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The relationship of the V_K1 gene family of the immunoglobulin light chain to autoantibody formation in the New Zealand Black mouse

Bailey, Naila Celesta, Ph.D.

City University of New York, 1991

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THE RELATIONSHIP OF THE VK1 GENE FAMILY OF THE IMMUNOGLOBULIN LIGHT CHAIN TO AUTOANTIBODY FORMATION IN THE NEW ZEALAND BLACK MOUSE

by

Naila Celesta Bailey

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

1991

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ABSTRACT

THE RELATIONSHIP OF THE VK1 GENE FAMILY OF THE IMMUNOGLOBULIN LIGHT CHAIN TO AUTOANTIBODY FORMATION IN THE NEW ZEALAND BLACK MOUSE

by

Naila Celesta Bailey

Advisor: Constantin A. Bona, M.D., Ph.D.

Autoimmunity results from the breakdown of tolerance to self antigens. Many mechanisms have been proposed to explain the breakdown of tolerance. These include 1) activation of autoreactive B or T cells that are not deleted from the immune repertoire, 2) polyclonal activation of B cells, 3) lack of specific T-suppressor (Ts) cells, 4) augmentation of T-helper (Th) cells, 5) deregulation of idiotypic network, 6) molecular mimicry of self components to external antigens and, 7) other genetic factors.

Numerous autoimmune mouse strains have been developed in the laboratory to study the underlying defects in autoimmune disease. The New Zealand Black (NZB) mouse is an excellent model in that it is genetically prone to the development of autoimmune diseases that resemble systemic lupus erythomatosus (SLE) and autoimmune hemolytic anemia in humans. These mice, as they age, spontaneously produce a variety of autoantibodies, such as anti-DNA, anti-thymocytes, anti-histones and anti-erythrocytes antibodies.

The various autoimmune traits are controlled by a limited number of unlinked genes. Many studies have revealed that multiple immune loci are involved in the pathogenesis of autoimmune disease. We conducted studies to determine whether there is a linkage between the occurrence of lupus nephritis and autoantibodies with some genes of the immunoglobulin (Ig) gene superfamily, namely, the variable region of the Ig light chain (V_K) the heavy chain (V_H), the variable region of the T-cell receptor ($V\alpha$) and the I-A and I-E loci of the major histocompatibility complex (MHC). Our results on two recombinant inbred strains (RI) NZBx129/J and NZBxSM/J revealed that the V_K1 NZB haplotype was significantly associated with the occurrence of anti-erythrocyte autoantibodies and glomerulonephritis.

Earlier studies have shown that NZB autoantibodies are encoded by several variable light chain genes with a high prevalence of the V_K1 gene family. In addition, restriction fragment length polymorphism (RFLP) analysis of this gene family in NZB mice showed a particular polymorphism that is not seen in non-autoimmune mouse strains.

The V_K1 light chain are subdivided into five subgroups (V_K1A-V_K1E). Amino acid sequencing of myeloma proteins from NZB mice have demonstrated that they belong only to the V_K1B subgroup. No V_K1A gene was present in the serum light chain of NZB (the V_K1A subgroup contributes approximately 2% to the total light chain pool in the serum of many mouse strains) or in a screening of over 125 plasmacytomas derived from NZB. It was, therefore; established that the lack of V_K1A expression in NZB is due to either a regulatory mechanism preventing its expression in antibodies, or the absence of this particular gene from the immune repertoire of NZB.

We sequenced 10 functional V_K1 germline genes, from NZB HindIII digested DNA, belonging to V_K1A, B, C, D and a new subgroup designated F. The structure of these germline genes were very similar to the non-autoimmune strain Balb/c, with the exception of a few allelic differences. Several V_K1 germline genes were isolated from a single fragment and identical genes were identified in two different RFLP migrating bands. Therefore, the complexity of the genes encoding the variable region of Igs cannot be determined by RFLP analysis alone.

Sequencing cDNA from NZB hybridomas secreting antibodies with specificities to self or external antigens revealed that they all expressed different V_K1 subgroups isolated from NZB at random with no bias towards a particular subgroup, and with no somatic mutations.

FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, and chapters may have specific introductory statements. Material and methods and result sections are in each individual chapter. Also, each chapter has a specific discussion section, and there is a general discussion as the final chapter of the thesis. The references for all chapters are pooled in order to avoid redundancy.

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I. INTRODUCTION

The immune system is able to distinguish self from non-self. However, there are many pathways for the breakdown of the central mechanisms underlying self recognition, leading to autoimmune responses. Autoimmunity can, therefore; be considered a consequence of the recognition of self by lymphocytic clones leading to immune responses to antigens normally present in the host's tissue. This response may be humoral (e.g., circulating antibodies) or cellular (e.g., delayed hypersensitivity). There are several mechanisms by which an autoimmune response is initiated. Fundamentally, there is a disruption of the normal pathways of interaction of T and B cells with autoantigens.

A. Hypothesis on the mechanisms involved in the breakdown of tolerance

1. T-cell subset imbalance

The immunological imbalance may arise from a disturbance of the suppressor and helper activities of regulatory T cells. Either an excess of T cell helper activity or a deficiency of suppressor activity can initiate the autoimmune reaction (Talal, 1976).

B cells with the potential to produce autoantibodies are held in a dormant state by the action of suppressor cells, a lack of "help" from inducer cells, or both. In autoimmunization, an imbalance between the two kinds of T cells perturbs the immunoregulatory network, thereby activating the dormant autoreactive B cells.

It appears that many autoimmune diseases such as systemic lupus erythematosus (SLE), Graves disease, autoimmune hemolytic anemia, progressive systemic sclerosis, and juvenile rheumatoid arthritis have depressed levels of T suppressor cell function (Miller and Schwartz, 1982). A key issue is whether the abnormality is a primary event or a secondary event. Soluble antigen-antibody complexes (Ag-Ab), a common finding in autoimmune diseases, can, as a secondary effect, impair both the function of suppressor cells and the expression of their surface markers (Moretta et al., 1978). An example of primary lesion caused by Ag-Ab reaction is found in multiple sclerosis, where a single kind of antibody against the shared antigen of suppressor cells and brain cells could cause both neurologic lesions and reduced suppressor cells (Oger et al., 1982).

Diseases such as progressive systemic sclerosis, procainamide-induced lupus and SLE-like syndrome in MRL/lpr/lpr mice show an increased helper T-cell functions (Miller and Schwartz, 1982). Furthermore, methydopa, a drug which can induce autoimmune hemolytic anemia (Miller and Schwartz, 1982) activates cAMP (cyclic adenosine monophosphate), an effect that inhibits suppressor cells (Kirtland et al., 1980). In SLE, the lymphocytes respond subnormally to adenosine, an activator of cAMP (Kammer et al., 1983). The inability to generate cAMP adequately may contribute to both impaired suppressor-cell function and excessive activation of helper T cells. Autoimmunity, may, therefore, result from either stimulatory or inhibitory effects from a drug on immunoregulatory cells or pharmacologic modification on these regulatory cells.

Suppressor T cell activity in autoimmune responses could be lost with age in several

ways. There may be an actual decrease in numbers of cells of suppressor/cytotoxic cell phenotype in the older autoimmune individuals (Morimoto et al., 1980; Tsokos and Balow, 1983). An age-associated increase in size of Lyt-2- positive cells was reported in NZB mice that could indicate a loss of functional capacity of the T suppressor cells (Manohar et al., 1984). Autoantibodies have been demonstrated in murine and human autoimmune disease that are specifically cytotoxic for T cells with suppressor functions (Julius et al., 1973; Mage et al., 1977) and that could cause a loss of T suppressor activity.

2. Molecular mimicry

Molecular mimicry is a term used to define the sharing of epitope on different antigens. It is well known that several microbial and viral agents share determinants with host proteins, however, an immune response against the infecting agent also occurs. Autoimmunity induced by molecular mimicry should occur only when the host and microbial determinants are similar, yet different enough to break immunological tolerance (Oldstone, 1987).

Molecular mimicry is determined by shared structures which are crossreactive for receptor of lymphocytes. Epitopes recognized by antibodies are usually defined by tertiary conformational structure (Atassi, 1984). T lymphocytes, however, recognize epitopes on many protein antigens defined by primary structure (Maizels et al., 1980).

An example of molecular mimicry between a virus and an inducible host cell component was the homology between the fusion protein of measles virus and a heat

shock protein (hsp) of 79,000 kD (Sheshberadaran and Nouby, 1984). Eukaryotic cells contain a number of genes of related stress proteins which may be induced by different stresses. An example in which autoantibodies to hsp may induce autoimmune disease is SLE. Autoantibodies to hsp90 and to a constitutively expressed member of the hsp70 family were found to occur frequently in SLE patients (Minota et al., 1988). Sera from SLE patients reacted to ubiquitin which is hsp found universally in eukaryotic cells (Muller et al., 1988).

Molecular mimicry has been described between a number of viruses and host proteins (Oldstone, 1987; Fujinami, 1988). Approximately 4% of 600 antibodies raised against viral polypeptides from many different viruses (such as herpesvirus group, vaccinia, myxoviruses, paramyxoviruses, arenaviruses, and coronaviruses) crossreacted with one or more host proteins from uninfected tissues (Srinivasappa et al., 1986). Infection with microorganisms may, therefore, alter the host immune response, cause the exposure of normally sequestered antigens, or induce an immunologic response to cross-reactive epitopes resulting in an autoimmune reaction (Fujinami et al., 1983).

Rheumatoid arthritis (RA) is an autoimmune disease with unknown etiology. Molecular mimicry between proteins found in synovial membranes of patients suffering from RA and Epstein-Barr virus (EBV) (Gaston et al., 1990). RA patients have a defective immune response to infection with EBV which may account for the high antibody titers in these patients against EBV antigens. A high percentage of RA patients (Furukawa et al., 1985) have the major histocompatibility complex (MHC) haplotypes HLA-DR4 and HLA-DR1 (Vaughan et al., 1988). A five amino acid match was found between a

glycoprotein of EBV and HLA-DR types associated with RA (Vaughan et al., 1988).

Autoantibodies need to be directed against biologically important domains of host cell proteins in order to mediate autoimmune disease (Dyrberg and Oldstone, 1986) since many natural antibodies to cell constituents are present in normal sera (Lahesmaa-Rantala et al., 1990). Unless the homology between sequences and subsequent cross-reactive immune response can recognize a host protein intimately involved in pathogenesis, autoimmunity is unlikely to occur.

3. Autoimmune Ig gene

Crucial questions concerning pathogenic autoantibodies are: a) whether they arise from germline V genes that contribute to the Ig repertoire in normal persons or animals, b) whether they stem from "defective" V genes that are peculiar to humans and animals with autoimmune disease, and c) whether they are encoded by unmodified or somatically mutated Ig genes.

Studies have demonstrated that B cells with the capacity to produce autoantibodies occur with high frequency in normal mice, newborn mice and humans (Avrameas et al., 1983; Cairns et al., 1984; Dighiero et al., 1985; Souroujon et al., 1988). These "natural" autoantibodies were encoded by unmutated germline genes (Trepicchio et al., 1987; Baccala et al., 1989; Hardy et al., 1989), mainly of the IgM isotype and they usually bind with low avidity to autoantigens (Souroujon et al., 1988). Data indicate that disease-related autoantibodies are of the IgG isotype and have been described for both rheumatoid factors (RF) and anti-DNA autoantibodies (Eilat, 1986; Carson et al., 1987;

Smeenk et al., 1988). Studies by Schlomchik et al. (1987) revealed that IgG anti-native DNA autoantibodies, considered to be pathogenic in SLE, arise through somatic mutation, with DNA as a selecting antigen.

Evidence that natural human autoantibodies can be related to pathogenic autoantibodies, comes from studies of idiotype (Id)-16/6, an idiotypic marker originally detected in an IgM anti-DNA antibody derived from an SLE patient (Shoenfeld et al., 1983). Monoclonal anti-DNA antibodies derived from unrelated patients have been found to cross-react strongly with Id-16/6, an indication that they might be related to germline-encoded antibodies. High levels of both IgM and IgG Id-16/6+ antibodies have been found in the serum of patients with active lupus (Isenberg et al., 1984) and in the immune deposits of the renal lesions of the disease (Isenberg and Collins, 1985). Id-16/6+ antibodies from normal subjects, fetus, cord blood and patients with SLE were encoded either by a gene identical to V_H18/2, or to a very closely related (at least 98% homology) V_H gene (Guillaume et al., 1990; Young et al., 1990).

The presence of lupus-specific autoantibodies in normal serum (Gaither et al., 1987) and the production of autoantibodies by lectin-stimulated B cells from normal subjects (Mumford et al., 1985) suggests that coding information for autoantibodies exists in the germline gene repertoire and that autoreactive B cells are not deleted from the repertoire (Naparstek et al., 1986b). There are studies that support the view that somatic mutations give rise to pathogenic autoantibodies (Diamond and Scharff, 1984; Schlomchik et al., 1990). Sequence analysis of a large number of anti-DNA autoantibodies from MRL/lpr/lpr (Schlomchik et al., 1990), revealed a series of mutations toward the

inclusion of arginine and asparagine in complementarity determining regions (CDRs) in association with increasing affinity for denatured DNA, the development of reactivity with native DNA, or both.

Molecular analysis of a group of anti-DNA autoantibody-producing hybridomas and EBV-transformed B cell clones has demonstrated that genes conserved in the human population contribute to anti-DNA autoantibody production in SLE (Sanz et al., 1989b; Souroujon et al., 1989). The V_H sequence of the lupus-derived anti-DNA antibody monoclonal Ab 18/2 is identical to the V_H26 germline gene (Chen et al., 1988) and also to 30 P1, a rearranged V_H gene derived from fetal liver (Schroeder et al., 1987). From a large panel of anti-DNA autoantibody producing cell lines, it has been shown (Logtenberg et al., 1989) that four distinct EBV cell lines used V_H6 germline gene. Moreover, anti-Sm (Smith antigen) autoantibody (4B4), which is one of the most specific markers of SLE was identical in V_H sequence to a germline gene (Sanz et al., 1989). It is important to know that the random combination of V_H and V_L genes, as well as D (diversity) genes, can result in binding sites that recognize autoantigens.

Recent evidence shows that CD5+ B cells as a source of disease-related anti-DNA autoantibodies in SLE (Zuzuki et al., 1990). This B cell subset produce both low and high avidity RF (Hardy et al., 1987; Burastero et al., 1988). In addition, restricted V gene usage and minimal somatic mutation seem to be characteristic of the CD5+ B cells (Kipps et al., 1989). One possible source of B cell hyperactivity and the autoantibodies which characterize lupus and other autoimmune disorders could be abnormal structural sequences of immunoglobulin genes which might have appeared during the generation

of the autoimmune prone strains. On the basis of sequence comparisons between the known autoantibodies H chains and the available sequences from other antibodies (Kabat et al., 1987), it is hypothesized that the expressed autoantibody V_H and V_K segments are encoded by unique germline genes that do not encode V-chains of antibodies with specificities to external antigens. Therefore, under normal conditions, B cells bearing receptors comprising these chains would be deleted (Nemazee and Burki, 1989) or inactivated (Goodnow et al., 1988), or would not proceed to class switching and IgG production. This mechanism may depend on the presence of autoantigen and may be facilitated by known regulatory mechanisms such as activation of T-helper cells (Kappler et al., 1987), inactivation of suppressor cells or regulation by idiotypic network (Kelose et al., 1980).

Studies on RFLP analysis of the immunoglobulin heavy and light chain variable region (Igh-V, and Igk-V) gene complex were carried out in various lupus and non lupus strains. This was done by using DNA probes corresponding to the V_H and V_K gene families. These comprise the majority of the known polymorphic murine V_H and V_K gene germline repertoire, including some encoding autoantibodies (Kofler et al., 1985; Kofler et al., 1989). Although no difference was observed between autoimmune NZB and normal mice in the V_H families (Kofler et al., 1985) except for the V_HQPC52 family (Painter et al., 1986), there appeared to be a unique polymorphism of the NZB in V_K1 family. The only mouse that was similar to NZB was C58/J (Moynet et al., 1985) which is a non-autoimmune strain but spontaneously develops a high incidence of thymic leukemia and anti-thymocyte autoantibodies (Datta et al., 1982).

Furthermore, studies in our laboratories and others (Schlomchik et al., 1986; Kasturi et al., 1988; Klotz et al., 1988; Fidanza et al., 1990) revealed a biased usage of V_K1, V_K8, V_K10 and V_K19 families among autoantibodies, such as rheumatoid factors (RF), and anti-DNA antibodies. These V_K families are used by 80% of autoantibodies secreted by hybridomas obtained from autoimmune motheaten mice (Kasturi et al., 1988), and nearly all B cells from these mice express the Ly-1 B cell marker (Kasturi et al., 1988) which is a B cell subset known to secret high levels of autoantibodies (Herzenberg et al., 1986).

4. Polyclonal activation

Another possibility is that self-specific antibodies are encoded by the germline V gene repertoire and are thus present in both normal and diseased individuals. In normal mice, self- reactive cells are suppressed, but in disease, an abnormal polyclonal B-cell activation occurs resulting in the pathological production of autoantibodies (Dziarski, 1982; Hang et al., 1983). This model predicts that autoantibodies would resemble those derived after polyclonal activation of normal B cells. This is in agreement with the view that autoantibodies are mostly in the germline configuration and lose their autoreactivity by undergoing somatic mutations (Naparstek et al., 1986b). Studies by Klinman et al. (1988) showed that the developing repertoire of "normal" Balb/c and autoimmune MRL/lpr and NZB were very similar suggesting that systemic autoimmunity arises when the normal B cell pool is hyperstimulated rather than when a restricted set of autoreactive B cells is specifically activated by antigen.

The development of the NZB.xid (x-linked immunodeficiency defect) congenic strain has demonstrated that a single gene, xid (Berning et al., 1980), can markedly retard the development of both anti-DNA antibody production and clinical autoimmunity (Nakajima et al., 1979; Taurog et al., 1981; Ohsugi et al., 1982; Steinberg et al., 1982). Klinman and Steinberg (1986) transferred a limited number of cells from NZB into NZB.xid/xid mice. A significant proliferation of B cells was seen in the recipient animals. These cells were characterized by anti-DNA autoantibody production. Their data suggests that purified NZB B cells, in the absence of autoimmune T lymphocytes (T cells alone transfered into recipient did not induce such a response) or other donor cells, are sufficient to transfer anti-DNA- producing capacity into non-autoimmune hosts and that such B cells preferrentially proliferate in xid recipients. Therefore, this indicates that the internal milieu of NZB.xid mice contain gene products that may facilitate the activation of Ag-stimulated lymphocytes.

Studies have indicated that autoimmune NZB, BXSB, and MRL/lpr/lpr mice have increased number of B cells producing antibodies reactive with at least nine different antigens (Klinman and Steinberg, 1987; Ishigatsubo et al., 1988). Furthermore, the B cell repertoires of autoimmune strains were not skewed toward the specific production of autoantibodies. The number of B cells secreting antibodies against DNA, TNP-KLH, myosin, ovalbumin, and Sm increased approximately fivefold in MRL/lpr/lpr mice between 6- and 12- week of age. This early B cell activation appeared to be polyclonal in nature because there was no detectable skewing of the repertoire toward reactivity against specific autoantigen and IgM production predominated over IgG. However, in

older MRL/lpr/lpr (12 to 38-wk of age) B cell repertoire was characterized by Ag-specific skewing and IgG autoantibody production. This pattern is consistent with autoantigen - driven immune responses (Eisenberg et al., 1987a, Eisenberg et al., 1987b) and which will be discussed in the next section. Polyclonal B cell expansion has been detected in many lupus patients with early untreated disease (Budman et al., 1977). Similarly, patients with well established disease tend to develop B cell repertoires preferentially skewed toward reactivity with specific autoantigens (Kofler et al., 1971; Klinman et al., 1990). Although the origin of the polyclonal stimulating substances present in autoimmune animals is uncertain, factors capable of causing the hyperproliferation and activation of lymphocytes have been detected at abnormally high levels in NZB (Jyonouchi et al., 1985) and MRL/lpr (Prud'homme et al., 1983) mice and in humans (Hirano et al., 1987) with autoimmune disease. Lipopolysaccharide (LPS), derived from gram-negative bacteria, is a potent in vivo and in vitro stimulator of B cells (polyclonal B cell activator) (Coutinho et al., 1975). A single injection of LPS into normal mice led to transient formation of auto-Ag-Ab immune complexes both within the circulation and also as deposits in glomeruli but without the development of glomerulonephritis (Fournie et al., 1974; Izui et al., 1977). In addition, the lipid A portion of LPS which is mitogenic and minimally antigenic (Andersson et al., 1973), can induce long - lasting production of both IgM and IgG autoantibodies (Andersson et al., 1973, 1978; Izui et al., 1981). Hang et al. (1983) have found that chronic stimulation with the mitogenic lipid A fraction beginning early in life greatly accelerated the onset of late - life SLE disease of BXSB, MRL/n and NZW mice, as evidenced by the early

increase of Ig-secreting splenocytes, hypergammaglobulinemia, autoantibody production, and fatal immune complex- mediated glomerulonephritis. However, similar mitogen stimulation had less effect in immunologically normal mice which developed a much milder form of disease and no effect in LPS nonreponder C3H/HeJ mice.

Bacterial or viral antigens may induce formation of some autoantibodies. Cross reactions of some anti-DNA antibodies, including IgG anti-DNA, with several subcellular, extracellular and cell surface components have been documented (Lafer et al., 1981; Jacob et al., 1984; Faaber et al., 1986) and may be involved in polyclonal B cell stimulation leading to the pathogenesis of autoimmune disease.

In conclusion, as Klinman and Steinberg (1986) postulated, a polyclonal activator is present in autoimmune-prone mice and it induces the hyper-proliferation of Ag-stimulated B cells resulting in the hypergammaglobulinemia characteristic of systemic autoimmune disease.

5. Antigen driven process

Another model suggests that self-antigens and not polyclonal activation, are the driving force for the production of autoantibodies (Nelson et al., 1987). This antigen driven process would give rise to autoantibodies derived from clonally related precursors for non-self antigen but are somatically mutated and isotype switched (Clarke et al., 1985; Caton et al., 1986). Anti-DNA antibodies which have been elicited by experimental animals seem to have different antigenic specificities from those of disease related anti-DNA autoantibodies and may express different V-genes (Zouali et al., 1987).

Therefore, the surveillance mechanism which precludes the proliferation of potentially harmful autoantibody producing cells may operate at the level of somatic mutation, rather than the level of germline gene expression. The DNA-specific germline gene is utilized for the expression of anti-DNA H chain, and the somatic mutations serve for affinity maturation, coupled with antigen selection (Manser et al., 1985). Arginine rich CDR3 of H chain has been found in anti-DNA antibodies (Ollis and White, 1987; Eilat et al., 1988). It has been proposed that this patch of positive charges acts as an electropositive "ionic trap" that may serve as an initial binding region for nucleic acids. The arginine-rich CDR3 may increase the affinity of autoantibody to DNA.

6. Autoimmune networks

The autoimmune network is the expanded framework for anti-self reactivity based on the positive and negative influences of receptor/anti-receptor and self-specific interactions within the idiotypic network proposed by Jerne (1974). Idiotypic determinants on the immunoglobulin or T cell receptor V regions are sites for recognition and regulation of immune responsiveness not only to exogenous or alloantigens, but also to self-antigens (Jerne, 1974). Studies have shown that many autoantibodies with same or different specificities express cross-reactive idiotypes (CRI). Zanetti et al. (1983a) prepared monoclonal antibodies in mice for thyroglobulin (TG) and showed that these antibodies were specific for a highly conserved TG epitope. Furthermore, the Buffalo rat strain spontaneously develops an autoimmune thyroiditis similar to Hashimoto's disease in man. The majority of rat autoantibodies shared a CRI (IdX), which was detected on the surface

of B cells from young and old rats (Zanetti et al., 1983a). It also appears that anti-TG autoantibodies share an interspecies IdX. Anti-Id antibodies against human anti-TG antibodies also bound to rat anti-TG. Conversely, anti-Id antibodies against rat anti-TG bound to human and mice anti-TG antibodies (McCoy et al., 1983; Zanetti et al., 1983b).

Several reports have demonstrated that anti-DNA autoantibodies from human SLE patients, as well as from mouse lupus strains share an IdX. Solomon et al. (1983) purified anti-DNA antibodies from an SLE patient that were used to prepare murine monoclonal anti-Id antibodies. One of the monoclonal anti-Id antibodies bound to eight of nine sera containing anti-DNA autoantibodies.

