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City University of New York PH.D. 1986

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IMMUNOCHEMICAL, CELLULAR AND MOLECULAR ASPECTS OF RHEUMATOID FACTORS AND OTHER AUTOANTIBODIES

BY

AUDREY J. MANHEIMER-LORY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1986

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ABSTRACT

IMMUNOCHEMICAL, CELLULAR AND MOLECULAR ASPECTS
OF RHEUMATOID FACTORS AND OTHER AUTOANTIBODIES

by

Audrey Jill Manheimer-Lory

Adviser: Professor Constantin A. Bona

The nature of the immune response to polysaccharide thymus-independent (TI) antigens was investigated in rheumatoid factor (RF) producing mouse strains, 129/Sv and MRL/lpr, and their normal congenic counterparts, 129/J and MRL +/+. An age-dependent variation of clones specific for bacterial levan (BL) and alpha 1, 3 dextran B1355 (Dex) was observed in 129/J mice. The anti-BL and anti-Dex responses observed for 1-month-old 129/Sv mice far exceeded those of their age-matched controls indicating an accelerated ontogenic development of these immune responses. A poor response was observed for both MRL+/+ and MRL/lpr mice after immunization with BL. MRL mice were unresponsive to Dex. These mice, however, could respond to the T-dependent (TD) form of this antigen, suggesting that perhaps these mice lack a subset of B cells required to respond to TI-2 antigens. The most striking observation was the occurrence of isotype-specific RF subsequent to immunization with these antigens in animals prone to develop RF, as well as in aged animals that do not spontaneously produce RF.

The secondary immune response to TNP-KLH and the anti-immunoglobulin response were studied in 1-, 3- and 6-month old 129/J, 129/Sv MRL+/+ and MRL/lpr mice. A profound inability of aging 129/Sv mice and MRL/lpr mice

to produce IgG_{2a} (129/Sv) and all IgG (MRL/lpr) anti-TNP antibodies was observed. This markedly low responsiveness could not be related to the presence of RF since their normal counterparts also produced RF after immunization.

We investigated the immunochemical and molecular characteristics of murine monoclonal RF and other autoantibodies. Although the specificities of these autoantibodies varied, many expressed an interstrain cross-reactive idiotype (IdX). Our panel utilizes a restricted set of the V_H repertoire representing the more 3' V_H families (V_H 7183, V_H Q52, V_H J558). The nucleotide sequences of two cloned RF V_H genes show no major differences between V_H genes encoding the heavy chain of autoantibodies and antibodies against foreign antigens. Finally, 60% hybridomas produced from BALB/c, NZB, or MRL/lpr which express V_H 7183 genes, are specific for self-epitopes and express IdXs.

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INTRODUCTION

It is generally accepted that the animal organism acquires the ability to distinguish between self and non-self in the immune response during ontogeny. Indeed, as early as 1900, Paul Erlich and Julius Morgenroth found that the injection of self-antigens into the autologous host did not elicit an immune response (Ehrlich, 1900). Similarly, when animal hosts were injected with their own red blood cells, no autoantibodies were produced. From these experiments, Ehrlich formulated a concept which he called 'horror autotoxicus.' This concept considered that the immune response against self-antigens was harmful.

Not until the mid 1900's did Ehrlich's original idea come to be expressed as Burnet's concept of 'self-tolerance' (Burnet, 1959). This hallmark theory implicated the thymus as an organ of surveillance which could prevent anti-self clones from arising. Nevertheless, even Ehrlich had recognized that a disruption of immunoregulatory mechanisms could lead to the manifestation of the autoimmune state. It is now clear that the phenomenon called autoimmunity is a result of multiple factors: genetic predisposition, immune incompetence, exogenous factors (viral, bacterial) and physiological factors associated with sex hormones and aging (Hirokawa, 1985).

The decline of age-related immune functions has been well documented in both humans and mice (Heimer, 1963; Hallgren, 1973; Makinodan, 1980). Although it appears that macrophage and dendritic cells function at capacity in older individuals, both B and T cell function suffers. Generally, B cells become less responsive in their ability to proliferate

after stimulation with B cell mitogens and their capacity to produce Ig after antigenic challenge diminishes (Abraham et al., 1977; Callard et al., 1977; Kishimoto et al., 1976). Furthermore, several T cell functions are impaired in the aged mouse or human. For example, in NZB mice there is an increase in polyclonal helper activity (Theofilopoulos et al., 1985). The data on suppressor cells (Ts) is controversial. One might expect that a decrease in Ts cells might provide the foundation for the autoimmune response, yet various investigators report an increase in this population as a function of age (Goidl et al., 1976).

It is well established that some autoimmune diseases involve genetic predisposition and susceptibility is most likely a multifactorial based process in both animals and humans. It may be that the genes of the major histocompatibility complex (MHC) (the HLA antigens) play a critical role in predisposition, immunoregulation and etiopathogenesis of autoimmunity. Many human autoimmune diseases have been found to be positively correlated with the HLA-B and HLA-D alleles (Svejgaard, 1983).

The contribution that viral infections make to the etiology of autoimmunity has also been pursued. Since viruses are especially capable of forming immunogenic units with surface antigens of infected host cells, some viruses such as Epstein-Barr virus may induce B lymphocytes to proliferate by themselves (Cruse and Lewis, Jr., 1985). In this way, virus-host cell surface antigens may initiate T lymphocyte helper effects leading to autoantibody formation. In particular, in NZB mice, type C retrovirus may be associated with autoantibodies produced against red cells and nucleic acids (Theofilopoulos et al., 1981). It is hypothesized that viral infections may stimulate the production of autoantibodies by the

creation of neoantigens in complexes with histocompatibility antigens (Allison, 1977), by nonspecific stimulation of B lymphocytes (EBV) specific for autoantigens and by inducing the expression of antigens not normally expressed by the host cells.

In the early days and in the years that followed, many advances were made by prominent physicians and immunologists in the recognition of the etiopathogenesis of autoimmunity. In fact, there were many claims that one type of disease process or another had an autoimmune basis. Donath and Landsteiner (Cruse and Lewis, Jr., 1985) observed that patients with a blood disease (hemolytic anemia) would develop a serum antibody that could adsorb to their erythrocytes at low temperatures and lead to lysis by complement upon warming. In an attempt to uncover the etiology of certain hemolytic disorders, Dameshek et al. (1938) injected guinea pigs with rabbit antibody prepared against guinea pig red cells. They could then induce experimental hemolytic syndromes similar to the human disorders. Although this advance allowed these investigators to eliminate the idea that there was an intrinsic erythrocyte defect in these diseases, it was not until the development of the Coombs test some years later for the detection of antiglobulin against red cells that further progress was made. At this time, the introduction of the term "autoantibodies" into the literature was made and the association of these globulins with other blood diseases soon followed (e.g., anti-platelet antibodies in idiopathic thrombocytopenia purpura (Cruse and Lewis, Jr., 1985).

Autoimmune diseases have been categorized as either organ specific e.g., Hashimoto's thyroiditis or as non-organ specific/systemic disorders e.g., systemic lupus erythematosus (SLE). Thyroiditis was considered an

autoimmune disease in the mid 1950's by Witebsky and Rose (1957). They showed that rabbit thyroglobulin (Tg) plus Freund's complete adjuvant (CFA) could elicit antibodies directed against thyroid extract in the autologous host. This response was shown to be T cell dependent. Roitt and his co-workers confirmed that the serum of Hashimoto's thyroiditis patients contained antibodies to human Tg (1956). Initial studies on anti-Tg autoantibodies were facilitated by the availability of the animal model of the disease, the Buffalo (BUF) rat strain. Zanetti and Bigazzi (1981) described this spontaneous disease in these rats and demonstrated that neonatal thymectomy increases the incidence of disease presumably by depleting T suppressor cells.

Another disease of endocrine origin, Insulin-dependent Type I Diabetes Mellitus was recognized as an autoimmune organ specific disorder because the inflammatory lesions in the pancreatic islets were a result of immunologic attack on the beta cells of the islets (Gepts, 1965).

Neurologic disorders have also been described as autoimmune in etiology. That is, immune reactions leading to myelin tissue injury could be attributed to reactivity to central nervous system (CNS) antigens (Kabat et al., 1947). Injection of homologous CNS antigens in monkeys induced allergic encephalomyelitis. This animal disease is reminiscent of the human autoimmune disease, multiple sclerosis, in which the principle antigen was shown to be myelin basic protein (MBP) (Paterson, 1977).

Another neurologic disorder, myasthenia gravis, is one in which the presence of antibodies to acetylcholine receptor is prominent. This results in the destruction of these receptors and could possibly account for the physiological tissue injury that is associated with the disease

(Ito et al., 1978).

Several autoimmune diseases of the skin have been described such as pemphigus and scleroderma. Beutner and Jordan (1964) found the presence of antibodies which could react with intercellular substances of stratified squamous epithelium in sera from patients diagnosed with pemphigus vulgaris. They demonstrated that the course of disease could be monitored by the rise and fall in antibody titer. The antibodies were considered to be autoantibodies since the serum antibodies from the patients were reactive with the individual's own skin. Scleroderma is a connective tissue disorder of unknown origin characterized by fibrosis and changes in the skin, arteries, and small intestinal organs. Its autoimmune nature is characterized by the presence of antinuclear antibodies, cryoglobulins and other autoantibodies (Kumar et al., 1985). Terato and co-workers (1985) have described an arthritic condition in mice induced by Type II collagen that serves as an animal model of this disease.

Although these diseases have been extensively studied, it is without a doubt that systemic autoimmune diseases such as SLE and rheumatoid arthritis (RA) have been the subject of the most intense investigations over the years. SLE was first described as a cutaneous disease in the early 1800's, but was later recognized as a multisystem disorder. It was not until the 1940's that SLE was established as an immunologic disorder. This was due primarily to the advances made in autoantibody detection tests beginning with the LE cell test (Hargreaves et al., 1948). Certainly, the association of anti-nuclear and anti-DNA antibodies with SLE has aided physicians in the diagnosis of this disease (95-98% of SLE patients are positive).

Although we have learned much about human SLE in recent years, our knowledge about this disease has come primarily from the available animal models. In fact, four spontaneous models of lupus have been extensively studied: NZB, (NZB x W) F1, BXSB and MRL. The disease process is not homogeneous in these animals, yet several common abnormalities exist among them. In particular, B cell hyperactivity is a feature common to all these SLE strains. In addition, these models are characterized by hypergammaglobulinemia, anti-DNA and anti-nuclear antibodies, anti-retroviral gp70 antibodies and circulating immune complexes (Theofilopoulos and Dixon, 1981). One other common feature among these strains is that when these mice are made congenic for the xid (x-linked immunodeficiency) gene from CBA/N mice, all exhibit markedly reduced levels of autoantibodies and a delay in onset and severity of disease (Steinberg et al., 1982; Steinberg et al., 1983), but this effect is not entirely complete.

The genetic derivation of these strains is quite different. NZB and NZW were spontaneously derived and inbred for color from a stock of undefined background (Theofilopoulos and Dixon, 1981). BXSB mice were generated by crosses between C57Bl/6 female mice and SB/Le male mice selected for a satin beige coat (Andrews et al., 1978). MRL mice were first described by Murphy and Roths (1979) and were the result of inbreeding among four strains: LG (75%), AKR (13%), C3H (12%) and C57Bl/6 (0.3%). All of these models exhibit two forms of the disease: a late-life type which usually appears during the second year of life (NZB x W male; BXSB female and MRL+/+ of both sexes) and an acute onset form in which phenotypic and functional abnormalities of the disease can occur within the first weeks of life (NZB) and death (usually from glomerulonephritis)

can occur within the first year of life (NZB x W female, BXSB male and MRL/lpr of both sexes). Male and female NZB mice develop a late form of lupus that is not influenced by sex. The development of autoimmune hemolytic anemia is characterized by the early appearance of anti-erythrocyte and anti-thymocyte antibodies by 3-4 months of age (Talal, 1976), but a low incidence of anti-nuclear antibodies. The F1 hybrids (B x W) F1 display symptoms quite reminiscent of human SLE. It should be mentioned that accelerated maturation and aging of immune functions in NZB mice has been reported. More specifically, the thymus prematurely involutes and there is prominent degeneration of epithelial cell vascularization (De Vries et al., 1967). In fact, young adult NZB mice seem to lack a thymic hormone activity. It is yet unclear what role the thymus plays in the autoimmune process in these mice. Some investigators have demonstrated that neonatal thymectomy accelerated disease in NZB x W females (Steinberg et al., 1980). Contrary to this, athymic (nude) NZB and (B x W) F1 mice have been reported to develop disease in a similar time frame to that of their nude (nu/+) counterparts (Gershwin et al., 1980). Theofilopoulos et al. (1980) have found that thymectomy had no real effect on the course of disease in NZB mice.

B cell function in NZB mice has been the subject of intense investigation by several groups (Moutsopoulos et al., 1977; Manny et al., 1979; Hayakawa et al., 1983). This work indicates that the primary defect in the NZB mouse is at the level of the B cell. Moutsopoulos et al. (1977) found that unlike normal mice, NZB and (B x W) F1 mice exhibit a hypersecretion of IgM even at birth, and by 6-8 weeks of age, spleen cells from these mice could produce as much as 40 fold more IgM than those of normal

mice. Manny et al. (1979) confirmed these studies and also showed that 18 day and 19 day fetal liver cells matured into IgM hypersecretors in the absence of T cells. It has been implied that a small subset of B cells, the Ly1.$^{+}$B cell may be responsible for autoantibodies seen in this strain and in other SLE strains (Hayakawa et al., 1983). These cells have a high ratio of sIgM to sIgD and are present in high frequencies in these mice unlike normal mice. Apparently, this subset is involved in high spontaneous (non-mitogen stimulated) IgM hypersecretion.

BXSB mice spontaneously develop a fatal disease characterized by high levels of serum immunoglobulins (Igs), including anti-nuclear antibodies and anti-thymocyte antibodies. Unlike the NZB strain, the male BXSB mice experience pronounced disease. This has been linked to an acceleration factor: a Y chromosome-linked gene (Eisenberg and Dixon, 1980), yet the mechanism is still poorly understood.

MRL/lpr mice probably have been the most widely studied of all the SLE strains. The onset of disease usually occurs at 3 months of age and is characterized by the features previously mentioned. A necrotizing polyarteritis and a RA-like disease is concommitant with the presence of RF in serum, anti-Sm antibodies and tissue deposits of the IgG_2 subclass (Theofilopoulos et al., 1980; Theofilopoulos and Dixon, 1981). In these mice the onset of symptoms depends upon two factors: the autosomal recessive lymphoproliferative (lpr) gene and the thymus. It is clear that the production of certain autoantibodies can be unique to a particular SLE strain and thus one might surmise that the production of these autoantibodies is under separate gene control. Furthermore, some autoantibodies (anti-red cell or anti-Sm) play very small roles (if any) in mediating the

lethal lesions of the disease. The lpr gene is a prime example of a gene which may be crucial in inducing disease unlike other genes. This was shown when Steinberg et al. (1983) introduced this gene onto the background of non-autoimmune mice (C57Bl/6 and C3H). Although autoantibodies were produced, the severity of disease was lessened and it occurred later in life. The role of the thymus has been clearly defined in these mice. It has been shown that neonatal thymectomy prevents lymphadenopathy and the lymphoproliferative disorder, delays the onset of disease and reduces the levels of anti-DNA and other autoantibodies (Steinberg et al., 1980). This, however, is not the case when thymectomy is at 1 month of age (Hang et al., 1984). Finally, when the thymus from a +/+ mouse was transplanted into a neonatally thymectomized lpr mouse, the result was early disease indicating that the genotype of the thymus is not critical (Theofilopoulos et al., 1985).

As mentioned above, RA is a general clinical manifestation in MRL mice and the presence of serum rheumatoid factors (RF) is quite common by 3 months of age. Although this spontaneous animal model was described in the late 1970's, it was over forty years ago in the human system that RF were discovered (Waaler, 1940). In these studies, Waaler was actually re-investigating an earlier observation in which it was seen that certain human sera were able to agglutinate sensitized sheep red cells when routine complement fixation tests were being performed for the detection of syphilis. Although Waaler's experiments found only limited agglutinating activity in normal sera, patients with RA had elevated activity. This factor was renamed 'rheumatoid factor' based on its association with RA. Although RA primarily affects the joints, like SLE, it

too is a systemic disorder which can be associated with malaise, fatigue, pericarditis, scleritis and vasculitis (Pope and Talal, 1985). At the peak of autoimmune discoveries in the mid 1950's, the demonstration that RF reacted best with heat-aggregated or denatured gammaglobulin led to the proposal that perhaps RF were autoantibodies. Franklin et al. (1957a, b) found both 19S RF and 7S gammaglobulin as a 22S complex when ultracentrifugation was used to study RA patients' sera. Sera from patients with advanced RA contained complexes which sedimented in the range of 9S to 17S and these complexes could readily dissociate to 7S units (Kunkel et al., 1961). This indicated that RF were generally IgM or IgG that could bind to antigenic determinants on autologous IgG. RF were not only found in association with RA, but later found in sarcoidosis, subacute endocarditis, hepatic diseases, diffuse scleroderma and Waldenstrom's macroglobulinemia (Carson et al., 1981).

The specific reactivity of human RF with antigenic determinants is located on the Fc fragment of IgG molecules, particularly on the CH2 and CH3 domains. These determinants include a) Gm allotypic determinants (Natvig and Kunkel, 1967), b) antigenic determinants shared by various IgG subclasses (Allen and Kunkel, 1966), or c) by IgG from various species (Williams and Kunkel, 1963). RF obtained from a mouse strain that spontaneously produces anti-Igs (129/Sv) recognize antigenic determinants located on the CH3 domain of IgG_1 and the C-terminal 8 residues of the CH2 domain plus the complete CH3 domain of IgG_{2a} (Stassin et al., 1983). The 129/Sv and MRL/lpr mice serve as models for the spontaneous production of RF (Van Snick and Masson, 1979; Andrews et al., 1978). Each of these strains has enabled investigators to study the age-dependent occurrence of

RF. Unlike MRL mice, the occurrence of RF in 129/Sv mice has not been associated with a well-defined autoimmune process. The high incidence of anti-Ig activity in the sera of these mice was shown to exist even prior to artificial induction (Van Snick and Masson, 1979). Dresser (1978) showed that most IgM-producing cells in the mouse secrete autoantibodies (RF) after LPS administration. RF from 129/Sv mice were characterized as Igs of the alpha and mu isotypes (Van Snick and Masson, 1979). IgA RF, a product of lymphocytes associated with gut lymphoid tissue was highly specific for IgG_{2a}, whereas IgM RF, a splenic lymphocyte product exhibited much broader specificity for various IgG subclasses (Van Snick and Masson, 1979; Van Snick et al., 1983). In a series of papers by Coulie and Van Snick (1983a, 1983b, 1985), the production of IgM RF after immunization with protein antigens was shown in 129/Sv mice. These RF were transient and later it was shown that the activation of RF precursors was induced by the interaction with immune complexes and carrier-specific helper T cells (1985).

The fundamental belief has been that a) RF are associated with chronic diseases and aging and b) pathological consequences may result from their presence in the circulation. That is, they can induce and/or maintain the disease state by forming immune complexes in sera and in the synovia. However, it is within the last few years that this concept has been questioned. Nemazee and Sato (1983) described the appearance of RF in A/J mice, a strain which does not produce autoantibodies normally. This autoimmune response was associated with hyperimmunization to a T-dependent antigen, Ars-LPH, inducing RF specific for IgG_1. Thus, there appears to be a paradox: why are RF (generally associated with adverse

effects to the host) produced during conventional antigenic stimulation? This phenomenon is not unique to mice. In fact, in humans it was shown that even after vaccination with Tetanus Toxoid, RF occur (Welch et al., 1983). It has also been demonstrated that RF exist transiently in the circulation of patients during the course of infections such as syphilis, tuberculosis, mononucleosis, chronic active hepatitis, subacute bacterial endocarditis, and rubella (Almeida et al., 1980; Tsoukas et al., 1980; Carson et al., 1981). Therefore, some investigators have ascribed beneficial roles for RF. More specifically, a RF-like activity was described in the serum of lactating rats which have never been infected with the parasite Trypanosoma lewisi (Clarkson and Mellow, 1981). This factor was shown to confer resistance to infected lactating rats and their suckling progeny by amplification of a specific gammaglobulin response. Furthermore, another rheumatoid-like factor which is produced late in the course of this infection, amplifies an earlier IgG response and is capable of terminating the infection in nonlactating rats.

It is apparent from the previous descriptions of the various autoimmune diseases, that the anti-self response in humans and in animals has been approached in various ways. In efforts to better understand the nature and binding properties of antibodies specific for self-molecules, investigators have used anti-idiotypes (anti-Ids) to explore the relatedness among molecules with specificity for the same or different antigens (Zanetti et al., 1986). The idiotypes (Ids) of two organ specific autoantibodies, anti-Tg (Hashimoto's disease) and anti-acetylcholine receptor (AchR) (Myasthenia gravis) have been well characterized.

Autoantibodies produced in Hashimoto's disease or in the experimental

models is limited. Therefore, this facilitates the study of the idiotypy of the Igs. Using rabbit anti-idiotype antibodies prepared against BUF rat serum affinity purified autoantibodies, it was reported that these antibodies express a cross-reactive idiotype (IdX) (Zanetti, 1986). In fact, this was evident in the majority of rats studied and was apparently up to 50% of the total serum autoantibodies. Zanetti et al. (1983b) could identify the same IdX on autoantibodies to Tg from other strains or species and even on human autoantibodies. This group has also documented a high degree of idiotype cross-reactivity among murine monoclonal autoantibodies directed against a highly conserved epitope on Tg (Zanetti et al., 1983a). This idiotype, Id 62, was found in serum of BALB/c mice immunized with Tg as well as in mice immunized with anti-Id antibody. Id 62 was also expressed on anti-Tg antibodies produced by BUF and BB rat strains. Zanetti et al. (1985) demonstrated that anti-Id antibodies specific for Id 62 react with the same idiotope on both heavy and light chains.

Antibodies to AchR have been extensively studied because of their involvement in myasthenia gravis. IdX was detected on anti-AchR antibodies from different strains and species when anti-Id antibodies were produced in mice after immunization with syngeneic T lymphoblasts educated in vitro with acetylcholine receptor (Schwartz et al., 1978). In humans, immunization with receptor leads to the production of specific antibodies and to muscle weakness. Lefvert (1981) and Lefvert et al. (1982) were able to demonstrate IdX on antibodies specific for AchR in sera of 60% of myasthenic patients tested. Interestingly enough, this Id was also found in the supernatant of PWM stimulated lymphocytes from healthy subjects.

This indicates that the precursors of anti-AchR antibodies are present in the normal population.

The second group is the non-organ specific autoantibodies which is exemplified by the systemic diseases SLE, RA and Sjorgen's syndrome.

In patients with SLE and in mice which produce SLE-like syndromes, autoantibodies to DNA are prevalent. In mice, many groups have shown that anti-DNA antibodies from NZB/W and MRL/lpr strains possess IdX (Andrejewski et al., 1981; Marion et al., 1982; Rauch et al., 1982; Tron et al., 1983; Hahn and Ebling, 1984). Shared idiotypy was demonstrated among monoclonal anti-DNA antibodies with different epitope specificity (Andrejewski et al., 1981; Marion et al., 1982). The idiotypy among human antibodies has also been studied. To examine the relatedness of autoantibodies to DNA among unrelated individuals, mouse monoclonal anti-Id antibodies were generated (Diamond and Solomon, 1983; Solomon et al., 1983). The expression of the IdX, 3I, is found in high titers among relatives of patients with SLE and usually occurs in the absence of anti-DNA activity (Halpern et al., 1985).

Another class of autoantibodies found in lupus is that specific for a cellular constituent - a nuclear glycoprotein, Sm. Several monoclonal anti-Sm antibodies have been prepared in MRL/lpr mice. Anti-Id antibodies against these monoclonal antibodies were prepared in rabbits (Pisetsky and Lerner, 1982). Dang et al. (1985) showed that anti-Id antibodies can inhibit the Sm specific proliferative T cell response in mice primed with the Sm antigen in CFA. This suggested that T cells recognizing the Sm antigen share the idiotypes of anti-Sm antibodies.

Probably the most widely occurring autoantibodies in both humans and

mice, the RF, was first established by Kunkel et al. (1973) to not only possess private idiotypic specifities, but also public determinants. The Wa specificity was found to account for approximately 60% of all RF whereas the minor IdX, Po, accounts for 30% of all RF. This group is considered to be IdXs of the germline type. Heavy chain variable region peptide analysis showed that two antibodies sharing IdX shared sequence identity (Capra and Kehoe, 1974) and thus this idiotype was likely to be a germline-determined one. The prevalence of the Wa IdX in RA was confirmed by the fact that PWM stimulated lymphocytes from RA patients express the Wa IdX at a higher rate than lymphocytes from healthy subjects (Bonagura et al., 1982). In the early studies by Kunkel's group (1973), it was also found that in most of the IgM RF proteins, the V_{KIIIb} light chain was used as opposed to control proteins which lacked RF activity. This suggested that the major contributor to the Wa IdX was the V_{KIII} light chain. Recent evidence from Chen et al. (1985b) supports this view. They prepared a synthetic peptide corresponding to the second complementarity determining region (CDR) of V_{KIII} light chain. Antibodies to this peptide could bind efficiently to all Wa^+ proteins and isolated kappa chain lending support to the idea that the IdX was largely dependent upon the primary sequence of the CDR2 segment of the light chain.

In contrast to this type of IdX on monoclonal RF, widely cross-reactive Ids on polyclonal RF from unrelated individuals were reported by Forre et al. (1979) and Fong et al. (1983). Fong et al. used a new method of purification in which rabbit anti-Id (RF) was isolated on an affinity column of an 'Id-equivalent' produced in the same species thus selecting specifically for anti-Id that recognized the antigen binding site

(internal image) (Colvin and Olson, 1985). This anti-Id could react with almost 80% of polyclonal RF and 100% of monoclonal RF even though differences in light and heavy chain variable region subgroups were present and differences in hypervariable regions were obvious. In 1980, it was shown by Agnello et al. that a particular epitope specificity could be correlated with idiotype. An IdX that was originally characterized on a monoclonalRF which bound to a DNA-histone complex and Fc IgG, was shared among other polyclonal RF of similar specificity. Chen et al., (1985a) presented data showing that anti-Id against RF reacts both with the Id of RF and the antigen recognized by RF. This type of antibody was designated epibody (Bona et al., 1982).

Recently, there has been much experimental evidence indicating that spontaneousautoimmune responses may be regulated through the immune network. Naturally occurring autoanti-Id antibodies specific for autoantibodies have been documented in several systems (DNA, Coombs, AchR, RF, Tg). Autoanti-Ids to DNA in serum of lupus patients could specifically block the binding of DNA autoantibodies to DNA during the inactive phase of disease (Abdou et al., 1981). In NZB mice, Cohen and Eisenberg (1982) showed that F1 hybrids and several normal strains which produce low quantities of anti-erythrocyte antibodies, spontaneously develop autoanti-Ids which agglutinate erythrocytes from Coombs positive NZB mice. These autoanti-Id antibodies are hypothesized to play some role in down regulation of Coombs autoantibody formation in mice. Dwyer et al. (1983) studied the incidence of autoanti-Ids in many patients with myasthenia gravis. 40% of the sera tested contained antibodies specific for the Id expressed on a mouse monoclonal antibody with anti-AchR specificity.

(There was an inverse relationship between anti-AchR antibodies and auto-anti-Id antibodies). Therefore, these reports provide evidence for the existence of naturally occurring antibodies to idiotypes expressed by self-reacting Igs. The true functional role(s) (down regulation) of these autoanti-Ids still needs to be evaluated further.

Thus, the anti-self response may be a manifestation of many complex processes including physiologic and genetic responses. All of the factors discussed above appear to contribute in some way to the transition from autoimmune response to autoimmune disease.

In the sections that follow, data is presented that focus on the immune response and anti-self response in two spontaneous murine models of autoimmunity. In particular, the in vivo immune response to various T-independent and T-dependent antigens in 129/J, 129/Sv, MRL+/+ and MRL/lpr mice was explored. The results on the kinetics of these responses and the anti-Ig phases of the response is discussed. Furthermore, the functional role(s) of the RF was investigated in an in vitro antibody synthesis assay through the utilization of monoclonal anti-Ig antibodies. The possible significance of RF production during conventional responses is outlined.

