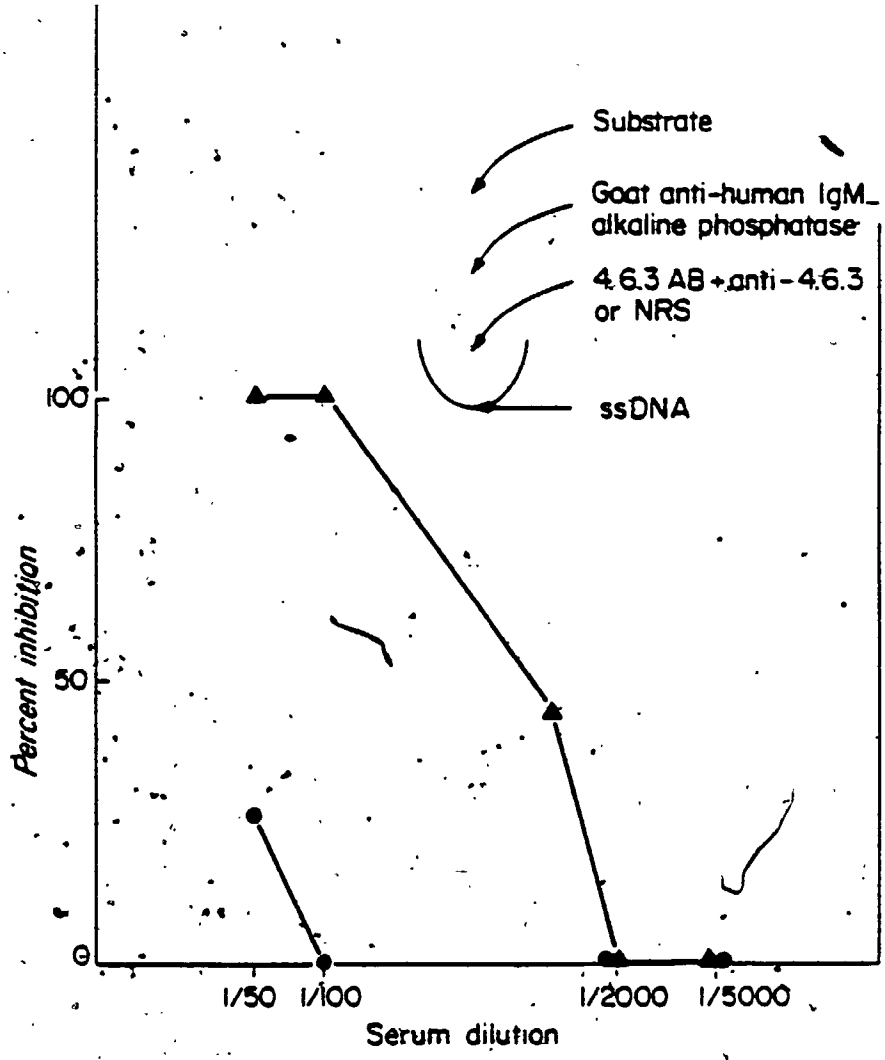
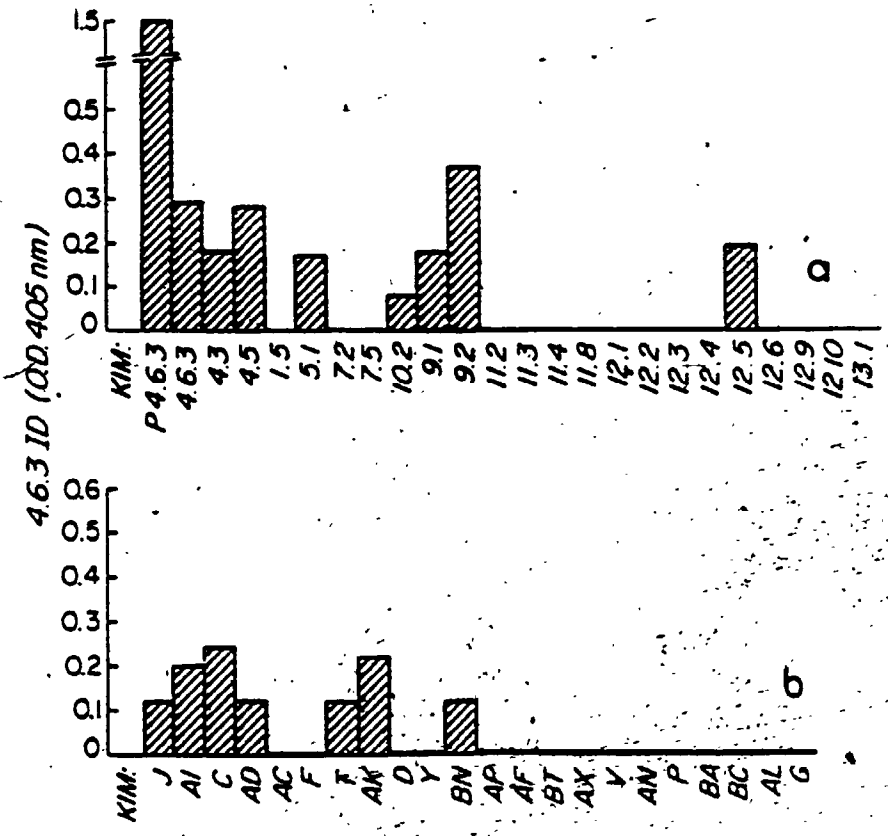

Substrate
Goat anti-human IgM-alkaline phosphatase
4.6.3 AB + anti-4.6.3 or NRS
ssDNA
100
50
0
Percent inhibition
1/50
1/100
1/2000
1/5000
Serum dilution

Figure 25.

The expression of 4.6.3 ID on monoclonal DNA binding (a) and non DNA binding (b) antibodies obtained in the same fusion.

(P 4.6.3 refers to the purified KIM 4.6.3 antibody; other numbers and letters refer to hybridoma supernatants.)


4.63 ID (ΔD 405 nm)
1.5
0.5
0.4
0.3
0.2
0.1
0
KIM:
P4.6.3
4.6.3
4.3
4.5
1.5
5.1
7.2
7.5
10.2
9.1
9.2
11.2
11.3
11.4
11.8
12.1
12.2
12.3
12.4
12.5
12.6
12.9
12.10
13.1
a
0.6
0.5
0.4
0.3
0.2
0.1
0
KIM:
J
AI
C
AD
AC
F
F
AK
D
Y
BN
AP
AF
BT
AX
V
AN
P
BA
BC
AL
G
b

The lack of 4.6.3 ID was not a function of low IgM expression in 4.6.3 ID-negative supernatants since some of them showed equal or higher IgM (KIM 7.2, 8 μg/ml IgM; KIM 11.4, 2.1 μg/ml IgM; KIM 12.10, 2 μg/ml IgM; KIM 12.1, 1.2 μg/ml IgM) than the 4.6.3 ID-positive supernatants (KIM 4.6.3, 2 μg/ml IgM; KIM 9.1, 1.2 μg/ml IgM; KIM 12.5, 0.93 μg/ml IgM; KIM 4.3, 0.77 μg/ml IgM; KIM 9.2, 0.74 μg/ml IgM; KIM 5.1, 0.58 μg/ml IgM; KIM 10.2, 0.512 μg/ml IgM; KIM 4.5, 0.45 μg/ml IgM). The concentration of IgM in affinity purified KIM 4.6.3 P preparation was 6 μg/ml. Some of the binding differences between the 4.6.3 ID-positive anti-DNA antibodies and anti-4.6.3 ID seen in Fig. 25a (KIM 4.3 vs KIM 9.2; KIM 10.2 vs KIM 4.5) could not be explained by the concentration of these antibodies since the pairs compared above exhibited almost identical IgM antibody levels.

These differences do not appear to be a function of the light chain expression in the 4.6.3 ID-positive IgM anti-DNA antibodies. Both lambda (KIM: 4.3, 4.5, 5.1, 10.2) and kappa (KIM: 9.1, 9.2, 12.5) light chains were expressed by these antibodies even though the original KIM 4.6.3 used for anti-ID production was of IgM lambda type.

Inhibition with anti-4.6.3 ID of some of the 4.6.3 ID-positive and 4.6.3 ID-negative anti-DNA antibodies binding to ssDNA was performed to further determine specificity of ID anti-ID interaction as well as to determine whether 4.6.3 ID is located at or close to the antigen binding site. There was no inhibition of 4.6.3 ID negative anti-DNA antibodies (KIM 11.4, 1.5 and 7.2) binding to ssDNA by anti-4.6.3 ID. Inhibition was observed with KIM 4.5 (48% inhibition) and KIM 4.3 (62%

inhibition) but not with KIM 5.1, KIM 9.2 and KIM 10.2. All the anti-DNA antibodies were tested over a similar concentration range of 50 ng/ml IgM - 500 ng/ml IgM and in the presence of anti-4.6.3 ID excess (1:100) dilution which produced 100% inhibition of homologous KIM 4.6.3 (6 µg/ml IgM) binding to ssDNA (Fig. 24). The observed lack of inhibition by anti-4.6.3 ID of 4.6.3 ID negative anti-DNA antibodies and inhibition of some 4.6.3 ID-positive anti-DNA antibody binding to nucleic acid antigen indicates specificity of anti-ID reagent for ID and indicates that anti-ID discriminated between different anti-DNA antibody variable regions. The lack of inhibition by anti-4.6.3 ID of 4.6.3 ID positive anti-DNA antibodies KIM 5.1, KIM 9.2 and KIM 10.2 binding to ssDNA suggests that either these antibodies may have lower affinity for anti-ID than for nucleic acid antigen, or that the anti-4.6.3 ID which is polyclonal in nature recognized not only the antigen binding site-related idiotope(s) but also idiotope(s) in the framework regions.