A high frequency of IdX was observed among anti-DNA antibodies produced by murine lupus strains. Rabbit anti-Id antibodies against H130, a monoclonal anti-DNA antibody obtained from MRL/lpr mouse was prepared (Rauch et al., 1982). Two-thirds of monoclonal anti-DNA antibodies from MRL/lpr mice shared the H130 IdX. Studies have shown that the administration of anti-H130 Id during pregnancy, after birth, or 3-wk old MRL/lpr mice had no effect on the synthesis of anti-DNA antibodies. By contrast, the synthesis of H130 Id+ antibodies devoid of anti-DNA activity was observed in both MRL+/+ (late-life SLE) and Balb/c mice (Teitelbaum et al., 1984). H130 Id was present in large amounts in MRL/lpr serum and is expressed on 40-60% of anti-DNA antibodies. Smaller amounts of H130 Id+ molecules were detected in the sera of MRL+/+, and was absent in Balb/c (Teitelbaum et al., 1984). These results suggest that H130 Id is an interstrain regulatory idiotype that is predominantly expressed in anti-DNA antibodies in MRL/lpr mice. The study of idiotypy of human RFs was initiated

by Kunkel et al. (1973). These authors studied the idiotypy of IgM monoclonal proteins exhibiting RF activity, and observed about 60% of those proteins shared an IdX (designated WaIdX). It has been documented that WaIdX is prevalent among the patients with rheumatoid arthritis and can also be identified in pokweed mitogen (PWM)-stimulated lymphocytes from healthy individuals (Bonagura et al., 1982). All IgM RF expressing WaIdX were encoded by VKIIIb light (L) chain (Kunkel et al., 1974), which suggests that VKIIIb L chain is the major contributor to WaIdX. In addition, it has been demonstrated by Chen et al. (1985) that WaIdX was largely dependent on the primary sequence of the CDR2 segment of the L chain.

Studies have shown that the majority of monoclonal RF from 129/SV, Balb/c and MRL/lpr mice were IgM/K and shared the idiotypes of 129-48 and LPS10-1 (14 out of 20 monoclonal RF), whereas, 7 out of 20 shared the Y19-10 idiotype (Manheimer et al., 1984).

Coombs' antibodies play an important role in autoimmune hemolytic anemia (Dameshek, 1965). These antibodies, which are prevalent in NZB causes a severe hemolytic anemia; a milder hemolytic disease is observed in F1 hybrids of NZB with normal strains (Burnet and Holmes, 1965). It has been shown (Cohen and Eisenberg, 1982) that F1 hybrids that develop a mild disease spontaneously produced anti-Id autoantibodies which recognized an IdX on NZB Coombs' anti-RBC (red blood cells) antibodies. Id62 is a monoclonal antibody obtained from Balb/c mice and is specific for TG (Zanetti et al., 1983a), and bears an interspecies regulatory idiotype (Zanetti, 1985). Rabbit anti-Id62 antibodies administered to Balb/c induced anti-TG autoantibodies in 50%

of the mice. This suggests that Id62 is a regulatory idiotype expressed on clones producing anti-TG autoantibodies. These clones can then be activated by the complementary anti-Id antibodies. This anti-Id stimulation could contribute to the breaking of self- tolerance and, hence, to autoimmune phenomena.

Hahn and Ebling (1983) have demonstrated that the immunization of (NZBxNZW)F1 female mice (which develop severe glomerulonephritis and SLE- like disease) in premorbid phase with a monoclonal anti-DNA antibody resulted in a delay of disease and a temporary suppression of nephritogenic anti-DNA autoantibodies. Survival in these mice were also longer. This effect was probably related to the production of anti-Id antibodies which downregulated the autoantibody response. In humans, the appearance of anti-Id antibodies against anti-DNA antibodies in SLE patients caused a decrease of serum levels of anti-DNA autoantibodies and clinical remission (Abdou et al., 1981).

Anti-Idiotypic antibodies carrying the internal image of the hormones or other biologically active substances could be an important factor in the appearance or maintenance of autoimmune diseases. Studies have shown that anti-Id antibodies against anti-insulin antibodies interacted with the insulin receptor (Sege and Paterson, 1978). Other studies on Graves' disease have also shown that anti-receptor antibodies observed in this disease may be anti-Id carrying the internal image of the receptor thyrotropin (TSH) (Farid and Lo, 1985). This anti-Id antibody not only bound to TSH receptor but also stimulated cAMP synthesis in throcyte- like thyrotropin hormone (Farid et al., 1982). Such antibodies can interact with autoreactive clones instead of autoantigens and induce their expansion, hence, initiating autoimmunity.

A recent study (Bailey et al., 1989) has demonstrated that immunization of 2-wk old MRL/lpr mice with a foreign antigen GT (glutamic acid-tyrosine homopolymer) or anti-Id antibodies carrying the internal image of GT activated clones producing antibodies that bound to GT and to self antigens. Some of these antibodies shared the idiotopes of anti-GT antibodies, RF, anti-DNA, and anti-Sm autoantibodies. The data demonstrated that B cell clones producing antibodies able to interact with a similar affinity constant with a foreign and self antigens may be expanded by the foreign antigen or anti-Id antibody carrying the internal image of the foreign antigen (Bailey et al., 1989). These autoreactive clones could, therefore; be activated in response to immunizations or infections, and such autoantibodies could be involved in autoimmune disease pathogenesis. It is worthwhile to mention that there is increased evidence showing that the immune repertoire of normal and autoimmune- prone animals contains clones bearing Ig receptors able to bind both self and foreign antigens (Zabriskie et al., 1970; Kabat et al., 1986; Naparstek et al., 1986a). In agreement with this evidence, studies by Monestier et al. (1987) have shown that 8 out of 20 heavy chain- V (V_H) J558+ monoclonal autoantibodies with various specificities also interacted with foreign antigens known to bind to antibodies encoded by genes derived from the V_HJ558 family, and a high fraction of such autoantibodies also shared the cross-reactive idiotypes of V_HJ558+ antibodies specific for foreign antigens, such as α1-3 dextran, arsonate, influenza virus and pGAT (glutamic acid-alanine-tyrosine homopolymer).

In conclusion, the alteration of the autoimmune network can be responsible for the onset of autoimmune diseases causing a breakdown of natural self tolerance.

7. Genetic factors

No single gene or group of related genes that code for a generalized autoimmunity or a predisposition for systemic autoimmune diseases can be identified. Rather, it appears that various individual genes or a cluster of genes contribute to the development of autoimmune disease (Howie and Helyer, 1968; Shirai and Mellors, 1971; Warner, 1973; Steinberg et al., 1981; Yoshida et al., 1981). Some of these genes appear to be linked to the MHC, and others are located on other chromosomes (Shirai et al., 1987). It has been shown that each autoimmune phenotype is under control of several genes (Raveche et al., 1978; Taurog et al., 1981) with the exception of autoimmune diseases developed by motheaten and tight skin mice, in which a single gene defect was identified (Green et al., 1976; Shultz and Green, 1976).

Studies have demonstrated the relationship between MHC structures to autoimmune disease susceptibility in humans. The structural correlates of HLA (human leukocyte antigen) with susceptibility to RA were investigated by Winchester and Gregersen (1988). RA was correlated with serologically diverse MHC alleles such as DR4 and DR1, DRW10 and DRW53. Nucleic acid sequencing studies showed that these diverse haplotypes associated with susceptibility exhibited sequence homologies in their DRβ1 third variability region (position 67 to 74). According to the authors, these homologies, attributed to the gene conversion events, might result in a conformationally- equivalent α-helical structure in these structurally different molecules that confer susceptibility to RA. It was also suggested that two different DR alleles may trans-complement each other to recreate this same set of susceptibility. Shared epitopes between the diverse MHC

alleles are important in conferring this susceptibility (Winchester and Gregersen, 1988).

A good correlation was found between DR3 and/or DR4 and, in particular, certain DQ molecules (mostly DQW3.2 and DQW2) with insulin-dependent diabetes mellitus (IDDM) (Todd et al., 1987). Gene segments encoding the amino-terminal polymorphic domains of these molecules were sequenced and found to be similar in IDDM patients and normal controls. That is to say, disease susceptibility is not caused by mutant class II alleles found exclusively in IDDM patients. However, nearly all DQβ chains ($>90\%$) found in diabetics had a neutral residue (Ala, Val or Ser) at position 57. In contrast DQβ chains, rarely ($<10\%$) found in IDDM caucausian patients, have Asp at this position. DQ- mediated IDDM susceptibility (non- Asp at position 57) was found to be recessive, and DQ- mediated resistance (Asp at position 57) was found to be dominant. This observation extended to the non-obese diabetic (NOD) mice, whereby its DQß homologue, I-Aβ, is unique at codon 57 (Ser in NOD, Asp in non-diabetic strains). In Japanese subjects, some Asp 57 seemed to be disease associated in DR4 and DRW9 (Erlich, 1989).

Other genetic factors can be found in certain B-cell clonotypes expressing high-affinity IL-2 receptors and responding to IL-2 (Waldmann, 1986). It has been demonstrated that several B-cell activities (B-cell growth and differentiation and induction of class II MHC molecules expression on resting B cells) can be mediated by a single lymphokine (Noma et al., 1986). Therefore, lymphokine interaction with a receptor in varying differentiative states of the B cell may activate "harmful" genes and cause autoimmune disease.

B. The New Zealand Black Mouse

In recent years, investigators have had available a sophisticated and highly rewarding system in their efforts to determine the genetic factors that contribute to autoimmunity. The NZB mouse is genetically predisposed to the development of autoimmune disease that resembles the human autoimmune SLE and autoimmune hemolytic anemia (Howie and Helyer, 1968; Theofilopoulos and Dixon, 1985b). NZB mice spontaneously produce anti-DNA, anti-histones, anti-erythrocytes, and anti-thymoctes autoantibodies (Talal and Steinberg, 1974). Many approaches were used to determine the genetic contribution to the NZB autoimmune disease, such as the generation of F1 hybrids from crossing NZB with a non-autoimmune strain, backcrossing the progeny to both parents, and using recombinant inbred lines of NZB with a non-autoimmune strain (Raveche et al., 1981b; Taurog et al., 1981; Datta et al., 1982; Shirai et al., 1987). The latter has been extremely useful, since individual RI lines are largely homozygous at most loci, and hence, recessive genes are observed as easily as dominant genes. The RI lines are obtained by mating an NZB mouse with a non-autoimmune mouse. The F2 mice are separately mated to initiate the RI lines and then brother-sister mated at each successive generation until full homozygosity is obtained. This usually takes between 17 to 20 generations (Bailey, 1971).

1. General characterization of NZB autoimmune disease.

The NZB mouse, which spontaneously develops an autoimmune disease resembling

human autoimmune hemolytic anemia and systemic lupus erythematosus, has been a commonly used model for the study of immunoderegulation in autoimmune disease. From an early age, these mice exhibit increasing immunologic abnormalities resembling those found in the human conditions (Howie and Helyer, 1968; Theofilopoulos and Dixon, 1985b), including B cell hyperactivity characterized by increased IgM, IgG and IgA production, diminished T cell function, immune complex glomerulonephritis with anti-DNA autoantibodies, anti-histones autoantibodies, anti-erythrocyte autoantibodies (Deeher and Edington, 1976; Moore and Calkins, 1985; Theofilopoulos and Dixon, 1985b), and anti-thymocytotoxic autoantibodies, as well as abnormalities of prethymic (Kincade, 1981) and thymic cells (Whittum et al., 1985).

2. Genetic studies in the NZB mouse.

It has been clearly demonstrated that autoimmune disease in NZB is genetically determined (Raveche et al., 1978; Taurog et al., 1981). Numerus studies suggest that several genes, rather than a single gene, are involved in the manifestation of the autoimmune abnormalities (Raveche et al., 1981b; Shirai et al., 1987).

a) *T cell function* Old NZB mice manifest impaired primary immune responses including impaired primary skin allograft rejection, impaired graft versus host disease in newborn recipients, and decreased primary antibody responses to thymic dependent antigens (Cantor et al., 1970, 1978; Stutman, 1972; Gelfand and Steinberg, 1973). Studies on young (NZBxNZW)F1 mouse (Steinberg et al., 1970) showed that the thymus contained some factor which suppressed the development of autoimmunity. Furthermore, decreased

numbers and activities of nonspecific Ts cells have been reported (Barthold et al., 1974; Primi et al., 1978). This led to the belief that an important factor in the pathogenesis of autoimmunity in NZB mice is inadequate suppressor function. However, other studies have demonstrated normal levels of concavalin A-induced Ts. Intact nonspecific, foreign or cross-reactive Ag specific suppressor systems have also been shown (Creighton et al., 1979; Cooke et al., 1980; Theofilopoulos and Dixon, 1985a). Although no common defect of Ts in NZB mice may be evident, failure of cell regulation at the level of the Th cell may lead to excessive Th activity, resulting in the B cell hyperactivity and an effective masking of Ts function. Increased number of cells bearing the Th phenotype and increased Th activity and other helper factors have been reported in NZB (Cantor et al., 1978; Theofilopoulos et al., 1980, 1983).

Recent studies by Moore and Hoover (1989) showed that a possible disfunction of an isotype-specific immunoregulatory circuit, is mediated by T cells via soluble Ig-binding factors (IgA-BF produced by IgA FcR [Fc receptor] bearing T cells), in the NZB mouse. NZB as well as (NZBxNZW)F1 mice produce natural thymocytotoxic autoantibody (NTA), which is cytotoxic for thymocytes and T cells and reacts with brain tissues of mice (Shirai and Mellors, 1971; Shirai et al., 1972). This NTA has preferential cytotoxic effects on some functional T cell subsets, including those involved in suppressor T cell function (Klassen et al., 1977). Maruyama et al. (1980) have shown that there is a significant association between the appearance of NTA and the development of early and severe renal disease in NZB/NZW F1xNZW backcrosses. They also demonstrated a correlation among anti-DNA antibodies, NTA, and the renal

disease in these backcross mice. Moreover, a single dominant gene in NZB mouse, contributes to the spontaneous appearance of NTA in the backcrosses. However, Raveche et al. (1981b) have shown that several strains of recombinant inbred lines of NFSxNZB that did not produce anti-T cell antibodies produced anti-DNA antibodies. In addition, studies by Taurog et al. (1981) using ZB.CBA/N that bear largely NZB autosomal genes, but have X chromosomes derived only from CBA/ N mice, developed T cell abnormalities of NZB mice but without any autoantibody production. Their results demonstrated that T cell aberrations in NZB mice represent independent defects. However, studies of NZB mice have demonstrated that maximal autoantibody production occurs only when autoreactive T cells are present (Laskin et al., 1986).

b) *B cell function and autoantibodies* NZBxNZC crosses suggested that at least two genes (one recessive and another dominant) are responsible for anti-erythrocyte autoantibody production (Raveche et al., 1981b). In addition , studies by Ozaki et al., 1983 suggested also two genes (Aia-1 and aem-1), both located on chromosome 4 are involved in anti-RBC (red blood cells) autoantibody production. Knight and Adams (1981) have obtained evidence that the lupus nephritis of the (NZBxNZW)F1 mice is determined by three dominant or co-dominant genes, Lpn-1, Lpn-2 and Lpn-3. Both Lpn-1 and Lpn-2 are located on chromosome 17 and Lpn-2 is tightly linked to the MHC. The investigators postulated that the third gene Lpn-3 may be either a k or λ light chain V gene. Furthermore, backcross studies of (NZBxNZW)F1 by Shirai et al. (1987) showed several putative loci involved in the develpment of each autoimmune feature, of these, Ads-1 (anti-double stranded DNA antibodies) and Ass-1 (anti-single stranded DNA

antibodies) found in NZB and Ads-3 and Ass-3 in NZW, both located on chromosome 17, while Ads-4 and Ass-3 of NZW are located on chromosome 6, the putative locations of Ass-2 and Ads-2 of NZB was not assigned. Both could also be located on chromosome 6, which has the V_K locus.

Raveche et al. (1981b) using recombinant inbred strains of NZBxNFS RI showed that the spontaneous hypersecretion of IgM in NZB is due to a single gene. It is assumed that there are loci in the NZB strain which determine the production of IgM anti-DNA antibodies. These loci are modified in (NZBxNZW)F1 mice by transacting genes of the NZW parent (Kohno et al., 1983; Shirai et al., 1987).

Studies by Boros et al. (1990), using a recombinant truncated Fc gamma RII molecule as a probe, have shown the presence of anti-Fc gamma R antibodies in several strains of autoimmune mice, including NZB, (NZBxNZW)F1 and motheaten mice. 16 micrograms of IgM per ml of serum (approximately 2% of total IgM) eluted from anti-Fc gamma R column was isolated from old NZB mice. No anti-Fc gamma R IgM was found in sera from C58/J mice. Moreover, a good correlation was found between the presence of anti-Fc gamma R Ig and impaired phagocytosis of immune complexes in NZB and (NZBxNZW)F1 mice. Few of the IgM monoclonal antibodies that bound to Fc gamma R were polyspecific and bound to DNA, histones, TG, and transferrin. According the the authors (Boros et al., 1990), the role of anti-Fc gamma R Ig in autoimmune mice may be to crosslink and activate Fc gamma Rs on neutrophils, macrophages, natural killer (NK) and mesangial cells, or it may desensitize Fc gamma R function of Fc gamma R- bearing cells.

Ly-1 B cell subset. There is ample evidence showing that a discrete subset of B cells bearing the CD5 antigen (Ly-1 in mice and Leu-1 in humans) is a major contributor to the production of autoantibodies (Hayakawa et al., 1984; Hardy and Hayakawa, 1986). This B cell subset is predominant in motheaten (mev) mice (Hardy and Hayakawa, 1986) and is significantly enlarged in NZB mouse strain (Manohar et al., 1982). Aged NZB mice show an anusual clonal expansion of Ly-1 B cells with premalignant properties (Seldin et al., 1987a; Tarlington et al., 1988). Certain human B cell malignancies which have a significant association with autoimmune phenomena (such as chronic lymphocytic leukemia) were shown to arise almost exclusively from CD5+ B cells (Boumsell et al., 1980). It has been suggested that this B cell subset is involved in the spontaneous production of RF, anti-DNA, anti-thymocytes and anti-bromelein treated mouse red blood cells autoantibodies (Hayakawa et al., 1984; Casali et al., 1987). Ly-1 is expressed on the majority of T cells and a small percentage of B cells (1-3%)(Manohar et al., 1982). Two Ly-1 transcripts of 2.9 and 2.1Kb were detected in thymocytes (Bailey et al., 1989), while Ly-1 transcripts of 2.9 and 1.6Kb were found in a small fraction of MRL/lpr hybridomas producing autoantibodies. Mayer et al. (1990), using S1-nuclease protection assay, have shown that the Ly-1 transcript detected in hybridomas and thymocytes were the products of the same gene despite the size difference. In addition, although studies have demonstrated that a high proportion of autoantibodies are derived from the Ly-1 B cell subset (Manohar et al., 1982), other experiments indicated that non-organ specific antibodies such as anti-DNA, anti-histones and anti-Sm were encoded by both Ly-1+ and Ly-1$^-$ B cells (Fidanza et al., 1990; Mayer et al., 1990) and pathogenic

autoantibodies such as Coombs' antibodies were found only in older (16-month old) NZB mice and were produced mainly by Ly-1$^-$ B cells (Fidanza et al., 1990).

Study of the expression of V genes in hybridomas that produced autoantibodies and were derived from Ly-1+ B cells showed no preferrential utilization of a particular V_H family, however, there seemed to be a preferrential usage of V_K1 family (Fidanza et al., 1990; Mayer et al., 1990).

c) *Major Histocompatibility Complex* Studies revealed significant association between the inheritance of both anti-dsDNA and anti-ssDNA antibodies and the H-2^d haplotype of the NZB strain in the (NZB/NZW) F1xNZW backcross progeny (Shirai et al., 1987). These studies assigned one of the 2 NZB genes responsible for each anti-ssDNA and anti-dsDNA antibodies on chromosome 17 (as mentioned earlier) and to some extent linked to the H-2^d complex of the NZB strain.

Genetic studies showed that NTA is determined by a single dominant locus (Nta-1) probably located on chromosome 17 of NZB mice and is to some extent linked to the H-2 complex (Maruyama et al., 1980).

Evidence was obtained by Furukawa et al. (1985) that a single dominant locus (Lbt-1) of the NZB strain is responsible for the appearance of immunoglobulin deposition at the dermal-epidermal junction in the NZB mouse. The skin lesion was significantly associated with the inheritance of the H-2^d haplotype, renal disease and anti-dsDNA antibodies in the B/W F1xNZW backcross mice. This suggested a linkage between Lbt-1 and Ads-1 on chromosome 17 of the NZB strain. These authors and others (Yoshiki et al., 1974) found evidence that the major glycoprotein constituent of the endogenous

retroviral envelope, gp70, was deposited, apparently as an immune complex in the renal glomeruli and vascular walls in the NZB and NZB/NZW F1 mice. Genetic studies revealed that there is a correlation between the appearance and the magnitude of the gp70 immune complexes in the serum, the development of renal disease, the appearance of anti-ds DNA and H-2^d haplotype in the NZB/NZW F1 x NZW backcross mice (Shirai et al., 1987). The putative location of the gene that is responsible for gp70 immune complex is located on chromosome 6 and linked to Ads-4 and Ass-4 of NZW and to Ass-2 and Ads-2 of NZB (Shirai et al., 1987).

Splenomegaly was found to correlate with the presence of splenic hyperdiploidy in RI lines and backcross progeny of NZB mice (Raveche et al., 1981a; Taurog et al., 1981; Seldin et al., 1987b). These studies have also shown that hyperdiploid spleen cells are derived from NZB bone marrow stem cells. These abnormal B cells have been found to be limited to the Ly-1+ subset which is elevated in NZB mice (Herzenberg et al., 1986). Therefore, there seems to be multiple genetic defects underlying autoimmune disease in the NZB mouse. The loci involved in the manifestation of different phenotypes in the disease appear to be unlinked. Thus, the predisposition of the disease cannot be assigned to the effect of a single genetic locus.

3. Polymorphism of V genes.

a) *T cell receptor (TCR) genes* The essential role of T cells in autoimmunity is most likely related to their interaction with other lymphoid cells through their antigen receptors. The T cell receptor is made up of α and β, or γ and δ chains which are

divided into V and C regions. The genes are divided into separate Vβ, Dβ, and Jβ gene segments that are assembled by recombination during T cell development to form a Vβ gene associated with either of 2 constant (Cβ1 and Cβ2) genes. There are 6 functional Jβ gene segments clustered just upstream of each Cβ gene and 2 Dβ gene segments. The Vβ segments are relatively few in number, and usually unique except Vβ8 and Vβ5, composed of three and two members, respectively. The α chain genes have similar organization, but differ from the β chain genes in that they contain a single Cα region gene segment, a larger Jα gene segment number, and a very large J-C intron. The Vα repertoire is larger than the Vβ repertoire, with the 11 Vα subfamilies identified thus far consisting of 1 to 10 members each. There is minimal somatic mutation involved in the generation of TCR diversity (Acuto et al., 1983; Hedrick et al., 1984; Yanagi et al., 1984; Kotzin et al., 1985). Studies by Noonan et al. (1986) showed no polymorphism among autoimmune strains, including NZB, and normal mice in the Cα gene region. There was also a high conservation in the Cβ and Jβ of different mice strains, except in the NZW mouse. Kotzin et al. (1989) showed that autoreactive T cells (Vβ17a and Vβ11, which both demonstrate reactivity to multiple alleles of I-E) were absent from peripheral lymphoid tissues of (NZBxNZW)F1 and (NZBxSWR)F1 mice. Their results indicated that T cell development in NZB mice is not affected by a global failure in self tolerance. Furthermore, their data demonstrated the existence of an NZB minor locus not present in other H-2^d strains that influence T cell repertoire and enhances stimulation of T cells potentially reactive to self class II MHC antigen. Autoimmune NZB mice have the same Vα genotype ($V\alpha^c$) as autoimmune susceptible PL/J, SJL, SWR and non-obese

diabetic (NOD) mice and they show the highest relative expression of the autoreactive Vβ8.2 gene (Theofilopoulos et al., 1988).

b) *IgV genes* The ability to mount a specific B-cell response resides partly in the polymorphic Ig germline gene repertoir. Abnormalities in these genes or particular Ig haplotypes, if present, may contribute to autoimmune disease. Ig heavy and light chains are encoded by 2 to 3 sets of germline gene segments: variable (V), diversity (D, heavy chains only), and joining (J), which are assembled during B-cell differentiation by stage-specific DNA rearrangements (Tonegawa, 1983; Alt et al., 1986). Murine V_H genes are organized in eleven multigene families of closely related members (Brodeur and Riblet, 1984; Kroemer et al., 1987; Hardy et al., 1989) that exhibit extensive genetic polymorphism among inbred strains of mice defining several Ig V_H haplotypes. The V_H of NZB (V_H^c) is rarely shared with other strains; only NZW mice have this haplotype (Klein, 1975) and a V_K1^b haplotype (Moynet et al., 1985). The complexity of individual families varied, with one family (V_HJ558) comprising 100-200 germline genes and another just a few (V_HX24) (Livant et al., 1986). The organization and complexity of the V_K gene families are not well defined. However, based on amino acid similarity up to the invariant tryptophan in position 35, mouse V_K light chains have been classified in 24 V_K groups (Potter et al., 1982).