In the latter half of this report, the molecular analysis of hybridomas which secrete RF and other autoantibodies is presented. More specifically, the variable region heavy chain gene families were identified for a panel of RF and other autoantibodies, two representative RF were genomically cloned and sequenced, and the signficance of cross-reactive idiotypes among various types of autoantibodies is considered.

MATERIALS AND METHODS

Animals. One, three, and five or six month old 129/J, 129/Sv, MRL +/+ and MRL/lpr mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Two and three month old BALB/c, C57Bl/6 and C58/J were also obtained from The Jackson Laboratories, whereas BALB.K and BALB.B were bred in our own facility. Young adult female New Zealand White (NZW) rabbits obtained from The Jackson Laboratories were used to prepare anti-idiotype antisera.

Antigens.

T-Independent Antigens: TI-2. Bacterial levan (BL) was isolated by alcohol precipitation from culture supernates of Aerobacter laevenicum (ATC15552) grown at 23°C in nutrient broth supplemented with 5-10% sucrose (Lieberman et al., 1975). Alpha 1,3-Dextran (B1355frS) (Dex) was a gift from A. Jeanes (retired from Northern Regional Research Center, Peoria, IL). A periodate-oxidized derivative of Dex was prepared according to Sanderson and Wilson (1971). Trinitrophenyl-aminoethylcarbamylmethyl-Ficoll (TNP-Ficoll) was prepared according to the method of Inman (1975).

TI-1. Trinitrophenyl (TNP)-Brucella abortus (BA) (TNP-BA) was a kind gift from Dr. J. Mond (Uniformed Services, University of the Health Sciences, Bethesda, MD). TNP-Nocardia Water Soluble Mitogen (TNP-NWSM) was prepared from Nocardia opaca as previously described by Ciorbaru et al. (1975) and was a gift from Dr. R. Barot (Universite Paris Sud, Orsay, FR).

T-Dependent Antigens. TNP-keyhole limpet hemocyanin (KLH) and TNP-ovalbumin (OVA) were prepared according to the methods of Rittenberg and

Table 1

Genetic Characteristics of Mice Utilized in These Studies

	H-2	Igh-V	Igh-C
BALB/c	d	a	a
BALB.B	b	a	a
BALB.K	k	a	a
C57Bl/6	b	b	b
C58/J	k	a	a
129	b	b	a
MRL	k	j	j
NZB	d	d	n

Pratt (1969) and Park et al. (1983). KLH was purchased from Calbiochem-Behring, Los Angeles, CA). B1355 Dex-KLH was prepared and donated by R. Ward (Roswell Park, Buffalo, NY).

Mitogens - Lipopolysaccharide (LPS). In vitro stimulation studies employed E. Coli LPS (50 ug/ml). In vitro anti-sheep red blood cell (SRBC) studies utilized 10 ug LPS. Yersinia enterocolitica was kindly donated by Dr. J. Rudbach (Abbott Lab., Chicago, IL).

Antigens Used to Test Binding Properties of Monoclonal Antibodies Generated in Some Molecular Studies. Myelin basic protein was a kind gift from Dr. G. Lewis (Institute for Basic Research Devel. Disabilities, Staten Island, NY), and Sm antigen was kindly donated by Dr. H. Dang (University of South Texas, San Antonio, TX). Thyroglobulin and cardiolipin were purchased from Sigma, murine transferrin was purchased from United States Biochemical Corp., (Cleveland, OH). IgG_3, IgG_1, IgG_{2b}, and IgG_{2a}, were purified from supernatants produced by hybridomas Y5606 (IgG_3), 88-C692 (IgG_1), BA6 (IgG_{2b}), and HOPC1 (IgG_{2a}).

Labelling of Sheep Erythrocytes (SRBC) with BL, Dex, Ars and TNP. An o-stearoyl derivative of BL was prepared according to the method of Hammerling and Westphal (1967). SRBC (Pocono Rabbit Farms, Canadensis, PA) were washed three times with saline and resuspended to a final concentration of 10% v/v. The reaction vessel contained 1 ml of 10% SRBC, 3 ml saline and 10 ul stearoyl levan. The mixture was allowed to incubate for 30 minutes at 37°C with gentle agitation. The SRBC were then washed (3 times) with saline to stop the coupling reaction and remove any uncoupled levan. TNP-SRBC were prepared according to the method of Rittenberg and Pratt (1969). Briefly, 20 mg TNP was incubated (stirred)

with 7 mls 0.2M Na Cacodylate Buffer and 1 ml packed washed SRBC for 10 minutes at 25°C. The reaction was stopped by adding a 1:5 dilution of Barbital Buffer (MBB). After pelleting (2000 rpm, 5 minutes), cells were washed 2x with MBB plus glycylglycine to remove unbound TNP. Cells were washed 2x and resuspended to 10% v/v in MBB. Dextran B1355S was coupled to SRBC according to Sanderson and Wilson (1971). SRBC were washed 2x with PBS and once with Borate buffered saline (BBS). To 200 ul of Dextran B1355 (10 mg/ml) in borate buffer, 2 mls of 10% SRBC were added. This was stirred in a scintillation vial at 37°C for 5 hours followed by 2 washes with BBS. Cells were resuspended to 10% v/v in saline. Ars-SRBC were prepared in three steps according to Isakson et al. (1979). To 1 mmol of 1M $NaNO_2$ was added on ice. One minute later, 5 ml cold 150mM NaCl was added and this mixture was incubated for 30 minutes on ice. 400 ul of methyl-p-hydroxybenzimidate-HCl (amidoester) in 5 ml of 35mM sodium borate was adjusted to pH 9.2 with 5N NaOH. 5 ml of the prepared arsonilic acid was slowly added over a period of 20 minutes on ice with stirring and was allowed to stir for 2 hours at room temperature. 1 ml of this mixture was added to 1 ml isotonic borate-NaCl buffer and was incubated overnight (shaking) with 0.5 ml packed SRBC. Finally, coupled cells were washed once with isotonic borate-NaCl buffer and once with saline.

Immunizations.

Age-Dependent Primary Responses: TI-1, TI-2 Antigens. 129/J, 129/Sv, MRL +/+, and MRL/lpr mice were immunized with BL (20 ug i.v.) or Dex B1355S (100 ug i.p.) in Complete Freunds Adjuvant (CFA) or saline. 129/J and 129/Sv mice were immunized i.p. with 0.1 ml of a 0.1% solution TNP-BA. 129/J or 129/Sv mice were immunized i.p. with 20 ug TNP-Ficoll in saline.

Age-Dependent Secondary and Tertiary Responses: TD Antigens. 129/J, 129/Sv, MRL +/+, and MRL/lpr mice were primed with TNP-KLH, 100 ug i.p. in CFA and were challenged 4 weeks later with TNP-KLH 10 ug i.p. in saline 1 week prior to sacrifice. For the tertiary response, mice received an additional boost of 10 ug TNP-KLH in saline. The plaque forming cell (PFC) response was measured 7 days later. 3 month old MRL +/+, MRL/lpr, BALB/c, BALB.K, BALB.B, C57Bl/6, C57Bl/lpr, and C58J mice were immunized i.p. with 100 ug Dex-KLH in CFA with a boost 4 weeks later i.v., with 15 ug Dex-KLH.

In Vitro Anti-SRBC Response: Coculture Experiments, 129 Mice. 129/J or 129/Sv were immunized with 100 ug TNP-OVA i.p. in CFA and boosted 1 month later i.p. with 10 ug TNP-OVA in saline. 129/Sv 6 month old mice were immunized i.p. with 10 ug LPS. 129/J 6 month old mice received 1 x 10^8 SRBC i.p. in saline.

Monoclonal Antibodies (MAb).

Selection of Rheumatoid Factors (RF). Rat anti-kappa antibody generated from the hybridoma 187.1, a gift from Dr. M. Scharff (Albert Einstein College of Medicine, Bronx, NY), was ^3H labelled and used to select hybridomas which exhibited RF activity.

Fine Specificity Assays. IgG_3, lambda (Y5606), IgG_{2a}, lambda (HOPC 1), and IgA, lambda (MOPC315) were donated by Dr. M. Potter (NCI, Bethesda, MD). IgG_1, lambda (88-C692) and IgG_{2b}, lambda (BA6) were donated by Dr. T. Moran (Mount Sinai School of Medicine, NY, NY).

Monoclonal Reagents Used To Test Serum Antibodies for Antigen Specificity and RF Activity. Monoclonal antibodies specific for 2,4-dinitrophenyl included: IgM, kappa (Dr. D. Katz, Medical Biology Institute, La Jolla, CA), 39-8, IgG_3, kappa (Dr. L.J. Rubinstein, National

Institute of Health, Bethesda, MD), 109.3, IgG_1, kappa, 10.12, IgG_{2b}, lambda (Dr. M. Zanetti, Medical Biology Institute, La Jolla, CA) and IC4, IgG_{2a}, kappa (Dr. Z. Ovary, New York University, NY). Monoclonal anti-arsonate antibodies included: IgG_3 (ascites); 36-71, IgG_1; 31-64, IgG_{2b}; and 31-62, IgG_{2a} (Dr. A. Marshak-Rothstein, Boston University Medical School, Boston, MA).

In Vitro Antibody Synthesis Studies: 129 and MRL Mice. Monoclonal anti-Thy-1.2 and anti-Lyt-1.2 antibodies (NE 1-017) were purchased from New England Nuclear, Boston, MA.

Induction of RF. BALB/c splenic lymphocytes cultured in RPMI supplemented with 20% FCS were stimulated with E. coli LPS (50 ug/ml) for four days. BALB/c mice were injected i.p. with 10 ug Y. enterocolitica. Y. enterocolitica was utilized since it is believed to be involved in several human autoimmune diseases: Y. arthritis, Graves' disease, and Reiter's Syndrome (Calin et al., 1976, Ford et al., 1977, Weiss et al., 1982).

Preparation of Monoclonal RF (MRF). Spleen cells were harvested from the spontaneously producing RF mouse strains (6 month old) 129/Sv and (5 month old) MRL/lpr and fused with the nonsecretory tumor line sp2/0 using standard protocol (Kohler and Milstein, 1975). LPS stimulated BALB/c lymphocytes 5×10^5 or Y. enterocolitica primed BALB/c lymphocytes were also fused with sp2/0.

Selection of RF and Fine Specificity Assays. Among the various MAb obtained, RF were selected via radioimmunoassay (RIA) in which microtiter plates were coated with 5 ug heat aggregated (15 min. 63°C) monoclonal antibody HOPC1 for 1 hour at 37°C. Plates were washed three times with

phosphate buffered saline (PBS) and subsequently incubated with 3% Bovine serum albumin (BSA) to block nonspecific sites (1 hour, 4°C). Plates were then washed as above and hybridoma supernatants were added to the plates (4°C, overnight). After washing, monoclonal ^{125}I labeled rat anti-kappa antibody was used as the developing reagent allowing us to measure 98% of antibodies with RF activity. Radioactivity was measured in a Beckman LS9000 scintillation counter after extensive washing. The fine specificity of the RF was also measured in a RIA. Microtiter plates were incubated overnight with 10 ug monoclonal heat aggregated (15 min, 63°C) Ig bearing lambda light chain. (See MAb). After washing with PBS (3 times) and post-coating with PBS-BSA (1%), the plates were incubated for 2 hours with 2 ug/ml RF monoclonal antibodies. After extensive washing with PBS-Tween 20 (0.05%), the plates were incubated for 2 hours with ^{125}I rat-anti-kappa monoclonal antibody (50,000 cpm/well). The radioactivity was measured in a Packard gamma counter. The background was subtracted (i.e., BSA coated plates incubated with RF MAb).

Hemagglutination Assays (HA). HA were utilized to determine the titer and fine specificity of serum RF. Sera were absorbed with SRBC prior to testing. SRBC were coupled to either TNP (Bona et al., 1982) to test pre-immune sera or with p-azophenylarsonate (Isakson et al., 1979) to test post-immune sera since the immunization protocol involved a TNP conjugate. The assay was carried out in a 96 U-bottom well flexible assay plate (Falcon 3911). Each well received 25 ul of 3% fetal calf serum (FCS) in saline. 25 ul of serum from the different age groups studied was added to the first well and twofold serial dilutions were made, followed by an additional 25 ul 3% FCS. 25 ul of subagglutinating amounts of MAb

directed against either of the above haptens was incubated with the coupled SRBC and the plates were covered with sealers and allowed to sit at room temperature 3 hours. HA titer was scored as $1/\log_2$ of the highest dilution giving agglutination.

Purification of MRF. All IgM MAbs were purified on a Sephacryl 300 Column in Tris buffer pH8.0.

Iodine (Rat anti-kappa) and Tritium (Igs) Labelling of Monoclonal Reagents. MAbs were iodinated by the chloramine T method (Greenwood et al., 1963). Briefly, to a plastic reaction tube containing 50 or 100 ul PBS, 100 ug protein to be iodinated is added. 10 ul of ^{125}I is added next with a syringe followed by 10 ul chloramine T (1 mg/ml in 0.1M Tris-HCl). The reaction is vortexed and allowed to sit for 2 minutes. 10 ul $Na_2S_2O_5$ (2 mg/ml in 0.1 M Tris-HCl) is then added to the reaction and mixture is vortexed again and allowed to sit for 2 minutes. 100 ul KI (5 mg/ml in PBS-BSA 1%) is finally added. The mixture is placed over a Sephadex G25 column (Pharmacia) and fractions are collected (5 drops/fraction). Two peaks of radioactivity are obtained: the first peak contains bound ^{125}I and the second peak has free ^{125}I. Monoclonal Igs were tritiated by the method of Wilder et al. (1979). Igs were radiolabelled via reductive methylation with tritiated [^3H] sodium borohydride (NaB_3H_4). Briefly, 0.2M Borate Buffer (pH9) was placed in a septum topped vial into which 0.1-1.0 ml of protein at 1-10 mg/ml was placed (4°C). After addition of formaldehyde (12-60 umol) from a 3.7% formaldehyde solution in water, the epsilon-amino lysine groups were reduced to the mono- and dimethyl (^3H) derivatives by the addition of NaB_3H_4 (1-15 umol of 10-60 Ci/mmol spec. act.) (prepared prior to use). Subsequently, this solution was added

immediately to the protein formaldehyde solution and allowed to react for 30 minutes on ice. The labelled protein was separated from unincorporated reactants on a Sephadex G25 column which had been pre-washed with 15% BSA and PBS (pH 7.4).

Kinetics of Anti-TNP Response. The kinetics of the anti-TNP response were determined by RIA. Microtiter plates were coated with 5 ug TNP bovine serum albumin (BSA) and incubated 1 hour at 37°C. Following three washings with phosphate buffered saline (PBS), plates were coated with 3% BSA for 1 hour at 4°C to block non-specific binding sites. Plates were washed as before and incubated overnight at 4°C with sera which had been collected from mice on day 3, day 10, day 20, and day 30 post second boost. After three additional washings, plates were reincubated overnight at 4°C with ^{3}H anti-IgM, ^{3}H anti-IgG_1, ^{3}H anti-IgG_{2b} and ^{3}H anti-IgG_{2a} (donated by P.K.A. Mongini, Hospital for Joint Diseases, NY). Radioactivity was measured in a Beckman scintillation counter LS9000 after extensive washing. The concentration of anti-TNP antibodies in the sera of immunized animals was determined from the linear part of a standard binding curve obtained with known amounts of unlabelled IgM, IgG_3, IgG_1, IgG_{2b} and IgG_{2a} monoclonal anti-TNP antibodies.

Detection of Plaque-Forming Cells (PFC). The number of cells secreting IgM antibody specific for BL or Dex was determined by a modification of the Jerne plaquing technique as previously described (Rittenberg and Pratt, 1969; Victor et al., 1983). Briefly, 50 ul of a suspension of immune spleen cells were added to 0.3 ml of 0.5% agarose that contained 50 ul of coupled erythrocytes (10%). Slides (precoated with 0.1% agar) were incubated initially for 2 hours at 37°C and then were reincubated for an

additional hour in the presence of guinea pig complement (1:20) (Flow Laboratories, MacLean, VA). Control slides were prepared by using a mixture of the same immune spleen cells and unsensitized SRBC. The anti-SRBC PFC response, which varied between 0 and 100 PFC/spleen, was subtracted from anti-BL and anti-Dex PFC responses. Anti-BL PFC bearing the E109Id and anti-Dex PFC bearing the J558Id were enumerated by incorporating A/J anti-E109Id antiserum (1:100 final concentration or goat anti-J558Id antiserum (1:500), respectively, into the agarose. The number of PFC secreting antibodies bearing a given idiotype was calculated as the difference between the number of PFC observed in the absence and presence of the anti-idiotype antiserum. The difference was considered to be the number of PFC secreting antibodies expressing the idiotype.

Isoelectric Focusing (IEF). IEF was performed according to detailed methods (Briles and Davie, 1975; Nicolotti et al., 1980). Ten ul of each serum were applied to the focusing gel. Radioactive antigens used for the gel overlays consisted of 20 x 10^6 cpm/gel of ^{125}I-labelled tyraminated BL or B1355S Dex. Autoradiograms were exposed for 7 days or 2 days, respectively. The number of cells secreting IgM antibody specific for TNP was determined in a Cunningham plaquing chamber (Cunningham and Szenberg, 1968). Two glass slides were taped together with double stick tape creating a channel between the two slides. A mixture containing 50 ul spleen cell suspension 10 ul 10% SRBC, 10 ul guinea pig complement (1:3) and when appropriate, 10 ul anti-Id antisera was added to the chambers. Slides were sealed with paraffin and incubated at 37°C for 1 hour. Cells secreting IgG were determined by the addition of developing rabbit anti-mouse Ig (1:640) (Dr. E. Bikoff, Mount Sinai School of Medicine, NY, NY)

and were enumerated by subtracting the number of direct (IgM) PFC from the total number of PFC. The background anti-SRBC PFC was scored in parallel and was subtracted from the anti-TNP PFC response.

In Vitro Antibody Synthesis Assay 129 and MRL Mice. TNP-specific B cells were obtained from mice immunized with 100 ug i.p. TNP-OVA in CFA and challenged 1 month later, i.p. with 10 ug TNP-OVA in saline. Two days before sacrifice, the mice received 0.2 ml of rabbit anti-mouse thymocyte serum (M. A. Bioproducts, Walkersville, MD). The spleen cell suspension was depleted of residual T cells by treatment with a cocktail of monoclonal anti-Lyt-1.2 and anti-Thy-1.2 and rabbit complement (1:20). The KLH-specific T cells were obtained from lymph nodes of mice primed with 100 ug KLH in CFA by injection s.c. at the base of the tail and in the hind foot pads. Seven days later, T cells from the popliteal, periaortic and inguinal lymph nodes were purified on a nylon wool column. Primed B cells (3×10^6) were cultured for 5 days at 37°C in a 7% CO_2 humidified incubator alone or with 1×10^6 KLH-primed T cells in 2 ml Mishell-Dutton media in 60-mm-diameter wells (3524, Costar Plates, Cambridge, MA). The mixture of cells was cultured not rocked) either in the presence or absence of 1 ug/ml or 10 ug/ml TNP-KLH or 10 ug/ml TNP-OVA, as an antigen-specificity control. Only the responses obtained with 10 ug TNP-conjugate were reported.

Regulatory Effects of RF on the Anti-TNP and Anti-SRBC Responses.

Anti-TNP Response. 2×10^6 spleen cells from BALB/c, 129/J or 129/Sv mice were cultured for 3 days in Mishell-Dutton medium containing 1 ug/ml TNP-NWSM. Either FD5-13 (anti-460Id), LP32-14 (IgM MAb) derived from J558 idiotype suppressed mice which is devoid of RF activity and is

of unknown specificity, 129-48 or LPS 10-1 (IgM RF) was added to the culture at concentrations of 0.01-10 ug/ml. After 3 days, cells were harvested and the direct and indirect PFC responses were measured as was the 460Id$^+$ component of the response.

Anti-SRBC Response. The secondary in vitro response of 129/J was measured by culturing spleen cells from primed 129/J mice at various concentrations with 3 x 10^5 SRBC per 1 ml culture. This response was studied after mixing 129/J spleen cells with either spleen cells from naive 129/J or cells from mice known to make RF, i.e., 129/J and 129/Sv boosted with TNP-OVA or cells from 129/Sv mice immunized with LPS. After 4 days of culture, the direct and indirect SRBC PFC responses were measured.

Preparation of MAbs Which Express VH7183 Genes. Splenocytes from BALB/c, MRL/lpr and NZB mice were cultured in RPMI (Gibco) supplemented with 10% FCS, 2-mercaptoethanol, pyruvate, non-essential amino acids, and glutamine. Cells were stimulated with E. coli LPS (50 ug/ml) for 3 days. Lymphocytes were then fused with the nonsecretory tumor line sp2/0 using standard protocol (Kohler & Milstein, 1975). MAbs were purified on Sepharose 4B-rat anti-murine kappa column except Z318 which was purified on a Sephacryl column.

Selection of Hybridomas Positive For VH7183 Gene Expression.

RNA Slot Blotting. RNA was prepared from cytoplasmic lysates of 1-3 x 10^6 hybridoma cells and was applied to nitrocellulose using a Minifold II apparatus (Schleicher and Schuell, Inc. NH). The filters were baked, hybridized to a ^{32}P labelled V_H7183 specific probe (V_H 81X probe donated by G. Yancopoulos, Columbia University, NY), washed and autoradiographed

(Yancopoulos et al., 1984).

Northern Blot Analysis of Total RNA. To confirm results obtained by slot blotting, total RNA was prepared from 3-5 x 10^7 cells using the guanidinium thiocyanate extraction procedure. Briefly, frozen or fresh cells (3-5 x 10^7) were added directly to 4M guanidinium thiocyanate solution and homogenized immediately using a polytron. This homogenate was carefully layered onto a 5.7M CsCl cushion and tubes were spun in a SW41 Beckman rotor at 32 K for 18 hours at 20°C. Supernatants were quickly suctioned off. RNA pellets were rinsed in 100% ethanol at 25°C, resuspended in 10 mM Tris 1 mM EDTA (pretreated with DEPC), extracted once with Phenol-SEVAG-(chloroform/Isoamyl alcohol, 24:1) solution and precipitated twice with 1/10 volume 4M NaCl (DEPC) and 2 volumes of ethanol. Northern blotting (used to confirm V_H gene family assignments) was performed by electrophoretically fractionating the RNA on a 1.2% agarose gel (6% formaldehyde) in 40 mM MOPS, 20 mM NaOAc, 2 mM EDTA. The gel was blotted without pretreatment onto nitrocellulose using 20xSSC (3M NaCl 0.3 M Na-citrate) (Maniatis et al., 1982).

Preparation of Anti-Idiotype Antisera. Female NZW rabbits were immunized subcutaneously with 400 ug MRF (129-48, Y19-10 or LPS10-1) in CFA and boosted monthly under the same conditions. The rabbits were periodically bled and tested for antibody titer. Finally, when anti-idiotype antibody titers were sufficiently high, animals were exsanguinated. Anti-idiotype antibodies were purified on a Sepharose 4B column that had been conjugated to the corresponding RF. The anti-RF antibodies were eluted from these columns and made anti-Id specific by extensive absorption on a Sepharose 4B column that had been linked to a pool of various MAb of the

IgM, kappa isotype that were devoid of RF activity.

Study of Idiotypy (Cross-reactive idiotypes - IdX).

Sandwich RIA to Detect IdX Among RF. Microplates were coated overnight with 2 ug/ml various RF MAbs. After washing and post-coating with PBS-BSA, the plates were incubated for 2 hours with 2 ug/ml rabbit anti-idiotypic antibodies (anti 129-48 Id, anti 129/Sv RF MAb, anti LPS 10-1 Id, anti Y19-10 Id specific for BALB/c RF) or normal rabbit Ig. After washing, ^{125}I donkey $(Fab)'_2$ anti-rabbit Ig (Amersham) was added as the developing reagent (50,000 cpm/well, 2 hour incubation).

Competitive RIA to Detect IdX Among VH7183+ Hybridomas or Autoantibodies of Various Specificities. The following anti-Id antibodies were used: anti-LPS 10-1 Id, anti-129-48 Id, anti-RF Ids; anti-Y2 Id, a MRL/lpr anti-Sm MAB; anti-62 Id, a BALB/c anti-thyroglobulin MAb; and anti-PY102 Id, an anti-PR8 influenza virus specific MAb. Py102 expresses a V_H gene from the 7183 family.

Competitive inhibition RIA was carried out as follows: Briefly, microtiter plates were coated overnight at 4°C with 10 ug/ml chromatographically purified anti-Id antibodies. After washing and postcoating with PBS-BSA, the plates were incubated for 2 hours at 25°C with various dilutions of MAbs (5,50,500 ng/well). After extensive washings, plates were incubated for 2 hours at 25°C with ^{125}I- labelled idiotype ligand (50,000 cpm), washed extensively and radioactivity was counted in a gamma counter. Antibodies yielding at least 40% inhibition were considered IdX positive.

Study of Antigen Binding Properties of VH7183+ Hybridomas.

Techniques: radioimmunoassay to assess the binding activity to IgG, cardi-

olipin, DNA, Sm, thyroglobulin, myelin basic protein, transferrin, and thymocytes; ELISA to assess the binding activity for murine collagen type II and immunofluorescence to study the binding activity for cellular antigens.

In a sandwich RIA microtiter plates were coated (overnight at 4°C) with 10 ug/ml antigen in 1M pH9 sodium carbonate buffer, washed with PBS and post-coated with PBS-BSA (1%) and sodium azide (0.02%). After several washings, the plates were incubated for 2 hours at room temperature with 10 ug/ml chromatographically purified monoclonal antibodies in PBS-BSA. After extensive washing, ^{125}I-rat anti-mouse kappa light chain (50,000 cpm) was added for 2 hours at room temperature as the developing reagent. The ability of our panel of monoclonal antibodies to bind to DNA was kindly tested by Dr. B. Diamond (Albert Einstein College of Medicine, Bronx, NY) according to a previously described technique (Solomon et al., 1983).

The binding specificity for various antigens was further studied by a competitive inhibition assay. In these experiments, 10 ug/ml of monoclonal antibodies (deemed positive by RIA) were incubated for 2 hours at 37°C with increasing amounts of antigen (0, 10, 50, 100 ug/ml), and then added to microplates coated with antigens. The binding of the antibody was studied using RIA as above.

Binding to collagen type II was kindly carried out by L. Klareskog (Uppsala University) by ELISA, according to a previously described technique (Holmdahl et al., 1985). Briefly, to test the binding properties to cellular antigens, 3 techniques were used: RIA for anti-thymocyte antibodies, agglutination tests for anti-red blood cell antibodies and immuno-

fluourescence for anti-tissue antibodies. Binding to thymocytes was tested using a cell surface assay as previously described (Waters et al., 1984). Binding to red blood cells was tested using an indirect anti-Ig test (American Association Blood Banks Technical Manual, 7th Edition, 1977. Am. Assn. Blood Banks, Wash., D.C.) and polybrene test (Lalezari, 1968). Binding to tissue antigens was studied using cryostat prepared sections from kidney and stomach. These were incubated for 1 hour at 4°C with various amounts of antibodies (0.1, 5, 10 ug/ml), washed extensively and then incubated for 1 hour at 37°C with FITC rat anti-mouse Ig as the development reagent.

Identification of VH Gene Families Utilized by RF, VH7183+ Selected Hybridomas, Autoantibodies. Briefly, RNA from cytoplasmic lysates prepared from 10^7 cells was fixed to nitrocellulose using a slot-blotting apparatus (White and Bancroft, 1982; Maniatis et al., 1982). The filters were baked, hybridized to V_H gene probes, washed and autoradiographed as described previously (Alt et al., 1982).

Preparation of VH and JH Specific Fragments. The J_H probe and V_H specific probes representative of the various families were prepared as described elsewhere (Yancopoulos et al., 1984; Blackwell et al., 1984). The V_H probes were kindly provided by G. Yancopoulos (Columbia University, New York, NY).

DNA Preparation. DNA was prepared from the tumor cell line sp2/0, cultured RF lines and germ line tissue (liver) as described elsewhere (Steffen et al., 1979; Alt et al., 1980).

Analysis of Heavy Chain Gene Organization in Cellular DNA. Approximately 10 ug genomic DNA was digested with Eco RI (New England Biolabs)

and fractionated on a 1% agarose gel (International Biotechnologies, Inc.). Following electrophoresis, DNA was transferred from the gel to nitrocellulose filter paper by the Southern method (1975). Subsequently, filters were baked, pretreated, hybridized, washed and autoradiographed as described by Alt et al. (1982).