The 4.6.3 ID was found to be expressed on 0 to 50% of the monoclonal antibodies obtained in fusions of tonsillar lymphoid cells from four different unrelated normal individuals (KIR, KEL, CAR, ROB) and again this idiotype was not restricted to antibodies with nucleic acid reactivity (Table 12).

3.17. The Expression of 4.6.3 ID in SLE and Normal Human Sera.

Human sera from 50 randomly chosen normal individuals and from 40 SLE patients were tested at different dilutions ($1/10^2$ - 2 x $1/10^4$)

Table 12. Expression of 4.6.3 ID in Different Tonsil Hybridoma Supernatants Obtained in Fusions from Unrelated Normal Individuals.

Source of Tonsil Hybridoma S/N	Anti-DNA Positive	Anti-DNA Negative	ID 4.6.3 Positive (%)
KIM	24	-	9 (37)
	-	22	7 (32)
KIR	1	-	0
C	-	17	4 (23)
KE	-	10	5 (50)
CAR	6	-	2 (33)
	-	16	5 (31)
ROB	-	7	2 (28)

for the presence of 4.6.3 ID by the ELISA method using anti-4.6.3 ID. The 4.6.3 ID was expressed in 43 - 90% of the SLE and in 0 - 24% of the normal sera (Fig. 26). To discriminate between the presence or absence of 4.6.3 ID in normal and SLE sera, an OD_{405nm} of 0.1 was chosen as the upper limit for 4.6.3 ID negativity. This upper limit demarked the line below which the majority of normal sera failed to express 4.6.3 ID at all dilutions tested. In contrast, 4.6.3 ID was detected over a wide range of serum dilutions ($1/10^2$ - $2 \times 1/10^4$) in SLE. The mean ± SD level of this ID in SLE was 0.32 ± 0.2 and in normals was 0.08 ± 0.05. These mean levels differed significantly ($p < 0.01$). They represent the highest levels of 4.6.3 ID which were obtained at optimal serum dilutions for a given individual.

In addition, the 4.6.3 ID was assayed with the immunoglobulin fraction (50% ammonium sulfate precipitable fraction) from 28 SLE and 17 normal sera at $1/10^3$ dilution after adjustment to equivalent immunoglobulin concentrations of unfractionated sera. The 4.6.3 ID was present in these immunoglobulin fractions of SLE and normal sera at frequencies of 70% (19/28) and 29% (5/17) respectively (Fig. 27) and was expressed at the levels producing mean values ± SD of 0.24 ± 0.18 in SLE and 0.092 ± 0.07 in normals. At the same dilution ($1/10^3$), the means ± SD in unfractionated corresponding sera were 0.26 ± 0.2 in SLE and 0.05 ± 0.04 in normals. The comparison of 4.6.3 ID means ± SD in the immunoglobulin fraction and in the unfractionated sera showed no significant differences in the SLE group ($p > 0.1$) and in the normal group ($p > 0.1$) and significant differences ($p < 0.01$) between the groups.

Figure 26.

Presence of 4.6.3 ID in SLE and normal human sera. (o SLE;

● normal sera)

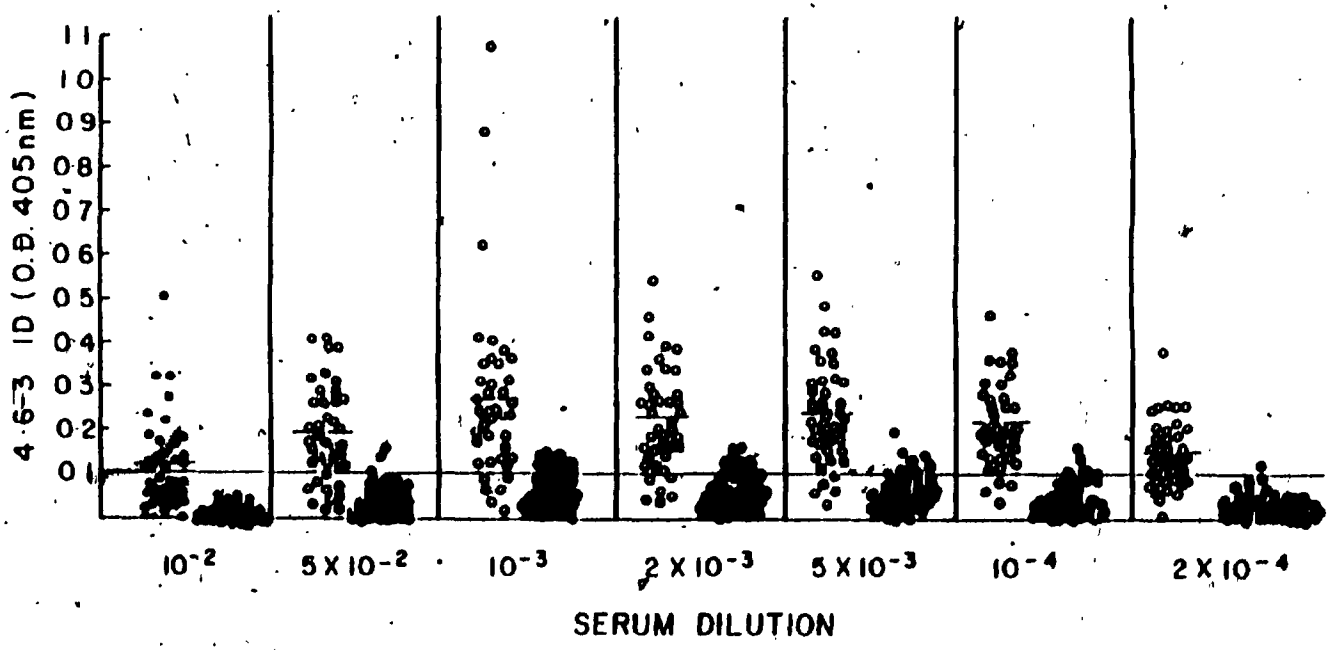
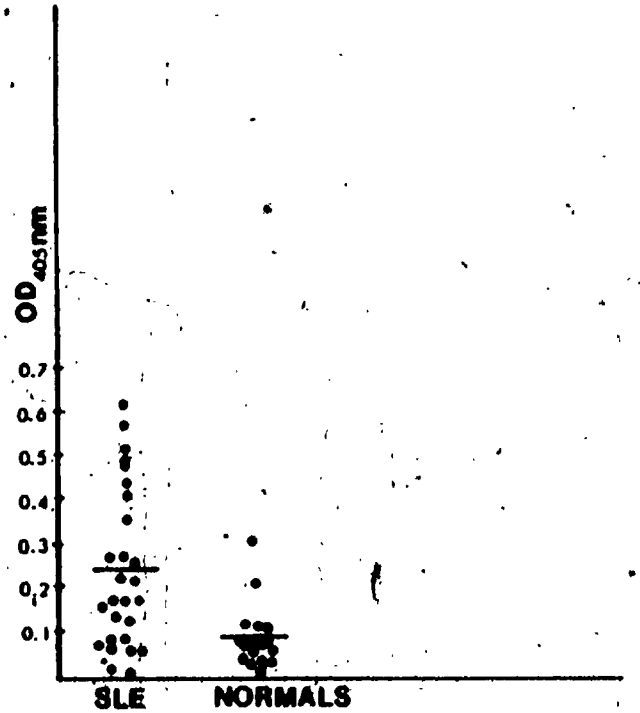

4·6-3 ID (O.D. 405nm)
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
10⁻²
5 X 10⁻²
10⁻³
2 X 10⁻³
5 X 10⁻³
10⁻⁴
2 X 10⁻⁴
SERUM DILUTION

Figure 27.

Presence of 4.6.3 ID in 50% ammonium sulfate fractionated SLE and normal human sera. (Sera diluted 1:1000 for testing)


OD_{405nm}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
SLE
NORMALS

The observed frequent expression and high levels of 4.6.3 ID in SLE were not merely a reflection of high serum immunolglobulin concentrations in these patients. This is indicated in Fig. 28 which shows the lack of correlation (r = 0.254) between serum IgM and IgG (mean ± SD = 25.6 ± 6.41 mg/ml) and 4.6.3 ID (mean ± SD = 0.32 ± 0.2). A lack of correlation (r = -0.0054) was observed between 4.6.3 ID (mean ± SD = 0.32 ± 0.2) and IgM (mean ± SD = 0.97 ± 0.76 mg/ml) or between 4.6.3 ID and IgG (mean ± SD = 24.6 ± 6.7 mg/ml) (r = 0.304) in the SLE sera tested. To further evaluate 4.6.3 ID expression in sera with increased total immunoglobulin, twelve sera from patients with unexplained polyclonal hypergammaglobulinemia were assayed for 4.6.3 ID. Only 3/12 (25%) of these sera were found positive for the presence of this idiotype (OD_{405nm} in these three sera = 0.12, 0.23, 0.166). The frequency of 25% and the low levels of 4.6.3 ID in these hypergammaglobulinemic sera were similar to that found in the sera obtained from normal individuals. (Fig. 26).

The expression of 4.6.3 ID in SLE sera could not also be correlated with total serum IgM and IgG anti-ssDNA antibodies (mean ± SD = 1.615 ± 0.927) (r = 0.131). This is presented in Fig. 29. No correlation (r = 0.107) was observed with total serum IgM and IgG anti-DNA antibodies (mean ± SD = 1.25 ± 0.888) and 4.6.3 ID (Fig. 30) in these SLE sera. Furthermore, no correlations were found in SLE sera between 1) IgM anti-ssDNA antibodies (mean ± SD = 1.16 ± 0.7) and 4.6.3 ID (r = 0.043), 2) IgG anti-ssDNA antibodies (mean ± SD = 0.53 ± 0.4) and 4.6.3 ID (r = 0.020), 3) IgM anti-DNA antibodies (mean ± SD = 0.89 ± 0.66) and 4.6.3 ID (r = 0.007), 4) IgG anti-DNA antibodies (mean ± SD

881

Figure 28.