Isoelectrofocusing IEF (Gibson and Maclean, 1979), peptide mapping (Edelman and Gottlieb, 1970), RFLP (Moynet et al., 1985), idiotype (Goldrick et al., 1985) and sequence analysis Lazure et al., 1981) suggest a high polymorphism of V_K gene complex, although its extent remained unclear as only a few number of inbred mice strains and V_K

loci were investigated. Kofler et al. (1989) have recently performed an extended RFLP analysis of the V_K gene complex of 33 inbred strains of mice using 16 V_K probes. They estimated between 60 and 120 discernable V_K gene-containing restriction enzyme fragment (REF). They also estimated the number of genes within each V_K family. However, to estimate the number of genes based solely on RFLP is misleading, since in one fragment, there may be more than one germline gene. Indeed, Livant et al. (1986) were able to isolate several V_HJ558 germline genes from a single fragment. The V_K1 group appears to represent an important group of k-chains in the mouse. V_K1 genes are exclusively used in the immune response to a variety of antigens, including dinitrophenol, flagellin, and, glutamyl-alanyl-tyrosine (GAT) terpolymer (Smith et al., 1977; Schiff et al., 1983; Hum et al., 1984). Furthermore, it has been shown that a high frequency of autoantibodies such as rheumatoid factors and anti-DNA are encoded by germline genes from this family (Schlomchik et al., 1986; Kasturi et al., 1988; Klotz et al., 1988).

Amino acid sequence studies on V_KI light chains have indicated the existence of five subgroups (V_K1A-E). Four of the subgroups (A, B C, and D) were found in Balb/c (Hum et al., 1984; Corbet et al., 1987). It was considered that all V_K1 genes of NZB mice belong solely to V_K1B subgroup (Loh et al., 1979). Studies on the IEF polymorphism of the light chain indicated that V_K1A subgroup is polymorphic in inbred strain, being absent from few strains (NZB, C58/J, BDP/J, CE/J, I/LnJ, and P/J). These strains have been designated $Ef2^b$ (Hum et al., 1984; Moynet et al., 1985). The locus controlling V_K1A expression (IgK- Ef2) has been shown to be closely linked to other k-chain genetic markers, namely, Lyt-2,3 and Hd loci located on chromosome 6 (Gibson and Maclean,

1979). Moynet et al. (1985) found that $Ef2^a$ strains of mice, which include most strains, possessed the same restriction fragments of V_K1 when BamH1 and EcoR1 digested DNA was hybridized with V_K1 5'-flanking probe. However, two of the $Ef2^b$ strains (NZB and C58) showed different V_K1 RFLP patterns, whereas the other four strains revealed RFLP patterns similar to those of the $EF2^a$ strains.

Many studies have been carried out to determine whether there are specifice V_H and V_K genes that encode autoantibodies. RFLP analysis on autoimmune strains, and several nonautoimmune control mice strains (D'Hoosteleare et al., 1988; Kofler et al., 1989) showed that the autoimmune mice, including NZB, were not associated with a particular IgH or IgK haplotype or abnormalities in the respective germline genes. Furthermore, sequences of the IgH enhancer region in two murine lupus strains (BxSB and MRL-/lpr/lpr) showed no abnormalities as compared to the normal Balb/c strain (Theofilopoulos et al., 1986).

Sequencing analysis on rheumatoid factors showed preferential usage of V_K1, 8 and 19 families in both secondary protein stimulation and LPS (lipopolysaccharide) stimulation of mice (Schlomchik et al., 1986). Other studies have also shown biased usage of V_K1 in autoantibodies (Klotz et al., 1988; Fidanza et al., 1990).

It might be worthwhile to mention that most autoantibodies that are encoded by V_K families other than V_K1 have over 75% homology to V_K1 family. Interestingly, V_K1, 8, and 19 genes that encode RF are highly similar in their Frameworks 1 and 2 compared with the same regions in V_K sequences from other families (Schlomchik, et al., 1986). This suggests that a specific B cell subset could be involved in the production of

autoantibodies such, as the Ly-1 B cells which is highly represented in NZB as discussed in an earlier section (Herzenberg et al., 1986; Mayer et al., 1990).

C. Significance

Autoimmunity, which is observed in clinical and experimental circumstances, can be defined by an apparent breakdown of unresponsiveness to self. Immunological tolerance is the result of many active physiological processes and is not simply the lack of immune response. One of these processes is mediated by antibodies that will react to self antigens causing tissue damage. These autoantibodies are either organ-specific, or non-organ-specific (Allison, 1977).

Pathogenic and normal antibodies are composed of H and L chains that contribute to the structure of the antibody combining site through interactions of their V_H and V_K domains (Amzel et al., 1974). Immunoglobulin germline genes need to be rearranged before expression; two genes recombine for the V_L domain (V_K and J_K, or $V\lambda$ and $J\lambda$), three genes for V_H (V_H, D_H, and J_H) and a leader sequence upstream of the variable region (Brack et al., 1978; Sakano et al., 1979; Kurosawa and Tonegawa, 1982). It is important to establish whether autoantibodies are encoded by the germline genes or result from somatic mutations in antibodies originally directed against exogenous antigens, since it defines the developmental stage at which a given B cell clone acquires self-specificity and escapes tolerance. Moreover, autoimmunity could result from the disturbed regulation of the mechanisms responsible for somatic mutations in the

maturation of normal immune responses. If somatic mutation is present, then it can determine that anti-self response is driven by autoantigen as opposed to polyclonal activation. Studies have shown that many autoantibodies are derived from germline genes with little or no somatic mutations. MRL- DNA10, an anti-DNA autoantibody from MRL/lpr mouse (an autoimmune strain) has a sequence which is identical to a Balb/c germline gene (Kofler et al., 1987). Furthermore, the human SLE-derived anti-DNA antibody (18/2) expresses the human germline V_H26 gene (DerSimonian et al., 1987). Sanz et al. (1989a) have documented that the V_H gene segment used in a human anti-Sm (Smith antigen, found only in patients with SLE or in certain inbred strains of mice that develop an SLE- like disease) monoclonal antibody has a germline encoded sequence. However, the presence of somatic mutations in J segments of some MRL-lpr/lpr autoantibodies clearly shows that somatic mutations can occur in spontaneous lupus autoantibodies (Kofler et al., 1987). Also, some antibodies seem to acquire self specificity after somatic mutations (Diamond and Scharff, 1984). No conclusive evidence has been shown yet in regard to germline versus somatic mutations in autoantibodies. Therefore, sequencing a large panel of autoantibodies from NZB mouse and comparing them to NZB germline genes will provide more definitive answers.

Preferential usage of certain V genes was clearly shown in humans. The predominance of the relatively uncommon V region subgroup isotype kIIIb among the light chains of human monoclonal (IgMk) anti-IgG antibodies (RF) was documented through sequence analyses of such autoantibodies isolated from IgM-anti-IgG cold-insoluble immune complexes (patients with mixed cryoglobulinemia) (Ledford et al., 1983; Pons-Estel et

al., 1984; Radoux et al., 1986). This gene, HumKv325, has been recently cloned and characterized (Chen et al., 1987). In addition, studies by Goni et al. (1989) demonstrated the same light chain restriction in autoantibodies with other specificities, such as anti-I (cold agglutinin), anti-low density lipoprotein, and anti-intermediate filaments. In contrast to the preferential association of the kIIIb subgroup with anti-self antibodies, only 8% of IgM-k antibodies devoid of self-reactivity have been found to possess this type of L chains (Moynihan et al., 1985). The absence or rare occurrence of isotype switch and somatic mutation in kIIIb encoded autoantibodies suggests that autoantibodies of the IgM class are necessary in the early stages of the development of the immune system and may not be subject to the same evolutionary pressure as the rest of the Ig genes.

Therefore, the study of rearranged V_K1 genes expressed in NZB hybridomas producing autoantibodies will allow us to determine if they are derived from germline genes unique to NZB or from V_K1 germline genes shared with other strains.

As mentioned earlier, many autoantibodies are encoded by V_K1 gene family. This is important since the phenotypic properties of the product of V_K1 genes showed that NZB mice belong to a particular phenotype rarely observed in other strains. Protein sequencing of V_K1 light chains permitted their classification into various subgroups (V_K1A-V_K1E) and showed that the NZB proteins belong to V_K1B subgroup which differs from V_K1A subgroup, abundantly represented in Balb/c mice (Moynet et al., 1985). V_K1B subgroup also exhibited a particular IEF profile characterized by disappearance of certain bands from the profile of V_K1A subgroup (Lazure et al., 1981).

Hence, genetic studies on NZB disease and its relationship to the inheritance of V_K1

may provide useful information on how the gene plays a role in the pathogenesis of the disease and how such gene functions can be manipulated. It is important to mention that V_K1 gene may be segregating with other genes involved in the pathogenesis of disease because various autoimmune traits are controlled separately by a limited number of genes (Howie and Helyer, 1968; Raveche et al., 1981b; Steinberg et al., 1981; Yoshida et al., 1981; Shirai et al., 1987).

D. Specific aims

1) To study the correlation between the polymorphism of V_K, V_H, TCR, MHC gene loci in NZB polymorphism and the occurrence of disease. This approach is done by studying the inheritance of NZB-VK1 polymorphism and its association with autoimmune manifestation in two recombinant inbred lines NZBx129/J and NZBxSM/J.

2) To determine whether there is a correlation between inbred mice carrying the V_K-Ef2^b haplotype and autoantibody production.

3) To sequence both V_K1 germline genes from liver of NZB mouse and a large panel of V_K1 rearranged genes from NZB hybridomas producing autoantibodies to determine whether genes that encode autoantibodies are derived from germline genes "unique" to NZB V_K1 or are derived randomly from all V_K1 germline genes. Study of structure of V_K1 genes will show evidence whether autoantibodies are encoded by unmutated germline genes, somatically mutated or both. This study will also permit the determination whether NZB mice have all V_K1 subgroups (V_K1A-V_K1E) present in Balb/c or just the V_K1B subgroup, as previously reported. Furthermore, sequencing autoantibodies will show if all V_K1 subgroups are expressed at random, or only one particular subgroup is utilized. This will determine if regulatory mechanisms are involved in preventing the expression

of V_K1A subgroup, or alternatively, if NZB mice do not possess the V_K1A subgroup in their germline repertoire.

II.

CORRELATION BETWEEN THE OCCURRENCE OF LUPUS NEPHRITIS, ANTI-ERYTHROCYTE AUTOANTIBODIES AND VK HAPLOTYPE IN NZBx129/J AND NZBxSM/J RECOMBINANT INBRED MURINE STRAINS

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Running title: Relationship Between V_K Haplotype and Autoimmune Disease in NZB Mice

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Abstract

The NZB mouse is genetically predisposed to the development of autoimmune disease that resembles the human autoimmune systemic lupus erythomatosus (SLE) and autoimmune hemolytic anemia, with increased titers of anti-DNA, and Coombs' autoantibodies. The various autoimmune traits are controlled separately by a limited number of genes. Genetic studies have shown that several immune loci are involved in autoimmunity: T-cell abnormalities, H-2 complex, and Ig genes have been implicated. In this paper, we present evidence for a significant correlation of NZB V_K1 haplotype defined by RFLP analysis with anti-erythrocyte autoantibodies in NZBx129/J and NZBxSM/J RI lines.

1 Introduction

NZB mice provide an excellent model of spontaneous autoimmune disease, with a high incidence of lupus glomerulonephritis and autoimmune hemolytic anemia (Howie and Helyer, 1968; Warner, 1973). Whereas lupus is associated with hypergammaglobulinemia and non organ-specific Abs (anti-DNA, anti-histones, anti-Sm and anti-RNA), autoimmune hemolytic anemia is associated with Coombs' Abs.

The genetic control of autoantibody production in NZB mice has been extensively investigated. It appears that IgM hyperglobulinemia is controlled by two loci Imh-1 and Imm-1 located on chromosome 4 (Manny et al., 1979; Hirose et al., 1984). The production of anti-DNA Abs is controlled by two unlinked genes in NZB (Ads-1 and Ads-2) (Shirai et al., 1987). These genes favor the age associated switching of IgM to IgG anti-DNA Abs in (NZB-NZW)F1 (Kohno et al., 1983). The production of anti-DNA Abs is associated with H-2 genes, and the Ads-1 gene is located on chromosome 17 (Shirai et al., 1987). Anti-E autoantibody production is controlled by three genes (Aia-1, Aem-1 and Aen-1) located on chromosome 4 (Ozaki et al., 1983). The appearance of severe lupus glomerulonephritis is under the control of three genes (Lpn1, 2, and 3) where Lpn 3 is most likely to be an Ig gene (Knight and Adams, 1981).

Based on this information, we conducted studies in our laboratory to determine whether there is a linkage between the occurrence of lupus nephritis and anti-E autoantibodies with some genes of the Ig gene superfamily, namely, V_K, V_H, $V\alpha$ of the

TCR and the I-A and I-E loci of MHC.

These studies have been carried out using NZBx129 and NZBxSM RI mice, since we determined that the haplotype V_K, V_K, TCR Vα and MHC genes of NZB (as shown by RFLP analysis) is different from 129/J and SM/J mice.

Our results show that V_K NZB haplotype was significantly associated with the occurrence of Coombs' Abs.

2 Materials and Methods

2.1 *Mice*

NZB, 129/J, SM/J mice and NZBx129/1, 2, 5, 10, 12, 16, 18 and NZBxSM/L, N, P, Q, W, X, Z RI strains were purchased from Jackson laboratories (Me). NZBx129 and NZBxSM RI strains were made by Dr. B. Taylor and Dr. E. Eicher (Jackson Labs), respectively. The genotypic and phenotypic characteristics of NZBxSM RI have been recently described (Eicher and Lee, in press).

2.2 *Measurement of Ig and autoantibody concentrations*

Concentration of IgK and Igλ was determined by RIA, using microtiter plates coated with various dilutions of serum and incubated with ^{125}I-rat monoclonal anti-murine K or λ Abs. Concentrations of anti-DNA and anti-histones Abs were determined by RIA, using plates coated with salmon sperm dsDNA, incubated with various dilutions of serum (1:10-1:1000) and consequently with ^{125}I-rat monoclonal anti-K antibody as previously

described (Fidanza et al., 1990). The concentration of antibodies was determined by using an interpolating program and standard curves constructed with chromatographically purified monoclonal antibodies: UPC10 (G2a/K) for IgK, HOPC1 (G2a/λ) for Igλ, HB2 for DNA. Coombs' antibodies were measured as previously described (Fidanza et al., 1990), and ZA1B8-1 a mAb of NZB origin was used as a positive control (Fidanza et al., 1990). The relative concentration of Coombs' Abs was expressed as log2 HA titer.

2.3 *Histopathology*

Kidneys were harvested from 1 year old mice, fixed in formalin, embedded in paraffin and sections were stained with Hematoxylin and eosin and periodic acid-Schiff (PAS). The pattern of glomerular injury was classified according to a modified World Health Organization of lupus nephritis (Churg and Sobin, 1982). The severity of the glomerular changes was graded from 0-3: 0: normal, +1: mild hyaline deposits and/or proliferation, +2: moderate hyaline deposits and/or proliferation and +3: severe hyaline deposits and/or proliferation. Detection of immune complexes was carried out on crystotat sections stained with fluorescinated rat anti-murine Abs. Measurement of proteinuria and hematuria was carried out by standard kits.

2.4 *Probes*

The V_K1 probe, generously provided by M. Fougereau (CNRS-Marseille), is a 330 bp Dde 1 fragment from LX1X27 cDNA. V_HS107 probe is a 213-bp Dde 1 fragment, isolated from a rearragement which was subcloned after addition of EcoR1 linkers. I-Aα

probe is a 1.3 kb genomic 3'-HindIII fragment in Gem4, and I-Eβ probe is a 1.0 kb cDNA fragment in EcoR1 site of PUC18, kindly provided by L. Glimcher (Harvard Medical School, Boston MA). Vα2 is a 400 bp fragment digested with EcorR1 and HindIII from PUC18, kindly given by S. Hedrick (USCD, San Diego CA). All probes were either nick translated or random primed with 750,000 cpm/ml.

2.5 *Nucleic acid*

Liver DNA was extracted and 10ug was digested with HindIII and ran on a Southern blot according to a previously described procedure (Berman et al., 1988). Filters were prehybridized for 1-2 hours and hybridized overnight with V_K1, V_HS107, Vα2, I-A or I-E probes. Filters were then washed three times, 15 minutes intervals in 2x SSC (0.3M NaCl, 30mM NaCitrate, 0.1% SDS) at 68°C, and three times, 15 minutes intervals in 0.2x SSC at 68°C. The blot was exposed to XAR film with intensifying screen for 5 days.

2.6 *Statistical analysis*

Data for NZBx129 and NZBxSM RI strains were analyzed separately. Six variables were used. An index of glomerulonephritis coded with integers 0-4 (for NZBx129) or 0-3 (NZBxSM) indicating increasing severity, and presence (coded 1) or absence (coded 0) of Coombs' Ab were considered independent variables. Each was modeled separately with four dependent variables, the haplotype at four loci: V_HS107, V_K1, Vα2, and H-2 (I-A and I-E). These were coded with 1=NZB and 0=129 (or SM). A logistic multiple

regression algorithm (SASR, PROC LOGIST (Hastings, 1986) was used to assess the ability of the haplotype at these loci to predict either the presence of Coombs' Ab or the severity of glomerulonephritis. Two groups of observations corresponding to the parent strains were included in each (NZBx129 and NZBxSM) analysis giving a total number of 9 sets.

3 Results

3.1 RFLP analysis of V_K, V_H, TCRα, and MHC class II genes of NZBx129 and NZBxSM RI murine strains

To correlate the occurrence of autoantibodies and autoimmune diseases with NZB haplotype, we used RFLP analysis to determine NZB origin of V_K1, V_HS107, TCRα and MHC class II genes in RI NZBx129 and NZBxSM mice.

V_K1: HindIII digested liver DNA hybridized with a V_K1 probe show 6 major bands of 0.9, 1.8, 3.0, 3.6, 7.9 and 9.0kb in NZB and 3.0, 6.2 and 11.1kb in both 129/J and SM/J. The data depicted in figure 1 show that NZBx129 RI-1, 5, 10, and 16 inherited the NZB RFLP pattern whereas NZBx129 RI-2, 12, and 18 have the 129/J RFLP pattern. In the case of NZBxSM RI mice, strains N, P, Q, W, and X have the NZB RFLP pattern whereas L inherited the SM pattern. NZBxSM RI-Z, probably represents a recombinant since it inherited the SM/J bands as well as a NZB 3.6Kb comigrating fragment.

V_HS107: Southern analysis of liver DNA from mice digested with HindIII and hybridized with a V_HS107 probe demonstrates that NZB has a different RFLP pattern than both 129/J and SM/J mice (Figure 2). The RI strains NZBx129-1, and 10 inherited the NZB RFLP pattern while RI-2, 5, 12, 16, and 18 inherited the 129/J RFLP pattern. RI strains NZBxSM-N, W, and Z show similar RFLP pattern as NZB and RI-L, P, Q, and X have

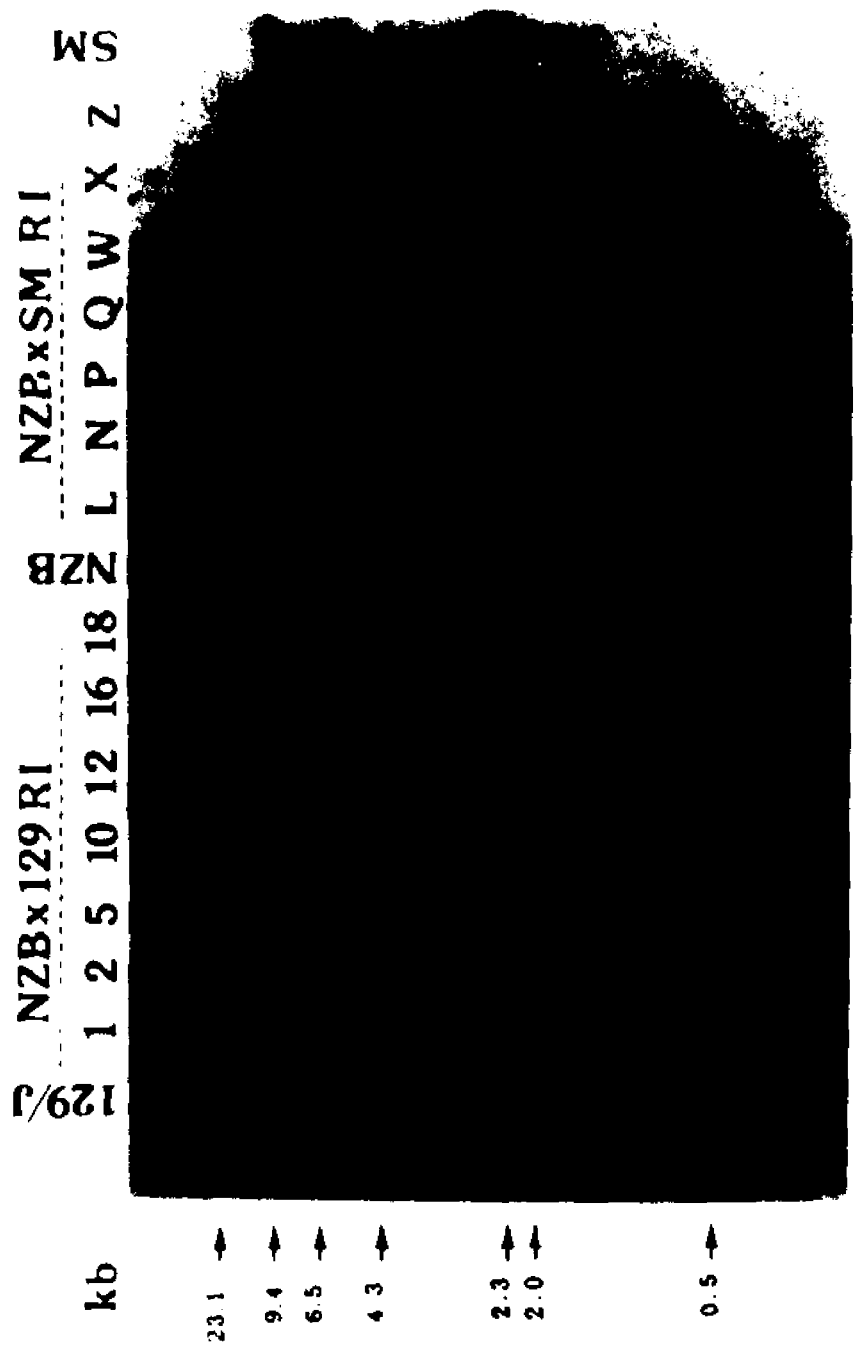

Figure 1
RFLP of V_k1 in NZBx129/J and NZBxSM/J RI strains

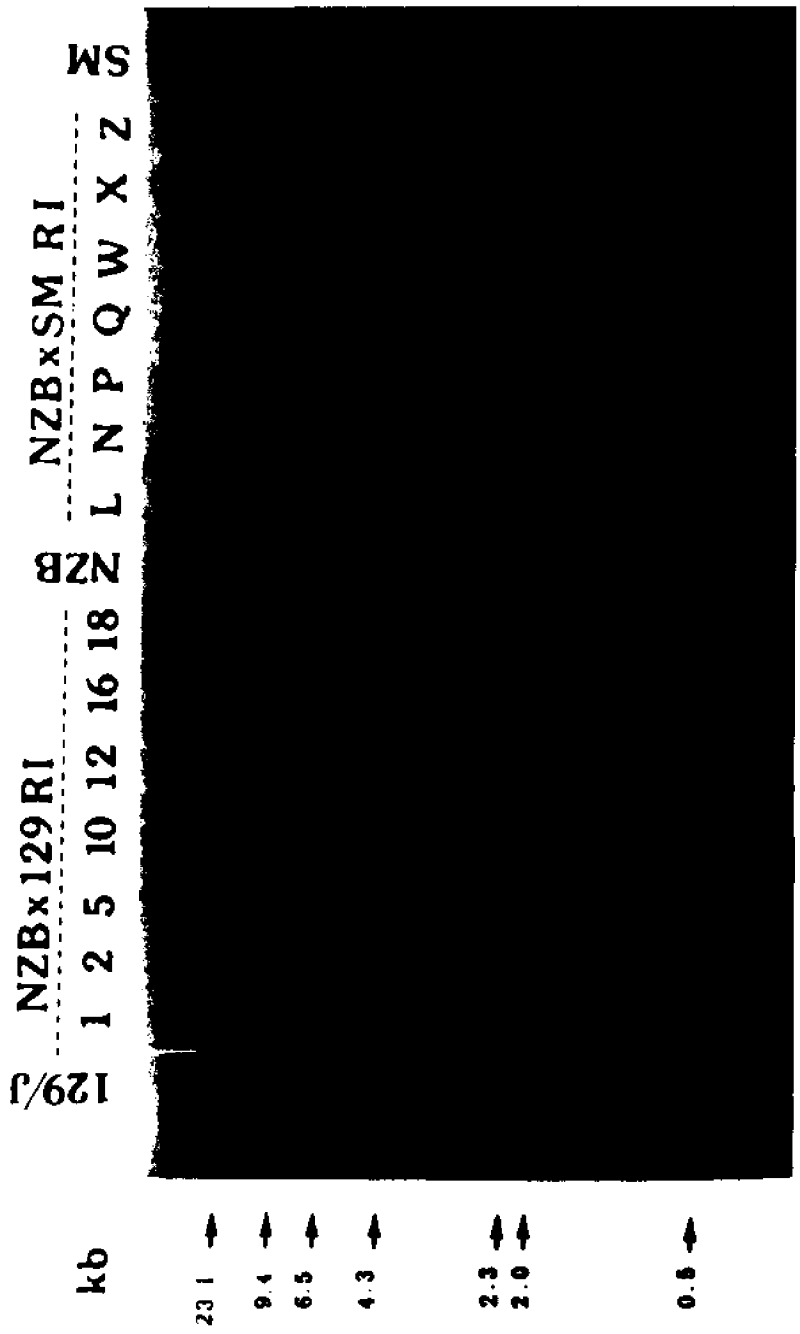

Figure 2
RFLP of V_HS107 in NZBx129/J and NZBxSM/J RI strains

the same RFLP pattern as SM/J strain.