Cloning and Sequence Analysis. Complete Eco RI digests of genomic DNA from two RF lines were cloned into the Eco RI site of Charon 16A (Blattner et al., 1977) and individual libraries were screened for phages containing inserts that hybridized to the J_H probe as described by Alt et al. (1982). J_H-positive phages were purified and subsequently subcloned into pUC9. Appropriate restriction fragments were prepared and sequence analysis was performed by the method of Maxam and Gilbert (1980).

Statistics. The two-sample t-test was employed to test whether differences exist between 1 month old 129/J and 1 month old 129/Sv animals, 1 month old MRL +/+ and 1 month old MRL/lpr animals for the anti-BL PFC response, and 1 month old 129/J and 129/Sv mice for the expression of the E109Id component of the response. This test was also used to determine if there were differences between MRL/lpr and C57BL6/lpr mice for the anti-Dex-KLH response. A single-factor analysis of variance (ANOVA) was utilized to test the hypothesis that u1 = u2 = uk. Chi square statistical analysis was calculated according to Zar (1974) and was used to determine significance of results of the frequency of expression of V_H7183 in hybridomas obtained from in vitro LPS stimulated lymphocytes and PR8 influenza virus immunized mice.

RESULTS

Age-Dependent Appearance of RF in 129 and MRL Mice. The spontaneous occurrence of RF was studied in 1-, 3-, and 6-mo-old 129 and MRL mice. The humoral level of RF was determined by using multiple systems and scoring hemagglutination of TNP or arsonate-labeled SRBC coated with subagglutinating doses of various monoclonal antibodies of distinct isotypes specific for these haptens. The specificity of this technique described by Nemazee and Sato (1983) was controlled for by testing sera from newborn, 1-mo-old 129/Sv, MRL/lpr, and BALB/c mice as well as other irrelevent monoclonal antibodies (MOPC104E and Ab_2A48-3). As indicated in Table 2, Part A, only the monoclonal RF antibodies and sera from 6-mo-old 129/Sv and MRL/lpr mice cause hemagglutination. In addition, we have shown that the inhibition of agglutination by RF monoclonal antibodies specific for IgG_{2a}-TNP-coated SRBC occurred with the Fc fragments of IgG_{2a} but not with those from IgG_3, IgG_1, IgG_{2b}, or the Fab fragment (Table 2, Part B) (Porter, 1959). Using this technique, we found that 129/J and MRL+/+ (1- to 6-mo-old) do not produce RF, whereas the 129/Sv mice produce high titers of RF beginning at 4 mo of age; MRL/lpr mice produce significant titers at 5 mo of age (Fig. 1). These results are similar to those previously reported for the age-dependent appearance of RF in 129/Sv (Van Snick and Masson, 1979) and MRL/lpr mice (Andrews et al., 1978).

Study of Anti-Polysaccharide Response.

TI-2 Antigens. BL, a beta 2-6 fructosan with beta 2-1 branch points, induces a vigorous TI response regardless of the MHC or Igh.C haplotype of the mouse strain (Rubinstein et al., 1982). Moreover, the

Table 2

Specificity of HA for detection of RF

A. HA using TNP-SRBC coated with a subagglutinating amount (5 μg/ml) of an IgG2a, TNP-specific monoclonal antibody (IC-4)

Ab or Sera Origin	Age	HA Titer (log_2 units)
BALB/c[a]	Newborn	0
BALB/c	6 mo	0
129/Sv	1 mo	0
129/Sv	6 mo	5
MRL/lpr	1 mo	0
MRL/lpr	6 mo	2
Monoclonal[b]	A1305A9-3	>8
RF antibodies	MRL 55-18	>8
Other monoclonal antibodies[c]	MOPC104E	0
	Ab_2 A48-3	0

B. Hemagglutination inhibition

Inhibitors (1 mg/ml)	HI Titer (log_2 units)	
	A1305A9.3	MRL55-18
Fcγ3[d]	>8	0
Fcγ1	>8	>8
Fcγ2a	3	7
Fcγ2b	>8	>8
Fab	>8	>8

[a]Undiluted serum in first well.

[b]RF monoclonal antibodies: A1305A9-3 is a 129/Sv monoclonal antibody kindly donated by Dr. Van Snick (Brussels) and MRL 55-18 was prepared in our laboratory.

[c]MOPC104E is an IgM monoclonal protein specific for Dex, whereas Ab_2A48-3 is a monoclonal antibody specific for A48-idiotopes. All monoclonals were used a 1 mg/ml concentration in first well.

[d]Fc IgG_3 was prepared from J606. Fc IgG_1 from MOPC21, Fc IgG_{2a} from MOPC173, and Fc IgG_{2b} from IDA23 according to methods described by Guyer et al.[2b] (1976) and the Fab fragment from IDA23 according to methods described by Porter (1959). These preparations were a kind gift from Dr. M. Stanislovsky (CNRS, Vielljuif, France).

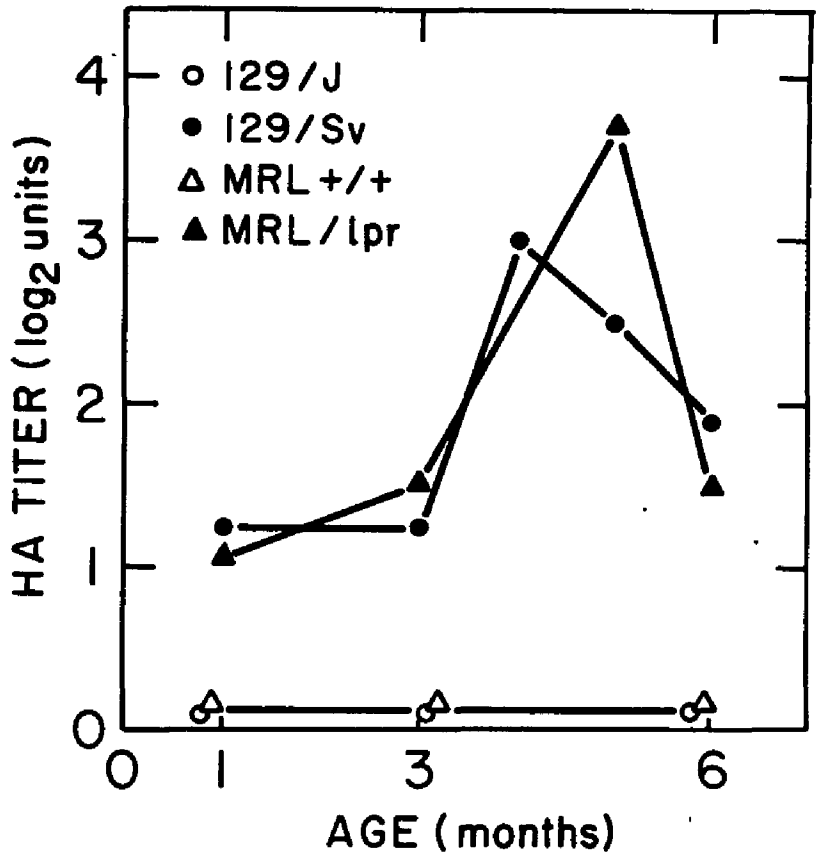

Fig 1. RF titers of nonimmunized 129 and MRL mice of various ages. Each point represents the mean of five mice for each age group with the exception of 129/Sv 4-mo. old and 5-mo. old mice of which there were two and four mice, respectively.

antibodies that bind beta 2-6 and beta 2-1-linked polyfructosans express a polymorphic cross-reactive idiotype shared by several inulin-binding myeloma proteins. The expression of these idiotypes is associated with the Igh^a allotype (Rubinstein et al., 1982).

Immunization of mice of the $Igh.C^a$ haplotype with Dex also induces a strong TI immune response. The majority of these antibodies express cross-reactive idiotypes identified on Dex-binding J558 and MOPC104E myeloma proteins (Riblet et al., 1975).

Because the 129 and MRL mouse strains also bear the a allotype,* it was therefore interesting to study the anti-BL and anti-Dex responses and the proportion of those antibodies exhibiting the IdX borne by EPC 109 (E109), an inulin-binding myeloma protein, and J558, a dextran-binding myeloma protein, as a function of the aging process.

Anti-BL PFC Response. The PFC response was studied in 1- to 6-mo-old 129/Sv and MRL/lpr mice as well as in the corresponding congenic strains, 129/J and MRL+/+. In the 129/J mice, an age-dependent increase in the anti-BL PFC response was observed (Table 3). This observation is in agreement with the results obtained in BALB/c mice in which it was shown that the magnitude of the anti-BL response was greater in older mice (Bona, 1980). This increase in responsiveness in older mice is probably related to environmental exposure during the lifetime of the animal. In contrast, a dramatic increase in the PFC response was observed in 1-mo-old

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*Footnote: At the time of these studies, MRL was classified on the basis of serology as Igh^a; it has now been shown to be Igh^j at the DNA level. (Trepicchio and Barrett, 1985).

Table 3

Age dependence of anti-BL response in 129 and MRL mice[a]

Strain	Age (mo)	Anti-BL Response: Total PFC/Spleen (log GM ± SEM (A.M.))	% E109 Id$^+$
129/J	1	3.06 ± 0.21 (1,668)	14.4 ± 0.5
	3	4.18 ± 0.16 (19,896)	17.3 ± 7.8
	6	4.69 ± 0.13 (56,850)	10.2 ± 6.7
129/Sv	1	4.23 ± 0.19 (22,161)	40.4 ± 18.7
	3	3.57 ± 0.21 (7,533)	31.4 ± 14.6
	6	4.30 ± 0.14 (23,500)	58.0 ± 5.6
MRL/++	1	2.56 ± 0.34 (912)	52.7 ± 16.0
	3	3.32 ± 0.22 (3,636)	58.3 ± 9.5
	6	3.42 ± 0.24 (3,720)	41.0 ± 0.7
MRL/lpr	1	3.60 ± 0.12 (4,610)	32.2 ± 3.4
	3	3.81 ± 0.25 (10,690)	7.0 ± 6.0
	6	3.39 ± 0.20 (2,195)	10.3 ± 6.2

[a] Log GM ± SEM (arithmetic mean) of five mice for each group. Mice were immunized i.v. with 20 μg BL and the PFC response was measured 5 days later.

129/Sv mice as compared with the 129/J age-matched controls ($0.002<p<0.005$). Although the magnitude of the E109Id$^+$ component was similar within the groups of 129/J ($p>0.25$) studied, the E109$^+$ component was significantly higher in 129/Sv vs. 129/J animals ($p<0.001$).

MRL+/+ and lpr mice develop a significantly lower anti-BL PFC response than the 129/J and Sv strain ($0.02<p<0.05$). Furthermore, in this group of animals, 1-mo-old MRL/lpr mice also exhibited a higher response than their MRL+/+ aged-matched controls ($0.002<p<0.05$). Unlike 129/Sv animals, a significant decrease of E109Id$^+$-producing clones was observed in 3- and 6-mo-old MRL/lpr mice ($p<0.001$).

The IEF patterns of the anti-BL sera of 129 mice (Fig. 2) were similar to those observed previously for BALB/c mice (Stein et al., 1980). In the sera of 129/J mice, three distinct spectrotypes could be seen, one of which was shared by all 3- and 6-mo-old mice. No 1-mo-old 129/J mouse made IgG anti-BL antibodies, which is consistent with the low anti-BL direct PFC response described above.

In 129/Sv mice, IgG antibodies to BL were observed in all age groups studied. The major IgG spectrotype seen in 129/J animals is seen in 129/Sv mice and is shared by all individuals; however, additional spectrotypes are observed in some individual mice independent of their age. It should be noted that an occasional 129/Sv mouse, regardless of age, made a poor IgG response.

The IEF analysis of the MRL sera (Fig. 3) revealed that most MRL/lpr and MRL+/+ mice made only very small amounts of IgG anti-BL antibody. Furthermore, the spectrotypes seen for both MRL/lpr and MRL+/+ mice were similar to those seen in 129/Sv mice, but more heterogeneous than in 129/J

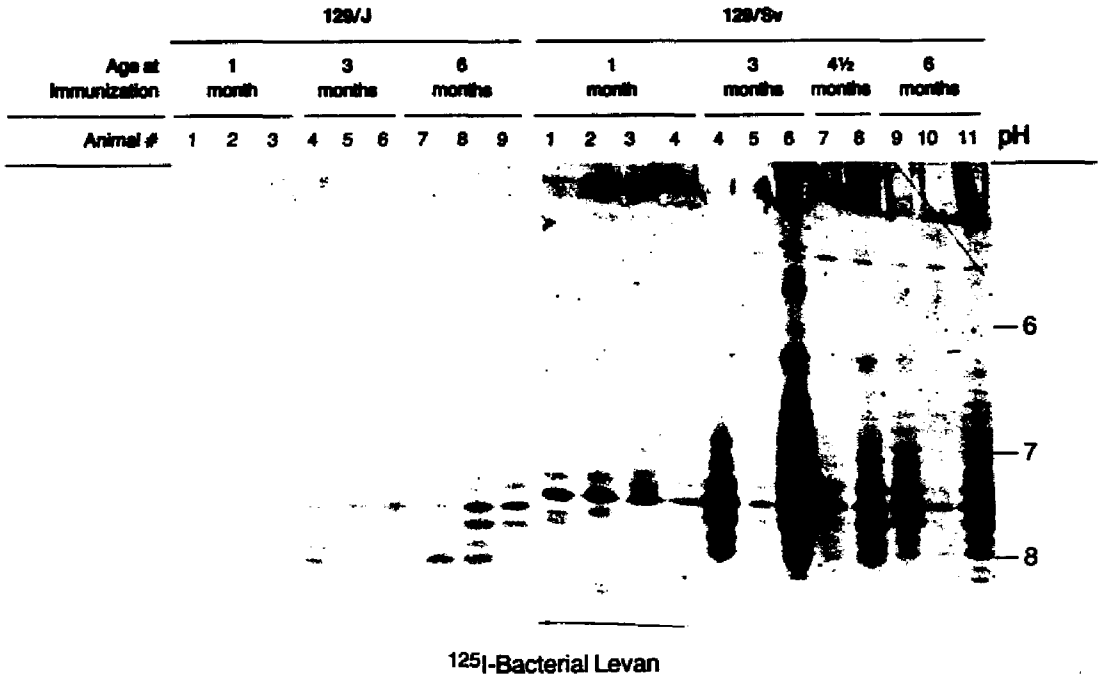

Fig. 2. IEF spectrotypes of anti-BL antibodies in 129 mice of various ages. The gel was exposed to ^{125}I-BL.

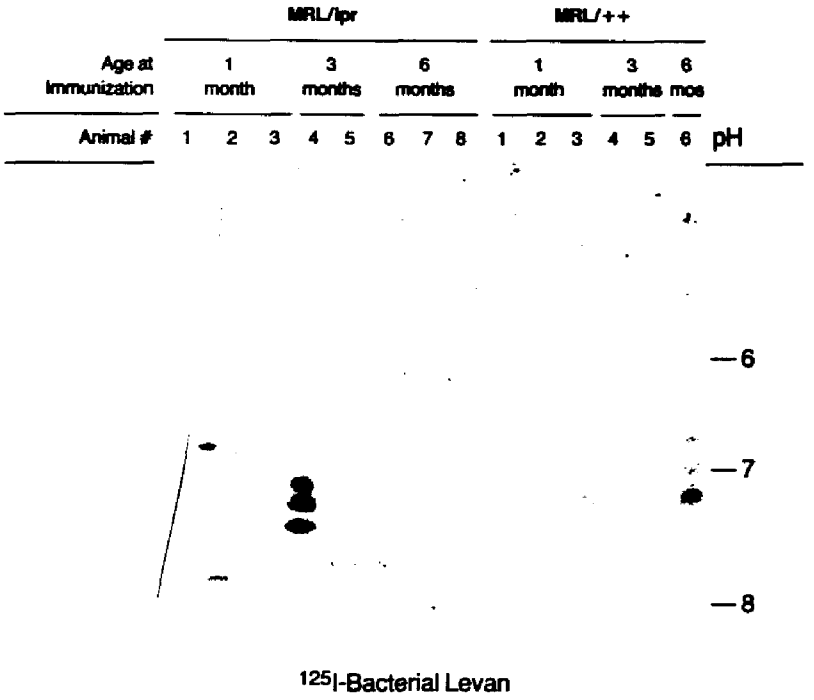

Fig. 3. IEF spectrotypes of anti-BL antibodies in MRL mice of various ages. The gel was exposed to ^{125}I-BL.

mice.

Anti-Dex PFC Response. The data presented in Table 4 illustrate an age-dependent increase of the anti-Dex response in 129 mice. In the anti-Dex response, we also observed that the total PFC is almost fourfold higher in 1-mo-old 129/Sv as compared to that of the 129/J congenic mice. Although 129/Sv and 129/J show a general decline in the expression of the J558 IdX, there are few, if any, clones that express this idiotype in all age groups of 129/Sv mice.

The spectrotypes observed for anti-Dex antibody in 129 mice (Fig. 4) are consistent with those published by Hansburg et al. (1976) for BALB/c mice. Both congenics show several spectrotypes that are shared between individuals. One of three 1-mo-old 129/J mice (No. 1, Fig. 4) failed to make IgG antibodies, as did 1-mo-old 129/J mice immunized at another time (data not shown). In 3- and 4-mo-old 129/Sv mice, two individual animals made weak IgG responses; otherwise, little or no differences were observed between the IEF patterns for the 6-mo-old mice of either 129 congenic. These data are consistent with a high PFC response to Dex in this age group (see Table 4). Most MRL/lpr and +/+ mice failed to produce IgG and anti-Dex antibodies as revealed by IEF. This concurs with our inability to demonstrate an anti-Dex PFC response for MRL mice in several experiments repeated at various intervals with the use of five individuals per age group. We investigated whether this unresponsiveness was related to the MHC gene complex or to the protocol of immunization. The data depicted in Table 5 show the results of the anti-Dex PFC response of various age-matched (3 mo) mouse strains. Whereas BALB/c mice develop a strong anti-Dex PFC response subsequent to immunization with B1355 Dex in saline or

Table 4

Age dependence of anti-Dex response in 129 mice[a]

Strain	Age (mo)	Total PFC/Spleen (log GM ± SEM (A.M.))	% J558 Id$^+$
129/J	1	2.76 ± 0.26 (803)	37.8 ± 20.7
	3	3.96 ± 0.22 (11,180)	22.7 ± 4.0
	6	4.11 ± 0.06 (13,140)	18.1 ± 12.9
129/Sv	1	3.54 ± 0.09 (3,545)	8.3 ± 8.2
	3	3.35 ± 0.10 (2,631)	9.3 ± 4.1
	6	4.59 ± 0.12 (40,480)	0.0 ± 0.0

[a] Log GM ± SEM (arithmetic mean) of five mice for each group. Mice were immunized with 100 μg of Dex in CFA and the PFC response was measured 5 days later.

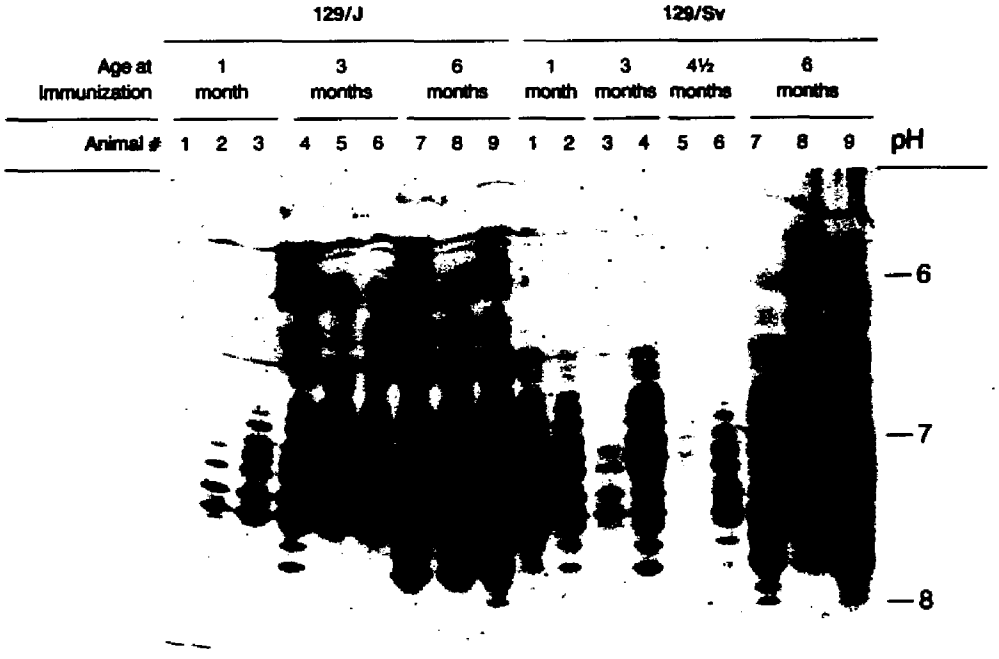

Fig. 4. IEF spectrotypes of anti-Dex antibodies in 129 mice of various ages. The gel was exposed to ^{125}I-dextran (1355).

Table 5

Anti-Dex response in various mouse strains bearing various allotypes[a]

Strain	H-2	IgC_H	Total PFC/Spleen (log GM ± SEM (A.M.))		
			Dex-Saline	Dex-CFA	Dex-KLH
BALB.c	d	a	4.88 ± 0.12 (82,272)	4.66 ± 0.04 (45,577)	2.87 ± 0.06 (751)
BALB.K	k	a	N.D.[b]	N.D.	4.36 ± 0.41 (34,835)
C58/J	k	a	4.15 ± 0.05 (14,200)	4.22 ± 0.10 (17,240)	N.D.
C57BL/6	b	b	2.99 ± 0.31 (7,144)	3.60 ± 0.27 (26,548)	3.84 ± 0.20 (6,300)
C57BL/1pr	b	b	N.D.	4.76 ± 0.43 (114,675)	3.43 ± 0.08 (2783)
BALB.B	b	a	5.28 ± 0.18 (206,238)	3.71 ± 0.37 (6960)	N.D.
MRL/++	k	a	N.R.[c]	N.R.	3.47 ± 0.38 (5716)
MRL/1pr	k	a	N.R.	N.R.	2.42 ± 0.50 (448)

[a] Log GM ± SEM (arithmetic mean) of three to five mice for each group. Mice were immunized i.p. with 100 μg of Dex in saline or in CFA or with 100 μg i.p. KLH in CFA. This group was immunized 4 wk later with 15 μg Dex-KLH i.v. Plaque assays were performed 5 days later.

[b] Plaque assay not done.

[c] No response (<400 PFC/spleen).

CFA, the MRL mice were unresponsive. This unresponsiveness,* however, was not related to the MHC gene complex because C58/J mice ($H\text{-}2^k$, $IghC^a$) developed a Dex PFC response. Furthermore, the unresponsiveness of MRL mice was not related to the lpr gene, because MRL+/+ mice did not mount a Dex response whereas C57Bl/lpr developed a strong response subsequent to immunization with Dex-CFA. These results clearly indicate that MRL strains have a particular defect in their ability to respond to TI antigens. The MRL mice have the precursors of Dex-reactive clones, since they did develop an anti-Dex PFC response subsequent to immunization with Dex-KLH. Nevertheless, it should be noted that even after immunization with dextran as a T-dependent antigen, MRL/lpr develop a significantly lower response as compared to C57Bl/lpr ($0.02<p<0.05$). These results suggest that the MRL mice may have a defect in the subset of B cells, which enables them to respond to TI antigens of the TI-2 type.

Titer of RF Produced in 129 and MRL Mice After Immunization with BL and Dex. The titer of anti-gammaglobulin activity was also measured in the serum of mice immunized with BL. The data depicted in Figure 5 show a marked increase of anti-IgG_{2a} antibodies in 6-mo-old 129/Sv mice and the de novo production of anti-IgG_{2a} antibodies in 6-mo-old 129/J mice. This increase is also evident for 3- to 6-mo-old MRL/lpr and MRL+/+ mice. A similar pattern was observed for anti-IgG_{2b} antibodies, whereas no apparent increase in titers of anti-IgG_1 and IgG_3 antibodies were observed

*Footnote: The most likely explanation for unresponsiveness is the fact that MRL mice are Igh^J and not Igh^a (see above).

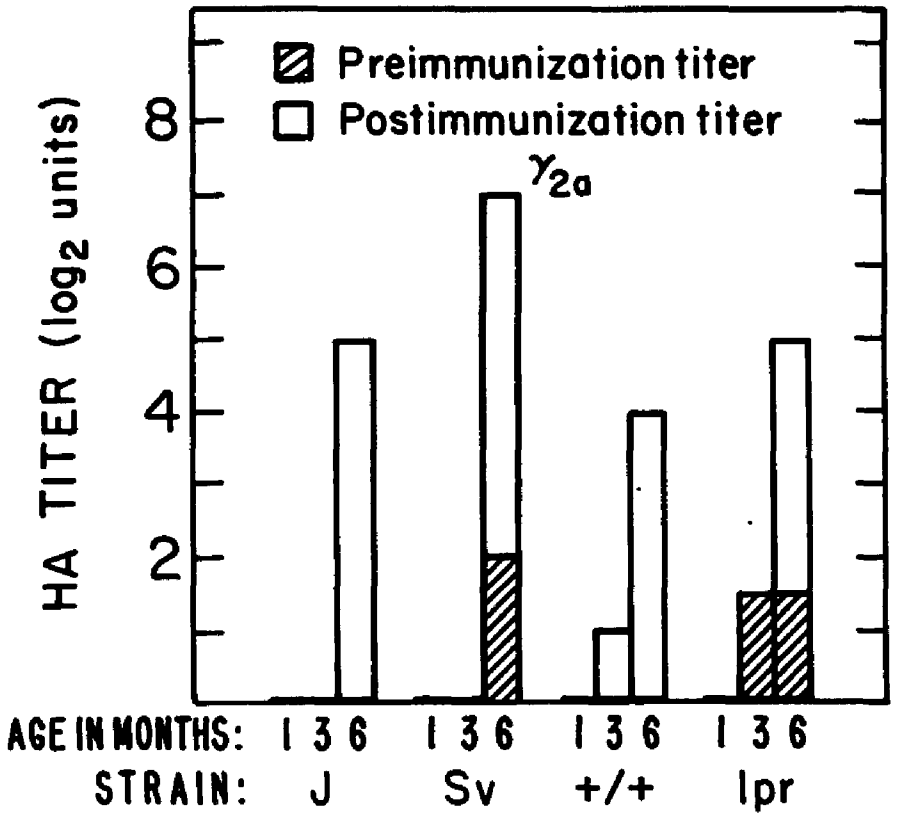

Fig. 5. Titers of RF produced by 129 and MRL mice after immunization with BL.

(data not shown). These data clearly show that the older mice develop anti-gammaglobulin antibodies subsequent to immunization with BL.

Anti-gammaglobulin antibodies in 129 mice immunized with Dex was also measured. The data in Figure 6 show that the anti-IgG_{2a} antibody titer increased in 6-mo-old 129/Sv as well as in 129/J mice. Only 3- to 6-mo-old 129/J mice appeared to make anti-IgG_1 antibodies after Dex immunization whereas 129/Sv 6-mo-old mice did not increase their preimmunization titer. No anti-IgG_3 or anti-IgG_{2b} antibodies were detected in 129 mice (data not shown).

Study of In Vivo Anti-TNP Response in 129 Mice.

Anti-TNP-BA PFC Response. In 129 mice, there was an age-dependent increase in the total PFC response (Table 6). In addition, the magnitude of the PFC response in 1-mo-old 129/Sv mice paralleled that of the adult 129/J animals. This is in agreement with our previous results in which an acceleration of the immune response against TI-2 antigens was shown. The 460 Id component of the response varied: the percentage of antibodies expressing the 460 Id declined by 6 mos. of age in 129/J mice while no change in this component was observed in 129/Sv mice.

Titer of RF Produced in 129 Mice After TNP-BA Immunization with TNP-BA. A significant increase in the titer of serum RF anti-IgG_{2a} was observed after immunization with TNP-BA in 6-mo-old 129/Sv mice (Fig. 7A). Most importantly, serum RF were induced in 6-mo-old 129/J mice.

Study of Anti-TNP-Ficoll Response. No remarkable age-dependent response differences were observed in 129/J mice immunized with TNP-Ficoll, except that at 3 mos. animals did not respond as well. A general decline in the magnitude of the anti-TNP PFC response was observed in 129/Sv mice.