Correlation between serum IgG + IgM concentration and presence of 4.6.3 ID in SLE sera.

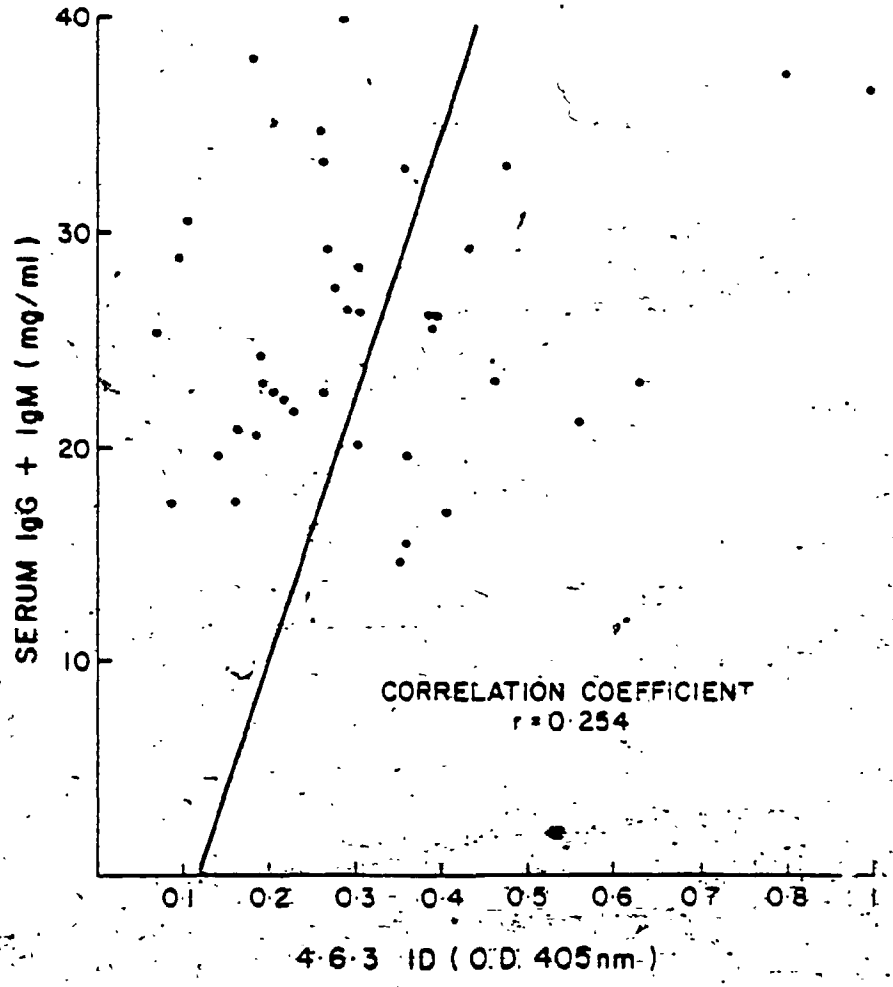

40
30
20
10
SERUM IgG + IgM (mg/ml)
CORRELATION COEFFICIENT
r = 0·254
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
1
4·6·3 ID (O.D. 405nm)

Figure 29.

Correlation between IgG + IgM anti-ssDNA antibody and 4.6.3 ID in SLE sera.

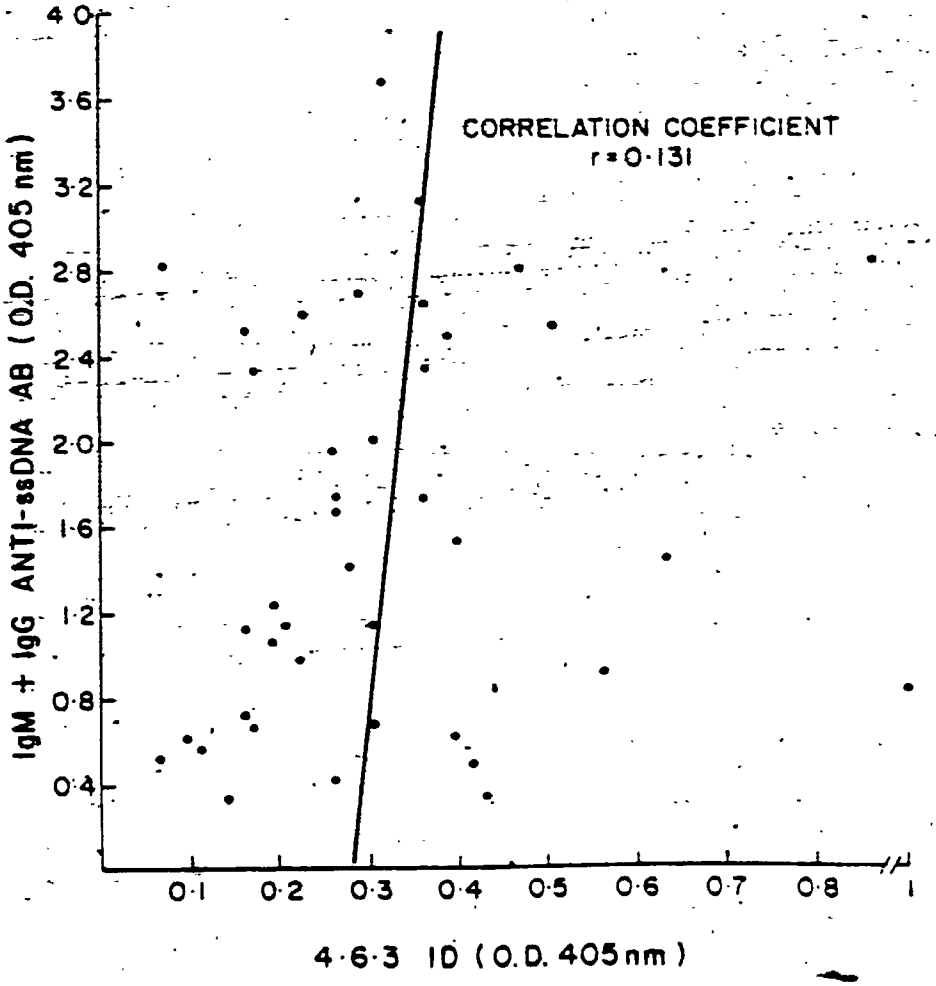
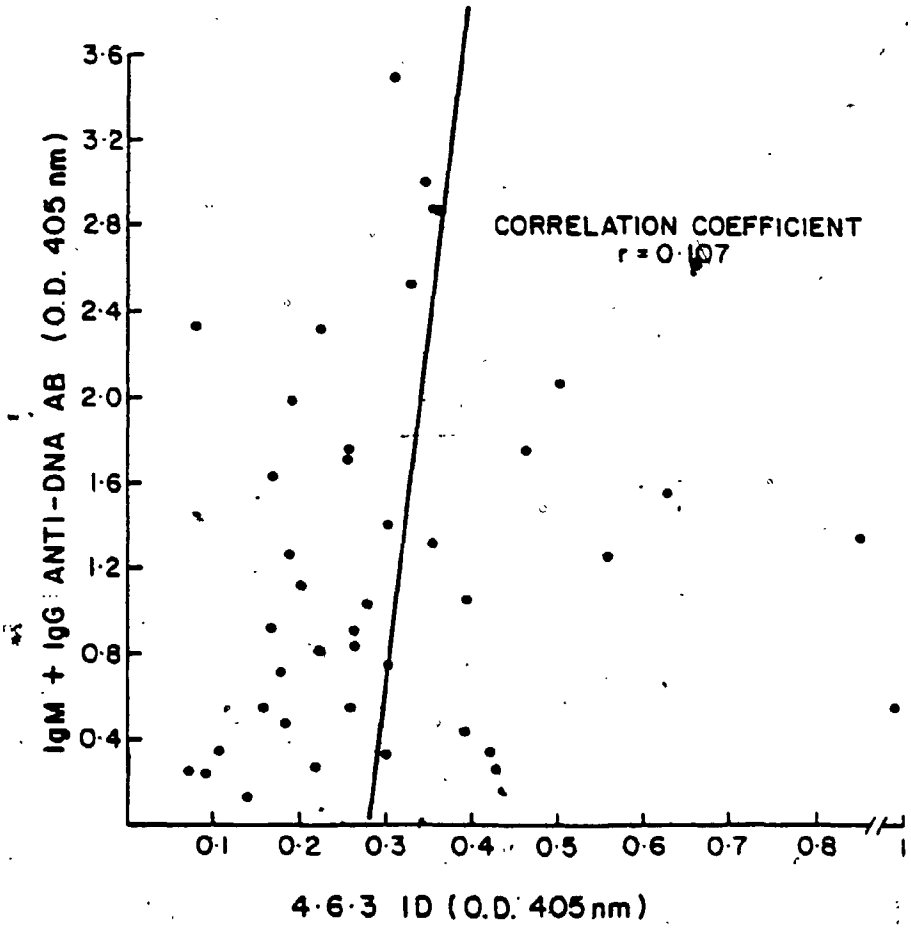

CORRELATION COEFFICIENT
r = 0·131
IgM + IgG ANTI-ssDNA AB (O.D. 405 nm)
4·0
3·6
3·2
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
1
4·6·3 ID (O.D. 405 nm)

Figure 30.

Correlation between IgG + IgM anti-DNA antibody and 4.6.3 ID in SLE sera.

~~157~~


3·6
3·2
2·8
2·4
2·0
1·6
1·2
0·8
0·4
IgM + IgG ANTI-DNA AB (O.D. 405 nm)
CORRELATION COEFFICIENT
r = 0·107
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
1
4·6·3 ID (O.D. 405 nm)

= 0.4 ± 0.4) and 4.6.3 ID (r = 0.159).