Vα2: HindIII digested liver NZB DNA and probed with Vα2 show four major bands of 6.3, 3.8, 4.0 and 1.5kb, while 129/J RFLP pattern is different with an additional band of 1.7kb. SM/J shows three bands of 6.3, 3.8 and 1.5kb fragments. NZBx129 RI-2, 5, 16 and 18 show NZB RFLP pattern (apparent differences are of a quantitative nature), and strains 1, 10 and 12 inherited the 129/J pattern. The strains NZBxSM RI-L, P, W, X and Z show NZB RFLP pattern whereas strains N and Q show the SM pattern (Figure 3). No polymorphism was observed among NZB, 129/J, SM/J and RI strains when HindIII digested DNA was probed with various Vβ families (data not shown).

I-A and I-E: HindIII digested liver NZB DNA and probed with I-Eß show that NZB, 129/J and SM/J have different polymorphism. RI strains NZBx129-2, 16, 18, and NZBxSM-P and W have NZB RFLP pattern whereas NZBx129 RI-5, 10 and 12 are similar to 129/J and NZBxSM RI-L, N, Q and Z inherited the SM RFLP pattern (figure 4). The same results were obtained with I-Aα probe (data not shown). These data are in agreement with the H-2 haplotype determined by serological methods.

3.2 Production of autoantibodies in NZBx129 and NZBxSM RI murine strains

The presence of autoantibodies in NZBx129 and NZBxSM RI strains was measured in one year-old mice. As expected, NZB mice showed an increased level of IgK and Igλ, as well as anti-DNA autoantibodies. A significant titer of Coombs' Abs was also detected (Table I). 129/J and SM/J mice show low levels of anti-DNA, and no Coombs' Abs. SM/J, a strain known to produce spontaneously anti-thymocyte Abs (Eicher and Lee,

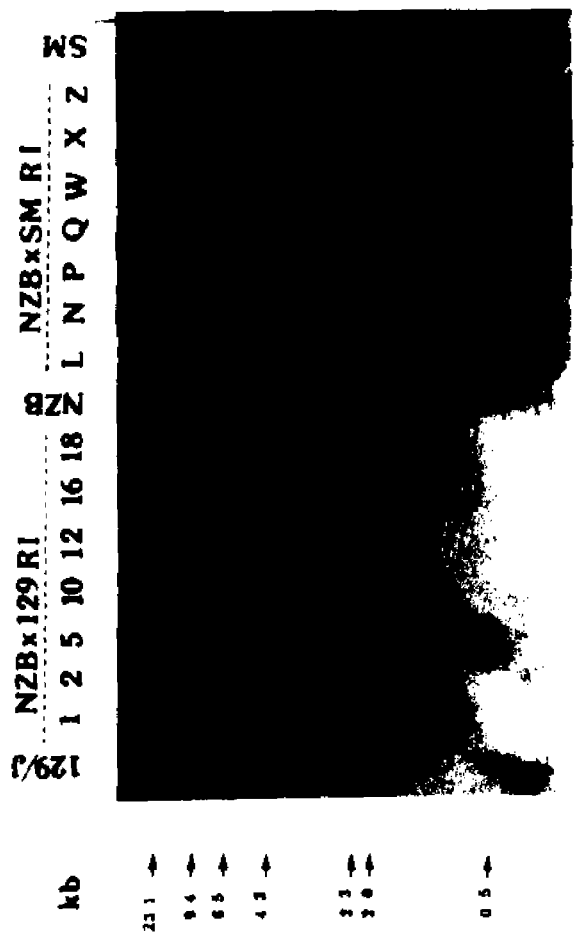

Figure 3
RFLP of T-cell receptor in NZBx129/J and NZBxSM/J RI strains

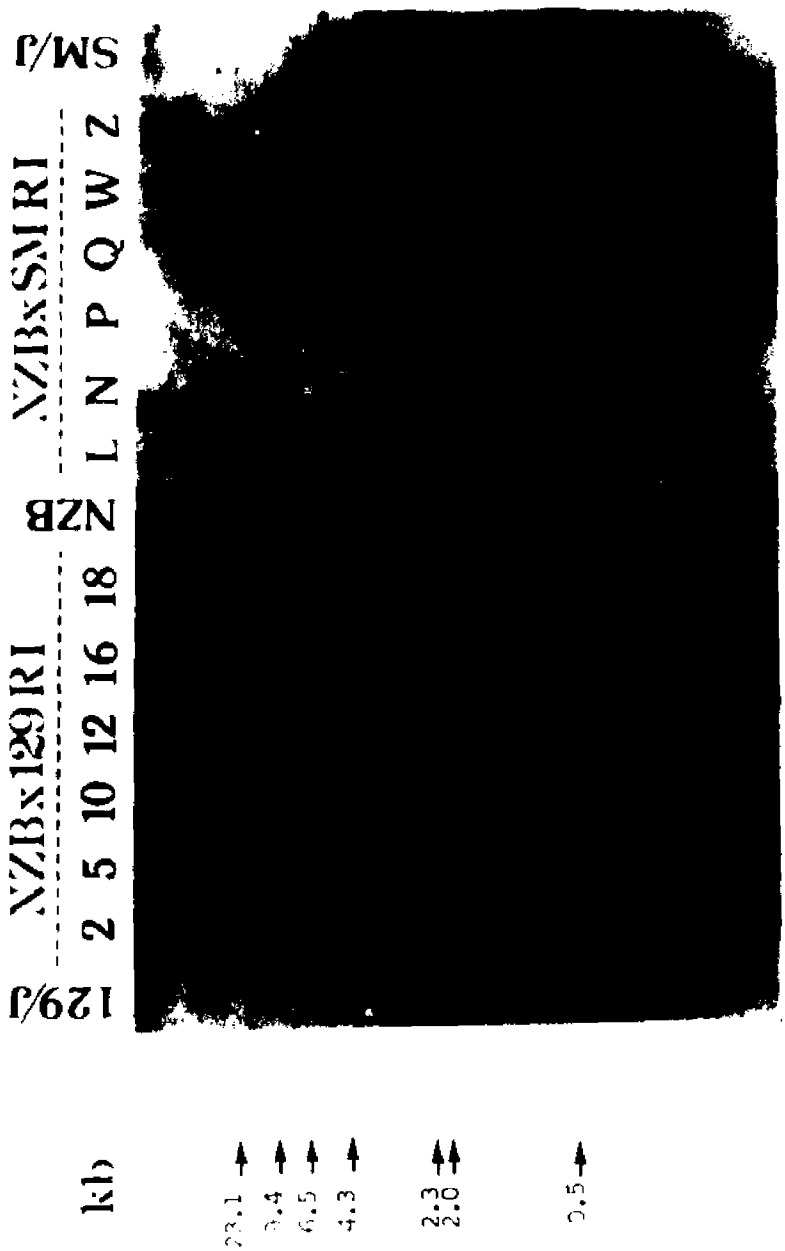

Figure 4
RFLP of MHC in NZBx129/J and NZBxSM/J RI strains

Table 1.

Immunoglobulins and autoantibody concentrations in NZBx129 and NZBxSM RI Strains.

Strain	IgK (mg/ml)	Igλ (ug/ml)	DNA (ug/ml)	Coombs (log2 HA)
129/J (5)	0.19* ±0.05	4.9 ±1.1	4.6 ±10.2	0
RI-1 (5)	0.34 ±0.1	2.9 ±1.3	95.4 ±35.7	2.2 ±1.3
RI-2 (4)	0.09 ±0.1	2.0 ±0.5	24.7 ±14.8	0
RI-5 (3)	0.20 ±0.03	7.0 ±1.3	22.0 ±15.0	1.0 ±1.4
RI-10 (4)	0.45 ±0.04	5.2 ±2.2	70.0 ±29.5	2.3 ±0.8
RI-12 (2)	0.13 ±0.04	3.0 ±0.0	20.0 ±5.6	0
RI-16 (2)	0.46 ±0.03	5.0 ±2.8	76.5 ±3.5	3.5 ±0.5
RI-18 (2)	0.14 ±0.02	3.3 ±0.3	21.5 ±2.1	0
NZB/J (11)	0.36 ±0.04	7.35 ±2.3	89.4 ±43.4	4.8 ±1.2
RI-L (3)	0.24 ±0.04	6.2 ±2.4	22.5 ±6.3	0
RI-N (3)	0.63 ±0.10	8.8 ±1.2	164.0 ±27.0	1.3 ±1.2
RI-P (4)	0.18 ±0.04	4.1 ±0.8	5.5 ±7.5	5.0 ±2.5
RI-Q (3)	0.24 ±0.02	3.9 ±1.5	25.0 ±9.1	3.6 ±2.3
RI-W (3)	0.24 ±0.03	7.3 ±1.5	54.0 ±18.0	0.6 ±0.4
RI-X (3)	0.30 ±0.05	4.4 ±0.9	134.0 ±24.0	1.3 ±1.5
RI-Z (3)	0.21 ±0.03	4.5 ±0.3	11.0 ±8.0	0
SM/J (5)	0.23 ±0.02	4.2 ±1.0	9.2 ±12.1	0

* = mean ± S.D. ( ) = number of mice studied

in press) show a high binding of serum Ig to thymocyte as compared to 129/J (data not shown) but no anti-DNA or Coombs' antibodies. The data depicted in Table 1 shows that NZBx129-1, 5, 10, 16 and NZBxSM-N, P, Q, W and X RI strains have increased amount of anti-DNA, and a significant titer of Coombs' autoantibody.

3.3 Occurrence of nephritis in NZBx129 and NZBxSM RI strains

The presence of nephritis in NZBx129 and NZBxSM RI murine strains was determined by measuring the proteinuria and hematuria and by determining histopathological lesions in the kidneys of one year-old mice. Histopathology was performed on two mice in each RI strain. Several hundred glomeruli on both H-E and PAS stained section were examined. The data presented in Table 2 shows that NZB mice, as well as NZBx129-1, 5, 10, 16 and NZBxSM-Q, N, P, W, X and Z exhibit lesions characteristic of acute glomerulonephritis. The patterns of glomerulonephritis were divided equally between diffuse and mesangial types. Diffuse prolerative glomerulonephritis consisted of variable diffuse endocapillary type proliferation usually accompanied by numerous hyaline deposits. Hyaline deposits were most numerous in the mesangium and contiguous subendothelial capillary wall forming wire loop lesions. Hyaline thrombi were present focally. Small, segmental areas of necrosis and neutrophils were infrequently identified within glomeruli. Crescents were infrequently observed. Hematoxylin bodies were not found. A few animals with severe glomerulonephritis showed parenchymal scarring with tubular atrophy. The histologic changes were classified as mesangial nephritis when there was widening of the mesangial stalk with increased cells, matrix or hyaline deposits and

Table 2.

Kidney histopathological alterations, proteinuria and hematuria in NZBx129 and NZBxSM RI strains.

Strain	Pattern	Severity	IC	Proteinuria (mg/dl)	Hematuria (RBC/λ)
NZB/J	diffuse proliferative	+3	+	50-300	5-10
129/J	normal	0	-	30	0
SM/J	mesangial	+1	-	30-100	0
Nx129-1	diffuse proliferative	+3	+	300-500	250
Nx129-2	normal	0	-	30	0
Nx129-5	diffuse proliferative	+2	+	50-300	10-50
Nx129-10	diffuse proliferative	+3	+	100	10
Nx129-12	normal	0	-	ND	ND
Nx129-16	mesangial	+1	±	30-100	50
Nx129-18	normal	0	-	30	0
NxSM-L	normal	0	-	30	0
NxSM-N	mesangial	+1	+	100-200	5-10
NxSM-P	mesengial	+1	+	30-100	0
NxSM-Q	mesangial	+1	+	30-100	5
NxSM-W	mesangial	+1	+	trace-30	0-50
NxSM-X	diffuse proliferative	+2	+	30-500	0-50
NxSM-Z	diffuse proliferative disease, scarring	+3	-	300	50

IC = immunocomplexes detected by immunofluorescence.
ND = not done

capillary wall extension was absent or small and rarely identified. Perivascular lymphoid aggregates were noted in some animals but interstitial nephritis was not present. Focal and pure membranous forms of glomerulonephritis were not encountered. Figure 5 shows a representation of mice with either normal or disease histopathology. Deposition of immunocomplexes in the glomeruli were identified by immunofluorescent staining in strains NZBx129 RI-1, 5, 10, 16 and NZBxSM RI-N, P, Q, W and X. Figure 6 shows a representation of a few strains with IC. No IC was detected by immunofluorescence in the rest of the animals tested (data not shown). The presence of glomerulonephritic lesions correlate with the presence of proteinuria and hematuria (Table 2). It should be mentioned that while 129/J mice do not exhibit any renal lesions, SM/J which develops high titers of anti-thymocyte autoantibodies as they age, show mild mesangial deposits, increased proteinuria but no hematuria.

3.4 Statistical analysis

The data for NZBx129 and NZBxSM RI strains were analyzed to assess the ability of genetic characteristics to predict the occurrence of autoimmune disease (see Materials and Methods). For both NZBx129 and NZBxSM RI mice, the presence of Coomb's Ab correlated perfectly with the NZB haplotype at the $V_{\kappa}1$ locus. Logistic regression yielded $p=0.0004$ and $p=0.0007$ for this association having occurred at random in NZBx129 and NZBxSM mice, respectively. There was no significant association of Coombs' Ab with haplotype at another locus.

The severity of glomerulonephritis was not significantly associated with haplotype at

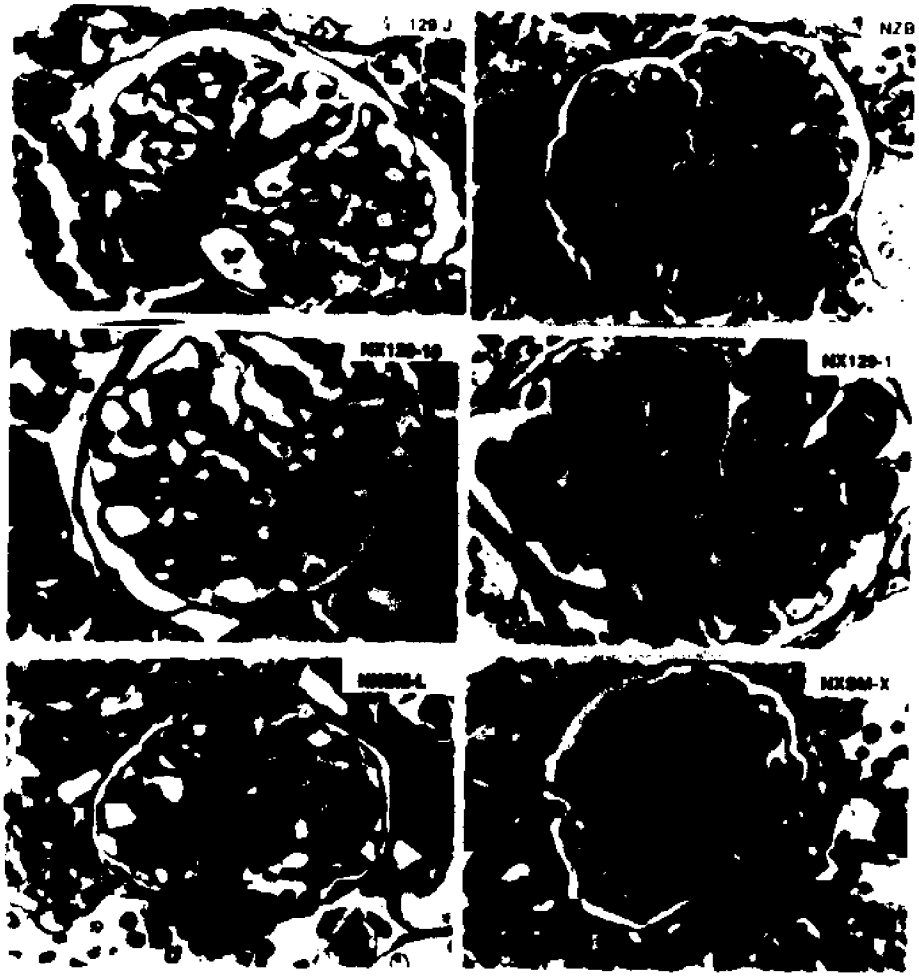

Figure 5
Kidney histopathology in NZBx129 and NZBxSM RI mice

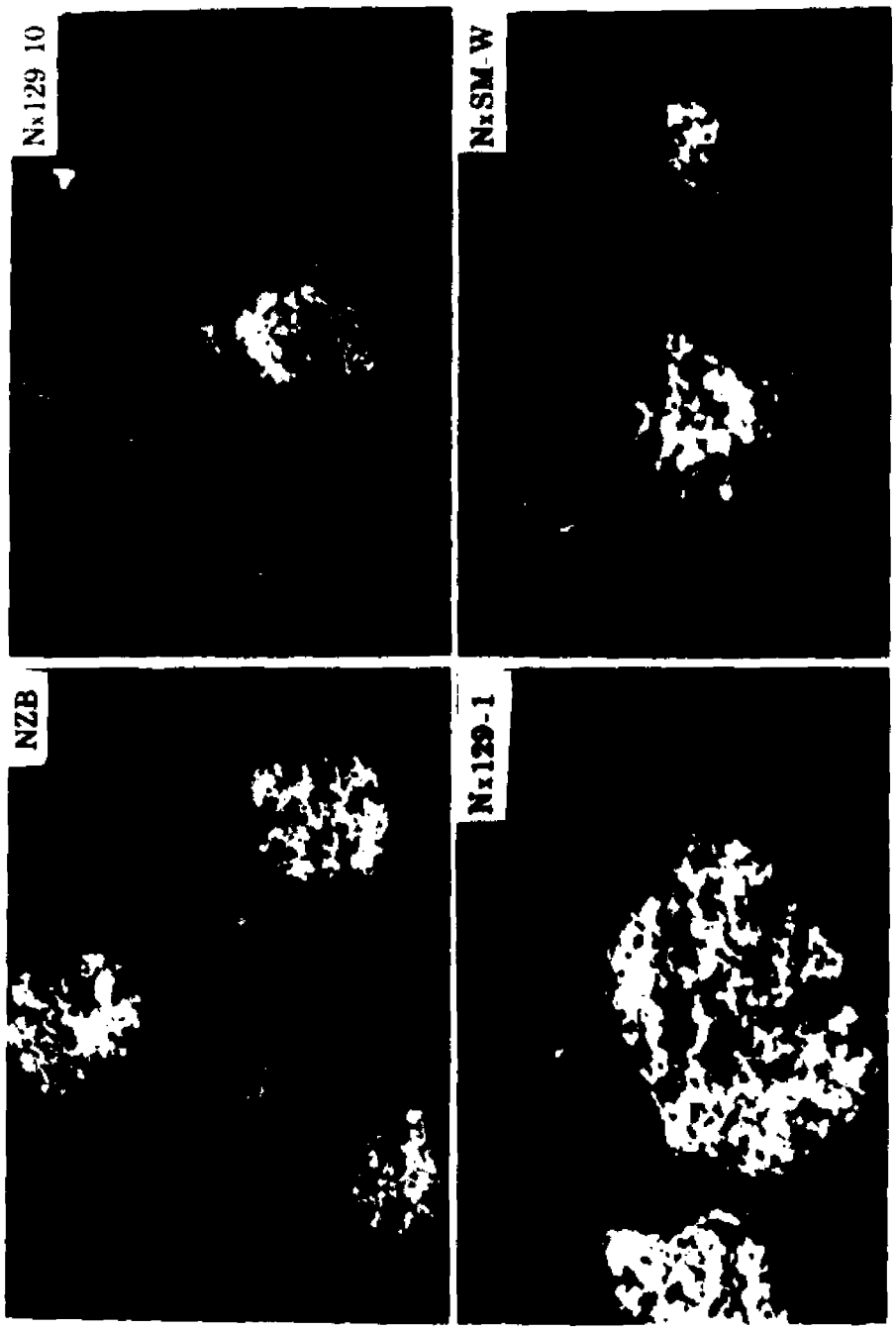

Figure 6
Immune complexes in NZBx129 and NZBxSM RI mice

Table 3.

Index of glomerulonephritis, Coombs' antibodies and V_K, V_H, V_α, I-A_α and I-E_α RFLP haplotypes in NZBx129 and NZBxSM RI strains.

			NZBx129 R1								NZBxSM R1						
	NZB	129	1	2	5	10	12	16	18	SM	L	N	P	Q	W	X	Z
GN	+3	0	+3	0	+2	+3	0	+1	0	+1	0	+1	+1	+1	+1	+2	+3
Coombs' Ab	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	+	-
V_HS107	B	9	B	9	9	B	9	9	9	S	S	B	S	S	B	S	B
V_K1	B	9	B	9	B	B	9	B	9	S	S	B	B	B	B	B	S
V_α2	B	9	9	B	B	9	9	B	B	S	B	S	B	S	B	B	B
H-2 (I-A_α and I-E_β)	B	9	9	B	9	9	9	B	B	S	S	S	B	S	B	S	S

GN: Glomerulonephritis
9:129/J
B:NZB
S:SM/J

any of the four loci examined in NZBxSM RI mice. Statistical analysis of NZBx129 mice suggested an association of glomerulonephritis with the NZB haplotype at the VK1 locus. The degree of correlation varied according to the order in which variables were added to the model, but p varied from <0.0001-0.0004 for any model including V_K alone or in combination with any or all of the other variables. We did not feel that more refined calculations were warranted.

4 Discussion

In this communication we present data demonstrating that the occurrence of autoimmune hemolytic anemia in NZBx129 and NZBxSM RI strains correlates better with a V_K1 NZB haplotype than wih V_HS107, VαTCR, I-A and I-E (MHC) NZB haplotype as determined by RFLP analysis. A summary table of this correlation is presented in Table 3.

Indeed, NZBx129-1, 5, 10, 16 and NZBXSM-N, P, Q, W and X which bear V_K1 NZB haplotype show high titers of Coomb's autoantibodies. These strains have 129/J or SM/J haplotypes of other loci (i.e. NZBx129-1 and 10 bear Vα and H-2, strain 5 has V_H and H-2, and strain 16 has V_H of 129/J haplotype, and NZBxSM-N bear Vα2 and H-2, strain P has V_H, Q has Vα2, V_H and H-2, and X strain bears V_H and H-2 of SM/J haplotype).