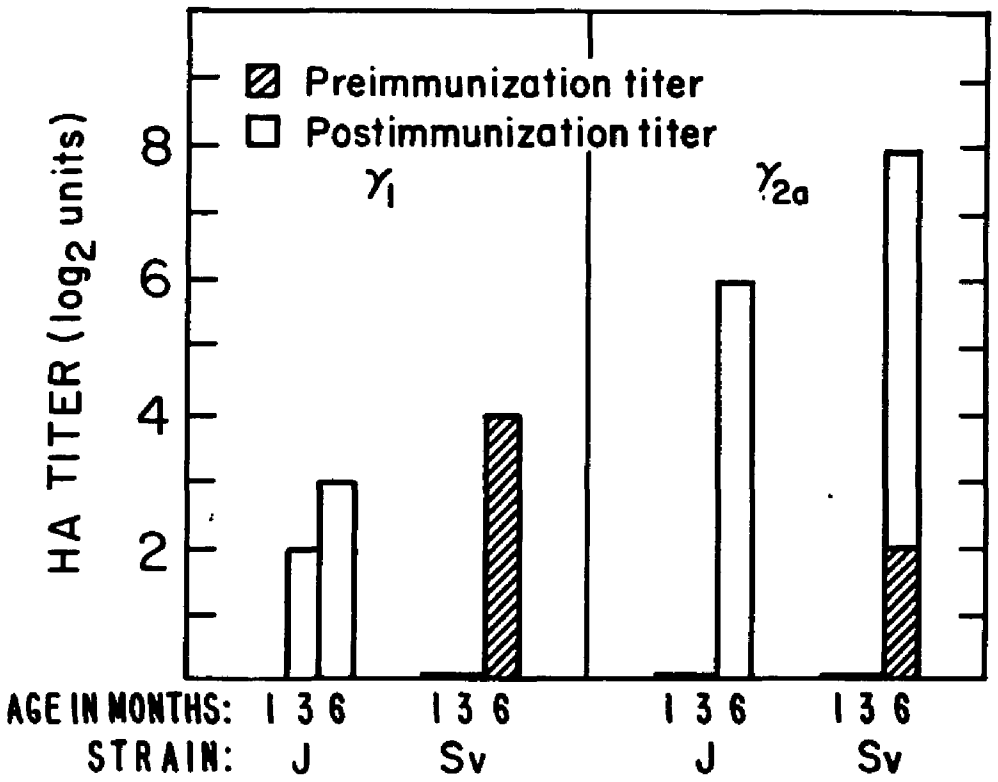

Fig. 6. Titers of RF produced by 129 mice after immunization with Dex.

Table 6. Age-dependence of anti-TNP response elicited by immunization with a TI-1 antigen in 129

Strain	Age(in mos)	anti TNP-PFC/Spleen[1]	
		Total	%460Id+[2]
129/J	1	3.46±0.39 (7,937)	41.8±14.9
	3	3.80±0.29 (10,380)	17.2±15.3
	6	4.50±0.09 (39,510)	17.6±4.6
129/Sv	1	4.62±0.21 (25,960)	12.1±3.6
	3	4.34±0.13 (25,650)	12.2±9.3
	6	4.40±0.22 (31,200)	N.D.

[1]Log GM±SEM(arithmetic mean) of 3-5 mice for each group. Mice were immunized i.p. with 0.1 ml of 0.1% TNP-BA. The PFC response was measured 5 days later.

[2]460-Id - The anti-TNP PFC carrying the MOPC 460-Id were enumerated by incorporating BALB/c anti-MOPC460-Id anti-serum (1:500) to the plaquing chamber. The number of PFCs secreting antibodies bearing this idiotype was calculated as the number of PFC observed in the absence and presence of the anti-Id antiserum. The difference was considered to be the number PFCs secreting antibodies expressing the idiotype.

[3]Not Done.

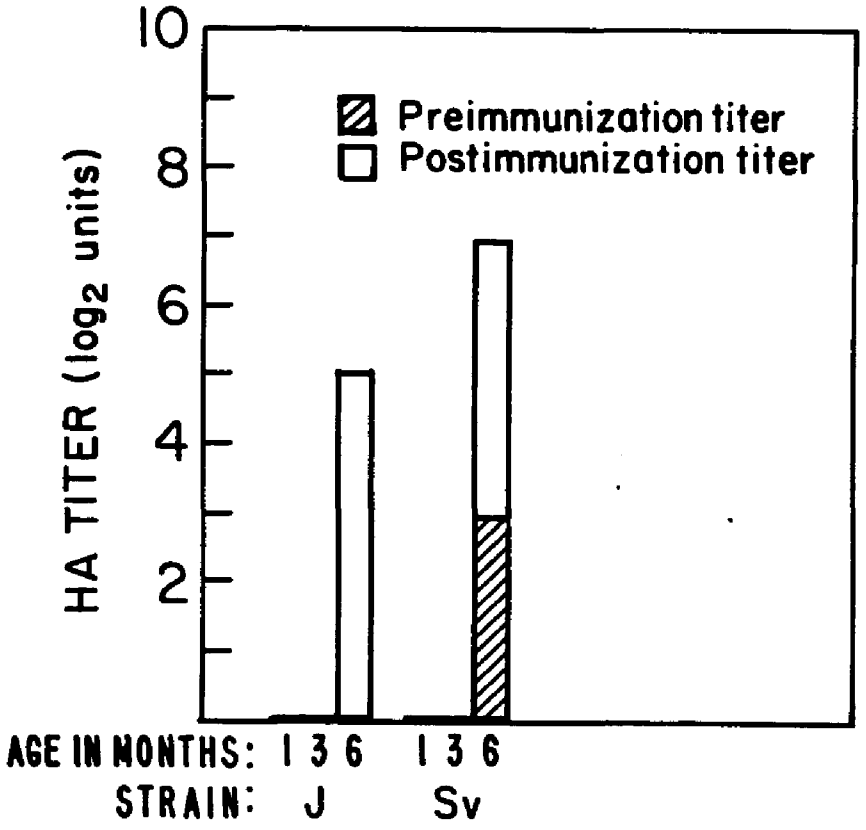

Fig. 7A. Titers of RF produced by 129 mice after immunization with TNP-BA.

Moreover, the response observed for 1 mo. 129/Sv mice far exceeded that of their age-matched controls or even adult normals (Table 7).

Titer of Anti-Gammaglobulin Antibodies in 129 Mice Immunized with TNP-Ficoll. A significant increase of RF anti-IgG_{2b} and -IgG_{2a} was observed in sera of mice immunized with TNP-Ficoll (Fig. 7B).

In Vivo Secondary and Tertiary Responses to a TD Antigen in 129 and MRL Mice.

Variation of Isotype of Anti-TNP Antibodies During the Secondary Response: 129 Mice. The age-dependent variation of isotypes of anti-TNP antibodies was studied in 1-, 3-, and 6-mo-old 129/J and 129/Sv mice during the TNP-KLH induced secondary response. Sera were collected on days 3, 10, 20, and 30 after challenge (1 month after priming). The amounts of TNP specific-antibodies were determined by RIA in which the concentration of isotypes was measured from standard curves constructed with TNP specific IgM, IgG_3, IgG_1, IgG_{2b} and IgG_{2a} MAbs. The kinetics of the IgM anti-TNP antibodies were similar between the congenics within each age group of mice studied. The concentrations of IgM anti-TNP antibodies decreased during the 20-day period as would be expected for a secondary response (Fig. 8A). In addition, profiles of the IgG_3 anti-TNP antibodies for the age-matched groups were similar in that high concentrations of IgG_3 antibodies were produced at 3 and 6 months of age. A sharp decrease in the concentration of IgG_3 antibodies occurred between days 20 and 30 of the response in 3 and 6 month 129/Sv mice (Fig.8B). The study of the kinetics of the IgG_1 anti-TNP response shows that there is a greater concentration of IgG_1 specific antibody in 1-month-old mice vs. 3 and6 month old 129/J and 129/Sv mice (Fig. 8C). The kinetics of the IgG_{2b}

Table 7

Age dependence of anti-TNP response elicited by immunization with TNP-Ficoll in 129 mice

Strain	Age (in mos)	TOTAL PFC/SPLEEN log GM ± SEM (A.M.)
129/J	1	4.22±0.30 (25,440)
	3	3.54±0.22 (4,306)
	6	4.28±0.34 (25,280)
129/Sv	1	4.86±0.10 (78,087)
	3	4.47±0.08 (29,950)
	6	3.76±0.29 (7,160)

Log GM ± SEM (arithmetic mean) of 3-5 mice for each group. Mice were immunized i.p. with 20 ug TNP-Ficoll. The PFC response was measured 5 days later.

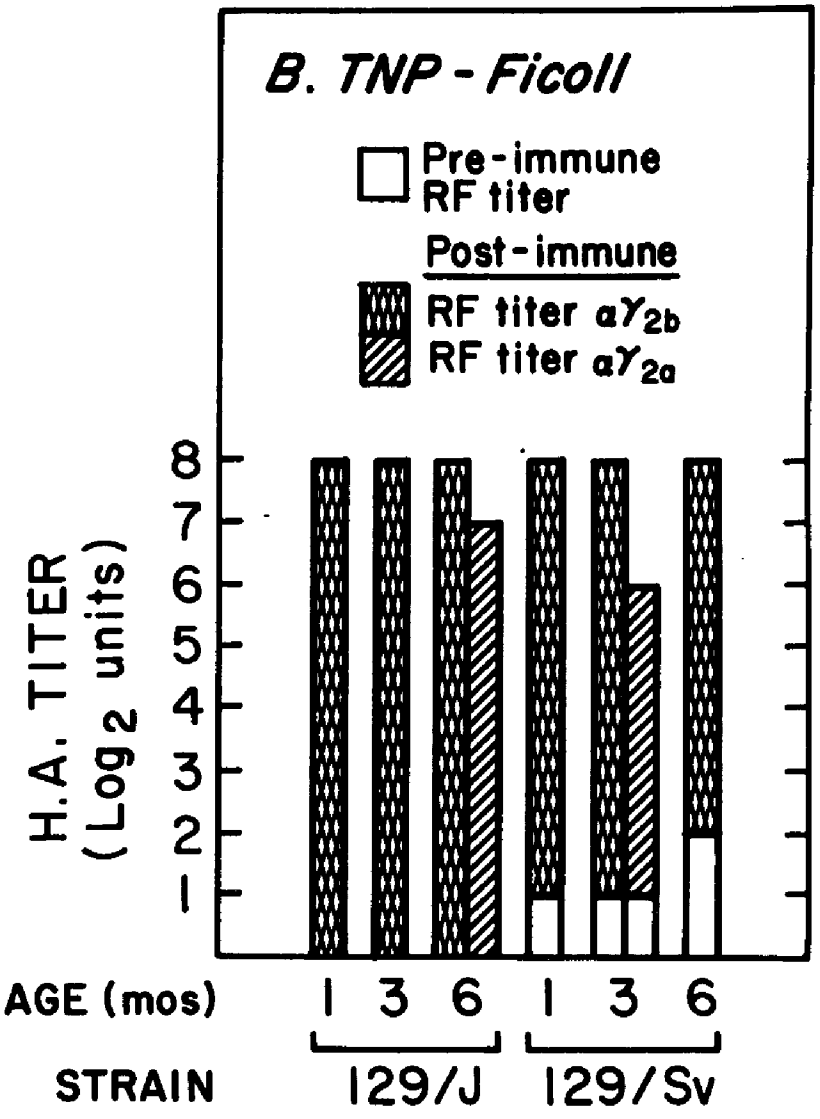

Fig. 7B. Titers of RF produced by 129 mice after immunization with TNP-Ficoll.

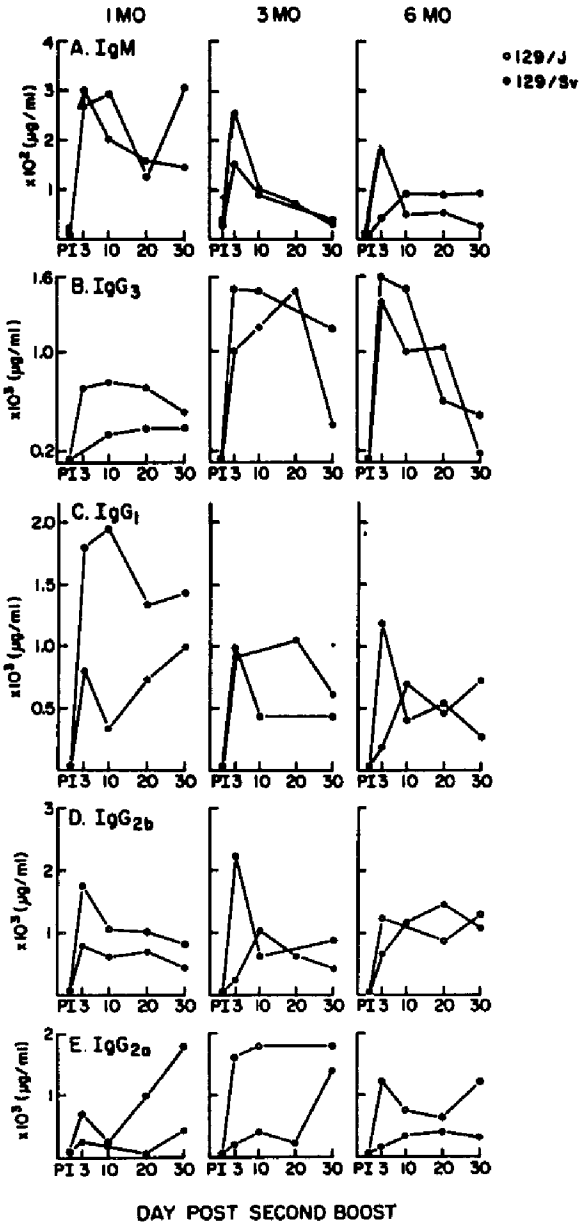

Fig. 8. Kinetics of the anti-TNP response. A) IgM anti-TNP, B) IgG_3 anti-TNP, C) IgG_1 anti-TNP, D) IgG_{2b} anti-TNP, E) IgG_{2a} anti-TNP. Sera were collected from immunized mice on days 3,10,20, and 30 after the second boost. PI(pre-immune). Concentrations of isotype specific antibody were determined from standard binding curves.

response is similar in both strains (Fig. 8D). However, the results depicted in Fig. 8E show that the concentration of IgG_{2a} antibodies in both 3- and 6-month-old 129/Sv mice was significantly lower than that observed in 129/J mice ($P<0.005$, Student's t-test). The decrease of IgG_{2a} anti-TNP antibodies in 129/Sv mice may be related to the spontaneous production of autoanti-IgG_{2a} antibodies which has been described in this strain.

Study of the Occurrence of Anti-Gamma Antibodies During the Secondary Immune Response. The sera from mice immunized with TNP-KLH collected at the previously designated timepoints, 1 month after priming, were also used to determine the titers of RF as well as the fine specificity of these anti-gammaglobulin antibodies. The titer of RF was determined by HA using Ars-SRBC coated with subagglutinating doses of Ars-specific monoclonal antibodies of various isotypes.

Both 129/J and 129/Sv mice produced anti-gammaglobulin antibodies during the secondary response (Fig. 9). The most striking finding was the observed long lasting production of RF and their broad specificity for various isotypes. The study of the kinetics of RF specific for IgG_3 shows that they are produced immediately after challenge and are maintained in 1- and 3-month-old mice, whereas a decrease can be noted between days 10 and 20 of the secondary response in 6-month-old mice (Fig. 9A). The concentration of anti-IgG_1 antibodies sharply increased in the first 10 days after challenge in both 129/J and 129/Sv mice (Fig. 9B). The titer of anti-IgG_{2b} antibodies was lower than that of anti-IgG_1 antibodies except at 30 days in 129/J mice when a sharp increase was observed (Fig. 9C). Little variation was observed in the kinetics of the anti-IgG_{2a} response between 1- and 3-month-old 129/J and 129/Sv mice. In contrast, the study

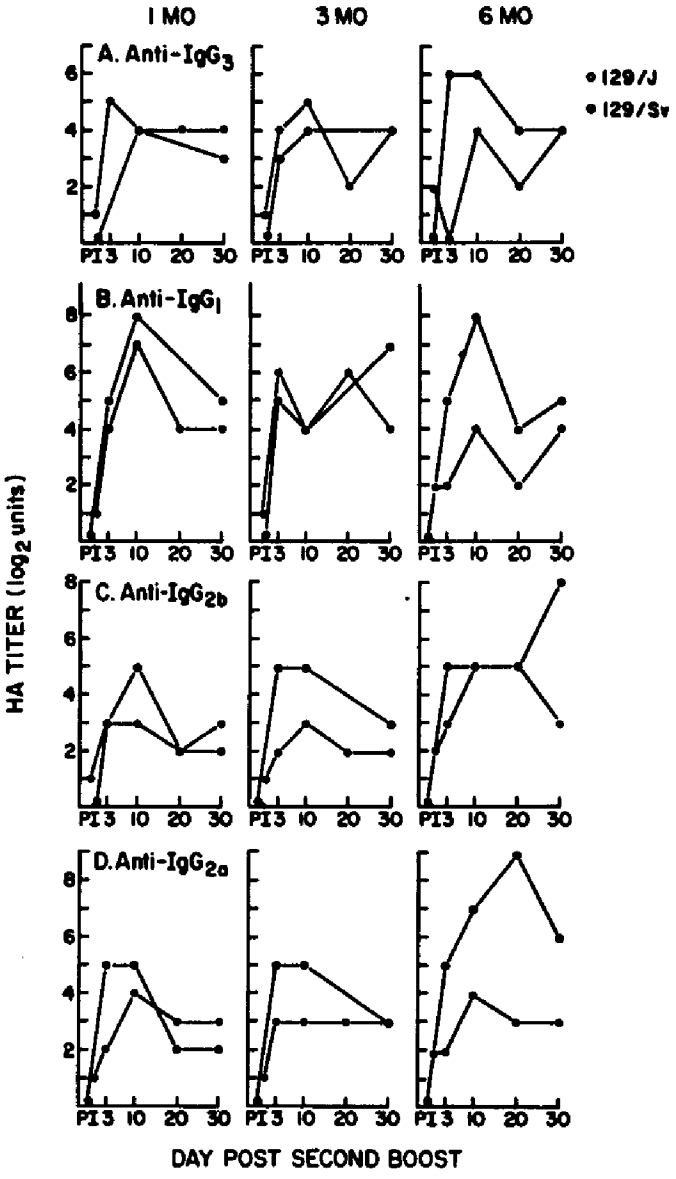

Fig. 9. Kinetics of the anti-gammaglobulin response during the secondary anti-TNP response (HA titer). A) anti-IgG_3, B) anti-IgG_1, C) anti-IgG_{2b}, and D) anti-IgG_{2a}. Titers were determined from sera obtained from the same mice utilized in the kinetic study.

of the kinetics of anti-IgG_{2a} antibodies during the secondary immune response showed a significant difference between 3- and 6-month old 129/J and 129/Sv mice (Fig. 9D) which may be related to the clearance of RF from circulation by interaction with IgG_{2a} anti-TNP antibodies.

Study of Secondary Anti-TNP PFC Response in 129 Mice. To more fully understand the basis of the observed isotypic variation that occurs in the secondary response, the PFC responses were studied in order to discern whether the decreased concentration of some isotypes in the sera is related to the clearance of antibodies by RF or to an interaction of RF with the receptor of antibody forming cells.

The direct and indirect PFC responses were studied in 1 to 6-month-old 129/J and 129/Sv mice 7 days after secondary or tertiary challenge (Table 8). In the case of the tertiary PFC immune response, the same group of mice utilized in the kinetic study of anti-TNP antibodies during the secondary response was used here.

An age-dependent increase of the direct secondary PFC response was observed in 129/J mice whereas the indirect PFC did not vary ($P>0.05$, ANOVA). In contrast, old 129/Sv mice make significantly low direct and indirect PFC responses as compared to the age-matched 129/J group ($P<0.01$, Student t-test). Similarly, the study of the indirect tertiary PFC response shows that 6-month-old 129/Sv animals cannot mount a tertiary response equivalent to even 3-month-old 129/J animals ($P<0.001$, Student's t-test).

In Vitro Study of Function of T and B Cells of Young and Old 129 Mice. The results of the study of secondary and tertiary anti-TNP PFC responses in the mice immunized with the TNP-KLH clearly indicated that the humoral

Table 8. AGE DEPENDENCE OF THE ANTI-TNP RESPONSE ELICITED BY IMMUNIZATION WITH TNP-KLH IN 129 MICE

TOTAL PFC/SPLEEN - Log GM±SEM (A.M.)

		SECONDARY RESPONSE		TERTIARY RESPONSE	
STRAIN	AGE(mos)	DIRECT	INDIRECT	DIRECT	INDIRECT
129/J	1	2.52±0.15(373)	2.91±0.38(1367)	3.18±0.03(1515)	3.82±0.33(7725)
	3	3.25±0.02(1770)	3.65±0.16(4680)	3.55±0.27(7020)	4.28±0.02(19020)
	6	3.48±0.04(2275)	2.95±0.20(2566)[a]	2.99±0.13(1210)	N.D.[b]
129/Sv[1]	1	3.23±0.28(2034)	4.30±0.35(26,916)	2.26±0.18(246)	3.63±0.31(5100)
	3	2.87±0.31(1163)	N.D.[b]	2.70±0.20(834)	3.89±0.06(7900)
	6	2.47±0.09(300)	2.36±0.06(230)	3.18±0.09(1632)	2.83±0.64(1125)

[a] only 4 of 5 mice expressed a γ PFC response.

[b] N.D. - not done.

log G.M. ± SEM (arithmetic mean) of 3-5 mice for each group

Mice were immunised i.p. with 100μg TNP-KLH in CFA and subsequently boosted i.p. with 10μg TNP-KLH in Saline. The PFC response was measured 7 days later for the secondary response. Mice utilised for the tertiary response received an additional boost (10μg i.p. in Saline) one month after the first boost. The PFC response was then measured 7 days later.

variation of isotypes of anti-TNP antibodies is not simply related to the clearance of certain isotypes by RF. It appeared, therefore, important to study the interaction of T and B cells in an in vitro hapten-carrier antibody synthesis system (Table 9). B cells were prepared from spleens of TNP-OVA primed mice and T cells were obtained from KLH primed mice. They were co-cultured with 10 ug TNP-KLH as well as 10 ug TNP-OVA, which served asa control for the carrier specificity of helper T cells.

It was apparent that when B and T cells were both derived from 1-month-old 129/J mice, low IgM and IgG responses were observed. In contrast, when 129/J B cells were cultured with 129/Sv T cells, the indirect PFC response dramatically increased. The 129/Sv B cells produced a significantly higher IgM PFC response when cultured with 129/Sv T cells than when they were cultured with 129/J T cells ($0.01<P<0.02$, Student's t-test).

The studies described above employing various T-independent antigens as immunogens revealed that 1-month-old 129/Sv mice display a PFC response equivalent to that of adult 129/J mice. The results obtained in vitro also show that 129/Sv mice may present an accelerated maturation of T and B cells. Perhaps this involves RF in the activation of B cell clones.

A completely different picture was observed with cells from 6-month-old mice. 129/J B cells incubated with T cells from 129/J or 129/Sv mice develop and excellent anti-TNP PFC response in which the IgG response was prevalent. In contrast, when the B cells from 129/Sv mice were cultured with 129/Sv T cells, the IgG PFC response was 2-fold lower than that when B cells were J derived. However, T cells from 129/J mice brought the response to within range of the normals (Table 9). These results also

Table 9. IN VITRO ANTI-TNP RESPONSE IN 129 MICE .

B Cells[a]	T cells[b]	Ag added	1 Month		6 Month	
			DIRECT μ PFC/Culture ± SEM	INDIRECT[c] μ PFC/Culture ± SEM	DIRECT μ PFC/Culture ± SEM	INDIRECT μ PFC/Culture ± SEM
129/J	-	TNP-KLH	0	11±11	5±1	1±1
		TNP-OVA	0	0	11±1	0
129/J	J	TNP-KLH	12±17	135±41	183±4	397±265
		TNP-OVA	0	27±13	5±1	0
129/J	Sv	TNP-KLH	56±23	602±248	256±35	690±340
		TNP-OVA	0	1±1	45±2	0
129/Sv	-	TNP-KLH	0	0	0	0
		TNP-OVA	0	2±2	0	0
129/Sv	Sv	TNP-KLH	2524±1164	248±98	476±152	305±61
		TNP-OVA	0	6±6	94±46	22±2
129/Sv	J	TNP-KLH	141±71	0	21±20	582±129
		TNP-OVA	67±3	0	1±1	5±2

[a] B cells 3×10^6 cells/ml

[b] T cells 1×10^6 cells/ml

[c] optimal response recorded for 10μg/ml TNP-KLH; PFC were enumerated by subtracting the number of PFC obtained in the absence of added antigen.

suggest that the production of RF in old 129/Sv mice may alter both B and T cells which cooperate in the production of IgG anti-TNP antibodies.

Variation of Isotypes Anti-TNP Antibodies During the Secondary Response: MRL Mice. The age-dependent variation of isotypes of anti-TNP antibodies was studied in 1-, 3- and 6-month-old MRL+/+, MRL/lpr or 129/J mice during the TNP-KLH secondary response. Sera (pooled from a group of 5 mice) were diluted 1:5, 1:10, 1:50 and 1:100 and the concentration of antibody was calculated by assigning the number of cpm corresponding to the linear portion of standard curves constructed using affinity-purified anti-TNP monoclonal antibodies. To control for nonspecific binding of mouse serum proteins, newborn mouse serum was incubated on TNP-BSA-coated plates giving a very low background (100-500 cpm) and this was subtracted from the cpm obtained for the binding of immune sera. The kinetics of the mu anti-TNP response (Fig. 10A-E) were similar between the MRL congenics and 129/J control within each age group of mice studied. The profiles of the kinetics for the IgG_3 anti-TNP response revealed that markedly low concentrations of these antibodies were present in sera of all MRL mice (except 3-month-old MRL+/+, and 3-6-month-old 129/J) and is consistent with previous data which have shown that IgG_3 is generally a negligable component of a thymus-dependent (TD) response in MRL mice while a significant IgG_3 response was observed in 3 and 6-month-old 129/J mice (Fig. 10B) (Park et al., 1983). In contrast, differences in the kinetics of the immune response were observed for IgG_1, IgG_{2b}, and IgG_{2a} anti-TNP antibodies. Although 1-, 3- and 6-month-old MRL+/+ displayed similar kinetics for IgG_1, IgG_{2b} and IgG_{2a} isotypes (with the exception of 1-month-old MRL+/+ mice), MRL/lpr mice had consistently lower IgG_1, IgG_{2b} and IgG_{2a}

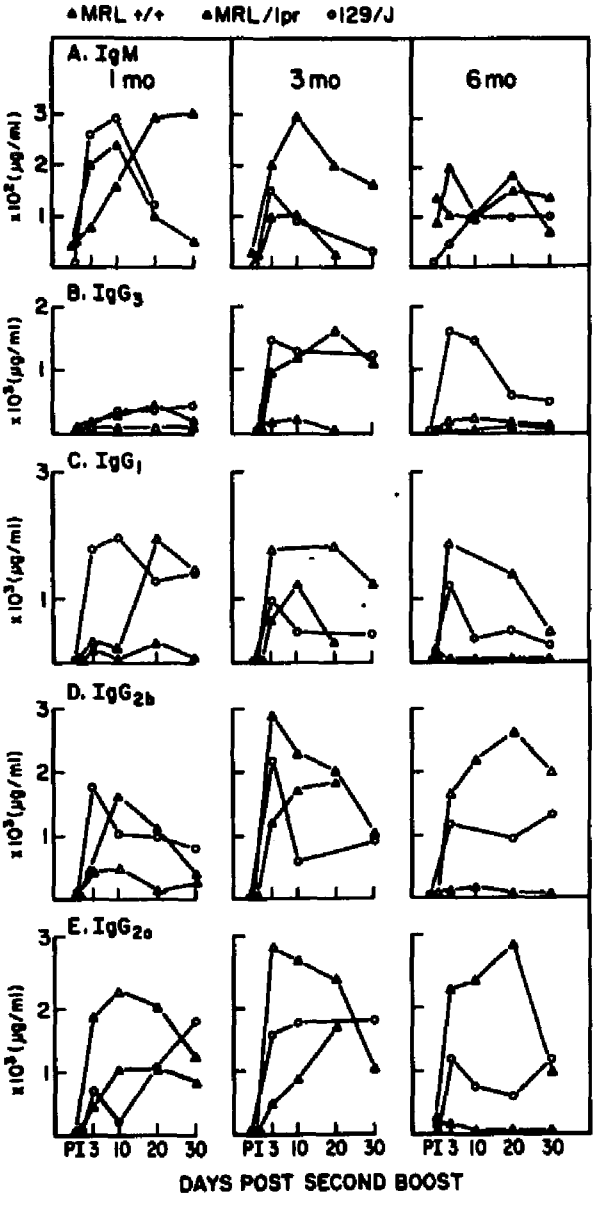

Fig. 10. Kinetics of the anti-TNP response. A) IgM anti-TNP, B) IgG_3 anti-TNP, C) IgG_1 anti-TNP, D) IgG_{2b} anti-TNP, E) IgG_{2a} anti-TNP. Sera were collected from immunized mice on days 3,10,20, and 30 after the second boost. PI(pre-immune). Concentrations of TNP-specific antibody were determined from standard binding curves.