In addition, the presence of 4.6.3 ID was revealed in 5/15 (30%) of the sera obtained from patients with monoclonal IgM (OD_{405nm} : 0.24, 0.14, 0.13, 0.375, 0.22). One of these sera also bound to phosphocholine antigen (OD_{405nm} = 0.431) when tested at 1/50 dilution by ELISA (personal communication with J. St. Germain).

CHAPTER 4 - DISCUSSION

These studies present evidence that the ability to produce anti-DNA autoantibodies similar to that of SLE is an inherent property of a normal immune system. The evidence includes: 1) the in vitro expression of anti-DNA autoantibodies by tonsillar lymphoid cells of normal individuals, 2) generation of anti-DNA autoantibody-producing human:human hybridomas which were derived from tonsillar lymphoid cells of a normal individual, 3) similarities of the antigen-binding characteristics of these normal-derived anti-DNA antibodies to the antigen-binding characteristics of SLE monoclonal anti-DNA antibodies, and 4) the existence amongst DNA-binding and non-DNA-binding monoclonal antibodies from different unrelated normal individuals of a cross-reactive 4.6.3 idiotype which is also shared with SLE serum immunoglobulins in a large population of patients.

The detection of anti-DNA antibodies was accomplished using a solid phase enzyme-linked immunosorbent asssay (ELISA). This method relies on the successful coating on a solid support of the antigen to which the specific antibody can bind. It was determined that SLE serum antibody binding to DNA occured only when this antigen was coated onto plastic which was pre-treated with the positively charged amino acid polymer, poly-L lysine (PLL). It was also determined that in the absence of PLL, DNA coating onto the plastic can not occur. Similar observations were reported by Aotsuka et al (1979), by Fish and Ziff (1981) and by Kavai et al (1982) who could not achieve adsorption of

DNA onto polystyrene surface without PLL or methylated bovine serum albumin. Engvall (1976) found that ELISA was only suitable for demonstration of antibodies against ssDNA. She considered this to be a result of very low direct binding of DNA to the polystyrene plates. These observations explain the extensive use of PLL in anti-DNA antibody solid phase ELISA (Shoenfeld et al, 1982; Dittman et al, 1983; Weisbart et al, 1984). Some investigators however, were able to obtain anti-DNA antibody binding to DNA which was directly coated onto the plastic (Epstein, 1975; Halbert et al, 1981; Pisetsky and Peters, 1981). The reasons for these discrepancies are not clear but may involve differences in source and type of plastic used or differences in the antigen coating conditions.

An attempt was made in this study to estimate the amount of DNA retained in the wells after PLL treatment of the polystyrene plastic which was used in anti-DNA antibody ELISA. The DNA binding to PLL-treated polystyrene wells could not be directly demonstrated by use of radiolabelled antigen due to the difficulties in measuring the retained radioactivity in the wells which are permanently embedded in the rigid plastic. A comparison was therefore made between serum anti-DNA antibody binding to DNA-PLL-polystyrene wells and to DNA-PLL-polyvinyl chloride wells coated with identical amounts of antigen (10 ug/ml). The binding under identical experimental conditions of three SLE sera to both DNA-PLL-coated plastics was similar with sera diluted $1/10^4$ and $1/10^5$, and binding was higher on DNA-PLL-polystyrene wells with sera diluted $1/10^2$ and $1/10^3$ (Table

3). This indicates that PLL-polystyrene wells absorb similar or higher amounts of CT DNA antigen than do PLL-polyvinyl chloride wells. The PLL-polyvinyl chloride retained 42-45 ng of DNA antigen (Tables 1 and 2). This estimate is identical to the values described by Aotsuka et al (1979) and Kavai et al (1982) for CT DNA adsorption onto PLL- or methylated BSA-polystyrene wells. A further indication that the anti-DNA antibody ELISA was dependent on the presence of nucleic acid antigen was obtained in the DNase I digestion study in which treatment of CT DNA-coated wells with this enzyme resulted in almost complete abrogation of SLE serum anti-DNA antibody binding (Fig. 2).

The specificity of anti-DNA antibody ELISA was additionally explored through inhibition of antibody reactivity with DNA or ssDNA. The binding of serum antibodies to DNA antigen was more effectively inhibited by pre-incubation of the serum with DNA than with ssDNA (Fig. 3). Conversely, the serum binding to ssDNA was inhibited to a greater extent by pre-incubation with ssDNA than with DNA. Although this shows that anti-DNA antibody ELISA could discriminate between anti-ssDNA and anti-DNA antibodies depending on the antigen used, it is of importance to note that ssDNA inhibited to some extent binding to DNA and vice versa, possibly due to single stranded regions in DNA (since S_1 nuclease was not used in preparation of this antigen) or to double stranded regions in ssDNA (Tan and Natali, 1970). A similar phenomenon was reported by others (Gripenberg et al, 1978; Casperson and Voss, 1983; Lie[illegible]g et al, 1985) and was attributed to a population of antibodies which recognize determinants

attributed to a population of antibodies which recognize determinants common to single and double stranded nucleic acid antigens. The presence of such DNA and ssDNA cross-reactive antibodies was confirmed using monoclonal anti-nucleic acid antibodies (Andrzejewski et al, 1980; Shoenfeld et al, 1983; Rauch et al, 1985; Hoch and Schwaber, 1986).

The sensitivity of the anti-DNA antibody ELISA was demonstrated by the ability to detect the relevant antibodies in highly diluted SLE sera ($1/10^4$, $1/10^5$) (Fig. 1), in supernatants from 7 day cultures of SLE or normal peripherheral blood lymphocytes, and in tonsillar lymphoid cell culture supernatants (Fig. 6,7). Moreover, anti-DNA antibody ELISA was used for detection of anti-DNA antibody production by human:human hybridomas derived from tonsillar lymphoid cells of a normal individual. These monoclonal antibodies could not be detected by the Farr assay even in the hybridoma supernatants containing the highest antibody concentration of 8 ug/ml. This indicates the differences in sensitivity between both methods even though ELISA and Farr assay showed significant correlation ($r = 0.821$, $p < 0.01$) when applied to anti-DNA antibody measurements in SLE sera.

The in vitro spontaneous production of antibodies to ssDNA and DNA of IgM class by SLE peripheral blood lymphocytes was demonstrated in 43% of the cultures (Fig. 8). Pokeweed mitogen (PWM) further increased the magnitude of these spontaneous anti-DNA antibody responses. At a cell concentration of 1×10^6/ml, the IgM antibodies reactive with ssDNA and DNA were equally represented in PWM-stimulated SLE cultures (88% of the cultures had anti-ssDNA antibodies, 73% of

the cultures had anti-DNA antibodies), while IgG antibodies were infrequent and were present in only 9% of the cultures (Fig. 6). These IgG antibodies were ssDNA reactive. The lack of finding IgG antibodies to DNA in SLE peripheral blood lymphocytes cultures was in disagreement with findings of others (Mach et al, 1984; Liebling et al, 1985) and was related to the culture cell concentration. At a higher density of 5 x 10^6 /ml, IgG antibodies to DNA were detected in 59% of SLE peripheral blood lymphocyte cultures in the presence of PWM (Fig. 7). Although the dependence on cell concentration for anti-DNA antibody production was noted, the factors responsible for dependence were not determined in these studies. Sekigawa et al (1986) found a requirement for T-B cell contact in LPS stimulated NZB/NZW F_1 spleen cell cultures for IgG but not IgM anti-DNA antibody production. Such cell to cell contact would have been better achieved in cultures at the higher cell concentration. The production of anti-DNA antibodies by SLE peripheral blood lymphocytes in vitro could be predicted, since the presence of DNA autoreactive B cells in SLE is well established (Bell et al, 1973; Bankhurst and Williams, 1975) and the in vivo expression of these cells characterizes SLE (Koffler et al, 1971; Swaak et al, 1979). The existence of such autoreactive B cells is not unique to SLE and can be demonstrated in normals as in the studies described here. Peripheral blood lymphocytes from normals were capable usually of anti-ssDNA antibody production in vitro upon pokeweed mitogen stimulation (Fig. 6,7). The majority of these antibodies were of IgM class. They occured at approximately half the frequency found in SLE cultures. Antibodies which bound DNA were

expressed in only 10% of normal peripheral blood lymphocyte cultures and were also IgM. IgG anti-DNA antibodies were detected in normal cultures, but only in high density cultures and at the low frequency of 17%. Rarely, in one out of 16 normal peripheral blood lymphocyte cultures, did anti-ssDNA antibodies arise spontaneously. The finding of IgM anti-DNA antibodies in normal peripheral blood lymphocyte cultures at a lower frequency than in SLE (and the finding mainly of IgM type) and only after mitogen stimulation is in agreement with the reports of others (Mach et al, 1984; Liebling et al, 1985). Anti-DNA antibody producing cells have also been shown to be expressed in vitro by the normal circulating B cells following EBV transformation (Hoch et al, 1983) or antigen stimulation (Bastian et al, 1985).

An important observation was made in the studies described here with tonsillar lymphoid cells of normals. These cells were capable of anti-nucleic acid antibody production and in that respect were similar to SLE peripheral blood lymphocytes. Firstly, they produced IgM anti-ssDNA antibodies at a high frequency (78%) similar to the SLE peripheral blood lymphocytes (88%) (Fig. 6). Secondly, these antibodies were generated spontaneously as often (44%) as in SLE peripheral blood lymphocyte cultures (43%) (Fig. 8). This may suggest a similar state of B cell activation in tonsillar tissue and in SLE circulation. This could be due to the exposure of tonsillar tissue to microbial agents on some previous occasion.