NZBx129 RI strains 1, 5, 10, and 16 developed typical glomerulonephritis lesions associated with deposition of immunocomplexes, autoantibodies and proteinuria and hematuria as they aged. Statistical analysis of NZBx129 RI strains suggested an

association of renal lesions with NZB haplotype at the V_K1 locus. In the case of NZBxSM RI strains, the occurrence of glomerulonephritis does not correlate to NZB haplotype of any loci studied. This can be related to mild nephritic lesions which develop in SM/J mice, a strain which spontaneously produce anti-thymocyte autoantibodies. However, it is interesting to mention that the strain NZBxSM RI-Z developed the most severe glomerulonephritis with no autoantibodies to the antigens tested in this study. RFLP analysis of HindIII liver DNA digest of Z strain shows SM/J pattern, in addition to a 3.6kb band similar to a NZB RFLP fragment (Figure 1). The gene cloned from the 3.6kb fragment was sequenced and was identical to the V_K1B germline gene isolated from NZB 1.8Kb band (Bailey et al., submitted). A recombination may have occurred in RI-Z strain either within the V_K locus or a linked gene.

Several explanations can be discussed concerning high significant correlation between the presence of Coomb's autoantibodies and V_K1 NZB haplotype in RI strains: First, VK1 locus is closely linked or in linkage disequilibrium to genes controlling the occurrence of Coomb's autoantibodies. Second, RI strains having chromosome 6 from NZB also inherited chromosome 4 of NZB where the genes controlling anti-E autoantibodies (Aia-1, aem-1 and Aew-1) are located (Kohno et al., 1983; Ozaki et al., 1983). These results are supported by data indicating that NZBx129 RI-1, 5, 10, 16 have Ly31, And-1, Anp-2, Gpu-1 and Xmmv-62 alleles of NZB whereas NZBx129-2 and 12 mice have the alleles of 129/J mice (Eicher and Lee, in press). Similarly, Mos, Galt, Mup-1 and Pgm-2 alleles of NZBxSM RI-N, Q, X and Z are of NZB origin and L and W are of SM/J origin. In the case of NZBxSM RI-P strain, only Pgm-2 is of NZB origin

(Eicher and Lee, in press). All these loci are located on chromosome 4 (Eicher and Lee, in press). Therefore, it is possible that a high correlation between V_K NZB haplotype and autoimmune hemolytic anemia represents a linkage disequilibrium between V_K locus and the loci controlling the occurrence of the disease in NZB. The lack of correlation between various loci containing genes of Ig supergene families and the occurrence of lupus nephritis can be related to multi-factorial origin of this disease. In fact, the genes controlling IgM hyperglobulinemia were located on chromosome 4 (Manny et al., 1979; Hirose et al., 1984), and those controlling anti-DNA antibodies are on chromosome 17 and 6 (Kohno et al., 1983; Shirai et al., 1987) and of nephritis on chromosome 6 (Knight and Adams, 1981). In our study there was no strain with identical V_K1 RFLP pattern to either 129/J or SM/J that developed the disease, but only one strain with NZB RFLP pattern showed only mild disease as SM/J (RI-P). In addition, there was complete segregation of Coombs' autoantibodies with V_K1 haplotype. NZBxC58 RI strains which also exhibit a B cell hyper- reactivity (Bocchieri et al., 1982) were not suitable for these studies since we found that NZB and C58/J display identical RFLP patterns when liver DNA was digested with 12 restriction enzymes and probed with V_K1 (Bailey and Bona, submitted) or a 5'-flanking V_K1 probe (Moynet et al., 1985).

Finally, it is possible that V_K locus of NZB mice and particularly V_K1 contain germline genes which are preferentially expressed in autoantibodies involved in the pathogenesis of NZB autoimmune diseases. This hypothesis is in agreement with data indicating that V_K1 encodes many autoantibodies (Schlomchik et al., 1986; Klotz et al., 1988; Fidanza et al., 1990). Similarly, in humans, it was shown that the V_KIIIb encoding the light chain

of rheumatoid factors are mainly derived from a unique germline gene namely, HumKv325 (Ledford et al., 1983).

Recently, we cloned and sequenced ten V_K1 germline genes from NZB (Bailey et al., submitted), and we are currently sequencing the V_K1 genes expressed in NZB hybridomas producing autoantibodies or devoid of self-reactivity to determine whether autoantibodies are derived from V_K1 germline genes present only in NZB. The identification of autoimmune genes can substantially contribute to our understanding the pathogenesis of autoimmune diseases and eventually to the development of new genetic-therapeutical approaches.

III.

STUDY OF SPONTANEOUS OCCURRENCE OF AUTOANTIBODIES IN VARIOUS INBRED MOUSE STRAINS SHARING THE EF-2^b HAPLOTYPE

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Running Title: Autoantibodies and the Ef-2^b Haplotype

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Abstract

We have shown in an earlier study that there is a high correlation between V_K1^b haplotype and the appearance of autoimmune disease in two recombinant inbred strains. We, therefore; extended this study to find out whether this holds true in strains of mice carrying the Ef-2^b haplotype which controls the V_K1A subgroup expression. Our results show that only 2 out of 5 mice with the Ef-2^b haplotype (NZB and C58/J) secrete autoantibodies as they age. Restriction fragment length polymorphism (RFLP) analysis reveals that these two strains belong to the V_K1^b haplotype.

1 Introduction

In a previous study carried out on NZBx129/J and NZBxSM/J recombinant inbred strains (RI), we investigated the association between V_H, V_K, TCR, and MHC gene with the occurrence of autoantibodies and the appearance of glomerulonephritis (Bailey et al., submitted). RFLP analysis of V_HS107, V_K1, Vα, I-A and I-E revealed that NZB, 129/J and SM/J mice belong to separate haplotypes. A significant association was observed between the presence of Coombs' autoantibodies and the inheritance of NZB V_K1 haplotype (V_K1^b) in NZBx129/J and NZBxSM/J RI strains. Furthermore, there was a good correlation between the inheritance of V_K1^b haplotype and glomerulonephritis in NZBx129/J RI strains (Bailey et al., submitted).

Compared to other murine inbred strains, V_K1 gene family is highly polymorphic in NZB mice (Kasturi et al., 1988; Bailey et al., submitted). Based on serological and amino acid sequence studies, V_K1 family is divided into five subgroups [V_K1A-V_K1E] (Hum et al., 1984). IEF analysis of V_K1 light chain showed differences between NZB and other murine strains (Hum et al., 1984). These differences have been attributed to the lack of expression of V_K1A subgroup in NZB mice sera. Mice lacking the V_K1A subgroup expression in their immunoglobulin light chain were given the Ef-2^b haplotype, whereas those that expressed this subgroup were given the Ef-2^a haplotype (Moynet et al., 1985). This polymorphism is under the control of Ef-2 locus located on chromosome 6 (Gibson and Maclean, 1979; Lazure et al., 1981). Since we have found a significant

correlation between V_K1^b haplotype and the appearance of autoimmune disease in NZBx129/J and NZBxSM/J RI strains (Bailey et al., submitted), we, therefore, studied the occurrence of autoantibodies in 1 year-old mice with the Ef-2^b haplotype (i.e. NZB, C58/J, BDP/J, CE/J and I/LnJ). Our results show that only NZB and C58/J secrete high titers of autoantibodies as they age. RFLP analysis show that only these two strains belong to the V_K1^b haplotype.

2 Materials and Methods

2.1 *Mice*

NZB/J, C58/J, BDP/J, CE/J and I/lnJ and P/J mice strains used in this study were purchased from Jackson laboratories (Me).

2.2 *V_K1 probe*

The probe is a generous gift from M. Fougereau (CNRS-Marseille). It is a 330bp Dde1 fragment from LX1X2 cDNA cloned into PBR322 and subcloned into PUC18.

2.3 *Nucleic acid*

Liver DNA was extracted and 10ug was digested with HindIII and ran on a Southern blot according to a previously described procedure (Berman et al., 1988). Filters were prehybridized for 1-2 hours and hybridized overnight with V_K1 probe at 750, 000cpm/ml. Filters were then washed three times, 15 minutes intervals in 2xSSC (o.3M NaCl, 30mM

NaCitrate, 0.1% SDS) at 68°C, and three times, 15 minutes intervals in 0.2xSSC at 68°C. The blot was exposed to XAR film with intensifying screen for 5 days.

2.4 *Measurement of Ig and autoantibody concentrations*

Concentration of IgK and Igλ was determined by RIA, using microtiter plates coated with various dilutions of serum and incubated with ^{125}I-rat monoclonal anti-murin K or λ Abs. Concentrations of anti-DNA and anti-histones Abs were determined by RIA, using plates coated with salmon sperm dsDNA, calf thymus histones (H1, H2b, and H4) incubated with various dilutions of serum (1:10-1:1,000) and consequently with ^{125}I-rat monoclonal anti-K antibody as previously described (Fidanza et al., 1990). The concentraton of antibodies was determined by using an interpolating program and standard curves constructed with chromatographically purified monoclonal antibodies: UPC10 (G2a/K) for IgK, HOPC1 (G2a/λ) for Igλ, HB2 for DNA, ZK2C8-1 for H1, ZL154-1 for H2b, and ZA2C2-6 for H4 (Fidanza et al., 1990). Coombs antibodies were measured as previously described (Fidanza et al., 1990), and ZA1B8-1 a monoclonal antibody of NZB origin was used as a positive control (Fidanza et al., 1990). The relative concentration of Coombs' Abs was expressed as log2 HA titer. Anti-thymocyte antibodies were determined by RIA according to a previously described procedure (Fidanza et al., 1990).

3 Results

3.1 RFLP pattern of V_K1 germline genes of Ef-2^b and Ef-2^a mice

The relationship between Ef-2^b and RFLP was studied by Southern blot analysis of HindIII digested DNA hybridized with V_K1 probe (Figures 7 and 8). Ef-2^b strains, BDP/J, P/J, I/LnJ and CE/J showed a RFLP pattern characteristic of Ef-2^a mice (Figure 8). Only NZB and C58/J mice displayed different V_K1 REF (restriction enzyme fragment) hybridizing bands. The only comigrating REF hybridizing band that appeared in all mice strains was the 3.0kb fragment (apparent differences are of a quantitative nature). These results show that there is no correlation between the IEF pattern seen in serum light chain Ig and RFLP analysis and is in agreement with other studies where DNA was digested with either EcoRI or BamHI and hybridized with a 5'-flanking V_K1 probe derived from V_K1A subgroup (Moynet et al., 1985). Since C58/J and NZB displayed identical V_K1 HindIII REF hybridizing bands, we used eleven restriction enzymes to examine possible allelic differences that can be detected by RFLP in these two murine strains. With each enzyme used, NZB and C58/J displayed similar REF hybridizing bands (Figure 9).

3.2 Production of autoantibodies in murine strains with the Ef2^b haplotype

The presence of autoantibodies was measured in 1 year-old mice (Table 4). As expected, NZB mice showed anti-DNA, anti-histones, anti-thymocyte and Coombs' autoantibodies.

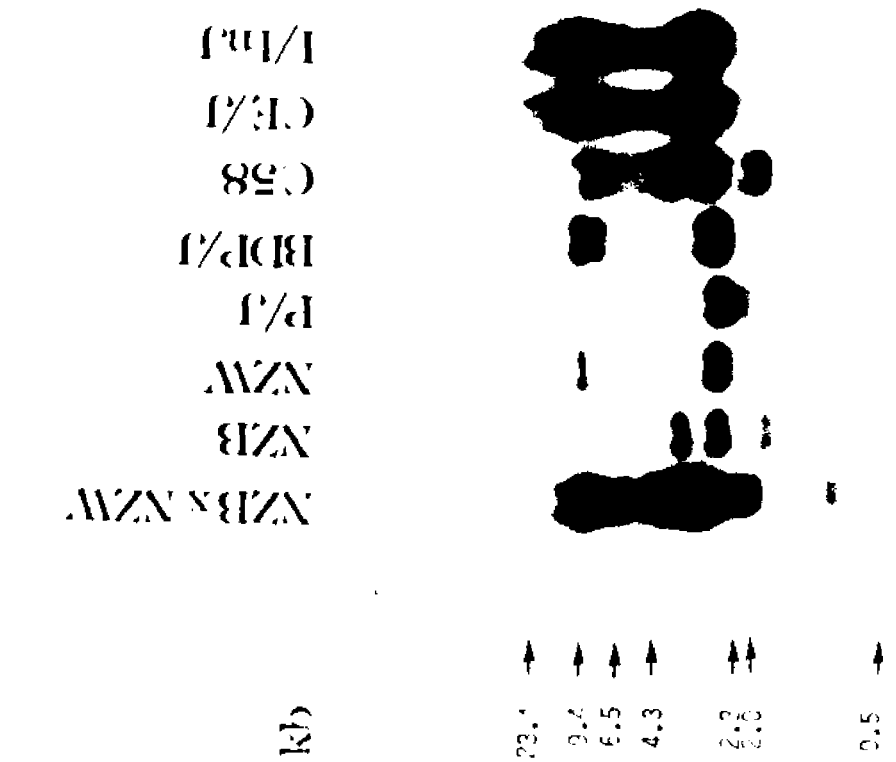

Figure 7
RFLP of V_k1 in mice sharing the Ef-2^b haplotype

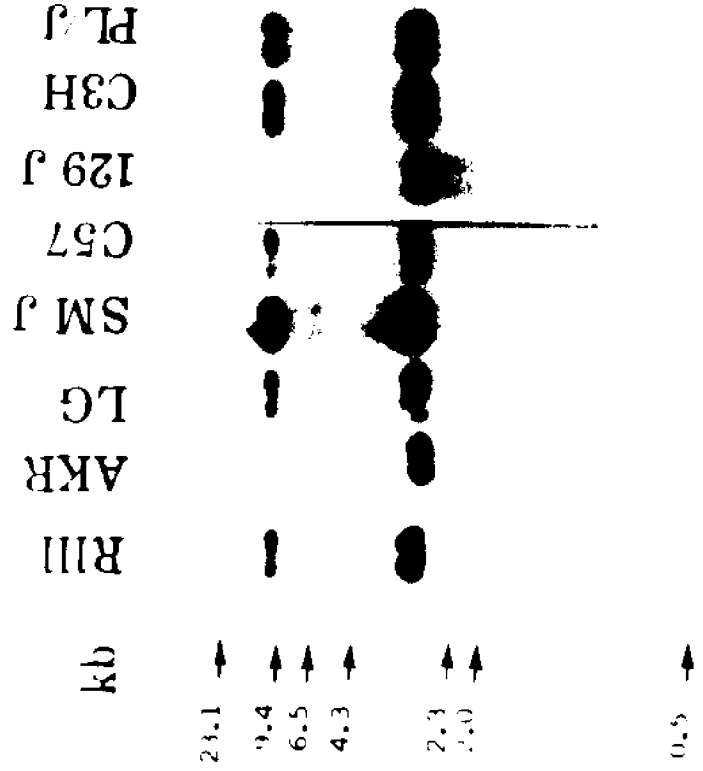

Figure 8
RFLP of V_K1 in mice sharing the Ef-2ᵃ haplotype

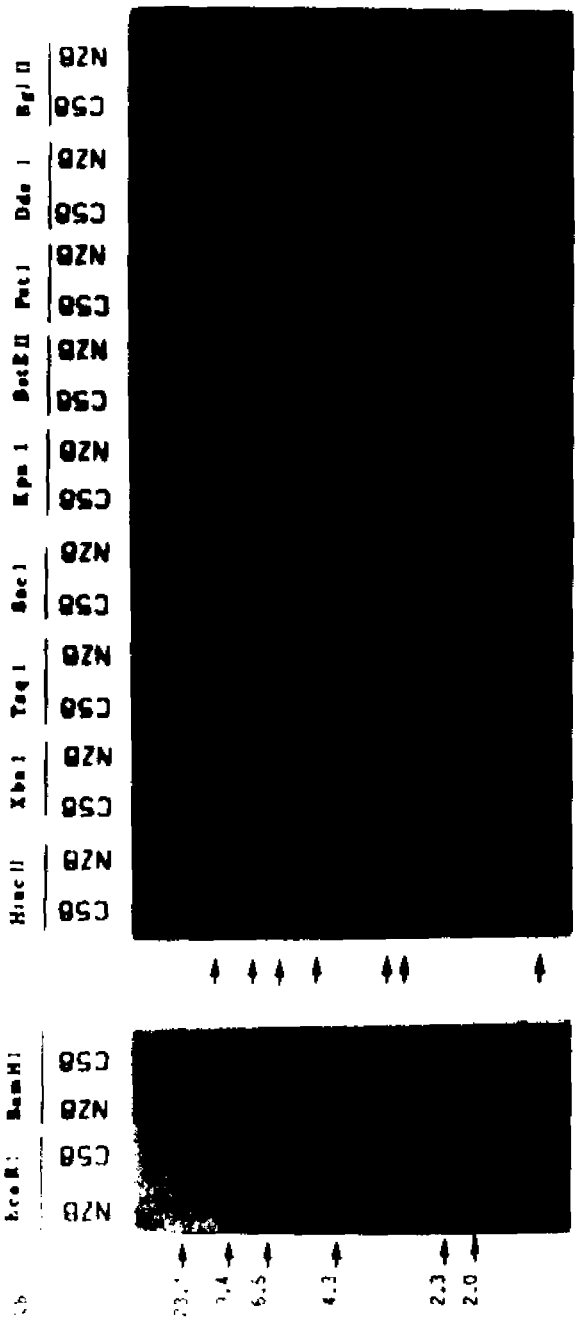

Figure 9

V_K1 polymorphism in NZB and C58/J mice with different restriction enzymes

Table 4.

Concentration of Immunoglobulins and autoantibodies in Mouse strains Bearing IgK-Ef2^a Haplotype.

Strain	Immunoglobulin		Autoantibodies					
	Kappa mg/ml	Lambda ug/ml	DNA ug/ml	H1 ug/ml	H2b ug/ml	H4 ug/ml	Coombs HA titer (log2)	Thymocytes (cpu)
NZB (11)	0.36±0.035	7.35±2.3	89.5 ±43.4	57.5 ±16.6	5.0 ±3.2	224 ±163	4.8±1.2	1,565±498
C58/J (8)	0.14±0.031	4.2±1.2	16.8 ±3.8	0	0	0	0	105±78
I/LnJ (2)	0.11±0.026	3.2±1.7	2±2	0.45 ±0.45	0	0	0	129±41
CE/J (2)	0.05±0.016	0.8±0.2	0	0	0	0	0	104±4
BDP/J (2)	0.07±0.015	0.75±0.2	0	0	0	0	0	185±18

( ) : number of mice studied. In these experiments the binding of allogenic anti-Thy 1.2 serum (1:25) to thymocytes was 5,612±381

There was also a high titer of kappa and lambda immunoglobulin indicative of hyperglobulinemia. C58/J showed elevated titer of anti-DNA autoantibodies with 16.8±3.8 ug/ml. There was no autoantibody secretion in either I/LnJ, CE/J or BDP/J.

4 Discussion

The data presented in this communication demonstrates that there is no correlation between the expression of V_K1A subgroup in serum light chains and RFLP analysis. Among 6 strains exhibiting the Ef-2^b haplotype, only C58/J and NZB mice displayed similar RFLP pattern when liver DNA was digested with HindIII and probed with V_K1 probe. Therefore, these results clearly demonstrate that the haplotype determined by IEF pattern does not correlate with RFLP defined haplotype.

Since we previously demonstrated that there is a high correlation between the V_K1^b haplotype and autoantibody production in NZB recombinant inbred lines (Bailey, et al., submitted), we attempted to determine whether this correlation holds true for the Ef-2^b haplotype since it controls the expression of a V_K1 subgroup (V_K1A). Our results show that hyperglobulinemia and spontaneous production of autoantibodies are not associated with the Ef-2^b haplotype. Only 2 out of 5 strains (i.e. NZB and C58/J) with this haplotype produced any autoantibody. Interestingly, these two strains carry the V_K1^b haplotype as defined by RFLP analysis.

C58/J mice develop leukemia and have a high titer of ectotropic and xenotropic viruses (Datta et al., 1982). Studies in NZB showed that they also have a high titer of xenotropic virus production (measured by the retroviral envelope gp70) which significantly

correlates with the appearance of anti-DNA autoantibodies and renal disease (Maruyama et al., 1980; Shirai et al., 1987). Furthermore, these authors showed in (NZBxNZW)F1 backcross studies that anti-DNA autoantibodies are controlled by at least four genes, two located on chromosome 17 and the other two are on chromosome 6. C58/J and NZB have the same V_K1 haplotype which is located on chromosome 6 but different H-2 (located on chromosome 17) haplotype (Datta et al., 1982; D'Hoosteleare et al., 1988); hence, this may account for the lower production of anti-DNA antibodies in C58/J compared to NZB mice. Alternatively, as the disease progresses in NZB mice a breakdown of tolerance could account for overt autoimmune disease which is absent in C58/J which does not reach that stage of the disease.

Bocchieri et al. (1982) have shown that many C58/J mice produce high levels of anti-thymocyte antibodies. This discrepency with our data could be due to our lower number of sampling compared to theirs (8 vs 25). Furthermore, in their studies, high anti-thymocyte antibodies was observed only in female mice.

No Coombs' antibody was observed in C58/J mice that would support our previous finding that Coombs' antibody correlates significantly with the V_K1^b haplotype of NZB (Bailey et al., submitted). This could be partly related to the low survival age of C58/J mice, which is about 12 months; or alternatively, the production of Coombs' antibodies in NZB is under the control of several unlinked gene loci which are not expressed in C58/J mice. Indeed, it has been shown by Kohno et al. (1983) and Ozaki et al. (1983) that at least three genes are necessary for the production of anti-erythrocyte autoantibodies. These genes (Aia-1, aem-1 and Aew-1) are located on chromosome 4.

NZB and C58/J have different Mup-1, Xmmv-62 and Gpd-1 alleles, all located on chromosome 4 (Taylor, 1988). Therefore, this may explain the lack of anti-erythrocyte antibody production in C58/J mice.

In conclusion, there are many genes involved in the production of autoantibody as previously reported (Maruyama et al., 1980; Raveche et al., 1981b). Each trait is under the control of separate loci. C58/J may carry some of the genes that are susceptible to autoantibody production (such as V_K1^b) but not enough to develop the disease. Identifying these loci is crucial in the understanding of autoimmune phenomena which occur in NZB mice.

IV.

COMPLEXITY OF THE IMMUNOGLOBULIN LIGHT CHAIN VK1 GENE FAMILY IN THE NEW ZEALAND BLACK MOUSE

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Abstract

The immunoglobulin V_K1 light chains are quite polymorphic in murine inbred strains, and are subdivided into five subgroups (V_K1A to V_K1E). The V_K1A subgroup contributes approximately 2% to the total light chain pool in serum immunoglobulin of many mouse strains, being absent in the serum of few strains, including New Zealand Black mice (NZB). Amino acid sequencing of myeloma proteins from these inbred mice has shown that they belong to the V_K1B subgroup. We report here the structure of nine functional germline genes from NZB mice that have high homologies to V_K1A, V_K1B, V_K1C and V_K1D subgroups. A novel germline gene representing the prototype of a new subgroup (designated V_K1F) was identified. Several V_K1 germline genes were isolated from a single RFLP fragment and identical genes were identified in two different RFLP migrating bands. Therefore, the complexity of the genes encoding the variable region of immunoglobulins cannot be determined solely by RFLP analysis. Sequence analysis of V_K1 genes expressed in 16 NZB hybridomas indicate that they originate from various V_K1 germline gene subgroups.

1 Introduction

Antibody specificity is encoded by rearranged V (variable) D (diversity) J (joining) genes which derive from 100-1000 V_H, 100-300 V_K and 3 Vλ germline genes (Cory et al., 1981; Brodeur and Riblet, 1984; Livant et al., 1986; Dildrop et al., 1987). Some V genes exhibit high degree of nucleotide and protein sequence homology which allowed their classification into V gene families (Brodeur and Riblet, 1984; Livant et al., 1986; Corbet et al., 1987; Dildrop et al., 1987; D'Hoosteleare et al., 1988; Kofler et al., 1989). Each V gene family is made up from one to several hundred closely related members [e.g. V_H24 has two members (Brodeur and Riblet, 1984) whereas V_HJ558 has more than one hundred (Livant et al., 1986)]. Based on germline gene polymorphism assessed by RFLP and hybridization with V_H or V_K gene probes, murine strains have been classified into six V_H (Brodeur and Riblet, 1984) and eight V_K (Kofler et al., 1989) gene haplotypes.

Early studies based on nucleotide sequencing, have shown that the V_K1 family in mice was composed of only two members (Corbet et al., 1987). However, later it has been shown by RFLP analysis that the V_K1 gene family displayed a higher degree of complexity consisting of four allelic groups among inbred mice strains (D'Hoosteleare et al., 1988; Kasturi et al., 1988). V_K1 light chains are highly represented in murine serum (Gibson and Maclean, 1979) and are frequently used by antibodies specific for foreign antigens such as DNP (dinitro-phenol), flagellin, GAT (glutamic

acid-alanine-tyrosine homopolymer), phosphoryl-choline (PC)(Jaffe et al., 1969; Smith et al., 1977; Tonnelle et al., 1983; Todd et al., 1984), self-reactive antibodies (Schlomchik et al., 1986; Bona, 1988), autoantibodies produced by NZB mice (Fidanza et al., 1990) and Balb/c myeloma proteins (Gibson, 1984). This large degree of representation is in agreement with high frequency of the expression of V_K1 family among LPS-induced B cell colonies in young and adult mice (Kaushik et al., 1988).