(except 1-month-old MRL/lpr IgG_1) responses (Fig. 10C-E). Furthermore, the decrease in the concentration of these isotypes was most profound at 6 months of age. The decrease in all gammaglobulin isotypes at this age in MRL/lpr mice may be related to the presence of auto-anti-gammaglobulin antibodies, generally of the IgM and IgG isotype (Hang et al., 1982; Theofilopoulos et al., 1980) that exhibit a broad specificity for Fc fragments of various subclasses of gammaglobulin. Since we had found that RF are produced in both MRL+/+ and lpr mice subsequent to immunization with polysaccharide antigens, the sera from mice immunized with TNP-KLH were also used to determine the presence and fine specificity of RF. Titers were determined via HA assays.

Study of the Occurrence of Anti-Gamma-Antibodies During the Secondary Response. Not only do MRL/lpr mice produce anti-gammaglobulin antibodies during the secondary response, but MRL+/+ and 129/J animals do so as well (Fig. 11). The most interesting finding was the observed long-lasting production of RF and their broad specificity for all IgG isotypes, even though some antigen-specific antibody was not present in the serum. We also studied the PFC responses to discern whether the decreased concentration of gammaglobulin isotypes in the sera is related to the clearance of antigen-specific antibodies by RF or to an interaction of RF with the receptor of antibody-forming cells.

Study of the Anti-TNP PFC Response in MRL Mice. The direct and indirect PFC responses (Table 10) were studied in the same group of 1 to 6-month-old 129/J, MRL+/+ and MRL/lpr animals utilized in the kinetic studies. The response was measured seven days after the second boost (tertiary challenge). Age-matched 1-month-old control mice (MRL+/+ and

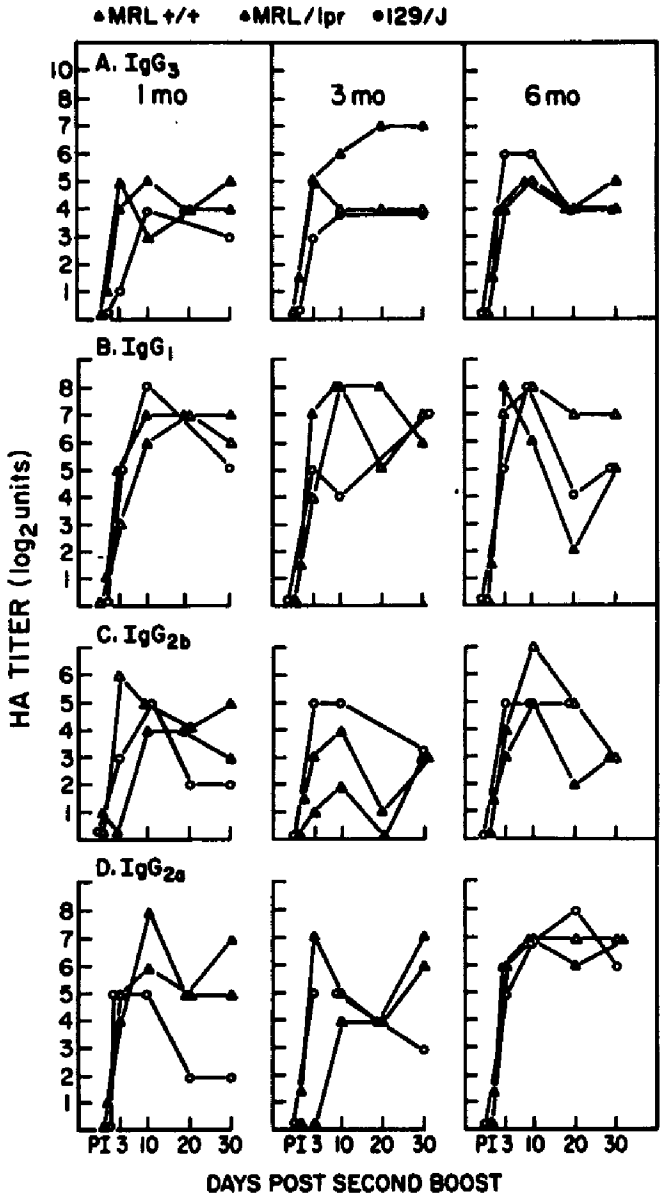

Fig. 11. Kinetics of the anti-gammaglobulin response during the secondary anti-TNP response (HA titer). A) anti-IgG_3, B) anti-IgG_1, C) anti-IgG_{2b}, and D) anti-IgG_{2a}. Titers were determined from sera obtained from the same mice utilized in the kinetic study.

Table XO. Age-dependence of the Anti-TNP response elicited by immunization with TNP-KLH.

		TOTAL PFC/Spleen (log G.M.±S.E.M.) (A.M.) TERTIARY RESPONSE	
STRAIN	AGE	DIRECT	INDIRECT
129/J	1	3.18±0.03 (1515)	3.82±0.33 (7725)
	3	3.55±0.27 (7020)	4.28±0.02 (19020)
	6	2.99±0.13 (1210)	N.D.*
MRL +/+	1	2.15±0.15 (148)	2.57±0.33 (650)
	3	2.25±0.24 (227)	3.51±0.10 (3350)
	6	1.48±0.00 (30)	3.24±0.81 (5610)
MRL/lpr	1	3.62±0.21 (585)	2.52±0.15 (380)
	3	2.34±0.10 (230)	2.43±0.00 (270)
	6	2.72±0.00 (525)	2.35±0.00 (225)

log G.M.±S.E.M.(arithmetic mean) of 3-5 mice for each group. Mice were immunized i.p. with 100 µg TNP-KLH in CFA and subsequently boosted 1 mo later i.p. with 10µg TNP-KLH in saline. Mice received an additional boost (i.p. 10 µg in saline) one month after the first boost. The PFC response was then measured 7 days later.

*N.D.- not done.

129/J) make similar IgM anti-TNP responses ($0.02<p<0.04$ two-tailed t-test), the IgM response for MRL/lpr mice is similar to the control group ($p>0.25$). Similarly, the IgG component of the response for 1-month-old mice was similar between MRL congenics and 129/J mice ($0.20<p<0.50$ ANOVA). However, by 3 months of age, the 129/J mice make a significantly stronger indirect response than do either 3-month-old MRL+/+ ($p<0.005$, two-tailed t-test), or MRL/lpr mice ($0.25<p<0.05$ two-tailed t-test). Finally, by 6 months of age, when MRL/lpr mice exhibit profound symptoms of the disease, the lowest IgG anti-TNP response was observed as measured by the PFC response. This is consistent with the isotypic profiles obtained for all subclasses of IgG anti-TNP antibodies in these mice at 6 months of age (Fig. 10B-E). Therefore, we cannot relate to the low anti-TNP IgG antibodies to simple clearance mechanism. Rather, this inability to produce gammaglobulins to a TD antigen may be the result of an innate lymphocytic defect in these mice.

We then studied the interaction of T and B cells in an in vitro hapten-carrier antibody synthesis system. The results depicted in Table 11 illustrate a representative experiment showing that MRL+/+ B and T cells could mount strong IgM and IgG responses in the presence of the appropriate antigen. However, although the lymphoproliferative defect in lpr mice causes an increased antigen-nonspecific help to B cells, these lpr T cells could not provide sufficient help to +/+ TNP-specific B cells in this antigen-specific response. Therefore, the response measured was only 35% of that observed for +/+ B and T cells co-cultured in the presence of TNP-KLH. In addition, 6-month-old MRL/lpr mice could not mount an in vitro antibody response to TNP when the source of the T cells

Table II. In Vitro anti-TNP Response in 6-mo-old MRL mice

B cells*	T cells**	Ag Added	6 Months DIRECT	6 Months INDIRECT
			PFC/culture	
MRL +/+	-	nil	3±1	6±2
		TNP-KLH	8±1	8±6
		TNP-OVA	6±3	6±0
MRL +/+	+/+	nil	0	0
		TNP-KLH	165±12	321±25
		TNP-OVA	24±4	33±10
MRL +/+	lpr	nil	3±3	6±1
		TNP-KLH	51±6	114±21
		TNP-OVA	9±5	12±2
MRL/lpr	-	nil	3±3	3±2
		TNP-KLH	30±11	46±10
		TNP-OVA	21±10	36±15
MRL/lpr	lpr	nil	0	0
		TNP-KLH	36±11	70±12
		TNP-OVA	3±1	6±2
MRL/lpr	+/+	nil	0	0
		TNP-KLH	81±11	172±6
		TNP-OVA	36±15	45±5

*B cells 3×10^6 cells/ml

**T cells 1×10^6 cells/ml

Cultures contain 2 mls Total Volume.

ND - Not Done.

was lpr derived. It is also interesting to note that +/+ T cells could provide only some help to lpr B cells but this was not sufficient to restore the IgG response. These results are consistent with the serum profile of gammaglobulins that we obtained by in vivo hyperimmunization of MRL mice with TNP-KLH. This suggests that MRL/lpr mice exhibit T and B cell defects and moreover, the production of RF may, in some way, hamper cooperation between T and B cells in the production of IgG anti-TNP antibodies in old MRL/lpr mice. Since one could induce RF in both normal and autoimmune mice after immunization with conventional antigens, it was important to more closely examine the nature and regulatory properties of RF produced during normal immune responses and during disease states. For this reason, monoclonal RF (MRF) were generated in a variety of ways.

Fine Specificity of MRF. A panel of hybridomas was obtained from several different mouse strains including: (1) splenocytes from unmanipulated 6-month-old 129/Sv and 5-month-old MRL/lpr mice which spontaneously produce RF upon aging, (2) splenocytes from BALB/c mice injected with Yersinia enterocolitica, or (3) BALB/c splenocytes stimulated in vitro with LPS. The majority of monoclonal RF were IgM, with the exception of 129-74, 129-77 (IgG_3, k) and 129-102 (IgA, k). Based on their fine specificity, our panel of murine RF monoclonal antibodies can be divided into three categories. The first group (4 of 20) binds with high affinity to IgG_{2a}, the second group (6 of 20) binds with high affinity to some IgG subclasses and/or with low affinity to others, and the remainder (10 of 20) bind with low affinity to one or more IgG subclasses (Table 12). It should be noted that a few RF selected on plates coated with a murine IgG_{2a} protein (i.e. HOPC1) exhibited low affinity for Fc (Table

Table 12. Specificity of rheumatoid factor monoclonal antibodies

RF	Binding to microtiter plates coated with: IgG3(Y5606 γ3,λ)**	IgG1(88-C692 γ1,λ)***	IgG2b(BA6 γ2b,λ)***	IgG2a(HOPC1 γ2a,λ)**
nil	225±1*	144±2	434±21	291±26
129-48	859±81	1,681±219	2,060±296	0
129-78	1,324±184	0	3,167±1	0
129-76	914±15	469±98	1,508±72	410±94
129-101	1,305±208	436±28	1,483±321	1,299±213
129-61	715±127	11,778±441	4,611±138	741±208
129-74	108±16	151±28	390±59	9,611±56
129-102	440±4	170±50	612±2	24,278±800
129 77	600±73	325±15	769±155	18,280±1,263
Y19-10	0	2,055±155	1,460±22	816±113
Y19-16	291±32	371±93	1,676±23	0
Y43-5	1,127±730	158±55	812±38	258±15
LPS10-1	720±716	21,476±595	9,958±1,305	6,758±612
LPS5-4	1,347±160	2,796±296	984±147	3,915±44
LPS5-7	4,327±347	12,503±289	4,509±141	1,413±141
LPS7-4	0	1,007±77	408±33	221±43
MRL5-31	1,001±60	814±12	1,143±225	513±10
MRL22-46	1,113±190	0	2,654±51	1,116±13
MRL30-8	518±33	915±125	3,078±127	1,089±112
MRL55-18	1,156±461	237±140	707±7	10,592±492
MRL55-23	558±28	2,853±33	1,679±154	783±101

*cpm-mean ± SD. Microplates were incubated overnight with 10μg heat aggregated (15 min 63C°) Ig.λ chain, washed, saturated with 3% BSA and incubated for 3 hrs at room temperature with 2 μg/ml RF monoclonal antibodies. After extensive washings with PBS-Tween 20(0.05%), the plates were incubated for 2 hrs with ^{125}I-rat anti-kappa monoclonal antibody (~50,000-cpm/well). Background (i.e. BSA coated plates incubated with RF monoclonal antibodies) was subtracted. **Kindly donated by Dr. H. Potter, NCI, Bethesda, MD. ***Kindly donated by Dr. T. Moran, Mount Sinai School of Medicine, NYC., NY.

12). This is explained by a modification of experimental procedure to study the fine specificity of RF in which extensive washings with PBS-Tween were employed to minimize a possible non-specific "stickiness" of RF of the IgM isotype.

In Vitro Effect of MRF on Anti-TNP and Anti-SRBC Responses.

Anti-TNP-NWSM Response. When spleen cells from BALB/c, 129/J or 129/Sv mice were cultured with both TNP-NWSM and anti-460Id MAb(FD5-13), 45-65% of the anti-TNP plaques were inhibited at high doses of FD5-13 (Fig. 12, left upper panel). Under these conditions 45% of the anti-TNP PFC from BALB/c spleens were $460Id^+$ whereas only 25% of the anti-TNP PFC from 129/J or 129/Sv spleens were $460Id^+$ (Fig. 12, right upper panel).

To determine whether MRF could modify the anti-TNP response, spleen cells from BALB/c, 129/J or 129/Sv mice were cultured with TNP-NWSM and LPS10-1 (IgM RF which also binds IgD, data not shown) or 129-48 (IgM RF which binds IgG_1 and IgG_{2b}). We compared the effect of a monoclonal anti-dextran antibody [LP32-14, (IgM)] with the effects of RF LPS10-1 and 129-48. At 10 ug/ml, LPS10-1 appeared to enhance the anti-TNP response elicited by 129/Sv spleen cells, but inhibited the 129/J anti-TNP response and to a lesser extent the BALB/c response. In contrast, at 10 ug/ml, 129-48 did not affect the responses elicited by 129/J and 129/Sv spleen cells, but slightly inhibited the BALB/c anti-TNP response (Fig. 12, lower panels).

Anti-SRBC Response. Since it had been reported that immunization with Ars-OVA (TD antigen) or LPS can induce RF production (Dresser, 1978; Nemazee and Sato, 1983), the effect of co-culturing spleen cells from 129/Sv or 129/J previously primed with LPS or TNP-OVA with 129/J SRBC-

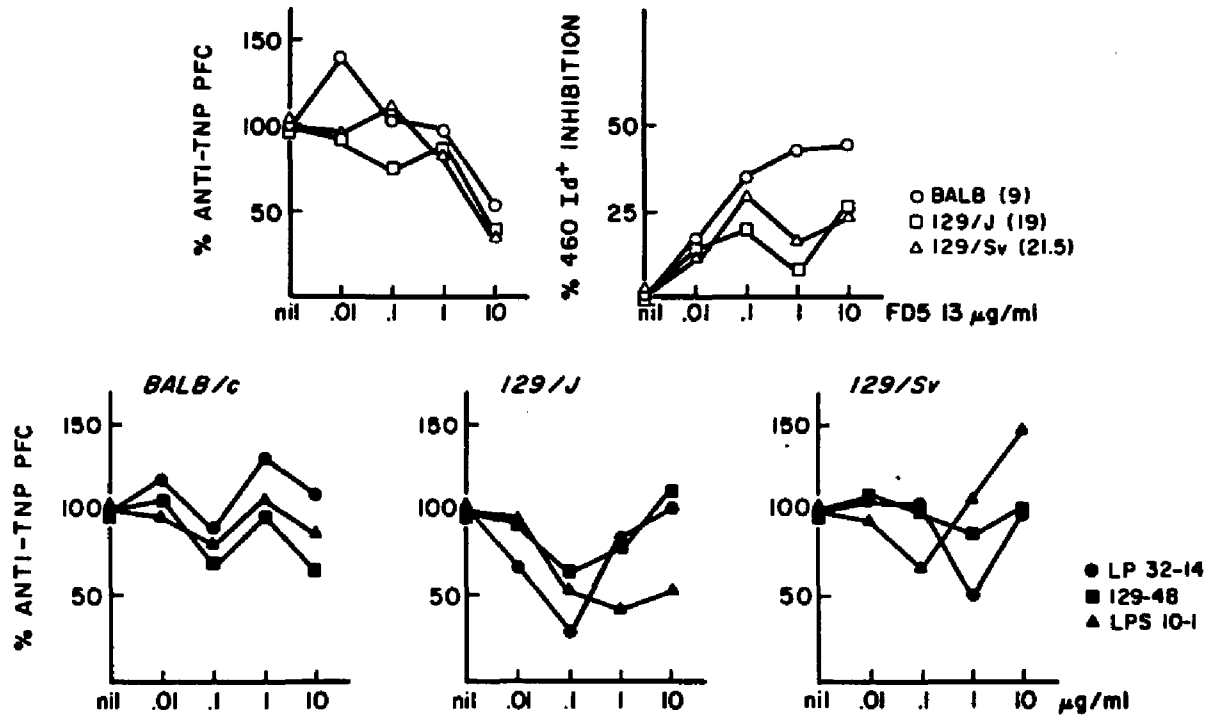

Fig. 12. In vitro effect of anti-idiotype and MRF on anti-TNP response.

primed spleen cells was studied. Only spleen cells from LPS immunized 129/Sv mice caused a significant decrease in the indirect PFC response (Table 13). No effect was observed on the direct PFC response under any circumstance.

Idiotype of MRF. Rabbit anti-Id antibodies against MRF 129-48, Y19-10 and LPS 10-1 were purified using a two-step procedure. First, anti-mu, anti-gamma, anti-kappa activities were removed by absorption on IgM, IgG, kappa sepharose 4B-columns. Subsequently, a column which contained bound-immunogen was used to purify the anti-Id antiserum. Our anti-Id antibodies are devoid of anti-IgM or anti-kappa activity as assessed by the lack of binding to a panel of IgM, kappa antibodies with various antigen specificities (Table 14).

Expression of Cross-Reactive Idiotypes (IdX) on MRF. The expression of IdX was studied using a sensitive sandwich RIA (described in Materials and Methods). 14 of 20 monoclonal RF share idiotopes of 129-48 and LPS10-1 while 7 of 20 share the Y19-10 idiotope(s) (Table 15). This suggests that the expression of IdX on monoclonal RF is neither related to strain nor to the fine specificity of the RF. It should be noted that three monoclonal RF obtained from 129/Sv mice specific for only IgG_{2a} (129-74, 129-77, 129-102) and one specific for IgG_{2b} (129-76) did not share idiotopes of 129-48, LPS10-1, or Y19-10 monoclonal RF (data not shown). Also, MRL 55-18 did not share idiotopes of any of the MRF 129-48, LPS10-1 or Y19-10.

VH Gene Family Utilization by RF. Because our immunochemical studies showed that MRF from various sources share IdX, it seemed possible that these monoclonal antibodies may be encoded by related V_H genes. To screen

Table 13. In vitro anti-SRBC PFC response

A	Spleen cells ($\times 10^6$) from SRBC primed, 129/J		SRBC – PFC[1]	
			direct	indirect
	2		6,559	2,642
	1.5		3,838	908
	1		1,358	167
	0.5		136	29
B	Spleen cells ($\times 10^6$) from		SRBC – PFC	
	SRBC primed, 129/J	nonimmune, 129/J	direct	indirect
	2	0	6,559	2,642
	1.5	0.5	3,538	1,108
	1	1	1,408	308
	0.5	1.5	253	17
C	Spleen cells ($\times 10^6$) from		SRBC – PFC	
	SRBC primed, 129/J	TNP-OVA primed, 129/J	direct	indirect
	2	0	6,559	2,642
	1.5	0.5	4,175	1,533
	1	1	1,863	563
	0.5	1.5	233	0
D	Spleen cells ($\times 10^6$) from		SRBC – PFC	
	SRBC primed, 129/J	LPS primed, 129/Sv	direct	indirect
	2	0	6,559	2,642
	1.5	0.5	3,467	542
	1	1	1,650	83
	0.5	1.5	196	2
E	Spleen cells ($\times 10^6$) from		SRBC – PFC	
	SRBC primed, 129/J	TNP-OVA primed, 129/Sv	direct	indirect
	2	0	6,559	2,642
	1.5	0.5	3,058	1,275
	1	1	1,396	308
	0.5	1.5	253	21

A SRBC primed, 129/J.
B, C, D Mixing experiment: SRBC primed, 129/J + nonimmune 129/J, TNP-OVA boosted 129/J LPS boosted 129/Sv, TNP-OVA boosted 129/Sv, respectively.
[1] SRBC PFC/culture.

Table 14. Specificity of anti-idiotypic antibodies

ANTIBODY	ANTI-129-48 Id	ANTI-LPS10-1 Id	ANTI-Y19-10 Id
2-8-2	355 ± 33	254 ± 36	231 ± 3
129-48	8948 ± 307	8292 ± 211	3899 ± 79
17-38	690 ± 42	411 ± 64	284 ± 60
LPS10-1	6543 ± 323	8109 ± 287	1908 ± 69
2-9-17	371 ± 49	262 ± 60	234 ± 43
2-11-1	315 ± 65	264 ± 43	265 ± 57
2-12-19	375 ± 90	232 ± 28	750 ± 10
Y19-10	6461 ± 335	4227 ± 91	4057 ± 177
3-14-19	1832 ± 244	885 ± 33	347 ± 22
3-76-4	296 ± 16	192 ± 16	243 ± 49
1-5-1	276 ± 30	256 ± 59	194 ± 16
3-76-42	351 ± 49	294 ± 66	321 ± 39
BSA	211 ± 30	222 ± 32	270 ± 80

Sandwich RIA. Microtiter plates were coated overnight with 2 μg/ml of 2-8-2, 2-9-17, 2-11-1, 2-12-9, 1-5-1 (anti-levan antibodies), 17-38 (anti-48-Id antibody;) 3-14-19, 3-76-4, and 3-76-42 (anti-anti-48-Id antibodies) and 129-48, LPS10-1 and Y19-10 (RF). All of them are μκ antibodies. After washing and post-coating, 2 μg/ml of rabbit anti-Id antibodies were added to the plates. ^{125}I-F(ab')$_2$ donkey anti-rabbit Ig was used as the developing reagent.

Table 15. Binding of rabbit anti-Id antibodies to monoclonal RF antibodies

RF	Anti129-48 Id	AntiLPS10-1 Id	AntiY19-10 Id
129-48	5,375±713	6,915±153	3,421±165
LPS10-1	4,748±270	6,812±106	1,355±43
Y19-10	5,323±288	4,742±600	3,728±78
129-78	505±369	193±75	277±24
129-101	3,409±900	3,857±349	780±28
129-61	5,969±190	6,377±728	2,679±109
Y43-5	1,196±215	1,266±90	0
Y19-16	2,793±188	2,375±332	1,753±332
LPS5-4	2,049±123	3,230±250	84±23
LPS5-7	4,474±395	7,880±150	2,209±70
LPS7-4	4,812±89	7,395±180	1,843±180
MRL5-51	3,719±227	4,163±426	605±116
MRL22-46	3,962±449	4,674±734	798±143
MRL50-8	3,340±417	1,072±88	416±69
MRL55-18	892±84	140±113	0
MRL55-23	1,086±118	1,584±71	0

Microplates were incubated overnight with 2µg RF monoclonal antibody, washed, saturated with 1% BSA and incubated with 2µg/ml rabbit anti-Id antibodies. After washing, plates were incubated for 2 hrs with ^{125}I F(Ab')$_2$ donkey anti-rabbit antibody (Amersham) (50,000 cpm/well). The results are expressed in cpm (mean±S.D.) after the background was subtracted. Pilot experiments showed no significant binding of RF to normal rabbit Ig (data not shown). The remaining 6 RF did not bind to any anti-Id antibody (data not shown).

monoclonal RF for V_H gene expression, cytoplasmic lysates were assayed for hybridization to V_H probes specific for each of the known families (Fig. 13). Of the 7 well characterized V_H gene families (Brodeur and Riblet, 1984), only three families are utilized by the RF tested: V_H J558, the largest family (4 RF), V_H QPC52, a more 3' family (2 RF) and V_H 7183, the most J_H proximal family (4 RF). Subsequent to these studies, three other RF were found to utilize a V_H gene from the 7183 family: 129-74, 129-76, 129-102 (data not shown). These results indicate that a restricted set of V_H genes encode the variable regions of murine RF. However, the data also show that the heavy chain V regions of many MRF which express cross-reactive idiotypes are encoded by divergent V_H genes.

Cloning of Expressed H chain VH Gene From Two RF Hybridomas. In order to study the precise structural nature of the V_H genes encoding RF, we have cloned the expressed V_H alleles from two hybridomas producing MRF: 129-48 (V_H 7183 family) and Y19-10 (V_H J558 family). Southern blotting analysis of Eco RI digested genomic DNA indicated J_H positive rearrangements of 2.0kb and 3.5kb from 129-48 and 3.8kb and 6.6kb from Y19-10 (Fig. 14). The rearranged fragments of 3.5kb from 129-48 and 3.8kb from Y19-10 were shown to be productive (data not shown). These fragments were cloned into the Eco RI site of lambda phage Charon 16A (Alt et al., 1982), subcloned, restriction mapped (Fig. 15) and the nucleotide sequences of the relevant regions determined. The J_H segments utilized by 129-48 and Y19-10 corresponded to the BALB/c J_H4 and J_H3 sequences, respectively. The D segments were also different from each other: DFL 16.1 in 129-48 and DSP2 in Y19-10. Comparison of the nucleotide sequence of the expressed V_H gene of 129-48 with germline genes in the V_H7183 family

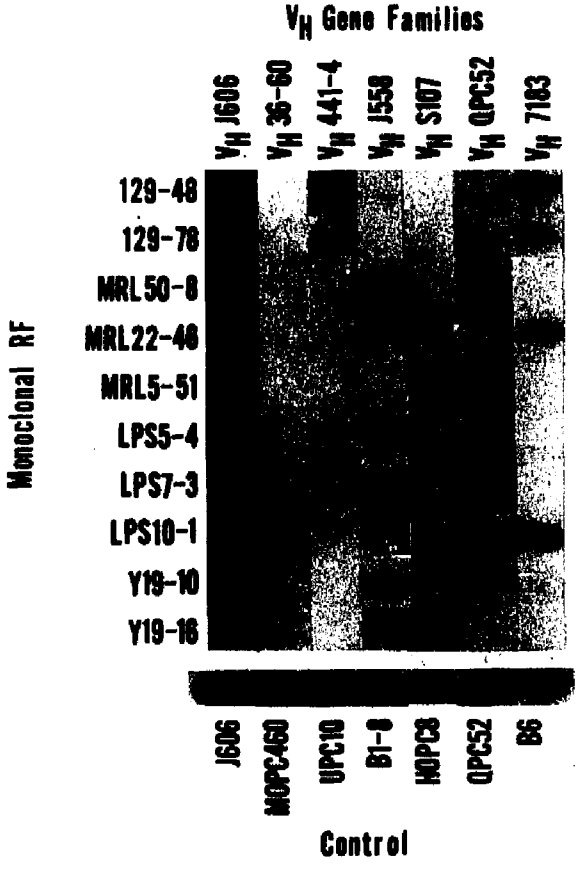

Fig. 13. Utilization of V_H genes by MRF. Autoradiogram composite of slot-blot analysis of cytoplasmic RNA lysates from RF-secreting hybridomas. Nitrocellulose was hybridized to ^{32}P nick-translated V_H gene probes under normal stringency conditions, washed and autoradiographed. Positive controls are shown at the bottom of the figure. Under normal stringency (42°C hybridization, 50% formamide; 68°C wash 2xSSC 0.1% SDS), V_H 7183 and V_H 441-4 cross-hybridize.