The presence of anti-ssDNA antibody-producing cells in the tonsillar lymphoid cell cultures stimulated with PWM was confirmed

using an anti-ssDNA PFC assay. The number of anti-ssDNA antibody-secreting cells varied between 55-240 per 10^6 mononuclear cells and correlated significantly ($p < 0.05$) with the number of immunoglobulin producing cells as measured by a reverse hemolytic PFC assay. The anti-DNA antibody PFC response, however, showed no positive correlation with the anti-ssDNA antibody detected in the supernatants by the ELISA. The lack of correlation between anti-nucleic acid antibody measurements by PFC and ELISA may be explained by the fact that these two methods differ in what they measure: the anti-ssDNA PFC method detects anti-nucleic acid antibody-synthesizing cells at the end of the culture, whereas ELISA determines the concentration of anti-ssDNA antibody cumulated during the culture.

The cellular requirements for the anti-nucleic acid antibody responses of cultured lymphoid cells in general indicate a requirement for T cells (Sagawa and Abdou, 1978; Takeuchi et al, 1984). This was also shown here in B and T cell co-culture experiments, especially those in which tonsillar lymphoid cells were employed, indicating anti-ssDNA antibody response variation depending on T cell concentration (Table 4). At the high T cell concentration of 5×10^5 cells/ml, an inhibitory effect on autoantibody production was observed. This may have been a result, for example, of the increased number of T suppressor cells and/or T helper cells regulating respectively idiotypic and anti-idiotypic anti-nucleic acid antibody responses.

The co-culture studies have also permitted a comparison of the

response of the B and T cells from the tonsil and circulatory system of normal individuals. A comparison of anti-ssDNA antibody responses produced by various combinations of B and T cells from the tonsil and peripheral blood of the same individual indicated that the tonsil may contain an enrichment of anti-nucleic acid antibody precursors B and/or T cells that can provide help for this response. Thus more tonsillar T cells were required to help the anti-ssDNA antibody of circulating B cells of the same individual, and considerably more peripheral blood than tonsillar T cells was required to provide help for the anti-ssDNA antibody response of tonsillar B cells and this response was lower.

Taken together, these data provide evidence that normal B lymphocytes have the potential as in SLE to produce anti-nucleic acid autoantibodies under appropriate conditions. The factors responsible for the increased anti-nucleic acid antibody response in tonsil compared with circulating normal lymphocytes were not determined in this study. Among the obvious possibilities are an enrichment of activated B and T cells (triggered by bacterial antigen) or a relative paucity of suppressor T cells in the tonsil compared with the circulation. A comparison of tonsillar T lymphocytes with peripheral blood T cells from the same individual and from peripheral blood T cells from an individual who had not undergone tonsillectomy was reported recently by Plum et al (1986). They showed that tonsillar T cells contain twice as many OKT 4 positive (helper) T cells and half as many OKT 8 positive (suppressor/cytotoxic) T cells as compared with T cells from the circulation of the same or different individuals. In

addition it was shown that tonsillar T cells responded better to PHA, PWM and Con A as well as to IL 2 than their counterparts in the circulation (Plum et al, 1986). If indeed tonsil is an enriched source of activated T helper cells, it remains to be determined what proportion of these cells cooperates with anti-nucleic acid B cells in vitro and whether this cooperation takes place also in tonsillar milieu in vivo resulting in production of autoantibodies which are not transported into the periphery. Tonsil may have physical or functional barriers which also prevent or block the export of autoreactive cells into the circulation preventing the emergence of anti-DNA autoantibody responses.

As a further proof of the existence of nucleic acid-reactive B cells in tonsil tissue of a normal individual, anti-DNA antibody-producing human:human-hybridomas were generated from this source of lymphoid cells. This was the first demonstration (Cairns et al, 1984) that hybridomas which synthesize anti-DNA autoantibodies can be obtained from a normal human lymphocyte source. The donor of tonsillar lymphoid cells for fusion with the lymphoblastoid cell line GM 4672 was a 7 year old female without any measurable levels of anti-DNA antibody in her serum, with normal immunoglobulin levels, and without a family history of a connective tissue disorder. The overall frequency of anti-DNA antibody-producing hybridomas (11.8%) under all culture and fusion conditions was similar to the frequency (9.7%) obtained by Shoenfeld et al (1983) and to the frequency (14%) obtained by Massicotte et al (1984) for anti-DNA antibody-producing hybridomas using the same GM 4672 cell line and pokeweed mitogen-activated

(Shoenfeld et al, 1983) or non-activated (Massicotte et al, 1984) circulating SLE lymphocytes. In comparison, even a higher frequency (21%) of anti-DNA antibody producing-hybridomas from tonsillar cell origin was obtained in the present studies when a 1:1 ratio of GM 4672:lymphocyte (unstimulated) was used. This possibly suggests an increased state of spontaneous activation of tonsillar cells and/ or that such cells are better fusion partners than peripheral blood lymphocytes. Glassy et al (1983) and Strike et al (1984) noted that the ability of tonsillar lymphoid cells to form hybrids exceeded that of peripheral blood lymphocytes. In hybridoma production, attention to fusion and culturing conditions might lead to an enhanced yield of specific hybridomas as data in Table 5 indicates, and as indicated by other investigators (Astaldi et al, 1982; Denis et al, 1983; Warenius et al, 1983; Massicotte et al, 1984). Based on the assumption that tonsillar lymphoid cells are pre-activated as indicated from the spontaneous anti-ssDNA synthesis of short term cultures, no additional activation with a mitogen of these tonsillar lymphocytes was performed prior to fusion. It is conceivable that even more efficient fusion of these tonsillar cells might have been obtained if mitogen stimulation had been employed prior to hybridization. It is also possible that a higher yield of anti-DNA antibody-producing hybridomas could have been detected had the hybridomas been screened for nucleic acid antigens other than ssDNA, since some clones that bound strongly to cardiolipin and poly dG.poly dC were barely reactive with ssDNA (KIM 12.5 - 12.9, 13.1) (Fig. 13).

The proof that the produced hybridomas are indeed true hybrids is

based on the observation that hybridoma culture supernatants bound to ssDNA and that this antibody was of IgM class. Neither IgM nor anti-ssDNA antibodies were producerd when the lymphoblastoid cell line GM 4672 (IgG_2 producer) was cultured alone. Furthermore, both IgG and IgM were demonstrated in the supernatants from the same hybridomas by ELISA (Table 8). The level of IgG was however, very low (OD_{405nm} range 0.02-0.09) in these hybridoma supernatants as compared with the level of IgG (OD_{405nm} = 0.36) in the supernatant from GM 4672 cells cultured alone. Low IgG concentrations (2-15 ng/ml) of GM 4672 origin in hybridoma supernatants also containing IgM anti-DNA antibodies have been observed (Shoenfeld et al, 1983). Hoch and Schwaber (1986) pointed out differences of at least three orders of magnitude in IgG produced by GM 4672 cell line alone and by hybridomas of which GM 4672 was a part. A low GM4672 Ig concentration was probably also responsible for the lack of simultaneous detection of kappa (GM 4672 origin) and lambda (tonsillar origin) light chains in the hybridoma supernatants by the double immunodiffusion method (Fig. 18).

The possibility of culturing Epstein Barr virus (EBV) transformed tonsillar lymphoctyes instead of generating true hybridomas was excluded since the antibody producing clones showed hyperdiploidy (Table 11), a feature rarely present in EBV transformed cells (Nilsson and Ponten, 1975). In addition to hyperdiploid cells, hypodiploid and diploid cells were also seen within each cloned hybridoma population. Others (Edwards et al, 1982; Shoenfeld et al, 1983) have made similar observations of chromosomal instability in cloned human:human hybridomas. Even stronger evidence against EBV transformation and for

true hybridoma production was brought about in the HLA typing. The clones expressed not only HLA antigens of the GM 4672 cell line (A-2, 3/B-12, 15) but also additional antigens (A-23/B-7, 22) which were presumeably derived from the tonsillar donor. All these HLA antigens appeared to be expressed by each cell in a given clone since 100% cytotoxicity was obtained with individual anti-HLA sera. The observed 100% cytotoxicity in the HLA-typed clones KIM 4.3 and KIM 4.5 which showed a high percentage (45-50%) of hypodiploid cells suggests that chromosome number 6 was preferentially retained in these chromosome depleted cells.

The anti-nucleic acid antibodies produced by hybridomas derived from tonsillar lymphoid cells were of IgM class and were initially screened for binding to ssDNA. In addition to ssDNA, the majority of the antibodies derived from primary hybridomas also reacted with DNA (Fig. 12). Similar ratios of ssDNA to DNA binding by supernatants from cloned and primary hybridomas were noted for the majority of hybridomas studied, except KIM 12 and KIM 13. KIM 12 clones did not bind DNA although the primary KIM 12 hybridoma had an equally good binding to DNA and ssDNA (Fig. 12, 13). Furthermore, the original KIM 12 hybridoma did not show reactivity with cardiolipin, although the clones did. KIM 13.1 bound readily to cardiolipin but not to DNA, and binding to ssDNA was almost undetectable. In contrast, its primary hybridoma showed excellent binding to ssDNA and slight reactivity with DNA. The observed discrepancy in antibody specificity between primary hybridomas of KIM 12 and KIM 13 and their respective clones could be due to the presence in the original hybridoma of two fusion products