Based on serological and amino acid sequence studies, V_K1 light chains were classified into five subgroups (V_K1A-V_K1E) (Hum et al., 1984). Sequence analysis of NZB myeloma proteins indicated that they belong to a single V_K1 subgroup, namely VK1B (Loh et al., 1979). This was surprising since V_K1 gene family exhibits highest degree of polymorphism in NZB mice compared to other inbred strains as assessed by RFLP analysis (Kasturi et al., 1988).

The aim of this study was to investigate the structure and organization of V_K1 germline genes in NZB mice and to determine whether the V_K1 genes expressed in NZB hybridomas are derived from a restricted V_K1 subgroups.

2 Materials and Methods

2.1 *Mice*

All mice used in this study were purchased from Jackson laboratories (ME, USA).

2.2 *V_K1 probe*

The probe was a generous gift from M. Fougereau (CNRS-Marseille, France). It is a 330

bp dde I fragment from LX1X27 cDNA cloned into PBR322 and subcloned into PUC18.

2.3 *Southern blotting*

Liver DNA was extracted and 10 ug was separated on a 1% agarose gel and analyzed by Southern blotting for hybridization to V_K1 probe as previously described (Berman et al., 1988). Hybridization with oligonucleotide probes were done as previously described (Ucla et al., 1987). The filters were hybridized at 43° or 49°C when CDR2 and FR1 specific probes were used, respectively. The filters were washed with 1x SSC at the above temperatures.

2.4 *Genomic DNA cloning and sequencing*

Liver DNA was digested with HindIII and cloned into the HindIII site of Charon 21A. Ligation mixture was packaged and titered with a final plating efficiency of 300,000 plaques/ plate. Total of 2x106 PFU were plated. Plaques were transferred to nitrocellulose by Benton-Davis method (Benton and Davis, 1977) and filters were prehybridized and hybridized with VK1 probe. phage minipreps were prepared from all positive clones, and a Southern analysis of HindIII digested DNA was carried out as to clone the desired fragments. Fragments pertaining to sizes of 0.9, 1.8, 3.0, 3.6, 7.9 and 9.0Kb were further purified, and subcloned into PUC18 for further amplification. DNA from plasmid minipreps was cut with either Alu1 or HaeIII and subcloned into M13 for single strand sequencing using the dideoxy method (Sanger et al., 1977).

2.5 *Polymerase Chain Reaction*

Several of the V_K1 clones were amplified according to method of Innis et al. (Innis et al., 1988) with few modifications. Briefly, either 20ul of plaque or 5ul from phage minipreps was used for amplification, using 50pmol of 5'-primer, few nucleotides upstream of V_K1 FR1 (Framework 1) (5'-ccggatccattgtttcagcttccagc-3') and 0.5pmol of 3'-primer, several nucleotides downstream of V_K1 CDR3 (5'-ccggatccttgttagggtctgtatca-3'), and subjected for 35 cycles. The amplified product was purified using centricon 30 (Amicon), precipitated with 2M ammonium acetate and ethanol, washed and resuspended in 10ul of H2O. 40-60% of the fragment was sequenced according to the dideoxy method using the limiting primer (i.e. 3'-primer). This procedure was repeated using the reverse ratios of the primers to sequence from both directions. Fragments that failed to clone into Charon 21A were directly amplified from agarose gel. Briefly, genomic DNA was digested with HindIII and separated on 0.8% low melting agarose overnight. Fragments of size range 7.0-9.4Kb from NZB and 3.6kb band from recombinant inbred line NZBxSM/Z were electroeluted, phenol, ether extracted, precipitated and washed. 1 ug of DNA was amplified using 100pmol of each primer at 25 cycles. The amplified VK1 product was further separated on 5% acrylamide gel. Fragment of size 350 bp (amplified V_K1 gene) was electroeluted, precipitated, washed and resuspended in H_2O. 1ng of DNA was asymmetrically reamplified (i.e. 50:0.5pmol ratios of primers), purified through centricon 30, precipitated, washed and resuspended in 10ul of H_2O. 50% of this material was used for sequencing. To isolate the new germline gene corresponding to the V_K1F subgroup,

fragments corresponding to all REF V_K1 hybridizing bands from NZB were amplified using two sets of specific primers. The first set is a 5'-primer from FR1 region 22-42bp (5'-ccacttttcctgcatgtcagc-3'), and a 3'-primer from CDR3 277-297bp (5'-acgaaggttgcatgtgtaaat-3') specific for a V_K1 genes expressed in NZB hybridoma differing from all known subgroups. The second set of primers are the introns flanking FR1 and CDR3 of V_K1 gene (the same as above). The amplified product was separated on a 1.5% low melt agarose (LMA) and the 360bp fragment hybridizing with CDR2 specific oligonucleotide probe, was cut, electroeluted and subcloned into PUC. Plaques were screened using specific oligos corresponding to the CDR2 region of V_K1F gene (5'-aaacgaaattctggggtt-3') and FR1. Positive plaques were sequenced using the dideoxy method.

2.6 *cDNA cloning and sequencing*

RNA was prepared according to the guanidinium thiocyanate method (Maniatis et al., 1982). cDNA was made using as 3'-primer an oligo made of C_K upstream region (5'-ccggatcctgttaactgctcactgga-3'). DNA was amplified using the polymerase chain reaction with the C_K primer and a primer corresponding to the leader sequence of V_K1 gene (5'-ccggatccatgaagttgcctgttagg-3'). The amplified product was cut with BamH1, separated on a 1.5% LMA and a band of about 450bp was cut, electroeluted and subcloned into the BamH1 site of M13 for dideoxy sequencing.

2.7 *Hybridomas and antibody specificity*

All hybridomas were prepared according to a previously described procedure (Fidanza et al., 1990). Antibody specificity was also determined as previously described (Fidanza et al., 1990).

3 Results

3.1 Structure of NZB V_K1 germline genes

In order to understand the molecular basis of the restricted expression of V_K1B subgroup in NZB repertoire which does not correlate with the high complexity of V_K1 gene family in this strain as demonstrated by RFLP analysis, we studied the gene organization and structure of V_K1 germline genes from NZB mice. The V_K1 genes from a 3.0Kb HindIII fragment common to other murine strains and other fragments unique to NZB mice (Figure 10) were cloned for structural analysis.

3.2 Structure of V_K1 germline genes isolated from the 3.0kb REF hybridizing band

Five clones were identified from the 3.0 Kb band and sequenced, and three independent germline genes were obtained. These clones were NZ1.2, NZ3.1 and NZ1.1 (NZ1.1 clone was PCR amplified and sequenced). These clones differed from each other by few nucleotides (Figures 11a,b,c,d and Table 5). Sequence alignment with V_K1A germline gene sequence (Moynet et al., 1985), showed that they have an identical FR1 segment. In CDR1, three clones showed differences compared to V_K1A subgroup, with two amino

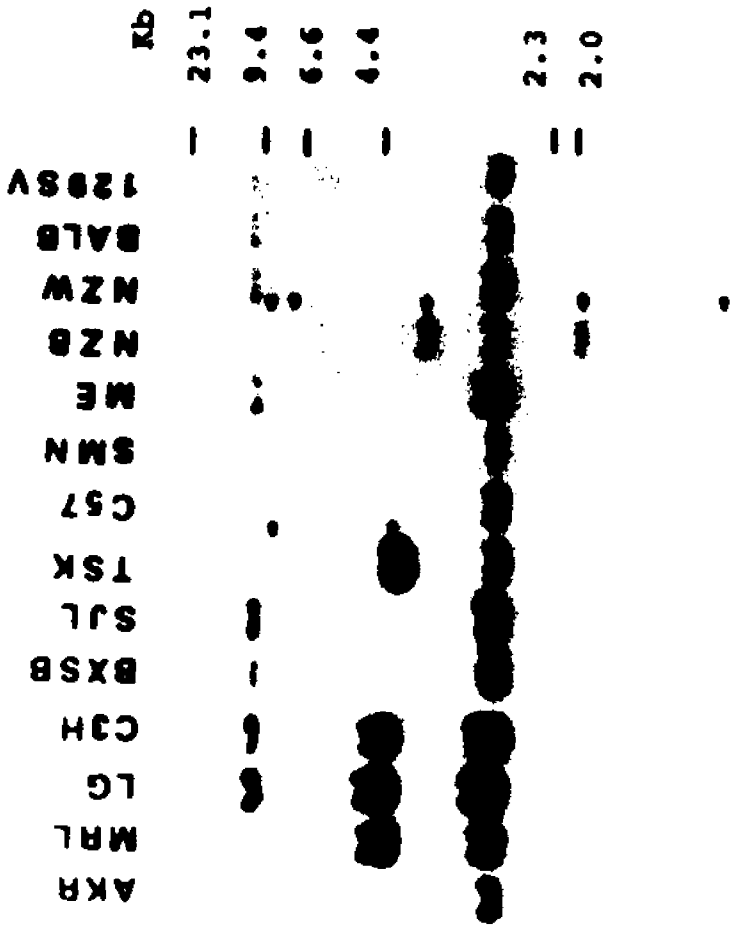

Figure 10
RFLP of V_K1 in various mouse strains

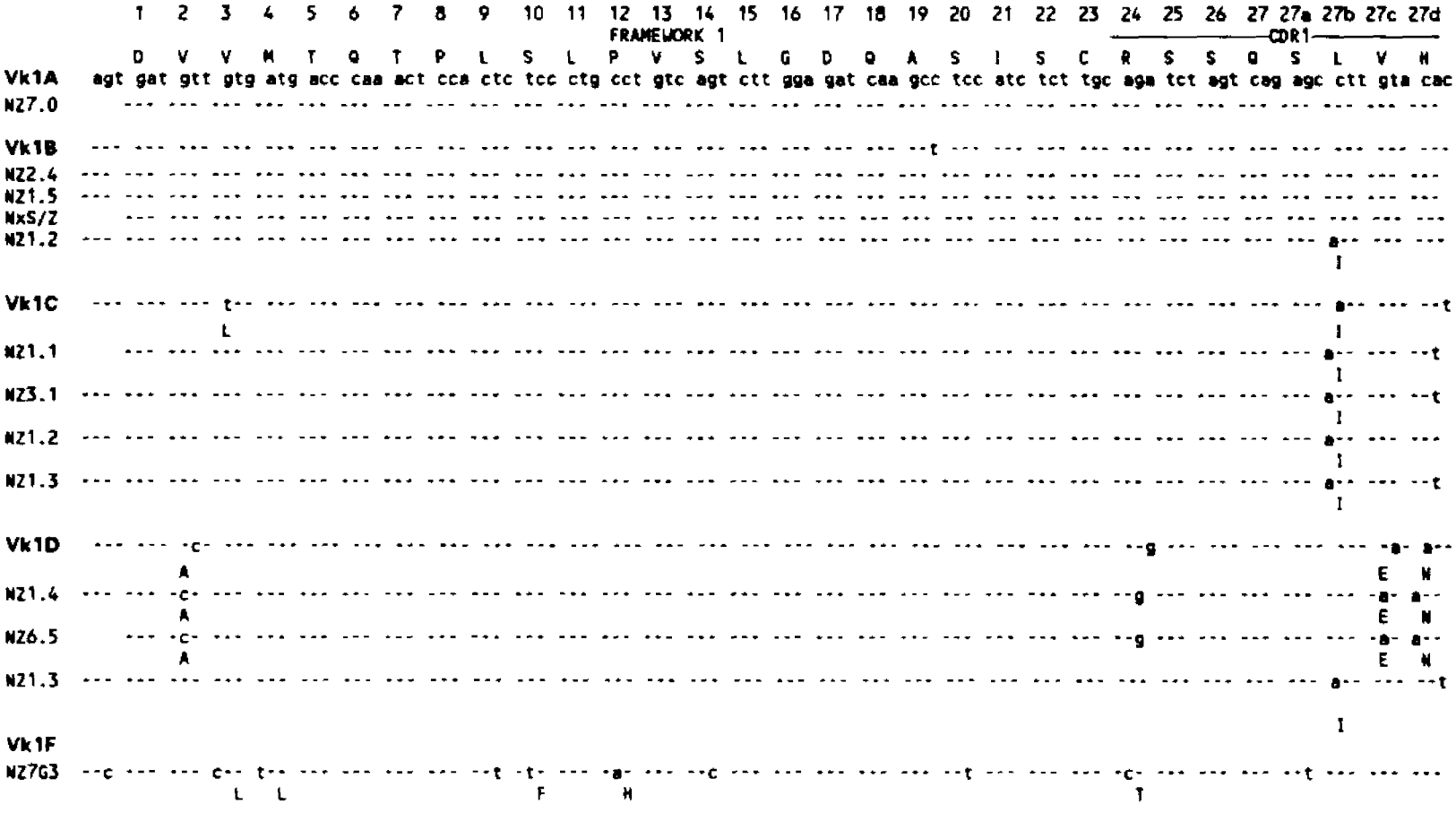

Figure 11a

NZB $V_\kappa 1$ germline genes

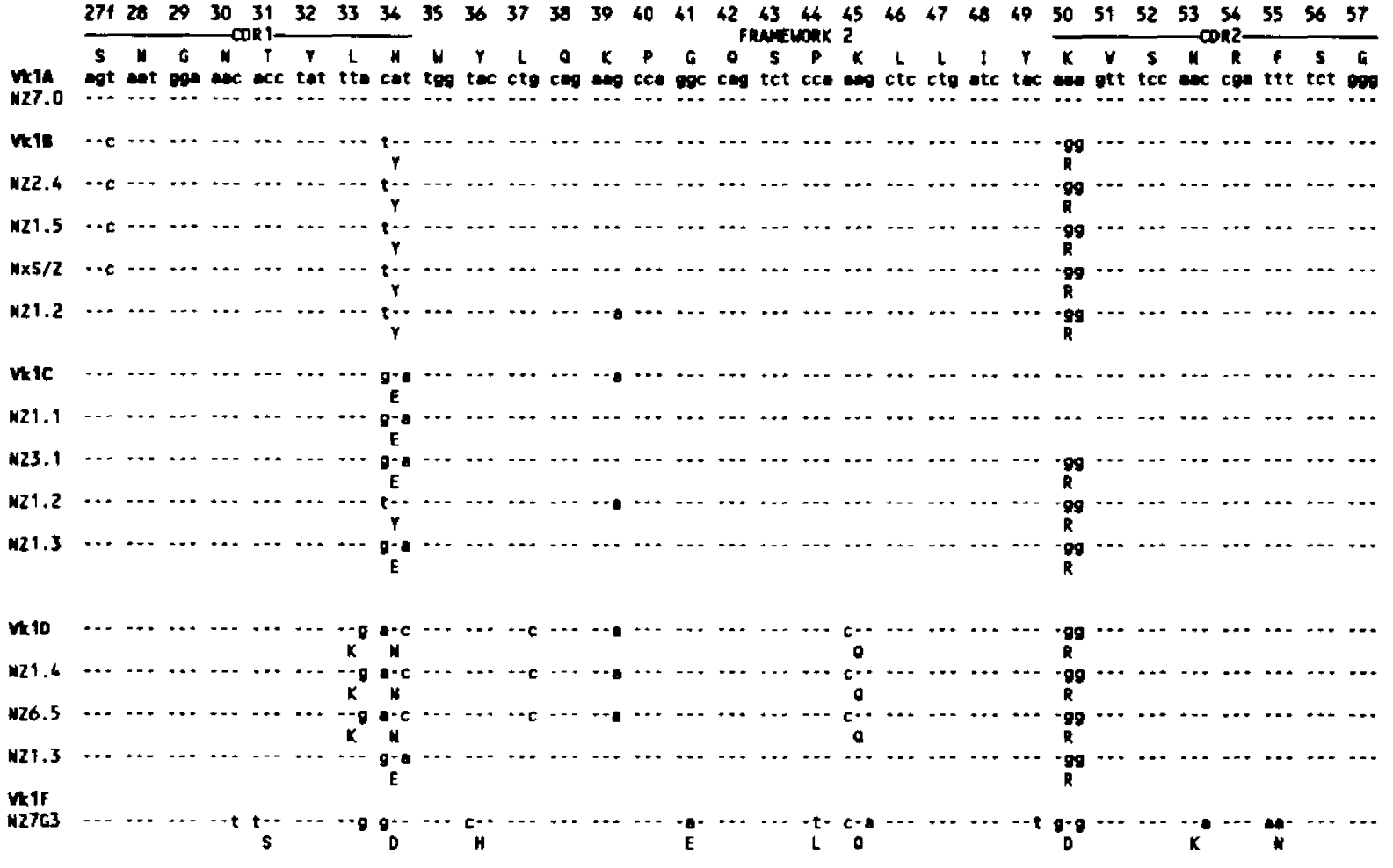
27f 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57
CDR1
FRAMEWORK 2
CDR2
S N G N T Y L H W Y L Q K P G Q S P K L L I Y K V S N R F S G
Vk1A agt aat gga aac acc tat tta cat tgg tac ctg cag aag cca ggc cag tct cca aag ctc ctg atc tac aaa gtt tcc aac cga ttt tct ggg
NZ7.0
Vk1B --c -gg
NZ2.4 --c -gg
NZ1.5 --c -gg
NxS/Z --c -gg
NZ1.2 -gg
Vk1C g-a
NZ1.1 g-a
NZ3.1 g-a -gg
NZ1.2 -gg
NZ1.3 -gg
Vk1D --g a-c c-- -gg
NZ1.4 --g a-c c-- -gg
NZ6.5 --g a-c c-- -gg
NZ1.3 g-a -gg
Vk1F
NZ7G3 t t-- --g g-- c-- -e- -t- c-a --t g-g --a aa-

Figure 11b

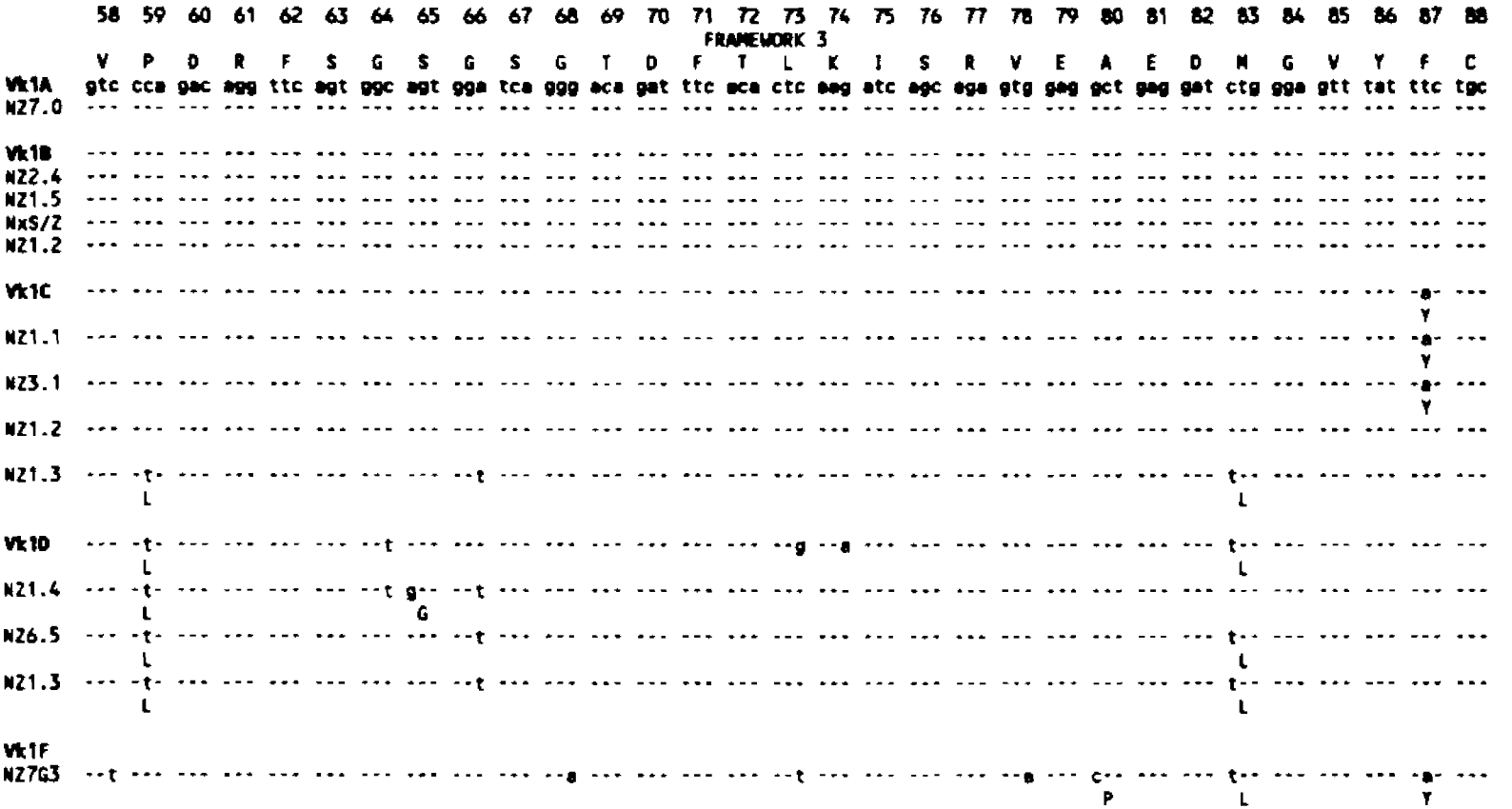
58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88
FRAMEWORK 3
V P D R F S G S G S G T D F T L K I S R V E A E D M G V Y F C
Vk1A gtc cca gac agg ttc agt ggc agt gga tca ggg aca gat ttc aca ctc aag atc agc aga gtg gag gct gag gat ctg gga gtt tat ttc tgc
NZ7.0
Vk1B
NZ2.4
NZ1.5
NxS/Z
NZ1.2
Vk1C
NZ1.1
NZ3.1
NZ1.2
NZ1.3
Vk1D
NZ1.4
NZ6.5
NZ1.3
Vk1F
NZ7G3

Figure 11c

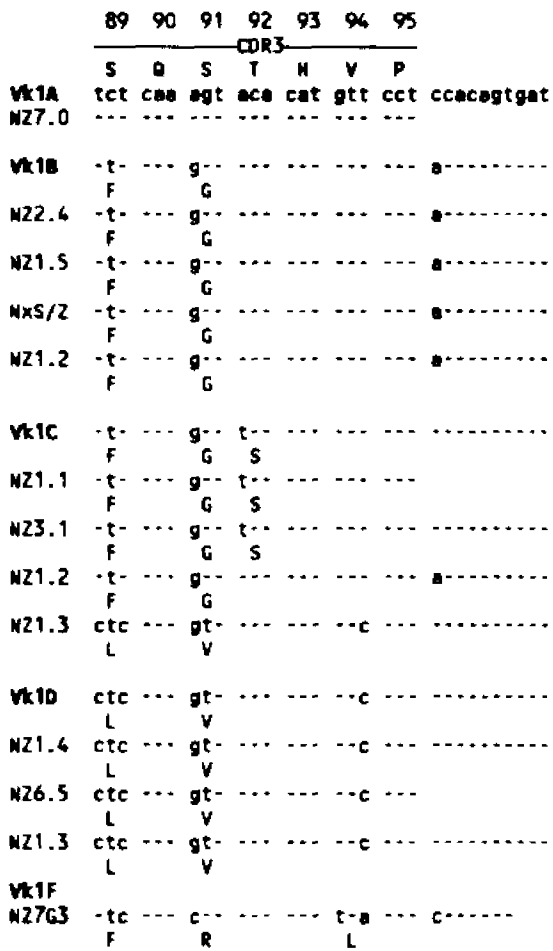
89 90 91 92 93 94 95
CDR3
S Q S T N V P
Vk1A tct cag agt aca cat gtt cct ccacagtgat
NZ7.0 --- --- --- --- --- --- ---
Vk1B -t- --- g-- --- --- --- --- a---------
F G
NZ2.4 -t- --- g-- --- --- --- --- a---------
F G
NZ1.5 -t- --- g-- --- --- --- --- a---------
F G
NxS/Z -t- --- g-- --- --- --- --- a---------
F G
NZ1.2 -t- --- g-- --- --- --- --- a---------
F G
Vk1C -t- --- g-- t-- --- --- --- ----------
F G S
NZ1.1 -t- --- g-- t-- --- --- ---
F G S
NZ3.1 -t- --- g-- t-- --- --- --- ----------
F G S
NZ1.2 -t- --- g-- --- --- --- --- a---------
F G
NZ1.3 ctc --- gt- --- --- --c --- ----------
L V
Vk1D ctc --- gt- --- --- --c --- ----------
L V
NZ1.4 ctc --- gt- --- --- --c --- ----------
L V
NZ6.5 ctc --- gt- --- --- --c ---
L V
NZ1.3 ctc --- gt- --- --- --c --- ----------
L V
Vk1F
NZ7G3 -tc --- c-- --- --- t-a --- c------
F R L

Figure 11d

Table 5.
Summary of comparison of V_K1B, C, D, F and NZB germline gene sequences with V_K1A germline gene.