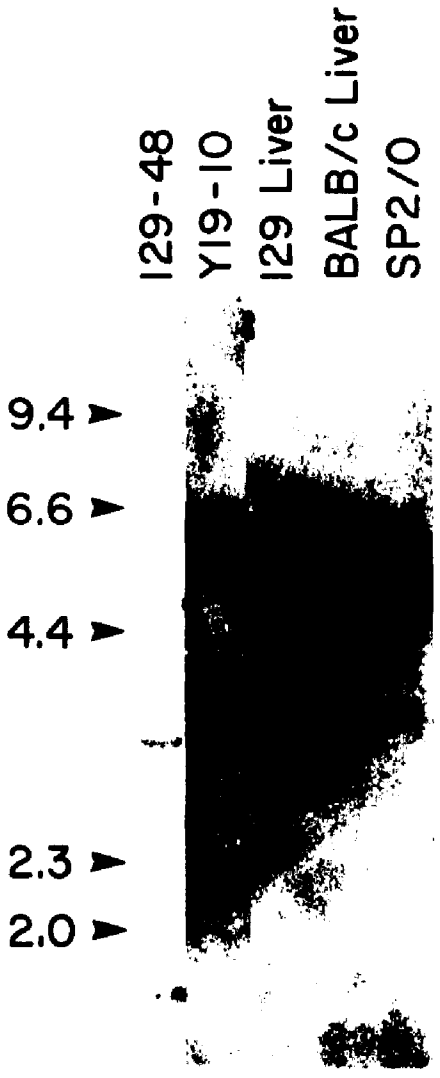

Fig. 14. Southern blot analysis of RI digested genomic DNA from RF lines 129-48 and Y19-10, germline liver DNA from 129 and BALB/c mice and parental fusion partner sp2/0. Nitrocellulose was hybridized to ^{32}P nick translated BamHI-RI J_H probe, washed and autoradiographed.

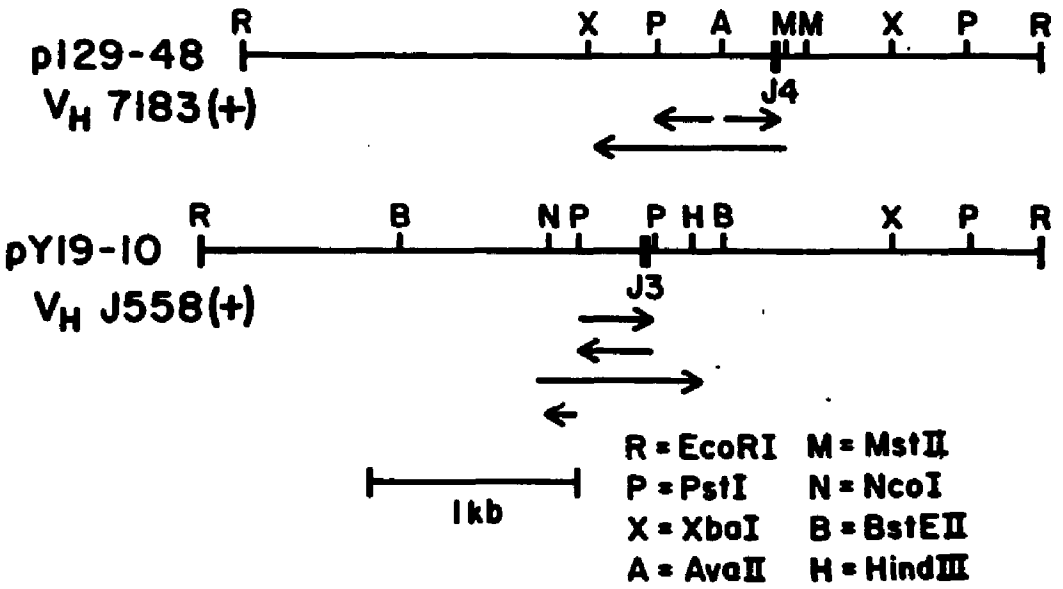

Fig. 15. Restriction enzyme analysis of RF clones. Plasmids p129-48 and pY19-10 were subjected to restriction mapping analysis. Sequencing strategy is shown.

showed that V_H 129-48 is very similar to the 37.1 germline gene (Hartman and Rudikoff, 1984) (19bp differences) and less homologous to V_H 81X (which is the most diverse member of the 7183 family (Fig. 16). Similarly, comparison of the sequence of the expressed gene V_H Y19-10 to various J558 germline genes of either BALB/c (V_H33) origin or C57Bl/6 (V_H23 and V_H186-2) origin (Fig. 17) indicated that V_H Y19-10 is closely related to germline genes in the J558 family. It should be noted that very few changes in the entire gene were observed for either V_H129-48 or V_H Y19-10 as compared to the most closely related germline V_H genes for which nucleotide sequence exists. However, if one compares the protein sequence of V_H 129-48 and V_H Y19-10, it is apparent that they share a tetrapeptide in framework 2. This common stretch of amino acids may contribute to the IdX one observes between these RF (Fig. 18).

Since we already determined that murine RF obtained from various sources were encoded by only certain V_H gene families, it was equally important to determine whether or not other types of autoantibodies also used a restricted set of V_H genes. The origin and specificity of monoclonal autoantibodies including the MRF used in these studies are shown in Table 16.

Cytoplasmic lysates were prepared from 10^7 hybridoma cells, transferred to nitrocellulose and assayed by hybridization to a V_H gene specific probe for each known family (White and Bancroft, 1982). With some autoantibodies, the results obtained with the slot-blotting technique were unclear, and, in those cases, the purified RNA was hybridized to the V_H probes using the Northern blotting technique.

Out of 43 autoantibodies tested, 17 used a V_H gene from the largest

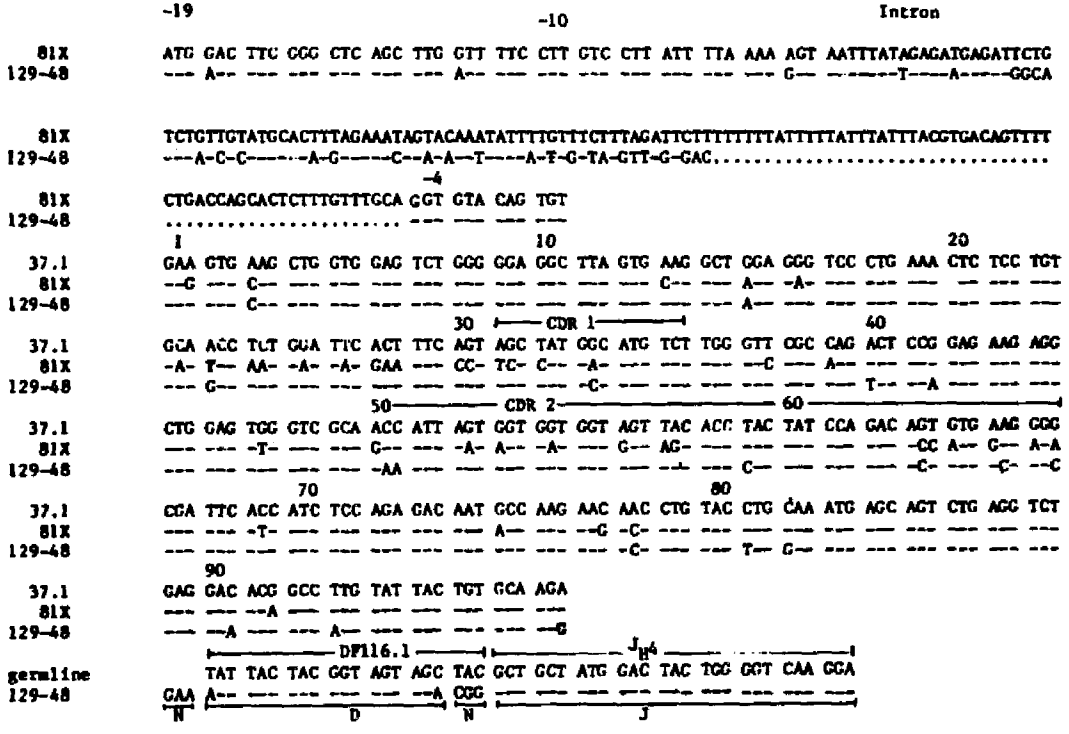

Fig. 16. Nucleotide sequence of RF clone V_H 129-48. Sequence of V_H 129-48 is compared to 2 germline genes in the 7183 family. 37.1 is a V_H gene isolated by A. Hartman (1984) and V_H81X was isolated by G. Yancopoulos et al. (1984).

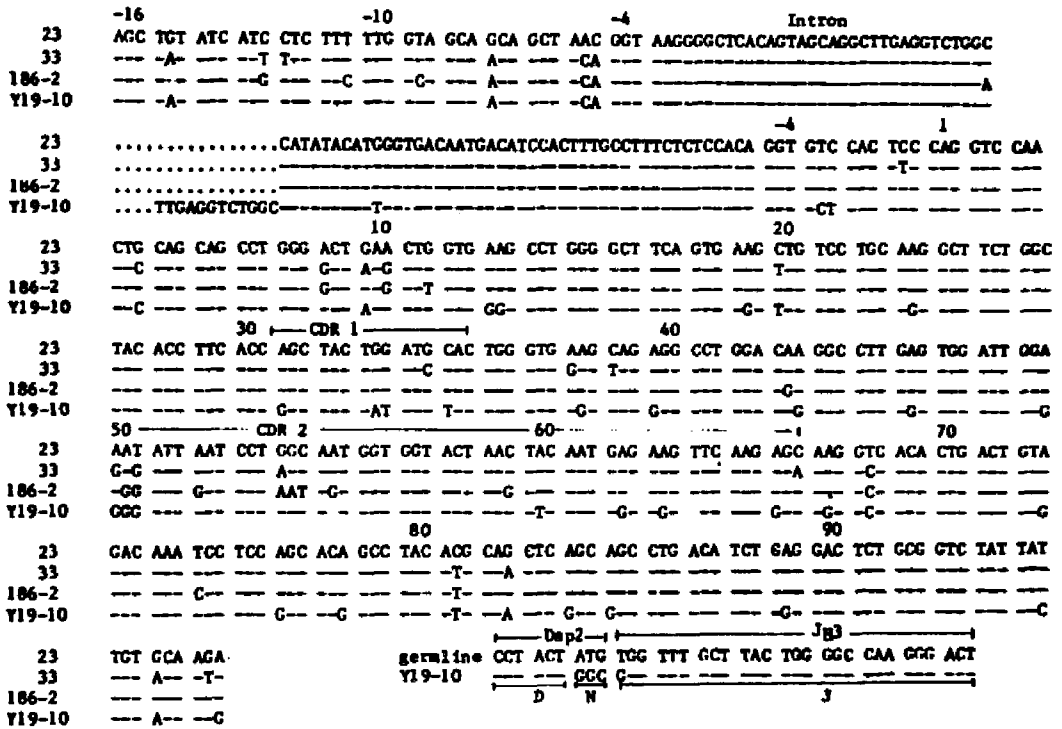

Fig. 17. Nucleotide sequence of RF clone Y19-10. Sequence of V_H Y19-10 is compared to germline genes of the J558 family incuding: V_H23 and V_H186-2, germline genes of C57Bl/6 origin, and V_H33, a germline gene of BALB/c origin.

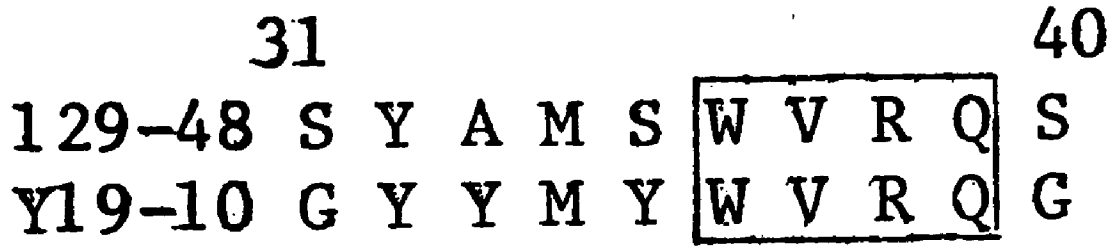

Fig. 18. Protein sequence of V_H 129-48 and V_H Y19-10 indicating shared tetrapeptide residues (36-39) in framework 2.

Table 16

Origin and Specificity of Autoantibodies

Strain	Origin	Designation	Specificity	Reference
129/Sv	S	129-48, 129-78, 129-74[a], 129-76 129-102, 129-66, 129-61, 129-77	Fc IgG	Manheimer-Lory
MRL/lpr	S	MRL50-8, MRL5-51, MRL22-46 MRL55-26, MRL18-68, MRL55-18		
BALB/c	LPS in vitro	LPS10-1, LPS7-4, LPS5-4, LPS7-3		
BALB/c	I	Y19-10, Y19-16, Y43-5		
MRL/lpr	S	H102[b], H130, H241[b], RL1-3	ss DNA	R. Schwartz
BALB/c	I	HB2	ds DNA	B. Roux
BALB/c	I	1-15[c], 62[c], B10H2A2[c], APDB6[c]	Tg	M. Zanetti
CBA/J	I	10VA2, 10IAIc, 84A3 8ID2, 8IB1, 84D1	Tg	N. Rose
MRL/lpr	S	Y2[b], 2G7[b], Y12[b], 6B6	Sm	D. Pisetsky
BALB/c	I	F8D5	AchR	B. Erlanger
BALB/c	I	Le4	TSHR	B. Erlanger
NZB	S	CP3, CP4	Br. treated	A. Bussard
CBA	S	CP5	MRBC	
DBA/1	I	A12[d], B11[c], E8[c], F9, C2[d], E5[c] F4[b], F10[c], E7[c], C1[b], B1	Type 2 Collagen	R. Holmdahl
BALB/c	I	HB8[c], HB9	micro-fibrils	J. Brochier
BALB/c	I	HB10, HB11, HB12	skin antigens	J. Brochier

S = spontaneous
I = immunized
All antibodies are IgM except [a]IgG_3, [b]IgG_{2a}, [c]IgG_1, [d]IgG_{2b}

family, V_H J558, 5 used a V_H gene from V_H QPC52 and 20 used a V_H gene from V_H 7183. Only one utilized a V_H gene from the V_H S107 family. Examples of slot blotting are illustrated in Fig. 19. The results of this study, summarized in Table 17 indicate that a restricted set of V_H genes encodes murine monoclonal autoantibodies.

Idiotype of Monoclonal Autoantibodies. Several idiotypic systems previously used to characterize IdX expressed on RF, anti-Sm and anti-thyroglobulin autoantibodies were used in this study. All the autoantibodies used were chromatographically purified on a Sepharose 4B-rat monoclonal anti-murine kappa antibody column.

a) ^{125}I-Y19-10 anti-LPS10-1 Id antibodies.

Y19-10 is a MRF induced in BALB/c mice by immunization with Yersinia enterocolitica. LPS10-1 is a MRF encoded by a V_H gene from the V_H 7183 family. In this system, a large number of monoclonal autoantibodies with various specificities gave >40% inhibition at a concentration of 10 ug/ml. The results (Fig. 20) show that the inhibition was dose dependent with the majority of the IdX positive autoantibodies with the exception of 129-61, LPS7-4, MRL22-46, MRL50-8, 10V A2 and CP5 for which an inhibition was not observed with 5ng of antibody/well.

b) ^{125}I Y19-10 anti-129-48 Id antibodies.

Monoclonal 129-48 is a RF obtained from 129/Sv mice, encoded by a V_H gene closely related to the V_H 37.1 germline gene, a member of the V_H 7183 family (Hartman and Rudikoff, 1984).

In this system, an IdX was detected among 4 monoclonal RF, 2 anti-thyroglobulin antibodies, one anti-collagen type 2 and 2 anti-skin antigen antibodies (Fig. 21).

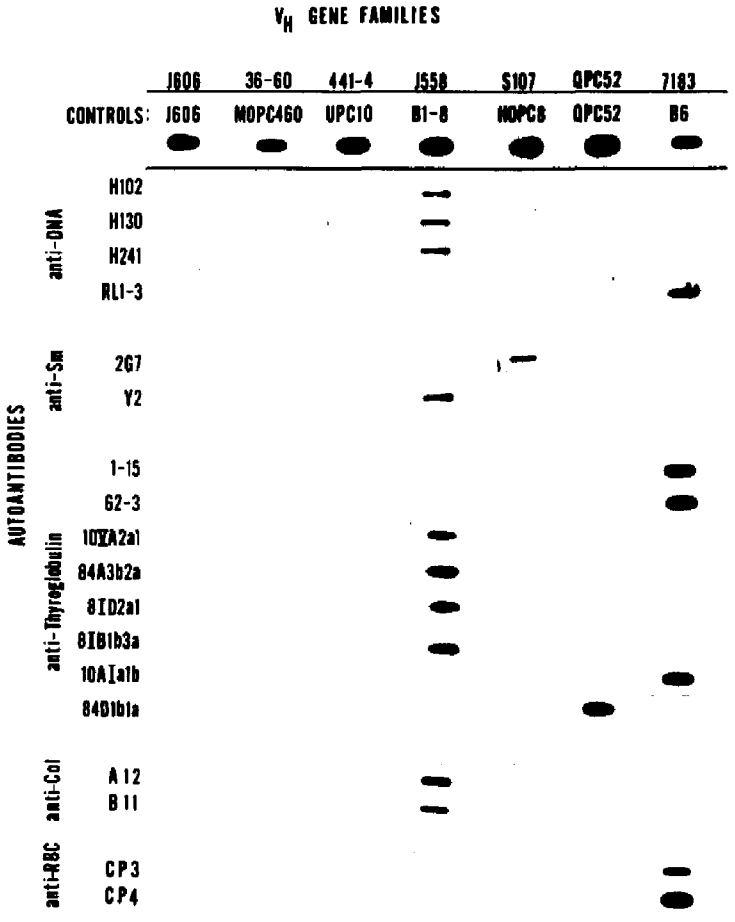

Fig. 19. Autoradiogram composite of slot blot analysis of cytoplasmic RNA lysates of autoantibodies. Nitrocellulose was hybridized to ^{32}P nick translated V_H gene probes under normal stringency conditions, washed and autoradiographed. Positive controls for each family are shown at the top of the figure.

Table 17. Summary results: frequency of V_H gene families expressed in autoantibodies

V_H family	Specificity									Frequency
	Rheumatoid factors	Thyroglobulin	DNA	Sm	br-MRBC	TSH	Collagen Type II	Micro-fibrils	skin antigens	
V_H X24										0/43
V_H J606										0/43
V_H36-60										0/43
V_H J558	MRL50-8 LPS5-4 Y19-10 Y19-16	$10\overline{A}_2$ 8.4A5 $8.\overline{D}_2$ $8.\overline{B}_1$	H 102 H 130 H 241	Y12,Y_2 6B6			A12,B11,E8			17/43
V_H S107				207						1/43
V_H QPC52	MRL 5-51 LPS7-3	$8.4D_1$					B1	HB 8		5/43
V_H 7183	129-48,129-78 MRL 22-46 LPS10-1,129-74 129-70,129-78	1-15,62Id $B10H_2A_2$ 10AI	RL1-3 HB 2		CP3 CP4 CP5	LE4	D3u		HB 10 HB12	20/43

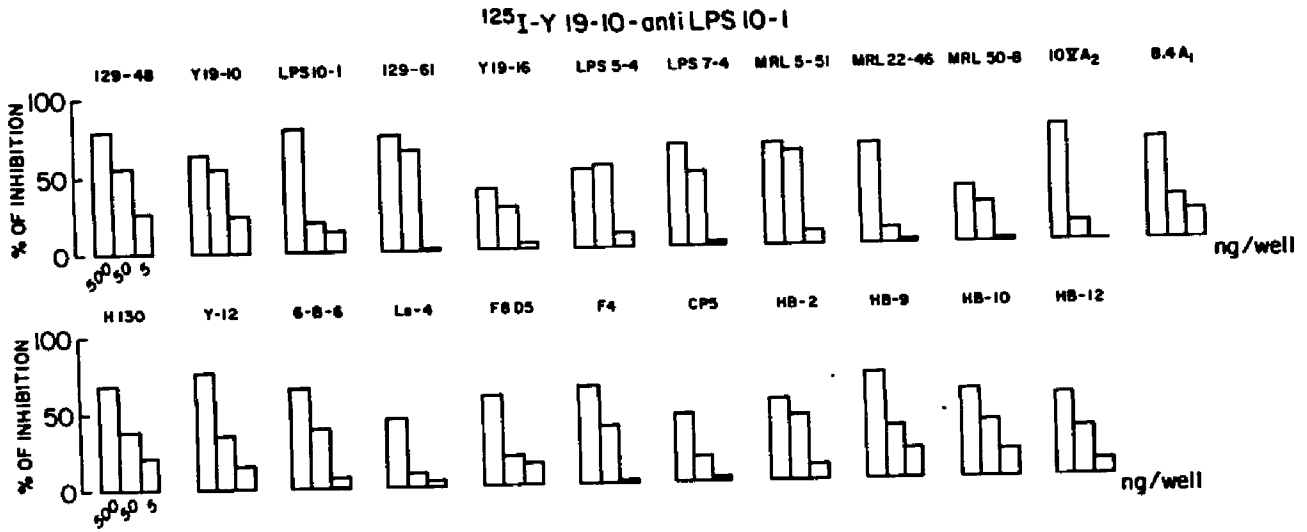

Fig. 20. Competitive inhibition of the binding of ^{125}I Y19-10 to anti-LPS10-1 idiotypic antibodies by various amounts (5-500 ng) monoclonal auto-antibodies.

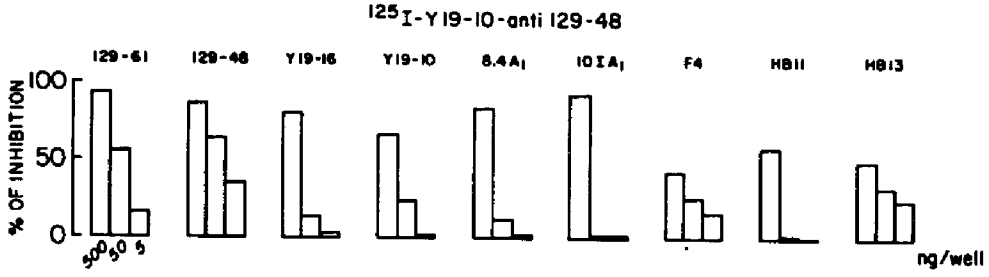

Fig. 21. Competitive inhibition of the binding of ^{125}I Y19-10 to anti-129-48 idiotypic antibodies by various amounts (5-500 ng) monoclonal auto-antibodies.

c) ^{125}I-Y2 anti-Y2 Id antibodies.

Monoclonal Y2 is an Sm binding antibody obtained from MRL/lpr mice encoded by a V_H gene from the V_H J558 family.

Idiotypic cross-reactivity was observed in this system among 3 anti-Sm antibodies and 8 RF. For some antibodies (LPS10-4, Y43-5, Y19-16, MRL55-26, MRL55-18 and MRL18-68), a strong inhibition was only observed with 1 ug of antibodies/well (Fig. 22).

d) ^{125}I-1-15 - anti-62 Id antibody.

Monoclonal 1-15 and 62Id were obtained from BALB/c mice that had been immunized with thyroglobulin. These antibodies are encoded by a V_H gene from the V_H 7183 family. In this system, an IdX was observed for four monoclonal anti-Tg antibodies obtained from BALB/c mice but not on other antibodies with different specificities, except for LPS10-1 which gave a weak inhibition (Fig. 23).

e) It was important to determine if the monoclonal autoantibodies also shared IdX with conventional antibodies specific for foreign antigens. For this purpose, we studied the inhibitory activity of 16 antibodies specific for PR8 or X31 influenza viruses in our RF idiotypic systems. These anti-viral antibodies were used as controls because they all use a V_H gene from 7183 family which is highly represented (20/43) among the autoantibodies.

Only one (VM114) inhibited weakly (at 500 ng/well) the binding of anti-129-48Id to labelled Y19-10. The other anti-influenza antibodies showed no inhibitory activity in any system used (data shown in Summary, Table 18).

Conversely, the ability of monoclonal autoantibodies to inhibit an

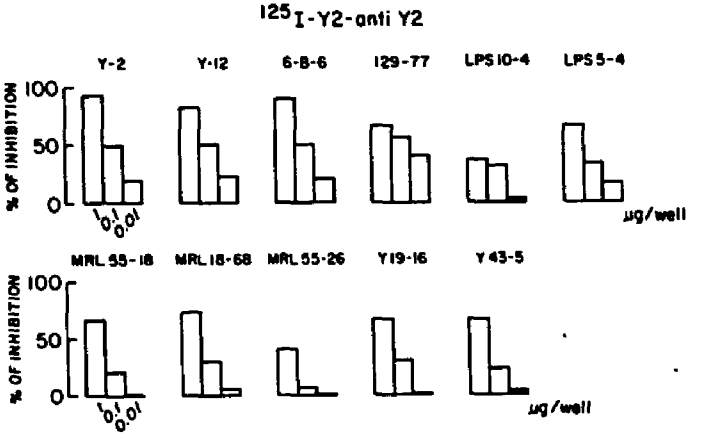

Fig. 22. Competitive inhibition of the binding of ^{125}I Y2 to anti-Y2 idiotypic antibodies by various amounts (0.01 - 1 ug) monoclonal autoantibodies.

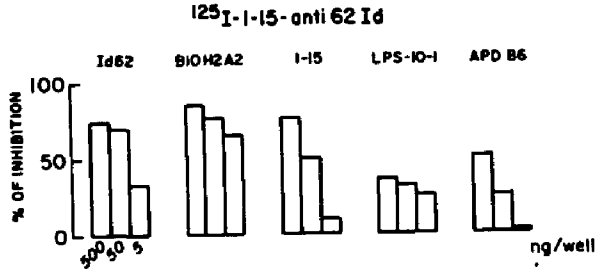

Fig. 23. Competitive inhibition of the binding of ^{125}I 1-15 to anti-62 idiotypic antibodies by various amounts (5-500 ng) monoclonal autoantibodies.

Table 8. Summary results: fraction of autoantibodies expressing cross-reactive idiotopes

Anti-Id antibodies	SPECIFICITY										
	Rheumatoid factor	Thyroglobulin	DNA	Sm	brMRBC	TSHR	Collagen type II	AcR	Micro-fibrils	Cytoplasmic Skin antigens	Influenza virus
Y19-10 anti-LPS10-1-Id	129-48, Y19-10 LPS10-1, 129-61 Y19-16, LPS5-4 LPS7-4, MRL5-51 MRL22-46, MRL50-8	$10^{9}A_2$ 8.4.A_1	H 130 H B2	Y12 6B6	CP5	LE4	F4	F8D5	HB9	HB10 HB12	
	10/20*	2/9	2/5	2/4	1/3	1/1	1/10	1/1	1/2	2/3	0/16
Y19-10 anti-129-48Id	129-48 Y19-10 129-61 Y19-16	8.4A1 10LA1					F4			HB10 HB11	VH114
	4/20	2/9	0/5	0/4	0/3	0/1	1/10	0/1	0/2	2/3	1/16
Y2- anti-Y2 Id	129-77, LPS5-4 MRL55-18 LPS10-4 MRL16-68 MRL55-26 Y19-10 Y43-5			Y2 Y12 6B6					ND**	ND	ND
	8/20	0/9	0/5	3/4	0/3	0/1	0/10	0/1			
1-15 anti-62 Id	LPS10-1	1-15 AFD B6 62 Id $B10H2A_2$									ND
	1/20	4/9	0/5	0/4	0/3	0/1	0/10	0/1	0/2	0/3	
VH202- anti-PT102 Id	0/20	0/9	0/5	0/4	0/3	0/1	0/10	0/1	ND	ND	6/16

*Fraction of antibodies expressing cross-reactive idiotypes.

**Not done.

IdX system delineated among anti-influenza antibodies was studied. In these studies, labeled VM202, antibody and anti-Py102 Id antibodies were used. Both (VM202, Py102) are MAb specific for PR8 influenza virus: they both use a V_H gene from the 7183 family and a V_K21 light chain (Moran et al., 1986).

None of the autoantibodies displayed any inhibitory activity in this system, whereas 6 of 16 monoclonal antibodies specific for influenza virus antigens gave a significant inhibition (data shown in Summary, Table 18).