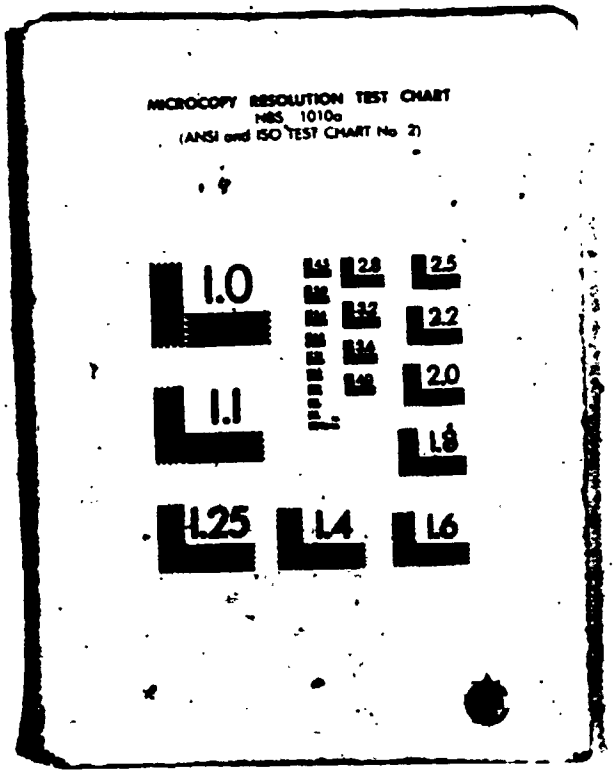
capable of secreting anti-DNA antibodies with different specificities of which only one was capable of being successfully cloned. It is also conceivable that these latter two hybridomas may have undergone somatic mutation and consequently the anti-DNA antibody which they produced changed specificity, or that the emergence of new antigen specificities relects the expression of a new silent clone. Somatic mutations of monoclonal antibodies including those of IgM class have been shown (Teillaud et al, 1983; Diamond and Scharff, 1984; Hartman and Rudikoff, 1984; Rudikoff et al, 1984).

As presented in Fig. 13 and in Table 8, a broad spectrum of anti-DNA antibody specificities towards various nucleic acids (ssDNA, DNA, poly(dA-dT), polydG.polydC, S/N DNA, RNA), cardiolipin, human IgG and vimentin or vimentin-related HEp2 cytoskeletal components were detected in the majority of cloned hybridomas derived from tonsillar lymphocytes. Some of these antibodies also reacted with human endothelial cells (Hashemi et al, 1986). Since the above observations were made, similar findings have been reported for anti-DNA antibodies produced by human:human hybridomas of normal peripheral blood lymphocytes (Rauch et al, 1985) and of normal splenic lymphocyte origin (Hoch and Schwaber, 1986). Interestingly, these normal-derived anti-DNA antibodies did not differ in their reactivity spectrum from hybridoma monoclonal or polyclonal anti-DNA antibodies of human and murine SLE origin (Lafer et al, 1981; Koike et al, 1982; Shoenfeld et al, 1983; Alcover et al, 1984; Andre-Schwartz et al, 1984; Rauch et al, 1985; Rauch et al, 1986), nor did they differ in specificity from monoclonal anti-DNA antibodies obtained by EBV transformation of

SLE PBLs (Sasaki et al, 1985).

The identification of the HEp2 cytoskeletal components to which the anti-DNA and/or cardiolipin antibodies reported here bound as being vimentin or a vimentin-related compound was based on the similarities of the immunofluorescence HEp2 staining patterns produced by these antibodies and mouse monoclonal anti-vimentin antibody, and by the observation that the HEp2 cytoskeletal reactivity of the anti-DNA and/or cardiolipin antibodies could be competitively blocked by mouse anti-vimentin antibody. Vimentin type intermediate filaments are typically present in cells of mesenchymal origin (Lazarides, 1980). Staining of HEp2 cells by mouse monoclonal anti-vimentin antibody suggests that this human epithelial cell line may express vimentin. This is in agreement with the report of Kataaha et al (1985) who obtained staining of the vimentin type in HEp2 cells and with the finding of vimentin in a variety of epithelial cells (Lazarides, 1980) or in the majority of vertebrate cultured cells regardless of their tissue origin (Franke et al, 1979). In view of the fact that a high degree of structural homology exists in all types of intermediate filaments (Fuchs and Hanukoglu, 1983), the observed reactivity with HEp2 cells' cytoskeleton by mouse anti-vimentin antibody and human monoclonal anti-DNA and/or cardiolipin antibodies may indicate that the reactivity is directed not to vimentin itself but to vimentin-related intermediate filaments.

No correlation was observed between any particular type of anti-nucleic acid antibody (e.g. ssDNA, DNA, poly dG.poly dC) and/or cardiolipin detected by ELISA, and HEp2 immunofluorescence staining.


MICROCOPY RESOLUTION TEST CHART
NBS 1010a
(ANSI and ISO TEST CHART No. 2)
1.0
1.1
1.25
1.4
1.6
2.8
2.5
2.2
2.0
1.8

Moreover, HEp2 cytoskeletal-reactive anti-DNA antibodies, regardless of their nucleic acid type reactivity, seemed to show preference for cytoskeletal determinants over nuclear determinants since no simultaneous staining of HEp2 nucleus and cytoskeleton was noted for these antibodies. The observed preference may be a reflection of the higher affinities of anti-DNA antibodies for cytoskeletal determinants or may be related to the concentration, conformation or display of these determinants in the nucleus and in the cytoskeleton. On the other hand, the lack of nuclear staining by these cytoskeleton-reactive anti-DNA antibodies may have been limited by steric hindrance caused by the formation of cytoskeletal-antigen-anti-DNA antibody complexes which may have occured in the nuclear vicinity. It has been recently described (McKeon et al, 1986) that lamins of the nuclear envelope show striking homologies in both primary and secondary structrue with intermediate filament proteins, including vimentin. Anti-DNA which binds to vimentin or intermediate filaments structurally related to vimentin may also recognize these nuclear envelope proteins and therefore concentrate on the surface of the nucleus. Thus intense staining at the nuclear/cytoplasmic interface could occasionally be seen.

Absorption of the majority of anti-DNA and/or cardiolipin antibodies by ssDNA or by cardiolipin resulted in the inhibition of cytoskeletal HEp2 staining. This strengthened the evidence that, in the majority of instances, the same antibody molecule recognized antigenic determinants present on ssDNA, cardiolipin and vimentin or vimentin-related antigens. In two cases however, the cytoskeletal

reactivity of anti-DNA antibody KIM 7.2.4 and mouse anti-vimentin antibody B 11.5.1 which showed ssDNA and cardiolipin activity could not be absorbed with these antigens. In these instances, such residual cytoskeletal staining could have resulted from the presence in the hybridoma supernatant preparations of a heterogeneous population of antibodies, some of which bound to ssDNA and cardiolipin and/or cytosketleton while others bound only to the cytoskeleton. This heterogeneity could reflect the emergence of mutant clones arising form a single cell during clonal expansion in vitro. Such mutations are frequent and have been estimated to occur at a frequency as high as 10^{-3} per base pair per cell division in some cases (Teillaud et al, 1983; McKean et al, 1984). The conversion of KIM 7.2.4 from a clone producing anti-DNA antibodies to one with cytoskeletal reactivity and B 11.5.1 from anti-vimentin to anti-DNA antibody, if such conversion indeed took place, could be of importance in the generation of a variety of new antibody specificities. It is of related interest that a single point mutation was held responsible for conversion of a non-autoantibody-producing myeloma clone to one producing anti-DNA antibody (Diamond and Scharff, 1984).

An important insight arising from these studies and subsequently confirmed by others (Rauch et al, 1985; Hoch and Schwaber, 1986) is that anti-DNA antibodies derived from the lymphoid cells of a normal individual display a wide range of specificities. The antigen binding specificities of these normal-derived anti-nucleic acid antibodies are essentially indistinguishable from the antigen binding specificities of monoclonal and polyclonal lupus anti-DNA antibodies (Lafer et al,

1981; Koike et al, 1982; Shoenfeld et al, 1983; Andre-Schwartz et al, 1984; Koike et al, 1984; Asano et al, 1985; Jacob et al, 1985; Rauch et al, 1986). The following question then may be asked: What is the common epitope on such apparently unrelated antigens as DNA, cardiolipin, IgG, vimentin (or vimentin related) and cell surface (e.g. epithelial cells) with which these normal and lupus polyspecific anti-DNA antibodies interact? Lafer et al (1981) first called attention to the importance of the backbone structure of nucleotides and phosphodiester-linked phosphate groups separated by an equal spacing on cardiolipin molecules. They suggested that these phosphate groups may represent a common epitope for antibodies recognizing DNA and cardiolipin. Their view was shared by others who found high anti-cardiolipin activity in SLE sera (Koike et al, 1982) and observed the induction of anti-DNA antibodies in normal mice after immunization with cardiolipin (Rauch et al, 1984). It is possible that some of the monoclonal anti-DNA antibodies described here bind to cardiolipin and DNA via a phosphate ester structure; however, the reactivity of the KIM 10 clones (Table 7) which react with ssDNA and cardiolipin but not DNA or RNA indicates that the deoxy-sugar of DNA and the conformation provided by randomly coiled or denatured DNA could be important in establishing the common determinant for both DNA and cardiolipin. Cardiolipin may not have a fixed conformation, but rather some flexibility that allows it to conform and bind to different anti-DNA antibody-binding sites. The cardiolipin conformation may also depend on the interaction with other cardiolipin molecules, and with the plastic-liquid interface in the assay system. Group 1 (KIM 11 clones)

(Table 7) on the other hand would appear to produce antibodies in which the binding site is such as to preclude any of the conformations of cardiolipin.

There is no example in the clones studied of anti-DNA antibodies reacting towards low molecular weight S/N DNA alone. This may be an indication that immunogenic low molecular weight S/N DNA (Pancer et al, 1981) may be derived from non-immunogenic high molecular weight DNA containing the same determinants. An exception was noted for group 8 (KIM 12 clones) (Table 7) which shows reactivity to S/N DNA but not to DNA. This group is however distinguished by its binding to RNA.