	FR1	CDR1	FR2	CDR2	FR3	CDR3
V_K1A						
NZ7.0 (7.0-9.4Kb)						
V_K1B	1S:(19)	1S:(27f) 1R:H-Y(34)		1R:K-R(50)		2R:S-F(89) S-G(91)
NZ1.2 (3.0Kb)		2R:L-I(27b) H-Y(34)	1S:(39)	1R:K-R(50)		2R:S-F(89) S-G(91)
NZ2.4 (1.8Kb)		1S:(27f) 1R:H-Y(34)		1R:K-R(50)		2R:S-F(89) S-G(91)
NZ1.5 (0.9Kb)		1S:(27f) 1R:H-Y(34)		1R:K-R(50)		2R:S-F(89) S-G(91)
NxS/Z		1S:(27f) 1R:H-Y(34)		1R:K-R(50)		2R:S-F(89) S-G(91)
V_K1C	1R: V-L(3)	1S:(27d) 2R:L-I(27b) H-E(34)	1S:(39)			2R:S-F(89) S-G(91) T-S(92)
NZ3.1 (3.0Kb)		1S:(27d) 2R:L-I(29) H-E(34)		1R:K-R(50)	1R:F-Y(87)	3R:S-F(89) S-G(91) T-S(97)
NZ1.1 (3.0Kb)		1S:(27d) 2R:L-I(27b) H-E(34)			1R:F-Y(87)	3R:S-F(89) S-G(91) T-S(97
V_K1D	1R: V-A(2)	1S:(24) 4R:V-E(27c) H-N(27d) L-K(53) H-N(34)	2S:(37,39) 1R:K-Q(45)	1R: K-R(50)	3S:(64) (73) (74) 2R:P-L(50) H-L(83)	1S:(94) 2R:S-L(89) S-V(91)
NZ1.4 (3.6Kb)	1R: V-A(2)	1S:(24) 4R:V-E(27c) H-N(27d) L-K(53) H-N(34)	2S:(37,39) 1R:K-Q(45)	1R:K-R(50)	2S:(64) (66) 2R:P-L(59) S-G(65)	1S:(94) 2R:S-L(89) S-V(91)
NZ6.5 (3.6Kb)	1R: V-A(2)	1S:(24) 4R:V-E(27c) H-N(27d) L-K(53) H-N(34)	2S:(37,39) 1R:K-Q(45)	1R:K-R(50)	1S:(66) 2R: P-L(59) H-L(83)	1S:(94) 2R:S-L(89) S-V(91)
V_K1F						
NZ7G-3 (7.0-9.4Kb)	3S:(9, 14,20) 4R: V-L(3) M-L(4) S-F(10) P-H(12)	3S(27a,30,33) 3R:R-T(24) T-S(31) H-D(34)	1S:(49) 4R:Y-H(36) G-E(41) P-L(44) K-Q(45)	3R:K-D(50) N-K(53) F-N(55)	4S:(58, 68,73,78) 3R: A-P(80) M-L(83) F-Y(87)	3R:S-F(89) S-R(91) V-L(94)
V_K1C/V_K1D NZ1.3 (3.6Kb)		1S:(27d) 2R:L-I(27b) H-E(34)		1R:K-R(50)	1S:(66) 2R: P-L(59) M-L(83)	1S:(94) 2R:S-L(89) S-V(91)

R: amino acid replacement
S: silent substitution
(-): residue changed to
(#): codon position

acid replacement at positions 27b and 34. In FR2 one silent substitution (codons 39) was identified in NZ1.2. One replacement substitution (codon 50) was found in both NZ1.2 and NZ3.1 in CDR2. FR3 had one replacement substitution (codon 87) in NZ1.1 and NZ3.1 genes. In CDR3 two replacement substitutions (codons 89 and 91) were observed in all three clones and an additional one in NZ3.1 and NZ1.1 genes (codon 92). Based on the replacement substitutions the structure of NZ1.1 and NZ3.1 genes is closely related to Vk1C prototype (Ng et al., 1989). Clone NZ1.2 is similar to subgroup V_K1C up to position 30 and to V_K1B subgroup in the rest of the gene sequence.

These results clearly demonstrate that several gene copies can be isolated from a single REF hybridizing band and that these genes can be polymorphic. Furthermore, two of the three germline genes (NZ1.1 and NZ3.1) exhibit closest homology with V_K1C subgroup from which rearranged genes expressed in antibodies with various specificities are derived.

3.3 V_K1 germline genes isolated from unique REF hybridizing band of NZB DNA Hind III digest

Study of the structure of V_K1 germline genes isolated from NZB mice showed that they are quite polymorphic and exhibited high homology with various V_K1 subgroups. We isolated and characterized three identical clones from the 1.8kb fragment. The 1.8kb fragment hybridized very intensely with V_K1 probe compared to other fragments (Figure 10) suggesting that 1.8kb fragment may contain several gene copies of the same gene. The nucleotide sequence of this gene differs from V_K1A, C and D subgroups by a unique

replacement substitution in CDR1 (codon 34) and shares a replacement substitution in CDR3 (codon 91)(Figures 11a,b,c,d and Table 5). This gene, NZ2.4 exhibited high homology with NZ6.2 V_K1 gene isolated from 5.5Kb BamH1 fragment of NZB (Ng et al., 1989), differing only by a single silent replacement at position 19. The NZ2.4 gene shows highest homology to V_K1B subgroup.

From the 3.6Kb HindIII fragment of NZB, three distinct germline genes were isolated. Two genes, NZ1.4 and NZ6.5 exhibit the highest homology with V_K1D germline gene sharing four replacement substitutions (codons 27c,d, 33 and 34), and one silent substitution (codon 24) in CDR1, two replacement substitutions in CDR3 (codons 89 and 91), one amino acid replacement (codon 50) in CDR2, one substitution (codon 45) and two silent substitutions (codons 37 and 39) in FR2. The germline gene from clone NZ1.3 resembles V_K1C subgroup up to position 58, but the rest of the sequence is identical to NZ6.5 gene exhibiting highest homology with V_K1D subgroup.

Since we failed to clone the 7.9 and 9.0kb fragments, we PCR amplified the region spanning from 7.0 to 9.4kb. This was done by separating NZB liver HindIII DNA digest on a low melting agarose (described in Materials and Methods). The gene isolated from this region was identical to V_K1A germline gene isolated from Balb/c mice.

One clone from the 0.9Kb fragment was isolated, NZ1.5, and the sequence was identical to the NZ2.4 clone which is found in the 1.8Kb fragment.

Studies of V_K1 RFLP polymorphism of recombinant inbred NZBxSM mice showed that strain NZBxSM/Z had a possible recombination in the V_K locus or within a linked gene. This strain showed a similar pattern of HindIII DNA digest to SM/J but an additional

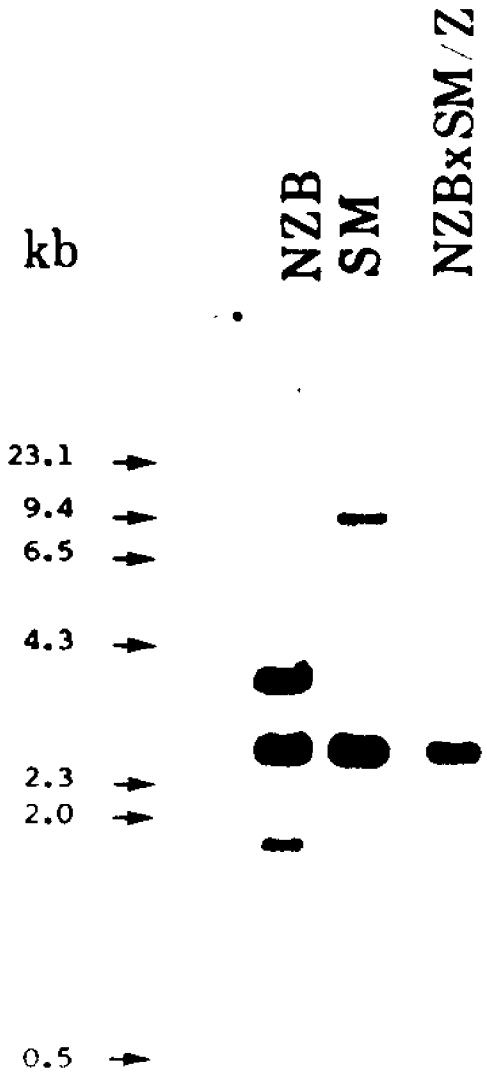

Figure 12
RFLP of V_K1 in NZB, SM and NZBxSM/Z RI strains

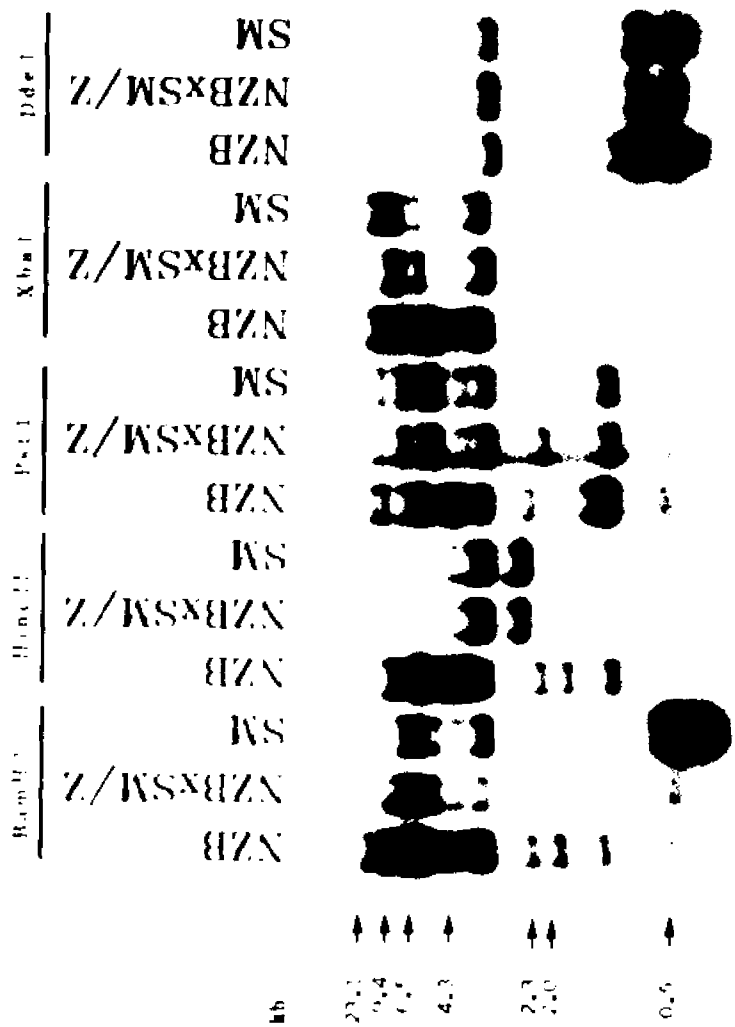

Figure 13
RFLP of V_k1 in NZB, SM and NZBxSM/Z RI strains with different restriction enzymes

band of 3.6Kb similar in size to that in NZB DNA (Figure 12). To confirm this, DNA was digested with several enzymes and probed with V_K1 gene. Figure 13 shows that NZBxSM/Z inherited the SM/J RFLP pattern; however, BamH1 8.4kb REF hybridizing band in Z comigrated with a NZB V_K1 band. Furthermore, a new restriction site in Z gave a Pst1 REF hybridizing band of 1.9kb that was not present in either NZB or SM/J. The gene from the NZBxSM/Z HindIII 3.6kb REF band was isolated by low melting agarose, PCR amplified and sequenced (details are in Materials and Methods). Comparison of NxS/Z germline gene isolated from the 3.6Kb fragment of the Z recombinant mouse with NZB germline genes showed that it is identical to NZ2.4 isolated from the 1.8Kb fragment of NZB (V_K1B subgroup) and is different from genes isolated from the 3.6Kb fragments (Figures 11a,b,c,d).

3.4 Identification of a new V_K1 subgroup germline gene

The nucleotide sequence of V_K1 genes coding two NZB autoantibodies ZK8E8-1 and ZK8E8-8 showed considerable differences from the four known V_K1 subgroups both in FR and CDR regions. These differences appear to be due to random substitutions (FR1-4 replacement substitutions and 3 silent substitutions; CDR1-3 replacement substitutions and 2 silent substitutions; FR2-3 replacement substitutions and 1 silent substitution; CDR3-3 replacement substitutions; FR3-1 replacement substitution and 3 silent substitutions; CDR3-2 replacement substitutions) suggesting that these rearranged genes might be derived from a distinct V_K1 subgroup. To confirm this and to identify the new germline gene, first, we hybridized genomic DNA Southern blots with specific

oligonucleotide probes for FR1 and CDR3 (see Materials and Methods) of the V_K1 genes encoding these antibodies. Unfortunately, we did not detect any specific hybridizing band. We, therefore, PCR amplified the DNA fragments extracted from NZB HindIII using two sets of primers: first- primers specific for FR1 and CDR3 of cDNA clones; second- primers specific for introns flanking V_K1 genes (see Materials and Methods). The amplified DNA was dot blotted and hybridized with CDR2 probe specific for the new gene. Only the DNA amplified from the 7.0-9.4Kb region hybridized with this oligo. Southern analysis confirmed the above results. Either a 280bp or a 360bp band hybridized when first and second set of primers were used for amplification, respectively (data not shown). The DNA amplified from the region spanning from 7.0-9.4Kb fragment using the second set of primers was cloned into the BamH1 site of PUC. The recombinant clones hybridizing with both FR1 and CDR2 specific oligonucleotide probes were selected for sequence analysis. About 4% of recombinants hybridized with both oligo probes. The small percentage of positive clones could be related the amplification of unrelated genes under the conditions used in PCR amplification. The 280bp amplified band (using the first set of oligo primers) represents less than 10% of the total amplified DNA (data not shown). The germline gene, NZ7G-3 (Figures 11a,b,c,d and Table 5), isolated represents the new V_K1 subgroup (V_K1F). This novel gene has 89.3% nucleotide homology with V_K1A subgroup and lower homology with other V_K1 subgroups (GenBank database).

3.5 NZB hybridomas are derived from various V_K1 germline gene subgroups

We previously showed that a high percentage of hybridomas obtained from 6 day, 1, 3 and 16 month-old NZB mice use V_K1 genes (Fidanza et al., 1990). Since a high representation of V_K1B subgroup appears in NZB myelomas, we examined whether V_K1 expressed in B cells producing antibodies with various specificities are also restricted. The sequence analysis of V_K1 genes expressed in 16 NZB hybrisomas show that they are derived from various V_K1 germline gene subgroups and are associated with J_K1, 2, or 4 genes (Table 6). Inspection of these sequences reveals that V_K1 genes expressed in our panel of NZB hybridomas use unmutated germline gene sequences (Figure 14a-f).

4. Discussion

Study of V_K1 gene family in NZB mice showed a high polymorphism of germline genes belonging to this family as well as phenotypic differences of gene products compared to other mouse strains (Moynet et al., 1985; Kasturi et al., 1988). However, based on the sequence of NZB myeloma proteins, it was established that the majority of NZB V_K1 immunoglobulins belonged to V_K1B subgroup (Loh et al., 1979; Lazure et al., 1981).

To understand the complexity of V_K1 germline genes in NZB, we sequenced V_K1 germline genes from NZB liver DNA digested with HindIII. From the 3.0kb fragment common to various murine strains, we isolated three different germline genes. The sequence of two germline genes (NZ1.1 and NZ3.1) showed highest homology with

Table 6.

Origin, specificity, V_K1 subgroup and J_K gene expressed in NZB hybridomas.

Designation	Origin	Specificity	V_K1 subgroup	J_K
A9A7	6 day-old	multispecific	B	4
A11G4		multispecific	B	4
B13D4		multispecific	B/D	2
B10B9		multispecific	C	2
ZK2H5-3	1 month-old	multispecific	B	1
ZK2A8-1		multispecific	A	1
ZK9C5-7		multispecific	A	1
ZK5F11-3		dsDNA	C	1
ZK3G11-1		unknown	A	2
ZK9A4-10		dsDNA	A	1
ZK2A7-3		dsDNA	B	1
ZK9B9-8		TNP	A	1
ZK8E8-8		multispecific	F	1
ZA4C3-1	16 month-old	multispecific	B	4
ZA4C3-7		unknown	B	4
ZA2C2-6		multispecific	C	4

Origin and antigen specificity of hybridomas have been previously reported (Fidanza et al., 1990)

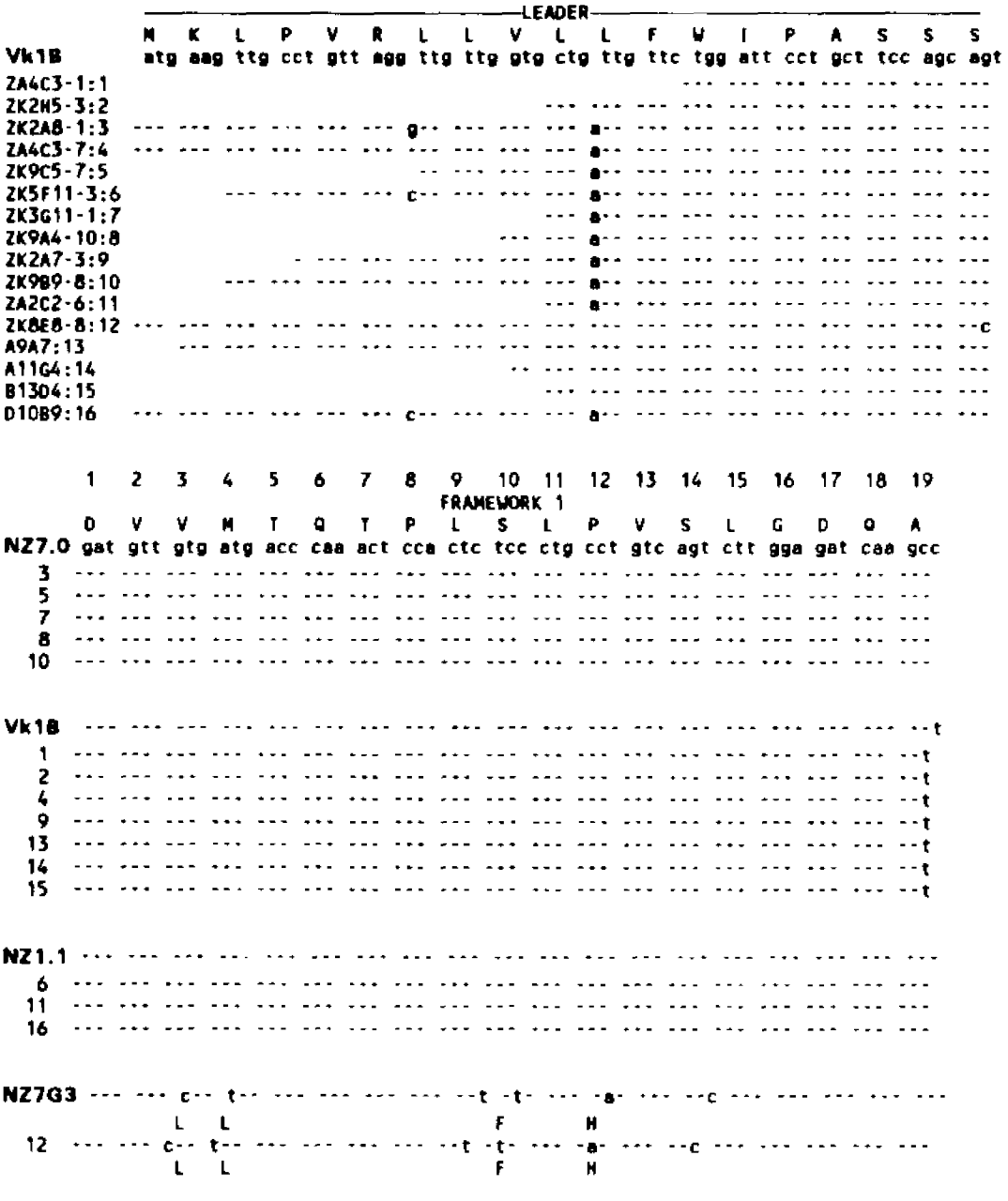

Figure 14a

Sequences of V_x1 antibodies from NZB hybridomas

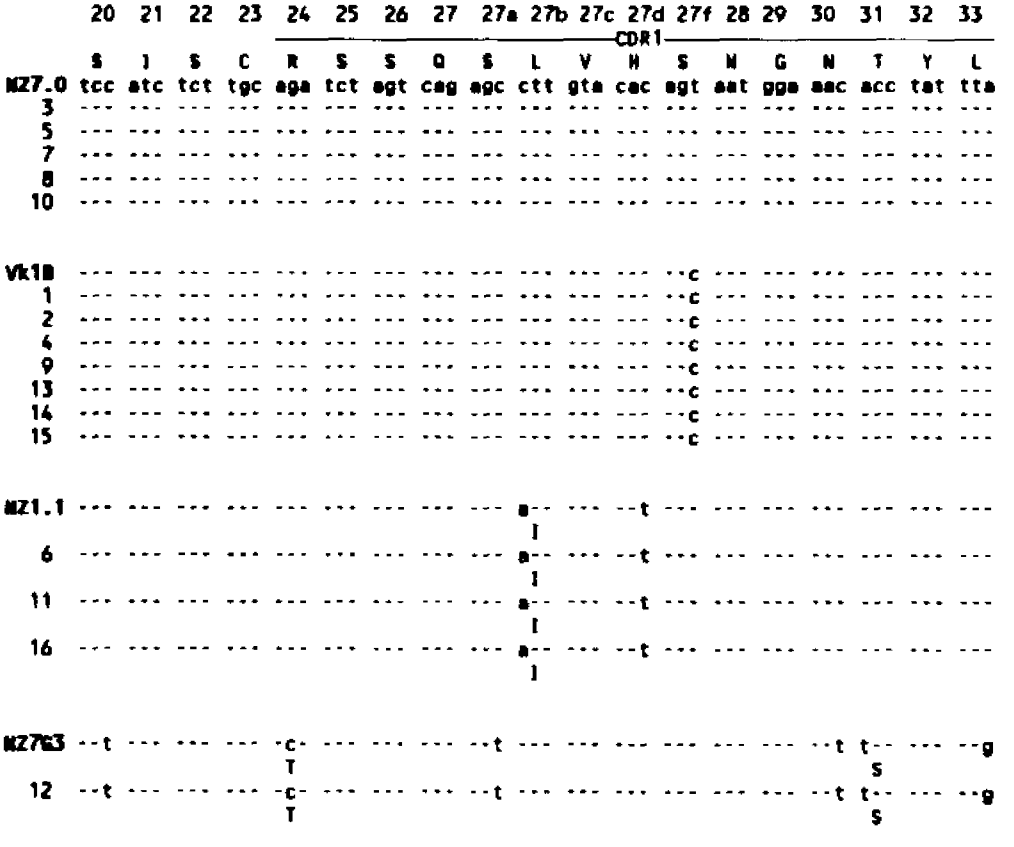
20 21 22 23 24 25 26 27 27a 27b 27c 27d 27f 28 29 30 31 32 33
CDR1
S I S C R S S Q S L V H S N G N T Y L
MZ7.0 tcc atc tct tgc aga tct agt cag agc ctt gta cac agt aat gga aac acc tat tta
3
5
7
8
10
Vk1B
1
2
4
9
13
14
15
MZ1.1
6
11
16
MZ763
12

Figure 14b

	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
								FRAMEWORK 2											
	H	W	Y	L	Q	K	P	G	Q	S	P	K	L	L	I	Y	K	V	S
NZ7_0	cat	tgg	tac	ctg	cag	aag	cca	ggc	cag	tct	cca	aag	ctc	ctg	atc	tac	aaa	gtt	tcc
3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Vk1B	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
1	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
2	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
4	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
9	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
13	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
14	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
15	t--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-gg	---	---
	Y																R		
NZ1_1	g-a	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	E																		
6	g-a	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	E																		
11	g-a	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	E																		
16	g-a	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	E																		
NZ7G3	g--	---	c--	---	---	---	---	-a-	---	---	-t-	c-a	---	---	---	--t	g-g	---	---
	D		H					E			L	Q					D		
12	g--	---	c--	---	---	---	---	-a-	---	---	-t-	c-a	---	---	---	--t	g-g	---	---
	D		H					E			L	Q					D		