Since molecular studies of the heavy chain of autoantibodies showed that the V,D,J segments used to encode the variable region are similar to those used by antibodies against foreign antigens (Andrews and Capra, 1981; Kofler et al., 1985a), and our previous studies on antibodies displaying various self-specificities clearly indicated that a restricted set of the V_H gene repertoire is utilized, we chose to examine the most 3' V_H family more closely. The V_H7183 family is of particular interest for several reasons: a) there is preferential utilization of a germline gene (V_H 81X) in pre-B cells and immature cells (Yancopoulos et al., 1984); b) Ly1 B, autoantibody producing B cells display the phenotype of immature B cells (Hayakawa et al., 1983); c) apparent overrepresentation of expression of the V_H7183 family by autoantibodies despite the small size of this family (12 members) relative to V_H J558 (60 members) and V_H QPC52 (15 members), and d) this family is rarely used by antibodies specific for foreign antigens (Brodeur and Riblet, 1984).

The goal of this study was to investigate the frequency of antibodies specific for self-epitopes among V_H7183^+ hybridomas obtained from normal mice versus autoimmune mice.

Selection of VH7183 Positive Hybridomas. Hybridomas from LPS stimulated lymphocytes which express a variable region gene from the V_H7183 family have been selected using the slot-blotting technique described above. A ^{32}P-nick translated V_H probe prepared from the most diverse member of the V_H 7183 family, V_H81X, was hybridized to nitrocellulose previously fixed with RNA from cytoplasmic lysates of these cells. Hybridomas positive for V_H7183 expression were cloned and 5-10 clones were retested using the same probe. One positive clone from each was then used for further study. By using this technique, we obtained 14/356 BALB/c, 16/101 NZB and 5/65 MRL clones positive for V_H7183 expression. The vast majority of these hybridomas are mu, kappa except for Z14 (IgG_{2a}, kappa and Z318 mu, lambda (Table 19). In order to confirm that hybridization to the V_H7183 probe was from the expressed allele, total RNA was prepared and analyzed in a Northern blot analysis using a ^{32}p nick translated probe V_H 129-48 (Fig. 24-26). (V_H 129-48 was isolated from a genomic library prepared in Charon 16A). This hybridoma secretes a mu, kappa RF. The sequence of V_H 129-48 is very homologous to a germline gene V_H 37.1, a member of the V_H7183 family (Hartman and Rudikoff, 1984). Total RNA from 2 BALB/c hybrids and one MRL hybrid which did not hybridize with the V_H7183 probe, hybridized with other V probes which indicating that they actually express a different V_H gene. For example, the MRL hyhybridoma M15 selected by slot blotting as V_H7183^+ actually hybridized with V_H441-4 probe in Northern analysis (data not shown), (These two probes cross-hybridize). 2 of the hybridomas from the NZB fusion did not secrete Ig, although they hybridized to the V_H7183 probe. Southern blot analysis performed 6 months later showed only the sp2/0 parental J_H EcoRI

Table IV. Origin and isotypes of monoclonal antibodies produced by hybridomas selected with V_H 7183 probe

Origin	Fusion	Designation and isotypes
3 mo old BALB/c	1	B57(μk), B6(μk), B93(μk)
	2	B56(μk), B34(μk), B36(μk), B68(μk) B61(μk), B76(μk)
	3	B28(μk), B64(μk), B48(μk), B38(μk) B53(μk)
3 mo old NZB	1	Z121(μk), Z250(μk), Z51(μk), Z318(μλ) Z317(μk), Z39(μk), Z26(μk), Z59(μk) Z218(μk), Z49(μk), Z14(γ2bk), Z232(μk) Z41(μk), Z113(μk). Z244 and Z132 nonsecretory hybridomas
3 mo old MRL/lpr	1	M 16(μk), M13(μk)
	2	M56(μk), M93(μk), M88(μk)

Fig. 24. Northern analysis of BALB/c hybridomas. 5 ug total RNA was electrophoresed through a 1.2% agarose gel (6% formaldehyde) blotted and hybridized to ^{32}P nick translated V_H 129-48 probe.

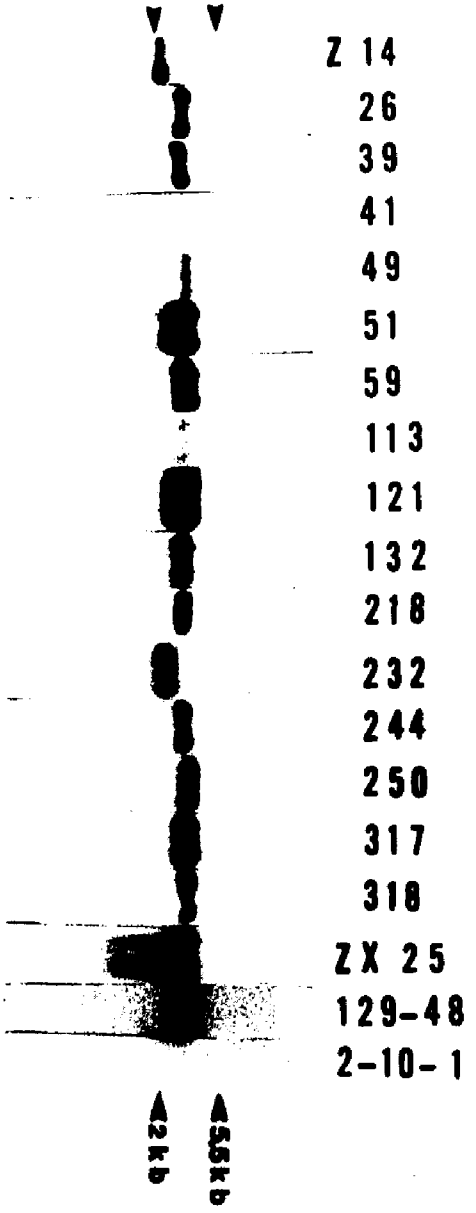

Fig. 25. Northern analysis of NZB hybridomas.

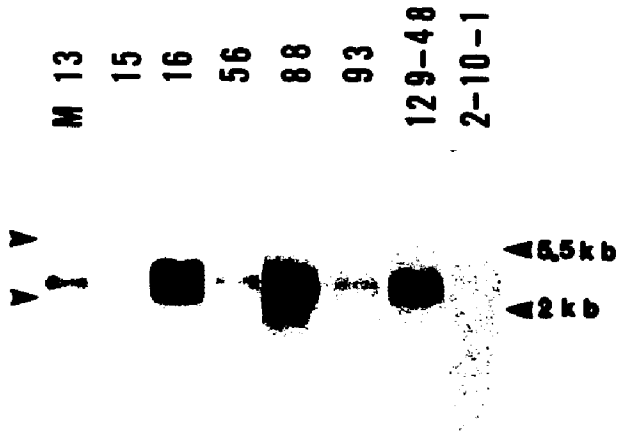

Fig. 26. Northern analysis of MRL hybridomas.

4.9kb band was present indicating that there may have been a loss of chromosomes in culture during this period (data not shown). These results show that slot blotting can only be used as a screening method and confirmation of V_H expression by Northern analysis is necessary. The results also show that the frequency of V_H7183 usage was significantly higher among hybridomas produced by NZB splenocytes than in BALB/c, whereas no significant difference was observed for MRL/lpr versus BALB/c (Table 20).

Since it is known that V_H genes used by BALB/c antibodies specific for Influenza A strain PR8 hemagglutinin also derive from this family (Hartman and Rudikoff, 1984), a fusion was carried out from NZB mice immunized with this virus. Among 15 antigen positive hybridomas, 2 antibodies utilize a V_H gene from the 7183 family as compared to 9/51 of our collection of BALB/c hybridomas. Although the sample of hybridomas from NZB was quite small, there was no significant difference between the two groups.

Antigen Binding Properties of MAbs. In previous studies, it was observed that the V_H7183 family is frequently utilized by autoantibodies of various specificities. Therefore, it was important to determine the binding properties of antibodies which had been selected by a V_H7183 probe. This was done using multiple techniques: RIA, ELISA, and IF. A large panel of available antigens was tested (see Materials and Methods). Our panel of antibodies can be divided into five major groups.

Antigen-Inhibitable Antibodies (Table 21).

Rheumatoid Factors. 4 antibodies obtained from BALB/c (B56, B57, B61, B68) and 1 from MRL/lpr (M88) exhibited RF activity. BALB/c antibodies exhibited specificity for the Fc fragment of 3 IgG subclasses

Table 20. Frequency of the expression of V_H7183 gene family in hybridomas obtained from in vitro LPS stimulated lymphocytes and PR8 influenza virus immunized mice

	Hybridomas from LPS stimulated lymphocytes				Hybridomas from PR8 influenza virus immunized mice			
Mouse Strain	Number tested	V_H7183^+	%	chi square test	Number tested	V_H7183^+	%	chi square test
NZB	101	16	15.8	p<0.001	15	2	13.3	NS
BALB/c	356	14	3.9	NS	51	9	19.6	
MRL/lpr	65	5	7.3		ND			

NS = not significant

Table 21. Antigen inhibitable antibodies secreted by V_H 7183$^+$ hybridomas

A. Rheumatoid factors

Antibody (10µg)	BSA	IgG_3	% Inhib.	IgG_1	% Inhib.	IgG_{2a}	% Inhib.	IgG_{2b}	% Inhib.
B56	971±139$^+$	14,419±1,667	78	8,119±1,016	21	5,473±211	43	622±107	0
B57	1,577±26	8,301±854	34	3,753±1,692	81	4,733±259	32	1,964±814	0
B61	533±30	3,610±140	69	9,422±2,087	70	3,204±562	46	1,048±278	0
B68	336±16	5,704±49	49	2,463±475	72	2,063±105	30	515±141	0
M88	160±3	1,020±60	0	2,818±122	65	491±19	0	283±52	0

B. Thyroglobulin

Antibody (10µg)	BSA	TG	% Inhib.
8.4D1	375±12	1,362±432	49
Z51	550±61	2,754±587	24

C. Sm

Antibody (10µg)	BSA	Sm	% Inhib.
Y2	576±44	10,097±982	60
B36	601±22	7,844±917	18
Z26	606±146	10,107±1,100	49
Z318	216±27	2,183±304	51
M13	553±35	2,534±387	19
M16	694±117	8,897±521	58
M56	703±165	8,887±702	29
M93	212±150	2,176±304	44

D. Cardiolipin and DNA

Antibody (10µg)	BSA	Cardiolipin	% Inhib.	ds DNA Exp.1	ds DNA Exp.2
H 241	95±7	2,643±508	45	2,377±72	4,673±24
Z 121	186±24	1,743±36	65	137±11	324±15
Z 317	71±12	823±61	28	368±51	944±22
Z 41	85±24	714±14	40	289±26	506±44
Z 49	88±12	1,142±2	36	313±22	520±2
B6(negative control)	113±16	127±3	0	53±5	102±10

*cpm = average ± S.D. **Inhibition obtained with 50µg of antigen. Antibodies were preincubated for 2 hrs at 37° with antigen before to be added to microtiter plates coated with antigen. 8.4D1 is an anti-TG monoclonal antibody, Y-2 is specific for Sm and H-241 for DNA(positive controls)

E. Glomerular basal membrane (immunofluorescence). B53;

(IgG_3, IgG_1 and IgG_{2a}), whereas the antibody from MRL/lpr bound to the Fc of IgG_1. This binding activity was inhibited by heat-aggregated Ig.

Thyroglobulin. One antibody from NZB (Z51) bound to thyroglobulin and the inhibition of this binding by antigen was weaker compared to a monoclonal antibody (84D1) obtained from CBA/J mice immunized with thyroglobulin.

Sm. One monoclonal from BALB/c (B36), 2 from NZB (Z26,Z318), and 4/5 (M13, M16, M56, M93) from MRL bound to Sm. However, only 4 of them (Z26, Z318, M16, M93) exhibited a level of inhibition similar to that obtained with a monoclonal antibody specific for Sm (Y2).

DNA. 4 antibodies from NZB (Z49, Z317, Z41) showed various degrees of binding to ds DNA and cardiolipin but only 1, Z121 bound as strongly as H241, an anti-DNA antibody obtained from MRL/lpr mice (Table 21).

Glomerular Basement Membrane. Finally, 1 monoclonal from BALB/c (B53) bound uniquely to the glomerular basement membrane of human kidney (Fig. 27).

Antibodies Exhibiting Multiple Binding Properties for Self-Antigens (Table 22).

5 MAbs. 3 from BALB/c (B28, B34, B93) and 2 from NZB (Z113, ZX25) exhibit various binding specificities for self-antigens. For example, B28 bound to myelin basic protein, Sm, IgG and collagen type 2, whereas Z113 bound to thyroglobulin, Sm, cardiolipin, smooth muscle and glomerular basement membrane. Immunofluorescence of antibody B76 binding to smooth muscle is shown (Fig. 28). However, we were unable to clearly inhibit the binding of these antibodies to the various antigens.

"Sticky" Antibodies. In this panel of monoclonal antibodies, 2 ex-

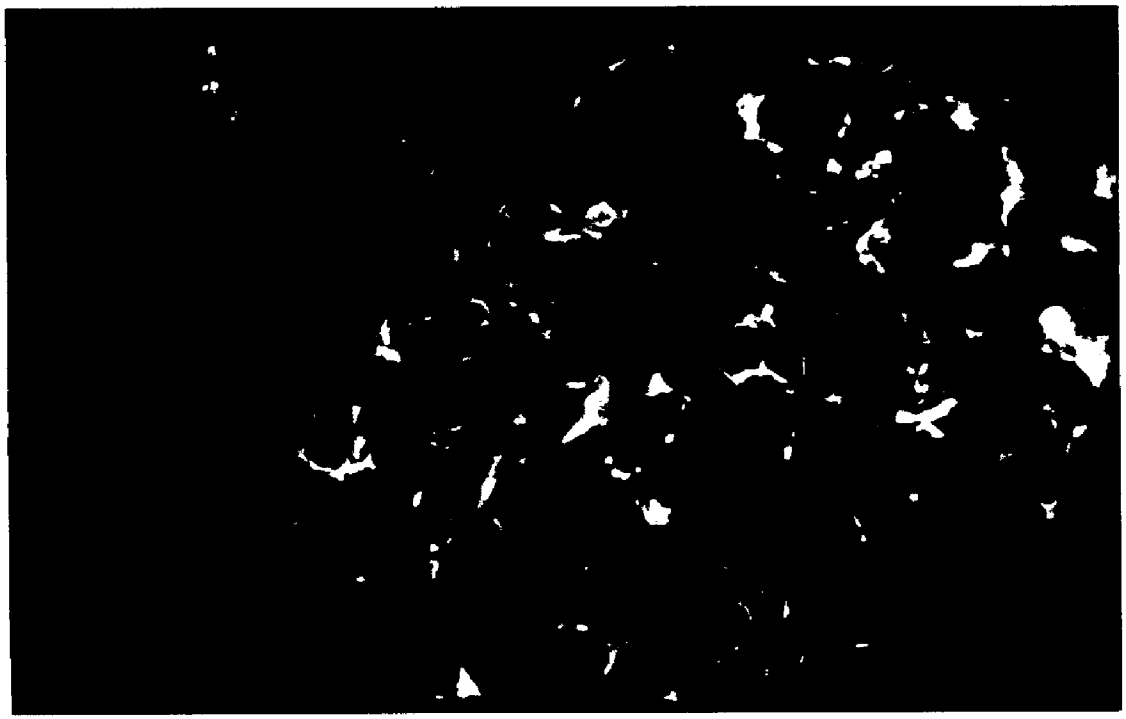

Fig. 27. Staining of kidney section with B53 monoclonal antibody.

Table 22. Antibodies secreted by Vμ 7183 hybridomas exhibiting multiple binding properties for self antigens

Method	Antigens	Antibodies (10μg)				
		B28	B34	B93	Z113	ZX25
RIA	BSA	476±26[a]	170±26	365±39	681±43	213±19
	Myelin Basic Protein	3,090±454 (17%)	557±36 (0%)	522±63 (0%)	822±42 (0%)	453±15 (0%)
	Thyroglobulin	763±131 (0%)	441±3 (0%)	1,276±56 (0%)	5,242±717 (15%)	674±58 (0%)
	Sm	4,982±517 (0%)	684±150 (0%)	978±284 (0%)	1,014±322 (0%)	5,295±93 (0%)
	Cardiolipin	866±202 (0%)	383±49 (0%)	1,267±11 (0%)	3,528±185 (0%)	647±17 (0%)
	IgG_{2a}	2,468±149 (0%)	298±24 (ND)	488±33 (ND)	2,028±373 (0%)	428±142 (ND)
ELISA	BSA	0.025[**]	0.025	0.025	0.025	0.025
	Type II Collagen	1.800 (ND)	0.327 (ND)	0.240 (ND)	0.109 (ND)	0.102 (ND)
	Mitochondria	+	-	-	-	-
	Smooth Muscle	-	-	-	+	-
	Nucleus	-	-	-	-	+
	Glomerular Basement membrane	-	+	-	+	+

[a] cpm=average±SD ( ) % of inhibition with antigen (see legend, Table 3)
[**] OD at 405 nm
ND - not done

In this experiment, F_2, a monoclonal specific for collagen type 2 gave an O.D. of 1.9.

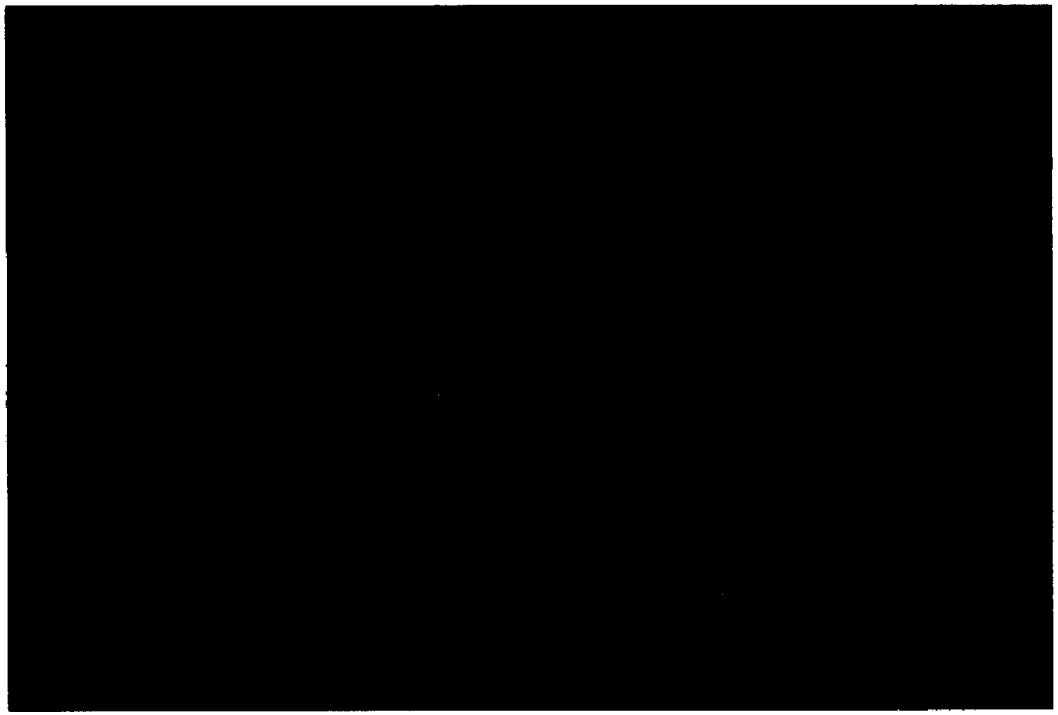

Fig. 28. Staining of smooth muscle section with B76 monoclonal antibody.

hibited an unusually high binding activity to BSA and all autoantigens tested (B76 and Z121). Obviously, the binding activity of these antibodies was not inhibited by antigen (data not shown).

Antibodies Lacking Anti-Self-Activity. Antibodies which did not exhibit binding activity for self-antigens were tested for their ability to bind to galactan and PR8 influenza virus. PR8 was chosen since antibodies specific for PR8 influenza virus are encoded by germline genes from the V_H7183 family (Hartman and Rudikoff, 1984; Clarke et al., 1985). Galactan is encoded by germline genes from the V_H441-4 family. It should be mentioned that only one antibody (B38) bound galactan and two antibodies bound to PR8 (B64, Z218) (Table 23).

Anti-Thymocyte and Anti-Red Cell Activity. No antibody exhibited anti-thymocyte or anti-red cell activity.

The summary of the study on binding activity is presented in Table 24. All MRL/lpr V_H7183 selected hybridomas (5/5) are autoantibodies, and 8/16 NZB and 9/14 BALB/c antibodies are specific for self-antigens.

Cross-Reactive Idiotypes. It was previously demonstrated that autoantibodies with various specificities share an IdX expressed on RF and anti-Sm antibodies. This IdX system was used to investigate the expression of this IdX by the monoclonal antibodies selected for V_H7183 expression. As a control in the study, the Id system used was one which can detect IdX expressed on PR8 influenza virus anti-hemagglutinin antibodies (Py102,VM202) which are also encoded by V_H genes derived from this family (Moran et al., 1986). The presence of IdX was studied by competitive inhibition RIA using various amounts of chromatographically purified anti-

Table 23. Antibodies secreted by V_H7183^+ hybridomas devoid of binding specificity for self-antigens and exhibiting binding activity for foreign antigens

Antibody (10μg/ml)	Microtiter plates coated with		
	BSA	Galactan - BSA	PR8 Influenza Virus
XRPC24	255±15*	6,772±330	ND
FY207	451±23	ND	19,965±2,509
B6	207±1	416±3	414±27
B38	238±7	5,712±588	424±104
B64	259±12	356±21	2,132±6
Z14	290±10	315±38	344±2
Z41	114±10	91±6	140±3
Z59	120±25	160±9	209±54
Z218	830±16	797±3	1,341±51
Z232	102±3	96±26	114±1
Z250	204±12	278±11	417±73

*cpm = average ± SD

Microtiter plates were coated for 3 hrs at 37°C with 5μg galactan-BSA or PR8 influenza virus, washed three times, and then incubated with monoclonal antibodies. The binding of monoclonal antibodies was measured with ^{125}I-rat anti-murine kappa monoclonal antibody.

XRPC24 is a galactan binding myeloma protein and FY207, a PR8 influenza virus hemagglutinin specific antibody.

Table 24. Summary results on binding properties of monoclonal antibodies produced by hybridomas selected with V_H7183 probe

Origin of Antibodies	Nbr of V_H7183^+	Nbr of V_H7183^+ Secreting Ig	Autoantibodies		"Sticky" Antibodies	Antibodies specific for foreign antigens	Antibodies with unknown specificity
			Antigen Inhibitable	Antibodies with Multiple Specificities			
BALB/c	14	14	6	3	1	2	2
NZB	16	14	6	2	1	0	5
MRL/lpr	5	5	5	0	0	0	0

bodies (5 to 500 ng/well). The binding of labelled Y19-10 (a RF) to anti-LPS10-1 Id (anti-RF-Id) antibodies was inhibited in a dose-dependent manner by a vast majority of the MAbs except for B28 and Z113 (Fig. 29).

A similar study was carried out employing other IdX systems, i.e., ^{125}I-LPS10-1 anti-129-48-Id (an anti-RF-Id), ^{125}I-Y19-10-anti-129-48-Id, and ^{125}I-VM202, anti-Py102-Id (data not shown).

The results of this study summarized in Table 25 show that 9/35 monoclonal antibodies share 129-48Id, and 27/35 share the LPS10-1 Id.

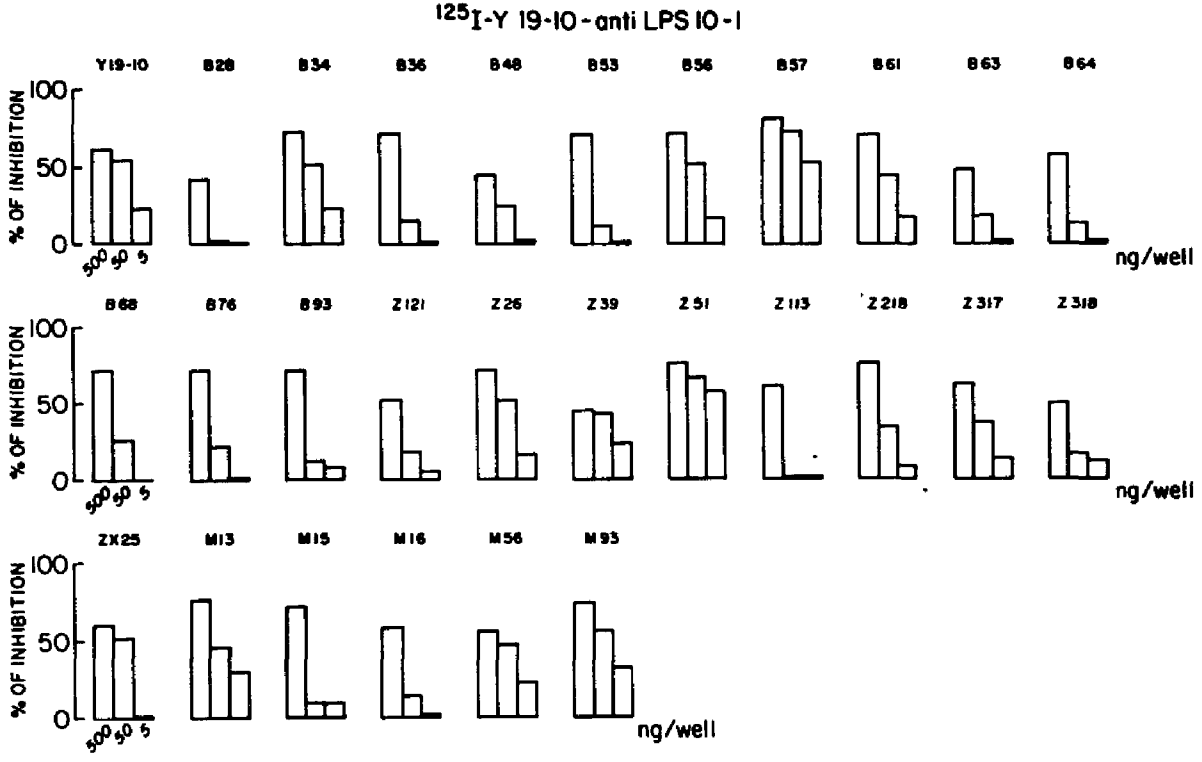

Fig. 29. Competitive inhibition of the binding of ^{125}I Y19-10 to anti-LPS 10-1 idiotypic antibodies by various amounts (5-500 ng) monoclonal antibodies.

Table 25. FRACTION OF ANTIBODIES PRODUCED BY HYBRIDOMAS SELECTED WITH V_H7183 PROBE EXPRESSING CROSS-REACTIVE IDIOTYPES

Idiotype systems	BALB/c	NZB	MRL/lpr	BALB/c antibodies specific for influenza virus (V_H7183$^+$)	
				PR8	X31
LPS10-1 anti-129-48	B34(49.3%)* B36(51.3%) B53(50.7%) B57(50.2%) B61(58.5%) B64(47%) B76(52%) 7/16**	Z218(51.8) 1/14	M88(43.8%) 1/6	0/12	0/4
Y19-10 anti-LPS10-1	B93(70.9%) B66(71%) B68(73%) B36(71%) B57(81.8%) B76(76%) B56(74%) B34(75%) B28(66%) B38(76.6%) B48(78.5%) B53(81%) B63(57%) B64(75%) 14/16	Z218(66%) Z113(61%) Z318(50%) Z317(62%) Z39(47%) Z51(75%) Z121(53%) Z26(71%) 8/14	M15(71%) M56(57%) M16(59%) M93(61%) M13(77%) 5/6	VM114(48%) 1/12	0/4
Y19-10 anti-129-48	B28(62%) B38(62.1%) B48(64.6%) B53(67.9%) B63(71.3%) B64(72%) 6/16	0/14	0/6	VM114(51%) 1/12	0/4
VM202 anti-PY102	0/16	0/14	0/6	VM202 PY102 VM113 PT109 4/12	XY101 1/4

*( ) = percentage of inhibition at concentration of 500 ng of chromatography purified antibody.

**fraction of antibodies expressing cross-reactive idiotypes.

DISCUSSION

Although Paul Ehrlich's early concept of 'horror autotoxicus' was accepted by many immunologists during the first half of this century, Paul Ehrlich was still able to recognize and appreciate the possibility that 'failure of internal regulation as well as the action of directly injurious exogenous or endogenous substances' could be involved in the etiopathogenesis of the autoimmune state.