Faaber et al (1984) proposed that the polyspecificity of some anti-DNA antibodies may be due to the recognition of similarities in repetitively spaced negatively charged groups presented on unrelated antigens. The basis for this was their observation of anti-DNA antibody binding to DNA and to proteoglycans, also recently described by Berden et al (1986). In the study presented here, polyspecific anti-DNA antibodies did not react with the negatively charged DNP hapten indicating that the recognition of charge may be restricted to a limited number of antigens. The binding to HEp2 cytoskeleton of monoclonal anti-nucleic acid antibodies may have occured via recognition of charge if the phosphate groups present on the intermediate filament proteins including vimentin (Lazarides, 1980) were to impose an antigen charge-restricted recognition by these antibodies.

Schwartz and Stollar (1985) suggested a most appealing explanation

for some anti-DNA antibody polyreactivity. Their theory implies the presence of multiple independent binding sites for different antigens on the same antibody. This may explain the reactivity of anti-DNA antibodies with cardiolipin, vimentin or IgG in which a common epitope (primary sequence, conformational or charge related) can not be established. The existence of a single antibody with different binding sites for different antigens has been reported (Kang and Kohler, 1986). Some of these unrelated binding sites may be located in the framework region as in the case of IgM mouse monoclonal antibodies with rheumatoid factor activity (Shlomchik et al, 1986). In this latter case, binding to IgG involves kappa light chain framework sequences while the complementarity determining regions bind still different antigens.

Efforts towards defining a common epitope on a variety of antigens which are targets of polyspecific anti-DNA antibodies have not been successful so far. An explanation of polyspecificity may become even more complicated as more complex antigens (platelets, erythrocytes, T cells, B cells, glomeruli, neuronal tissue, bacteria, etc.) which cross-react with DNA are discovered to bind anti-DNA antibodies (Asano et al, 1985; Carroll et al, 1985; Jacob et al, 1985; Rauch et al, 1986).

The ligand binding properties of monoclonal anti-DNA autoantibodies indicates that the original antigenic stimulus evoking their response may not necessarily be DNA. This would be in accordance with the finding that DNA is not ordinarily immunogenic (Madaio et al, 1984). What is then the antigen that induces anti-DNA antibody formation?

Jacob et al (1985) implicated protein cross-reacting with DNA, rather than DNA itself as the triggering antigen. Their evidence was based on the ability of monoclonal antibody derived from lupus prone mice to recognize identical membrane polypeptides from different cells (T cells, erythrocytes, platelets, neuronal cells) in addition to DNA.

Recently, attention has been devoted to bacterial antigens as being responsible for at least part of the anti-DNA antibody responses. Different bacteria can bind anti-DNA antibodies suggesting a common determinant (Carroll et al, 1985) and bacteria can stimulate production of these antibodies in cultures of normal peripheral blood lymphocytes (Shoenfeld et al, 1986). It may be possible that the generation of a large number of hybridomas producing anti-DNA antibodies from tonsillar lymphoid cells as described here, may have been due to the effects of a specific bacterial stimulation of the tonsil on some previous occasion as opposed to polyclonal stimulation.

Molecular analysis of lupus derived monoclonal anti-DNA antibodies has been initiated and shows a direct link between anti-DNA antibodies and anti-bacterial antibodies. These studies revealed that the amino acid sequence of the heavy chain of lupus anti-DNA antibodies, except for a single amino acid, was entirely homologous to antibodies to phosphocholine, an important cell wall component of many bacterial agents (Eilat et al, 1984). This provoked speculation that anti-DNA antibodies may originate from anti-phosphocholine genes which had undergone somatic mutation. Diamond and Scharff (1984) subsequently showed that such a scenario may indeed take place. They isolated an anti-phosphocholine myeloma mutant which lost binding to this antigen

but acquired binding to DNA. A single amino acid change in the variable portion of the anti-phosphocholine antibody was responsible for the antigen binding specificity change. Thus bacterial antigens may initiate an immune response against infection and this response may be converted to reactivity against self.

Other groups of investigators (Atkinson et al, 1985; Naparstek et al, 1986) gathered evidence indicating that an anti-DNA antibody response may not necessarily be a by-product of somatic mutation. Instead, this response may be an integral part of the anti-bacterial response and can simultaneously occur on the same antibody molecule. These authors supported their view by showing that a marked amino acid homology existed between the variable portion of the light chain of different lupus cross-reactive anti-DNA antibodies and the light chains of IgM antibodies isolated from patients with Waldenstrom's macroglobulinemia. One of the latter which was idiotypically related to lupus anti-DNA antibodies, bound DNA as well as Klebsiella (Kabat et al. 1980). It was proposed therefore that anti-DNA antibody variable genes may be conserved in the germ line primarily because of this anti-bacterial rather than their anti-DNA antibody properties.

The above suggestions (implicating antigens other than DNA as being in part responsible for an anti-DNA autoantibody response) have initiated new concepts regarding the origin of anti-DNA antibodies.

The finding of identical antigen binding characteristics for normal-derived monoclonal anti-DNA antibodies and for SLE anti-DNA antibodies provided the first clues that these autoantobodies may be derived from a common germ line gene. The availability of monoclonal

antibodies makes it possible to investigate the genetic elements encoding the variable portion (idiotype) of individual antibody molecules using anti-idiotypic antibodies. As the idiotype determinants represent serological markers of antibody variable region genes (Weigert and Riblet, 1978; Rajewsky and Takemori, 1983), the finding of cross-reactive idiotype determinants on different antibodies indicates the utilization of the same or closely related variable (V) region gene(s) in the expression of these antibodies.

Anti-idiotypic antibodies have helped identify cross-reactive idiotypes in many antibody systems; for example, antibodies to hepatitis B surface antigen (Kennedy and Dreesman, 1983), anti-dextran antibodies (Haba et al, 1983), anti-myoglobin antibodies (Kawamura et al, 1984), anti-casein antibodies (Cheung and Cunningham-Rundles, 1985), rheumatoid factors (Carson and Fong, 1983) and anti-DNA antibodies of murine and human SLE origin (Rauch et al, 1982; Tron et al, 1982; Solomon et al, 1983; Pisetsky, 1983; Isenberg et al, 1984).

In these studies a similar approach was undertaken using anti-idiotypic serum (anti-4.6.3 ID) to determine the idiotypic relatedness amongst monoclonal anti-DNA antibodies derived from tonsillar lymphoid cells of normal individuals. This anti-4.6.3 ID was directed towards idiotypic determinant(s) (4.6.3 ID) expressed by IgM lambda anti-DNA antibody KIM 4.6.3. To ensure specificity for 4.6.3 ID, rabbit anti-4.6.3 antiserum was extensively absorbed with normal human IgM and IgG. It should be noted that the absorptions of anti-4.6.3 antiserum with normal IgM failed to abolish its reactivity

with normal human IgM and IgG. The need for absorption with normal human IgM and IgG even though IgM monoclonal anti-DNA antibody was used as an immunizing antigen is not clear, but has also been noted by others (Shoenfeld et al, 1983). Among the possible reasons for this are: 1) inability of normal human IgM to efficiently absorb a strong anti-light chain antibody response in the rabbit serum, 2) contamination of the immunizing antigen by human IgG below the threshold of detection by the IgG ELISA and 3) the presence of antibodies cross-reactive with human IgG in rabbit 4.6.3 antiserum induced by Freund's adjuvant. Since human IgG could not be detected by ELISA in the purified concentrated IgM monoclonal antibody KIM 4.6.3 used for immunization, the possibility of the rabbit 4.6.3 antiserum reacting with the variable regions in IgG seems highly unlikely. It is possible however, that the observed reactivity of this antiserum with human IgG was induced by the adjuvant injection, since even the control rabbit injected with adjuvant alone showed binding to human IgG. This reactivity was removed after the absorptions of 4.6.3 antiserum and control rabbit serum with human IgG in addition to IgM. Specific anti-4.6.3 ID and control (normal rabbit serum, NRS) reagents were thus prepared.

The specificity of anti-4.6.3 ID for 4.6.3 ID was indicated by 1) its binding to KIM 4.6.3 antibody, 2) lack of binding to normal human IgM and IgG, 3) inhibition of anti-4.6.3 ID binding to KIM 4.6.3 antibody by homologous affinity purified antibody and by homologous hybridoma supernatant antibody and 4) by anti-4.6.3 ID interference with KIM 4.6.3 antibody binding to ssDNA. In comparison, these KIM

4.6.3-anti-4.6.3 ID and KIM 4.6.3-ssDNA interactions were not affected by normal human IgM and NRS respectively. The inhibitory effect of anti-4.6.3 ID on KIM 4.6.3 antibody reactivity with ssDNA suggests that anti-4.6.3 ID recognized an idiotypic determinant(s) located in or near the antigen binding site of KIM 4.6.3.

Anti-4.6.3 ID detected a cross-reactive determinant which was represented on 3[illegible]% monoclonal anti-DNA antibodies as well as on 3[illegible]% monoclonal non-DNA binding antibodies obtained in the same tonsillar lymphoid cell fusion as KIM 4.6.3. Furthermore, this idiotype was shared with antibodies with and without nucleic acid reactivity produced in four different fusions of tonsillar lymphoid cells from unrelated normal individuals. The 4.6.3 idiotypic determinant(s) was (were) not always associated with the ssDNA binding site of 4.6.3 ID positive anti-nucleic acid antibodies. Only in two cases closely related to KIM 4.6.3 (KIM 4.3 and KIM 4.5) by common derivation from the primary hybridoma KIM 4, did anti-4.6.3 ID inhibit binding to ssDNA. The failure of anti-4.6.3 ID to inhibit binding in the remaining 4.6.3 ID-positive anti-DNA antibodies (KIM 5.1, KIM 9.2, KIM 10.2) may be explained by 1) a higher affinity of the latter antibodies for ssDNA than for anti-4.6.3 ID, 2) expression of amino acid sequences and/or conformational determinants similar to 4.6.3 ID located in the variable region outside the antigen binding site, 3) recognition by anti-4.6.3 ID which is polyclonal in nature of private antigenic determinants within the antigen binding site of KIM 4 clonally-related antibodies and cross-reactive antigenic determinants in the framework region.