Figure 14c

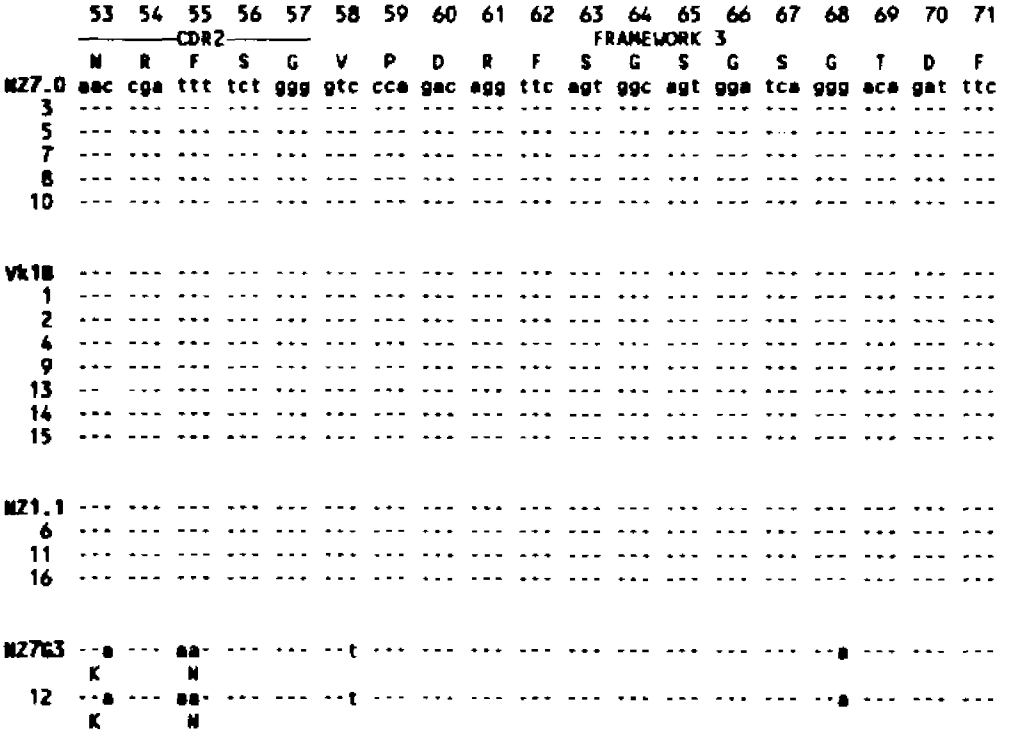
53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71
CDR2
FRAMEWORK 3
N R F S G V P D R F S G S G S G T D F
MZ7.0 aac cga ttt tct ggg gtc cca gac agg ttc agt ggc agt gga tca ggg aca gat ttc
3
5
7
8
10
Vk1B
1
2
4
9
13
14
15
MZ1.1
6
11
16
MZ7G3
K N
12
K N

Figure 14d

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
								FRAMEWORK 3											
	T	L	K	I	S	R	V	E	A	E	D	M	G	V	Y	F	C	S	Q
MZ7.0	aca	ctc	aag	atc	agc	aga	gtg	gag	gct	gag	gat	ctg	gga	gtt	tat	ttc	tgc	tct	caa
3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-c-	---	---	---
																S			
Vk1B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
13	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
14	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-t-	---
																		F	
MZ1.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-a-	---	-t-	---
																Y		F	
6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-a-	---	-t-	---
																Y		F	
11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-a-	---	-t-	---
																Y		F	
16	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-a-	---	-t-	---
																Y		F	
MZ6.5	---	---	---	---	---	---	---	---	---	---	---	t--	---	---	---	---	---	ctc	---
												L						L	
15	---	---	---	---	---	---	---	---	---	---	---	t--	---	---	---	---	---	ctc	---
												L						L	
MZ7G3	---	--t	---	---	---	---	--a	---	c--	---	---	t--	---	---	---	-a-	---	-tc	---
									P			L				Y		F	
12	---	--t	---	---	---	---	--a	---	c--	---	---	t--	---	---	---	-a-	---	-tc	---
									P			L				Y		F	

Figure 14e

```
        91  92  93  94  95
       ----------CDR3----------
        S   T   H   V   P
NZ7.0  agt aca cat gtt cct
   3   --- --- --- --- --g tgg acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1
   5   --- --- --- --- --g tgg acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1
   7   --- --- --- --- --g tac acg ttc gga ggg ggg acc aag ctg gaa ata aaa c Jk2
   8   --- --- --- --- --g tgg acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1
  10   --- --- --- --- --g acg tcg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1


Vk18   g-- --- --- --- ---
        G
   1   g-- --- --- --- --a ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa c Jk4
        G
   2   g-- --- --- --- --- cgg acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1
        G
   4   g-- --- --- --- --a ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa c Jk4
        G
   9   g-- --- --- --- --- cgg acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk1
        G                  ca
  13   g-- t-- --- --- ---/ttc acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk4
        G   S
  14   g-- --- --- --- --- cac acg ttc ggc tcg ggg aca aag ttg gaa ata aaa c Jk4
        G


NZ1.1  g-- t-- --- --- ---
        G   S
   6   g-- t-- --- --- --- cgg acg ttc ggt gga ggc acc aag ctg gaa ata aaa c Jk1
        G   S
  11   g-- t-- --- --- --a ttc acg ttc ggc tcg ggg aca aag ttg gaa ata aaa c Jk4
        G   S
  16   g-- t-- --- --- --- cag acg ttc ggt gga ggc acc aag ctg gaa atc aaa c Jk4
        G   S


NZ6.5  gt- --- --- --c ---
        V
  15   gt- --- --- --c --g tac acg ttc gga ggg ggg acc aag ctg gaa ata aaa c Jk2
        V


NZ7G3  c-- --- --- t-a ---
        R           L
  12   c-- --- --- t-a --- cgg acg ttc gtt gga ggc acc aag ctg gaa atc aaa c Jk1
        R           L
```

Figure 14f

V_K1C subgroup isolated from Balb/c genes. The germline genes isolated from HindIII digested fragments not found in other strains show a rather high polymorphism. The germline gene which showed highest homology with V_K1B subgroup was isolated from the 1.8kb and 0.9kb fragments of NZB and from the 3.6kb fragment of recombinant inbred NZBxSM/Z DNA. The nucleotide sequence of NZ2.4 (1.8Kb band) is identical to the germline gene isolated from NZB liver DNA digested with BamH1 (Ng et al., 1989) except for a silent nucleotide substitution at position 19. The finding of NZ2.4 gene of NZB located in 1.8kb fragment of NZB and in 3.6kb fragment of NZBxSM/Z may be related to generation of a new restriction sites subsequent to recombination process which may have taken place in NZBxSM/Z recombinant inbred strain. Studies by Tutter and Riblet (Tutter and Riblet, 1988) suggested that high RFLP in the Igh-V locus can be due to recombination mechanism. A gene was isolated from the fragments spanning from 7.0 to 9.4kb in size showed highest homology with V_K1A subgroup. Two of the three germline genes isolated form the 3.6Kb fragment show highest homology to V_K1D subgroup. The sequence of the third gene showed highest similarity to V_K1C subgroup in FR1 and CDR1, and to V_K1D subgroup in the rest of the gene.

Finally, we isolated a new V_K1 germline gene (NZ7G-3) which shows highest homology to V_K1A (89.3%). This novel V_K1 gene represents the prototype of a new functional subgroup that we designated V_K1F.

From these data several points may be discussed:

First, we have shown that in a single REF hybridizing band, several germline genes can be isolated (e.g. three genes in each of the 3.0kb and 3.6kb fragments) and that an

identical gene can be found in two different fragments (e.g. NZ1.5 and NZ2.4 in NZB and NxS/Z in the recombinant inbred strain). This is important since it confirms that comigrating REF bands found in many strains (such as 3.0kb band in figures 1 and 2) does not necessarily contain the same allelic groups. Indeed, one would expect to find that all V_K1 germline genes from Balb/c mice [which has all five V_K1 subgroups (Ng et al., 1989)], distributed between the two HindIII REF hybridizing bands (3.0kb and 9.5kb). Furthermore, these results are different from those reported for V_K21 family where a single gene copy was found in each fragment (Heinrich et al., 1984), but are similar to results reported by Livant et al. (1986) where several genes were isolated from a single REF hybridizing band of DNA from the V_HJ558 family. Many efforts have been devoted to the study of individual V- gene family repertoire based on RFLP analyses (Cory et al., 1981; Brodeur and Riblet, 1984; Huppi et al., 1985; Kofler et al., 1989). However, our data clearly demonstrate that the analysis of V gene complexity based solely on RFLP pattern is potentially misleading.

Second, we identified in NZB a V_K1 germline gene which based on nucleotide sequence homology represents a novel V_K1 subgroup. This germline gene (NZ7G-3) is functional in NZB B cells since one of the antibody in our panel of 16 hybridomas is encoded by this gene (Figure 14a-f).

Third, it was reported that the majority of V_K1 immunoglobulins in NZB belong to the V_K1B subgroup (Loh et al., 1979; Ng et al., 1989). If this is correct, then there must be a regulatory mechanism that favors the expression of the V_K1B subgroup in this mouse strain. NZB mice are known to develop autoimmune diseases and malignant lymphomas

with the concomitant expansion of clones that are self reactive (Howie and Helyer, 1968; Theofilopoulos and Dixon, 1985a; Seldin et al., 1987b). Moreover, NZB mice develop proliferation of Ly-1+ B cells, a subset of B lymphocytes that are involved in autoantibody production (Herzenberg et al., 1986; Hardy et al., 1989). This may account for the clonal expansion of a subset of V- genes in these mice, hence, the predominant expression of V_K1B subgroup in myelomas. However, sequencing of V_K1 genes expressed in a panel of 16 hybridomas obtained from 6-day, 1, and 16 month- old NZB mice (Fidanza et al., 1990), showed that V_K1 expressed genes are derived from various V_K1 subgroups (V_K1A, V_K1B, V_K1C, V_K1D and V_K1F).

These data demonstrate that no particular regulatory mechanism is involved in the expression of various germline gene subgroups in NZB B cell repertoire. It is noteworthy to add that the lack of somatic mutations among sixteen V_K1 genes expressed in hybridomas obtained from 6- day, 1 and 16 month-old NZB mice can be due to the isotypic origin of these antibodies, majority are of the IgM class. Previous sequence studies on various autoantibodies have demonstrated that IgM antibodies such as anti-DNA, anti-IgG rheumatoid factors (RF) or anti-bromelain-treated mouse red blood cell (BrMRBC) autoantibodies are encoded by germline genes (Radoux et al., 1986; Schlomchik et al., 1986; Reininger et al., 1987), and that many somatic mutations in the CDR regions can be found in IgG anti-DNA autoantibodies and RF (Schlomchik et al., 1986). There are numerous data indicating that Ly1+ B cells use unmutated V_H and V_L germline gene sequences (Pennel et al., 1985; Forster and Rajewsky, 1987). In six out of sixteen hybridomas studied here, we detected Ly1 gene transcripts by Northern

blotting and a 67Kd protein interacting with anti-Ly1 mAb by Western blot (Mayer et al., 1990).

Finally, the complexity of V_K1 gene family in NZB mice, a strain of unknown origin, clearly demonstrates that it does not differ from other inbred strains such as Balb/c from which germline genes belonging to various subgroups have been identified (Corbet et al., 1987; Ng et al., 1989). Comparison of the divergence of V_K1 genes with other V_H or V_K families (Perlmutter et al., 1985) suggests that V_K1 genes were more recently duplicated V region subgroups. Inspection of sequence differences of V_K1 germline genes, summarized in Table 5, shows that replacement substitutions are clustered in a small number of residues in position 34 of CDR1, 50 in CDR2, 89 and 91 in CDR3. The majority of replacement substitutions have been observed in CDRs and not FR regions, suggesting that a selective pressure to conserve framework region, which is responsible for the tertiary structure of the Ig, has been maintained during evolution (Novotny and Franck, 1975; Litman et al., 1983). However, the CDRs are important for antigen binding (Sablitzky et al., 1985) and, hence; changes within these regions would contribute to the generation of antibody diversity. Inspection of the nucleotide sequences reveals that the highest degree of homology is between NZ2.4 (V_K1B) and V_K1A gene. The V_K1B subgroup of NZB differs from V_K1A subgroup by one replacement substitution in each CDR1 and CDR2 and two replacement substitutions in CDR3. It differs from VK1C subgroup by two and one replacement substitutions in CDR1 and CDR3, respectively. This strongly suggests that V_K1B have occurred after divergency of V_K1A and V_K1C, probably by gene conversion or recombination events between V_K1A and

V_K1C subgroups. V_K1D and V_K1F germline genes of Balb/c and NZB show a lesser homology with the three other subgroups suggesting that these genes diverged earlier during evolution. While the replacement substitution in CDR2 is common to V_K1B, NZ1.2, NZ3.1, NZ2.4 and NZ1.5, the most important differences are seen in CDR1 and CDR3 which differs by six replacement substitutions from other V_K1 subgroups.

Interestingly, the nucleotide sequence of NZ1.2 clone with isoleucine at position 27b is similar to V_K1C subgroup, while at position 34 (tyrosine), position 50 (arginine), and position 92 (tryptophan) is identical to V_K1B subgroup. This suggests a possible gene conversion event that took place between V_K1C and V_K1B subgroups in NZB. Furthermore, clone NZ1.3 is identical to NZ3.1 (a V_K1C subgroup) up to position 27d, and to V_K1D subgroup in FR3 and CDR3. This also suggests a possible gene conversion event may have taken place between V_K1C subgroup and V_K1D gene. The distribution of putative conversion events is directed towards a particular member in a V_K1 subgroup. Since the majority of substitutions are not silent, this may reflect a selective advantage for the antibodies using those germline genes.

While we cannot determine the frequency of gene conversion among our data with respect to V_K1 germline gene sequences, it appears that gene conversion has occurred during a relatively short time frame involved in divergence of subgroups (alleles) within a single strain.

Therefore, the high polymorphism of V_K1 family observed in NZB mice by Southern analysis is due to the presence of several distinct V_K1 germline genes. Recombination, gene conversion, and nucleotide changes in the CDR regions are necessary mechanisms

which contribute to the evolution of IgK-V gene diversity.

V. DISCUSSION

A basic feature of the immune system is its ability to recognize and interact with any antigen. This enormous recognition potential includes interactions with self components. Some of these interactions appear to be important for the regulation and control of immune function, whereas others can contribute to the pathogenesis of autoimmune diseases.

Pathogenic expression of self- reactivity may result from abnormalities in the generation of the T and B cell receptor repertoire. Such autoreactive receptors may represent normal components of the immune system that, under physiologic conditions, remain unstimulated or suppressed. Alternative possibilities can be distinguished by defining the genetic origin of, and somatic mechanisms generating, the B and T cell receptors responsible for anti-self responses that give rise either to pathogenic or normal autoantibodies. Important questions are whether the autoimmune receptor germline gene repertoire is abnormal, or if anti-self receptors are encoded by unique gene segments, and whether the anti-self response is genetically, structurally or idiotypically restricted.

A. Linkage of NZB V_K1 haplotype to autoimmune disease

Our aim in this study was to find out whether there is a correlation between the Ig supergene family of the NZB haplotype and disease manifestation in two RI lines

(NZBx129/J and NZBxSM/J). Our data shows that there is a correlation between the V_K1^b haplotype of NZB and anti-erythrocyte autoantibodies in both RI lines, and between the V_K1^b haplotype and glomerulonephritis in NZBx129/J RI lines. This correlation was not seen in TCR (Vα), IgH variable region (V_HS107), and MHC products (I-Aα or I-Eβ). This raised the possibility that "unique" V_K1 genes with autoreactive properties may exist in the NZB mouse. Perhaps, through a skewed immunoregulatory mechanism, autoreactive B cell clones are either not deleted from the mouse immune repertoire or they continue to differentiate into plasma secreting cells with antibodies that bind to self-antigens. Furthermore, several studies have shown that there is a preferential usage of a limited set of V_K genes, including the V_K1 gene family, among autoantibodies (Schlomchik, et al., 1986; Kasturi et al., 1988; Fidanza et al., 1990). Moreover, it has been shown that NZB mice have elevated levels of the Ly1+ B cell subset, which is programmed, primarily, to secrete autoantibodies (Mayer et al., 1990). A fraction of these autoantibodies use V_K1 genes (Fidanza et al., 1990).

However, we did not find any Coombs' antibody encoded by V_K1 gene family, as assessed by Northern analysis (data not shown). In a recent study by Reininger et al. (1988), five V_K families encoded pathogenic anti-MRBC (mouse red blood cells). These were V_K8, 9, 19, 21 and 28. There was no clear evidence for somatic mutations in the V_K genes, however, several mutations in the J_H gene segments of both IgM and IgG anti-MRBC autoantibodies, whether pathogenic or not, suggested that their V_H regions may be highly mutated (Reininger et al., 1990).

NZB has an unusual and possibly recombinant IgK-V haplotype (Theofilopoulos et al.,

1988). Some of the NZB V_K families belong to the "b" haplotype, including V_K1, whereas others belong to the "d" haplotype.

Many studies have shown that each autoimmune phenotype is under the control of independent genes. It is possible, therefore, that:

1) The V_K1 family belonging to the "b" haplotype is not the locus necessary for the production of Coombs' autoantibodies, but is linked to other genes responsible for susceptibility to autoimmunity. Indeed, it has been shown recently by Noonan et al. (1990) that the NZB TCR β chain with the B/B haplotype correlated strongly with glomerulonephritis-associated mortality. Furthermore, studies by Theofilopoulos et al. (1988) have revealed that NZB mice have the highest relative expression of autoreactive Vβ8.2. Both IgK and the TCR β genes are located on chromosome 6 in the mouse. It is difficult to assess the relationship between the Vβ haplotype and disease manifestation in our two RI lines because all mice showed identical RFLP patterns. This, however, does not exclude allelic differences that may be overlooked with RFLP analysis.

2) V_K1^b is in linkage disequilibrium with genes located on the same chromosome (e.g. TCRβ) or other genes located on other chromosomes, such as those controlling the anti-erythrocyte autoantibodies and IgM hypergammaglobulinemia. These genes are located on chromosome 4 (Manny et al., 1979; Kohno et al., 1983; Ozaki et al., 1983; Hirose et al., 1984). This is in agreement with our data that RI strains having chromosome 6 genes from NZB also inherited chromosome 4 of NZB.

We extended our studies to see whether there is a correlation between the IgK-Ef2^b haplotype (that controls the expression of V_K1A subgroup) and autoantibody formation

in several strains of mice (including NZB). There was no correlation between the V_K1^b and $Ef2^b$ haplotypes as assessed by RFLP analysis. Furthermore, no association was between the $Ef2^b$ haplotype and autoantibody production. Interestingly, among five strains with the $Ef2^b$ haplotype, only C58/J mice, had identical V_K1^b haplotype as NZB and showed elevated levels of anti-DNA autoantibodies. C58/J mice are known to develop a high incidence of leukemia by the age of 12 months of age. In addition, other studies have shown that these mice also have high levels of anti-thymocytes autoantibodies (Datta et al., 1982).

B. NZB V_K1 germline genes

Studies on V_K1 gene family in NZB mice have revealed high polymorphism when compared with other mouse strains. Based on sequencing analysis, NZB myeloma proteins expressed the V_K1B subgroup (Loh et al., 1979). No other V_K1 subgroup has been identified in the NZB mouse. IEF analysis of NZB serum light chains and of over 125 plasmacytomas derived from NZB did not detect any V_K1A subgroup, which is highly represented in most mouse strains. It was, therefore, established that the majority of NZB V_K1 Igs belonged to the V_K1B subgroup (Loh et al., 1979; Lazure et al., 1981).

It was also suggested that, either a) V_K1A subgroup is not present in the germline gene repertoire of NZB mice, b) that there are allelic differences of V_K1A subgroup between NZB and most other strains, or c) that V_K1A is present in the germline gene pool of NZB mice, but is not expressed due to some regulatory mechanisms. Sequencing 10 germline genes from NZB have shown that they belong to V_K1A, B, C, D and F

subgroups. There was no allelic differences between NZB and Balb/c V_K1A germline genes, and the V_K1A subgroup was expressed in antibodies derived from NZB hybridomas. The high polymorphism of the V_K1 gene family, observed in NZB mice by Southern blot analysis, is due to the presence of many distinct germline genes, several of which have resulted from gene recombination and gene conversion.

C. Sequences of V_K1 antibodies from NZB

It has been established that the majority of NZB V_K1 Igs belonged to the V_K1B subgroup, as mentioned earlier. This is very interesting, as it would suggest that a particular B-cell subset, such as Ly1+ B cells, with autoreactive properties are activated. Sequencing 16 cDNA from NZB hybridomas secreting both autoantibodies and non- self-reactive antibodies revealed that they belong to all of the V_K1 subgroups found in the germline gene pool. A new V_K1 subgroup (designated V_K1F) has been identified in one multispecific autoantibody. Only 4 out of 16 antibodies were Ly-1 positive. Therefore, this rules out the possibility of an oligoclonal activation of B cells in NZB mice, at least in the earlier antibody repertoire, since only three antibodies from 16 months- old NZB were sequenced. Most of the antibodies were of the IgM isotype, which would explain the lack of somatic mutations in these antibodies.

NZB mice have a high propensity to secrete IgM rather than IgG autoantibodies throughout life (Klinman, 1990). 77% of the NZB-derived antibodies are of the IgM isotype (Gavalchin et al., 1985). Klinman (1990) has shown that B cells from NZB mice were resistant to lymphokines IL-4 and IFN-γ (both cause isotype switching) and did not

induce increase in IgG production. In contrast, an abnormally high percentage of B cells from adult MRL-lpr/lpr mice produced IgG2a antibodies. According to the author, NZB B cells cannot undergo class switching. Resting B cells isolated from NZB mice were surface IgM+ and surface IgG- at the initiation of culture, suggesting they had not undergone isotype switch in vivo. These cells proliferated normally (but produced little IgG) when stimulated with mitogen in vivo.

Many studies have shown that autoantibodies of the IgG and not IgM are pathogenic (Slack et al., 1984; Eilat, 1990). IgG autoantibodies have high affinity receptors and are somatically mutated. However, in vivo studies by Reininger et al. (1990) have demonstrated that IgM anti- mouse red blood cells (MRBC) autoantibodies derived from NZB mouse, when transferred into Balb/c mice were capable of inducing severe anemia and death in the recipient animal. In addition, Caulfield and Calkins (1989) have shown that an IgM monoclonal anti-mouse red blood cells autoantibody with the G-8 idiotype isolated from NZB mice and grown as tumors in Balb/c mice, developed autoimmune hemolytic disease characterized by a decrease in the number of erythrocytes and the development of Coombs'- positivity in Balb/c mice. A recent study by Roudier et al. (1990) have shown that IgM rheumatoid factors secreted from chronic lymphocytic lymphoma (CLL) patients were pathogenic. Therefore, IgM autoantibodies cannot be excluded from being directly involved in the etiology or pathogenesis of autoimmune disease.

Therefore, NZB IgM autoantibodies with unmutated germline genes may encode anti-self specificity that, given under normal circumstances would switch to IgG isotype

and undergo somatic mutation physiologically associated with maturation of antibody response to foreign antigen (Naparstek et al., 1986b).

"Natural" autoantibodies can be the source of pathogenic autoantibodies in autoimmune disease. These "natural" autoantibodies of normal mice and humans may be maintained in a state of equilibrium by numerous idiotypic connections and other regulatory factors. Trans-activating factors such as polyclonal activation may be present in the young NZB mouse which activates B cells to give rise to hypergammaglobulinemia which, in itself, may cause tissue damage. However, in the adult NZB mouse, autoantibodies are activated by the selected antigen (antigen-driven process). Indeed, studies by Fidanza et al. (1990) have shown that no Coombs' autoantibodies were seen in 1- month or 6-month old NZB mice, but many were secreted by 16-months of age.

In summary, our results are in agreement with previous studies, in that many gene loci are involved in the pathogenesis of autoimmunity in NZB mice. A complex set of immunoregulatory forces can explain the enhanced autoreactivity that occurs in these mice, although no specific mechanism can yet be implicated. Indeed, the normal immune system is endowed with many intricate processes with each process crucial in maintaining the homeostasis and well being of the organism.

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