The results of a comparative study of the age-dependent variation of clones specific for polysaccharide TI antigens in 129/Sv and MRL/lpr mice, which spontaneously develop anti-IgG antibodies, and in 129/J and MRL+/+ congenic strains which do not produce anti-IgG antibodies show that there is the concommitant induction of RF in both groups of animals subsequent to this immunization.

The analysis of the specificity of RF in multiple systems showed that they bind to various classes of Ig. The specificity of RF produced in 129/Sv mice is IgG_{2a}, IgG_{2b}, and IgG_1, RF produced in MRL mice exhibit a broader specificity in that they strongly bind to IgG_{2a} and IgG_{2b}, but also bind (although less well) to IgG_1 and IgG_3.

The study of the PFC responses to bacterial antigens, BL and alpha 1,3 Dextran, in 129/J mice revealed an age-dependent increase in the response that is probably related to the increase in the frequency of antibody-responding cells by exposure to environmental antigens. In 129/Sv mice, however, we observed that the magnitude of the PFC responses to these antigens in 1-mo-old mice was several fold higher than that of their age-matched controls. These results are consistent with our other

studies that indicate the in vitro PFC responses to TNP-TI or TD antigens are higher in the 1-mo-old 129/Sv mice relative to their age-matched controls (TNP-BA, TNP-KLH responses). This unexpected observation suggests that 129/Sv mice exhibit either an accelerated maturation of their immune apparatus or perhaps an increased intestinal permeability to environmental antigens. The last hypothesis is supported by the observation of Van Snick et al. (1979) who showed that RF in 129/Sv can be of the IgA type, and in fact, it is well known that the majority of the precursors of IgA are located in the gut-associated lymphoid tissue system.

This accelerated maturation of 129/Sv mice is also reflected in IEF data, which showed an increased IgG component of BL and Dex-specific antibody in these mice vs. the age-matched 129/J mice. The analysis of clonotypes by IEF showed that Dex and BL clonotype patterns are similar to those reported for BALB/c mice sharing the same IghC genes. However, a significant decrease of the E109Id$^+$ component of the anti-BL response and of the J558$^+$ Id component of the anti-Dex response was observed in old MRL/lpr and 129/Sv mice, respectively. These results suggest that in animals prone to develop RF, there is an alteration of dominant clones. (The effects of RF monoclonal antibodies on idiotype expression (in vitro) subsequent to immunization with TI antigens will be discussed later.)

Furthermore, we observed that both MRL+/+ and MRL/lpr mice develop a low BL response and are unresponsive to immunization with Dex in saline or CFA. Because the Dex response is genetically well defined, we investigated whether the unresponsiveness of MRL mice was related to the H-2 gene complex or to the effects of the lpr gene. At the time of these experiments, serologic analysis showed that MRL mice shared the same haplotype

as C58/J and BALB.K mice ($H\text{-}2^k$ and $IghC^a$) and thus should be capable of responding to Dextran. We investigated whether the unresponsiveness was related to H-2, IghC or lpr gene complexes by testing several strains of common haplotypes and also a strain congenic for the lpr gene. Responses were observed for the other strains (Table 5), yet the MRL mice remained unresponsive. Not until this past year was it established that MRL mice are actually of the Igh^j haplotype (Trepicchio and Barrett, 1985; Kofler et al., 1985b). Thus, their inability to respond is in agreement with results obtained for other strains which are not $IghV^a$ and $IghC^a$ of BALB/c (CBA/J^j, AKR^n) (Blomberg et al., 1972).

The very poor response to BL in these mice suggests a defect in the Lyb-5 subset, which is required for the response to TI-2 antigens (Mosier et al., 1983). Indeed, Mond et al. (1983) found that CBA/N mice and $C3H^{xid}$ mice, a line that exhibits a synergistic defect responsible for the complete unresponsiveness to TI-1, TI-2, and B cell mitogens also develop low or no IgG_3 and IgG_1 antibodies in response to the immunization with TNP-KLH.

These results suggest that MRL mice exhibit a genetic defect in the Lyb-5 subset despite the fact that the defect is not as profound as it is in CBA/N or $C3H^{xid}$ mice. Actually, the MRL defect for TI-2 antigens more closely resembles the defect observed in RIIIS/J mice. The RIII mice develop a good response to TNP-Ficoll (J. Mond, personal communication) but not to Dex, SIII, E. coli 0113 LPS antigens (Hiernaux et al., 1982), or BL (J. Hiernaux, personal communication).

The most striking observation in this study was the occurrence of RF subsequent to immunization with polysaccharide TI-2 antigens in the older

animals. In the 129/J and MRL+/+ mice, RF were first detected after immunization whereas in the older 129/Sv and MRL/lpr mice, an additional increase in the preimmunization RF titer was observed. Interestingly, the specificity of RF varied with the antigen. Whereas RF were specific for IgG_{2a} after BL immunization, they were specific for IgG_1 and IgG_{2a} after immunization with Dex. There is no clear explanation for the induction of Fc isotype-specific RF after immunization with these antigens, and at this time it is not known whether this is related to the nature of immunogen or to the class of antibody produced after immunization.

Several explanations can be entertained regarding the mechanisms for the occurrence of these RF. a) It is known that substances endowed with B cell mitogenic properties can stimulate the RF-producing cells (Dresser, 1978; Bona et al.,1979a; Izui et al., 1979). One may imagine that these RF produced after immunization with BL and Dex occurred because these substances are B cell mitogens (Andersson et al., 1972). This explanation is not sufficient, however, in that recent data from Nemazee and Sato (1983) demonstrated the induction of RF during the secondary immune response elicited by TD antigens. b) The stimulation of RF-producing cells can occur by antigen-antibody complexes in a TI manner as was recently proposed by Nemazee and Sato (1982). c) The stimulation of RF-producing cells may occur by anti-Id antibodies. Recently, Bona et al. (1982) delineated a particular category of anti-idiotype antibodies designated "epibodies" which exhibit a high affinity for idiotypes and secondarily bind with a lower affinity to human Fc gamma fragments (Bona et al., 1984).

Although the mechanism(s) responsible for the activation of RF-pro-

ducing cells is not understood and needs further investigation, it clearly appears that RF can occur during various immune responses. In humans, RF were detected in the circulation of patients during various infections (syphilis, tuberculosis, subacute bacterial endocarditis, mononucleosis) and even subsequent to vaccination (Welch et al., 1983; Carson et al., 1981). Thus, our comparative studies in the autoanti-IgG-producing 129/Sv and MRL/lpr mice and their normal congenic counterparts, the 129/J and MRL+/+ mice, suggest that two categories may exist: one associated with autoimmune diseases, leading to the deposition of immune complexes with its deleterious consequences, and a second category of RF beneficial for the host. This second category designated as "enhancing antibodies" (Nemazee and Sato, 1982) could have beneficial properties for the host by accelerating the process of clearing antigen-antibody complexes or even by manifesting protective roles in parasitic diseases (Carson et al., 1981; Clarkson and Mellow, 1981). Such enhancing antibodies can be beneficial and aid defense reactions in the clearance of infectious agents, particularly when a decrement of T cell functions occurs during aging (Tyan, 1977).

In the age-dependent study of the immune response in 129 mice to a TI-1 antigen, TNP-BA, it was clear that the 1-mo-old 129/Sv animals displayed a 3-4 fold increase in the PFC response (Table 6). This accelerated response was similar to what was observed for TI-2 antigens. Although this response exceeded that of the age-matched control group, there was no decrease in the frequency of anti-TNP precursors in the 129/Sv mice as a consequence of aging. This is in agreement with an observation made by Hooijkaas et al. (1983). There was a significant increase in the titer

of RF against IgG_{2a} in 6 mo. 129/Sv mice and 129/J mice indicating that RF can be induced in vivo with TI-1 antigens.

Immunization with another TNP conjugate, TNP-Ficoll (a prototypic TI-2 antigen) also resulted in the production of RF in all age groups of both 129/J and 129/Sv mice.

Since various TI responses had already been studied in these strains, it was important to examine the response elicited by a TD antigen (TNP-KLH) in vivo.

Several points can be made from these studies: In 129 mice: (1) RF can also be produced in these strains in response to TD antigens. (2) Although results from prior studies showed that immunization with a particular TI antigen induced the production of RF against a specific isotype in both 129/J and 129/Sv mice, immunization with TNP-KLH led to the production of multispecific RF. (3) The kinetics of the secondary response for TNP-KLH was basically similar for all isotypes except IgG_{2a}, in which case, 3- and 6-month-old 129/Sv mice showed markedly depressed concentrations of IgG_{2a} anti-TNP specific antibody. (4) The study of the direct and indirect secondary and tertiary PFC responses indicated that although 129/J mice show an age-dependent increase in the response, older 129/Sv mice made significantly lower IgM and IgG responses. This may indicate that the depletion of serum IgG_{2a} anti-TNP antibodies may not be a consequence of the clearance of this isotype from the sera but rather may be related to an innate lymphocyte dysfunction and/or the interaction of RF with the receptor of antibody forming cells. The in vitro studies utilizing T and B cells from young or old 129/J or 129/Sv in coculturation experiments confirmed the in vivo assays.

Analysis and comparison of the kinetics between the antibody-specific response and the RF response revealed that the production of RF in 129 mice was long lasting and occurred in parallel to peak reactive antigen specific antibody titers except in the case of IgG_{2a} anti-TNP antibodies. In this case, 129/Sv mice were incapable of making an equivalent antigen specific antibody response compared to their age-matched controls. This is somewhat in opposition to the short-lived effect of RF produced by 129/Sv or C57Bl/6 mice reported by Coulie and Van Snick (1983b). However, this variation may be due in fact to the nature of the initial immunogen, the route of immunization or even to the dose of the antigen.

Study of the fine specificity revealed that no single IgG subclass was the target of RF produced by either 129/J or 129/Sv mice. This is in agreement with monoclonal RF that we have prepared from 6-month-old 129/Sv animals which bind to all IgG subclasses. High titers of RF against all isotypes could be demonstrated for all age groups of each subline. This differs from results reported by others in which production of RF anti-IgG_1 was elicited by several different T-dependent antigens (Coulie and Van Snick, 1983; Nemazee and Sato, 1983). Here, however, the predominant antigen-specific isotype produced by 3- and 6-month-old 129/J and Sv mice was IgG_3 and not IgG_1-anti-TNP antibody. Still, it seems feasible that certain isotypes may be more immunogenic than others in stimulating the production of RF (Nemazee and Sato, 1982). This might occur by the stabilization of antigen-antibody complexes on surfaces of cells which might in turn act in a T-independent fashion in stimulating the precursors of RF-producing clones.

It occurred to us that the depletion of the IgG_{2a} TNP-specific anti-

body could have resulted by clearance of this isotype by RF. This clearance mechanism in which RF are considered to be enhancers of the immune response was proposed in an attempt to explain the presence of RF in response to conventional immunogens, vaccines, or bacterial and viral infections (Clarkson and Mellow, 1981). That is, enhancing RF may be produced in order to amplify an earlier response or aid in the recovery from a disease (Welch et al., 1983) by contributing to the clearance of antigen-antibody complexes through cytolysis or even phagocytic mechanisms (Van Snick and Coulie, 1983a). However, the fact that we could demonstrate significantly low secondary and tertiary IgG responses in the 129/Sv strain indicated that the low level of IgG_{2a} anti-TNP antibodies was not merely due to clearance but rather may have been a consequence of the interaction of spontaneously produced RF with antigen-specific antibody precursor cells or perhaps a result of an inherited lymphocytic defect.

In order to more clearly examine the components of the specific antibody response, we studied the age-dependent in vitro synthesis in a hapten-carrier system in the 129/J and 129/Sv mice. As was observed for several TI responses, here too, in a T-dependent response system, 1-month-old 129/Sv mice exhibited markedly accelerated direct and indirect responses to TNP. This was especially apparent when both T and B cells were derived from 129/Sv animals. Evidently, 129/J T cells enabled antibody levels to remain within the range of their normal counterparts. However, 129/Sv B cells were not sufficient to produce an adequate IgG response even when help was provided by 129/J T cells. In older mice, a similar accelerated response was obtained. That is 129/Sv T cells pro-

vided more help to 129/J B cells for the IgG response, but could not provide sufficient help to autologous B cells. In addition, 129/Sv B cells produced an enhanced IgM response. Several explanations can be entertained here. It may be that B cells from 129/Sv mice, like that of NZB and BXSB mice, have a heightened excitability to triggering stimuli such as antigens and mitogens, but the T cells from 129/Sv are unable to provide sufficient help for an IgG response. Furthermore, RF production in 6-month-old mice may affect the ability of B and T cells to cooperate which is necessary for IgG antibody synthesis to occur.

The data reported for MRL mice show a low IgG_3 anti-TNP response at various ages (except 3-month-old) and the absence of IgG_1, IgG_{2b} and IgG_{2a} components of the response in 6-month-old MRL/lpr mice while the IgM response does not differ from that of MRL+/+ and 129/J mice.

The lack of a IgG_3 response may be related to a Lyb5 B cell subset defect. It is well-documented that CBA/N or C3H/HeJ^{xid} mice develop a low IgG_3 response during the secondary response elicited by various TD antigens and they lack this B cell population entirely (Scher et al., 1976; Mond et al., 1983). The most striking difference between MRL/lpr and MRL+/+ and 129/J was the absence of IgG_1, IgG_{2b} and IgG_{2a} responses in 6-month-old MRL/lpr mice. This was also confirmed by the study of the indirect anti-TNP PFC response. The study of function of T and B in a hapten-carrier type (TNP-KLH) in vitro response indicated that while T and B cells from MRL+/+ produce a relatively good anti-TNP response, the T and B cells from MRL/lpr produce a significantly lower response. In addition, the mixture of B cells from MRL/lpr mice with T cells from MRL+/+ mice did not restore the response to the MRL+/+ level. Taken together, these

results suggest that a cellular defect of both T and B cell compartments is more prominent in aged MRL/lpr mice and is responsible for the lack of IgG anti-TNP antibodies. This interpretation is supported by data which demonstrate a hypo-reactivity of B cells from 20-25 day old MRL/lpr mice to various stimuli such as anti-u, lipopolysaccharide, concanavalin-derived spleen supernatants, B cell growth factor and their inability to develop an indirect SRBC PFC response upon in vitro culture with antigen, lipopolysaccharide or various factors (Prud'homme et al., 1983; Scott et al., 1984). The polyclonal lymphoproliferation of T cells prominent in older mice probably contributes to this defect by not allowing antigen (KLH)-induced expansion of the precursors which have already proliferated. It may also be that once activation of B cells occurs in these mice, clonal expansion is inhibited by virtue of the low production of IL2 in this strain (Theofilopoulos et al., 1985).

The study of the fine specificity of RF produced during the secondary response shows that RF were produced against all of the IgG isotypes. One may imagine two mechanisms by which antigenic stimulation could expand the precursors of RF. a) via immune complexes: antigenic stimulation induces the synthesis of specific antibodies that bind to circulating antigens and ultimately leads to the formation of immune complexes. These immune complexes can bind to the receptor of RF precursors (since the Fc fragment is altered) and therefore trigger their proliferation in a T-independent manner. This mechanism was favored by Nemazee and Sato (1983) to explain the rapid synthesis of RF during the secondary response and by Welch et al. (1983) to explain the rapid expansion of IgM-RF producing cells in individuals vaccinated with tetanus toxoid. b) Via direct antigen stimula-

tion. One of the most basic axioms in immunology is that antigen specificity of the receptor of immuno-competent cells governs the response. The variable region of these receptors has a three-dimensional structure which allows it to interact with the antigen. However, this does not imply that a receptor can only interact with one antigen. Indeed, it can bind to an antigen which occupies the entire cleft with high affinity and it can also bind to another antigen which interacts with only a small area of the combine site that has a different affinity. A classic example of such a multispecific antibody was given by Czaja et al. (1976). They studied the binding of MOPC 460, a myeloma protein to menadione and the DNP hapten. Similar findings were made by Davies et al. (1975) on using the Fab' fragments of the 'New' protein which bind a hydroxy derivative of vitamin K and phosphocholine. Therefore, the Ig receptor of RF precursors can be likened to a multispecific antibody which binds with low affinity to the Fc fragment of IgG and with high affinity to other antigens which can subsequently trigger their expansion. This possibility is strongly supported by a recent observation in which Bona et al. (1984) showed that rabbit anti-Id antibodies specific for anti-a or b series allotype antibodies can also bind with a low affinity to human Fc and therefore they displayed a RF-like activity. Thus, it appears that some protrusions of idiotypes or anti-idiotype antibodies represent "infidele" copies of the three-dimensional structure of Fc domains and can stimulate the expansion of RF precursors subsequent to binding to the receptor of these precursors. At this point in time, we do not know the precise mechanism of the activation of the RF precursors during physiological immune responses. However, the data discussed above suggest several potential candidates such as B

cell mitogens, immune complexes, anti-idiotypes or even the foreign antigens themselves.

Since RF production can be induced in a physiologic response, one may ask if in general, RF exhibit a regulatory role on lymphocytic functions or contribute to the maintenance of physiological cellular homeostasis. Because the penetration of foreign molecules into a body causes a dramatic alteration of homeostasis, multicellular organisms have developed various mechanisms to maintain this physiologic balance. The immune apparatus represents one of the most highly evolved systems by which vertebrates are able to recognize foreign molecules, eliminate them from the bloodstream, inactivate their foreign character by secreting specific products and finally destroy them.

It has been suggested that RF may aid in the clearance of antigen-antibody complexes from the blood and even promote the endocytosis of such complexes by neutrophils and macrophages (Coulie and Van Snick, 1983a). This function may be compared to the "scavenger" function of phagocytes which eliminate and catabolize antigen-antibody complexes and altered autologous cells that result from aging, chemical injuries, etc. However, one must keep in mind the fact that RF are antibodies specific for antigenic determinants of immunoglobulin molecules. Indeed, there is evidence which indicates that antibodies specific for antigenic determinants of immunoglobulin molecules play an important regulatory role on immunocompetent cells. Thus, antibodies specific for isotypic determinants such as anti-mu or anti-delta antibodies can under certain conditions, stimulate B cells to proliferate (Finkelman et al., 1979). Conversely, anti-mu or anti-k antibodies administered in vivo can induce

suppression of Ig synthesis or even promote the expansion of the lambda repertoire as a compensatory effect for the suppression of the k repertoire (Weiss et al., 1984). Similarly, anti-allotype antibodies administered at birth or transferred via placenta from the mother functionally delete the expansion of the corresponding allotype in heterozygous progeny (Eskinazi et al., 1979). Furthermore, anti-Id antibodies administered at birth or during adult life, or transferred via placenta or colostrum from the mother can either suppress or expand the clones bearing the corresponding idiotype on their receptor (Bona et al., 1979b). Therefore, one may ask whether or not RF produced during the course of infection or during an immune response elicited by various antigens exhibit regulatory properties on immunocompetent cells. The study of the effect of a MRF which binds IgD (LPS10-1, data not shown) on the in vitro anti-TNP response elicited by a TI-1 antigen which stimulates mature lymphocytes expressing both surface IgM and IgD receptors revealed that at high concentrations, the MRF (LPS10-1) inhibited the TNP PFC response in normal amimals (BALB/c and 129/J) but enhanced the response in 129/Sv mice. Thus, it appears plausible that RF which can bind IgD, can bind to IgD-IgM bearing cells and may then expand mature lymphocytes in vivo. Subsequently, one may observe an accelerated maturation of the immune response in 129/Sv mice. Hyperreactivity of B cells does occur in other autoimmune strains (NZB and BXSB) (Theofilopoulos et al., 1985) and it has been shown that parenteral administration of anti-delta antibodies stimulates B cells leading to the increased synthesis of immunoglobulins (Finkelman et al., 1979).

Since monoclonal antibodies specific for Fc of IgG were generated, it

facilitated our attempts to define more precisely the origin and molecular nature of autoantibodies by studying the idiotype and V_H genes encoding RF produced by hybridomas from several mouse strains. The study of the fine specificity of the monoclonal RF indicates a wide degree of heterogeneity among these antibodies. However, despite this apparent combining site (paratope) heterogeneity and different strain origin, these RF share interstrain cross-reactive idiotypes. The expression of IdX in our system was independent of H-2 and C_H gene complexes and was not found on a variety of antibodies with other specificities.

Molecular data showed that the shared idiotype cross-reactivity does not result from a common utilization of heavy chain variable region segments (V_H, D or J_H) as is observed in some systems (Margolies et al., 1983); thus, 129-48 and Y19-10 which share IdX, use totally different V_H, D and J_H gene segments. However, there is a shared tetrapeptide in framework 2(residues 36-39) (Fig 18) which may explain the IdX we observed. Recently, Chen et al. (1984, 1985a) using a synthetic peptide showed the structural correlate of a human RF idiotype corresponds to a few amino acid residues on the light chain. The possible contribution of the light chain to IdX expression in these RF will be elucidated via Western blotting analysis, hybrid molecules and the preparation of monoclonal anti-Id antibodies.

A restricted number of V_H gene families is utilized among murine RF (J558, QPC52, and 7183). It appears that this restriction is not unique to anti-Ig antibodies because a study of 10 murine anti-DNA monoclonal antibodies from the MRL/lpr strain has also shown that these families are preferentially utilized (K. Barrett, personal communication). Our other

results indicate that autoantibodies of various specificities preferentially use these V_H gene families as well. One possible explanation for the biased family usage among these autoantibodies is that the other V_H families may not contain V_H genes encoding autoantibodies. However, it is interesting to note that the three families used are 3' V_H gene families; preferential expression of 3' V_H genes is known to occur early in development (Yancopoulos et al., 1984). Thus, it seems possible that the preferential utilization of 3' V_H gene families in the anti-Ig and anti-DNA responses may reflect the repertoire of the B cells from which these clones are selected; for example, the clones may arise early in ontogeny. Two representative RF were genomically cloned and the V_H regions were sequenced. The data demonstrate that the genes encoding the heavy chain variable region of autoantibodies and antibodies against foreign antigens are not obviously different. A similar conclusion was reached from the analysis of an anti-DNA antibody (Kofler et al., 1985a). Comparison of the nucleotide sequence of 129-48 with the 7183 germline gene 37.1 revealed that the expressed V_H gene is closely related to this gene and other members of this family. Similarly, V_H Y19-10 is very closely related to germline genes in the J558 family. It is especially homologous to the 23, 33, and 186-2 germline genes which encode the NP response. In addition, CDR sequences are relatively conserved between Y19-10 and J558 germline genes and base pair changes, when observed, usually can be found in other germline genes from this family. Thus, little somatic mutation is apparent. This result is consistent with the finding that these RF are IgM antibodies and frequently, somatic mutation does not contribute to IgM diversity.

The study of the idiotypy and V_H genes encoding a panel of monoclonal autoantibodies produced by hybridomas from several mouse strains revealed similar findings. Some hybridomas were obtained from mice which spontaneously produce autoantibodies, including RF obtained from old 129/Sv or MRL/lpr mice, anti-DNA and anti-Sm antibodies from MRL/lpr, and anti-red blood cell antibodies from NZB or CBA mice. Others were obtained from normal immunized animals: e.g., RF from BALB/c mice injected with Yersinia enterocolitica, anti-Tg antibodies from BALB/c or CBA/J mice injected with thyroglobulin, and antibodies against type 2 collagen from DBA/1 mice.

Because the panel of monoclonal antibodies covers various antigenic specificities, one might expect that all the V_H gene families would be used at random. However, as found for RF, only a restricted number of V_H gene families are used among these autoantibodies, namely J558 and the most 3' families: QPC52 and 7183. This restriction is totally independent of combining site specificity and is not related to a spontaneous or induced origin. As proposed for RF, these V_H genes used by autoantibodies may reflect an immature repertoire of B or pre B cells or alternatively, these V_H genes may be representative of a subset of the B cell lineage such as Ly1 B cells which secrete a high percentage of IgM autoantibodies (Hayakawa et al., 1984). This lineage is highly represented in autoimmune strains such as NZB or (NZB x NZW) F1 mice (Hayakawa, 1985) and is also frequently found in patients with rheumatoid arthritis.

The various autoantibodies studied express IdX despite the fact that they are heterogeneous with respect to combining site and utilize different V_H genes. For example, the presence of IdX originally borne by

RF LPS10-1 and 129-48 and Y2 (a monoclonal anti-Sm antibody) was identified among these autoantibodies. The presence of these IdXs is independent of their specificity, MHC, and C_H gene complexes.

At first, the presence of an IdX on antibodies encoded by various V_H gene families is surprising, since it is accepted that IdX are often markers of a particular V_H family as was shown for anti-dextran, anti-NP or anti-arsonate antibodies (Green and Nisonoff, 1984). However, several exceptions to this rule have been reported. Anti-arsonate antibodies produced by BALB/c mice express IdX despite the fact they use a V_H gene which does not derive from the V_H 36-65 germline gene (Leo et al., 1985). In addition, cross-reactive idiotypes were also observed for antibodies of various specificities that were derived from different members of the same family of V_H germline genes (Victor-Kobrin et al., 1985).

These IdXs shared by autoantibodies of various specificities may be a clue to the genetic and immunoregulatory basis of autoimmunity. It is clear that the cells that produce these autoantibodies are present in normal animals where they can be induced at times into clonal expansion by hyperimmunization as in the case of RF. They may also emerge spontaneously during the course of an autoimmune disease or perhaps even during the aging process.

Since it appeared that the 7183 family was utilized by various kinds of autoantibodies and that this family is not extensively used to encode specificities for foreign antigens frequently, the most striking aspect of the study on the properties of antibodies secreted by hybridomas selected for 7183 expression was the very high percentage (60%) of V_H7183+ antibodies that was shown to bind to various self-antigens regardless of

whether they were generated from autoimmune mouse strains or a normal mouse strain. It was surprising that among the NZB V_H7183 antibodies, there were none which could bind red blood cells, in spite of the fact that this mouse strain develops an autoimmune hemolytic anemia later in life, and that we previously showed that 3 of 3 monoclonal antibodies specific for bromelain treated mouse red blood cells are encoded by V_H7183 genes. This suggests that either the frequency of these autoanti-RBC antibody forming cells is very low at 3 months or they lack the LPS receptor.

A major portion of these autoantibodies were antigen inhibitable. However, 3 antibodies obtained from BALB/c and 2 from NZB exhibited multiple binding activities which were not antigen inhibitable. This group of antibodies may be similar to that described by Dighiero et al. (1985), obtained from newborn BALB/c. The inability to inhibit the binding of these antibodies by antigen suggests that they have low avidity for the corresponding self-antigens. It should be mentioned that all antibodies with multiple binding specificities are IgM. Their multiple binding property can be related either to a common 3 dimensional structure shared by various self-antigens or to a protein-protein interaction of these antibodies with the antigens rather than a paratope-mediated recognition.

Two IgM antibodies (B76 and Z121) exhibited a strong non-specific binding for BSA in addition to binding to self antigens. Such antibodies have been also reported by other groups (Serban et al., 1985). The origin of this "stickiness" is unknown and needs to be investigated further.

The precise mechanisms that could down regulate the potentially auto-

reactive B cell clones are not known but they probably involve both intracellular (i.e., genetic) and extracellular (i.e., suppression) events. One such intracellular event, based on findings in this report, might be the utilization of a restricted set of V_H genes. The presence of an immature B cell subset (e.g., Ly1 B cells) may be associated or even necessary for this to occur.

Extracellular mechanisms leading to polyclonal B cell activation and autoimmunity may involve failure of T cell suppression, interference with network regulation based on idiotype recognition, failure of natural killing (Schoenfeld et al., 1984; Talal, 1978; 1984), or some combination of these. If IdX is present on the surface of autoreactive B cells and is recognized by other cells, then its over-abundance in autoimmunity may contribute in some way to immunoregulatory failure and disease pathogenesis. It is quite likely that the response to autoantigens involves all the factors mentioned in this report. One's genetic predisposition, environmental influences, endogenous substances, regulatory failures, and aging are all presumed to be part of the basis for self-anti-self reactivity. Manipulation of the idiotype network through Id or anti-Id therapy has provided some promise in controlling disease in animals (Colvin and Olson, 1985). Nevertheless, this type of immunotherapy does not always improve the condition and is not yet understood completely. As we gain a greater understanding of the receptors on B and T cells, the genes which contribute to anti-self processes, the role of the idiotype network in regulation and homeostasis, it is anticipated that future studies will provide an improved understanding of autoimmunity and disease pathogenesis.

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