The 4.6.3 cross-reactive ID, although expressed by normal tonsil lymphoid cells in vitro, was either not expressed or infrequently expressed and at low levels in normal individuals in vivo. Only 24% of normal sera contained this idiotype and the mean level ± SD of 4.6.3 ID was 0.08 ± 0.05 OD_{405nm} (Fig. 26). Amongst normal sera lacking 4.6.3 ID was serum from the donor whose tonsil lymphoid cells were used to generate hybridomas and from which the 4.6.3 ID described here was originally derived. These findings argue that the expression of this anti-DNA antibody cross-reactive idiotype in vivo may be suppressed in normal individuals. In contrast, the normal-derived in vitro anti-DNA antibody 4.6.3 ID was found in 85-90% of sera from unselected SLE patients and its mean level ± SD of 0.32 ± 0.2 OD_{405nm} was significantly higher than the level of 4.6.3 ID in normal sera. The high frequency of 4.6.3 ID in SLE sera was not merely a reflection of the high serum immunoglobulin concentrations often seen in SLE, since sera from patients with unexplained polyclonal hypergammaglobulinemia expressed 4.6.3 ID at the similar low frequency (25%) found in normal sera and there was no correlation in SLE sera between IgM and IgG concentration and 4.6.3 ID. The differences of 4.6.3 ID expression in normal and SLE sera suggest that 4.6.3 ID may relate to the SLE disease process. In isolated cases, the presence of 4.6.3 ID in SLE sera paralleled the clinical course of SLE and was independent of serum levels of anti-DNA antibody (Bell et al, 1985). In randomly tested SLE sera, no correlation of 4.6.3 ID and IgM and IgG antibodies to ssDNA and DNA was found (Fig. 29 and 30). This however, does not exclude a possibility that some SLE serum anti-DNA

antibodies bear 4.6.3 ID. Some 4.6.3 ID-positive anti-DNA antibodies may have escaped detection by anti-4.6.3 ID by being complexed with antigen (Fish and Ziff, 1982) or by being lodged in different tissue sites. These possibilities can be supported by recent reports describing the presence of a SLE major anti-DNA antibody cross-reactive idiotype designated 16/6 in renal and skin tissue lesions (Isenberg and Collins, 1985; Isenberg et al, 1985) and by the inability to detect anti-DNA antibody reactivity on antibodies bearing cross-reactive idiotype by conventional assays (Halpern et al, 1984). On the other hand, the 4.6.3 ID-positive DNA negative antibodies in SLE sera may represent: 1) other autoantibodies frequently found in SLE, for example, antibodies to histones (Stollar, 1969; Tan et al, 1976), antibodies to small ribonucleoproteins (Sm, nRNP, Ro, La) (Northway and Tan, 1972; Sharp et al, 1976), anti-lymphocyte antibodies (Winfield et al, 1975), 2) idiotypically related antibodies to exogenous antigens (Atkinson et al,1985; Kofler et al, 1985; Naparstek et al, 1986), 3) "antigen-negative, idiotype-positive" regulatory idiotypes .

The frequent expression of 4.6.3 ID in SLE sera and its absence in normals in vivo suggests the possibility of either differential utilization and/or expression of the genes encoding this idiotype in normals and SLE or an immunoregulatory defect in SLE. The latter may involve for example anti-idiotypic antibodies, key elements in the immune network. Such antibodies directed against cell surface receptors or secreted idiotypic molecules have been shown to modulate specific antibody responses (Geha, 1982; Goidl et al, 1983; Thomas

et al, 1983; Pollok and Kearney, 1984; Tamate et al, 1986). The absence , low level, or too high level of anti-4.6.3 ID in SLE sera and/or under/over responsiveness of 4.6.3 ID-bearing cells to regulatory signals provided by anti-4.6.3 ID, could result in excessive 4.6.3 ID synthesis.

The involvement of anti-idiotypic antibodies in lupus has attracted some attention. Abdou et al (1981) and Silvestris et al (1984) found that sera from active SLE patients, but not sera from normal individuals, lacked anti-DNA anti-idiotypic antibodies. Hahn and Ebling (1984) introduced passive therapy of anti-cross-reactive anti-DNA antibodies in NZB/NZW lupus mice. This resulted only in transient improvement in the level of cross-reactive ID, anti-DNA antibody level and disease activity. In contrast, Teitelbaum et al (1984) using a similar approach had noted a potentiating effect of anti-cross-reactive anti-DNA antibody (anti-H-130 ID) on anti-DNA antibody and H-130 ID levels in MRL mice. Interestingly, majority of H-130 ID-positive antibodies were not reactive with DNA.

The expression of normal-derived in vitro anti-DNA antibody cross-reactive idiotype 4.6.3 by monoclonal DNA-binding and non-DNA-binding antibodies from different normal individuals, by serum immunoglobulins of the majority of SLE patients and 30% of patients with monoclonal IgM, some of which also bound ssDNA and had cold agglutinin activity (Bell et al, 1986), strongly suggests that the 4.6.3 ID is encoded by a common or closely related germ line gene(s). The same suggestion was put forward by other investigators (Andrzejewski et al, 1981; Rauch et al, 1982; Datta et al, 1983;

Shoenfeld et al, 1983; Pisetsky, 1983; Solomon et al, 1983; Isenberg et al, 1984; Datta et al, 1986; Jacob et al, 1986) who studied genetic elements encoding SLE anti-DNA antibodies using anti-idiotypic probes. Their experiments, complementary to the experiments presented here, revealed the presence of major cross-reactive idiotypes on anti-DNA antibodies form human and murine lupus origin. For example cross-reactive idiotype H-130 expressed by anti-DNA antibody from MRL/lpr/lpr autoimmune strain of mice was described by Andrzejewski et al (1981). In this strain of mice H-130 was represented in about 50% of the serum immunoglobulins and less than half of H-130 positive Ig had anti-nucleic acid reactivity. Different strains of autoimmune mice shared H-130 ID (Rauch et al, 1982). Correlation of this ID expression and the severity of the disease in MRL/lpr/lpr mice was noted. Lipopolysaccharide (LPS) stimulated production of anti-DNA antibodies bearing H-130 in normal spleen lymphocyte cultures (Datta et al, 1983).

In man, a public idiotypic marker termed 16/6 derived from a SLE monoclonal anti-DNA antibody was shared with monoclonal anti-DNA antibodies from different SLE patients (Shoenfeld et al, 1983), with serum immunoglobulins from more than half of the SLE patients with active disease, and with 4% of normal human sera (Isenberg et al, 1984). Some pathogenic relevance was assigned to 16/6 ID since its presence could be demonstrated in skin and renal biopsies from SLE patients (Isenberg et al, 1985; Isenberg and Collins, 1985). Peripheral blood lymphocytes from normal individuals were capable of 16/6 ID expression in vitro after stimulation with pokeweed mitogen

(Datta et al, 1986), with bacteria (Shoenfeld et al, 1986) or α - interferon (Schattner et al, 1986). Some of the 16/6 ID-positive antibodies in the cultures with α - interferon were DNA reactive.

Another public idiotype, 3I, was described for human anti-DNA antibodies (Solomon et al, 1983). It was identified by mouse monoclonal antibodies which were obtained from spleen cells of a mouse immunized with affinity-purified human SLE serum anti-DNA antibodies. This 3I idiotypic marker was expressed at high levels in 85% of the sera from SLE patients. The sera from unaffected SLE relatives also contained antibodies with 3I ID but without DNA binding properties. (Halpern et al, 1985). Ten percent of the myeloma proteins had 3I of which about a third had anti-nucleic acid reactivity. Although this ID was represented on IgM, IgA and IgG myeloma proteins, the anti-DNA antibody was predominantly on IgG (Diamond and Davidson, 1986).

Taken together a very uniform picture emerges from all these findings. An anti-DNA autoantibody response formerly thought to be unique to SLE is part of the B cell repertoire of a normal immune system. This normal anti-DNA antibody response can be demonstrated under permissive conditions in vitro where the immunoregulatory mechanisms may be disturbed. Mitogen (PWM) stimulate in vitro production of anti-DNA antibodies by normal tonsil lymphocytes and to a lesser extend by circulating normal lymphocytes.

The anti-DNA antibodies of normal origin are qualitatively identical to the anti-DNA antibodies of SLE. Their first striking feature is polyreactivity with a variety of antigens, some of which include single stranded DNA, double stranded DNA, synthetic double

stranded polynuleotides enriched in GC or AT, cardiolipin, IgG and vimentin. It has not been defined what common epitope, if any, on these apparently unrelated molecules is being recognized by these polyspecific anti-DNA antibodies.

The other remarkable property of anti-DNA antibodies of SLE and normals is that they are idiotypically related to one another and that they are also idiotypically related to non-DNA binding antibodies. It is strongly suggestive therefore that these cross-reactive antibodies are encoded by common or highly homologous germ line genes. These autoantibody encoding genes may have been conserved in the germ line primarily because of their anti-bacterial function and/or regulatory function. The molecular analyses which have already been initiated supports the idea of these anti-nucleic acid autoantibodies being encoded by the germ line gene (Barrett and Trepicchio, 1986; DerSimonian et al, 1986; Naparstek et al, 1986).

The genes giving rise to 4.6.3 ID cross-reactive DNA and non-DNA binding antibodies are expressed in the majority of SLE patients, in some patients with Waldenstrom macroglobulinemia and in myeloma patients; however, they are not expressed or infrequently expressed in vivo in normal individuals. The stimulus for activation of these genes in SLE and the mechanisms leading to their suppression in normal individuals are yet to be determined.

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