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Molecular characterization of the CD5 B cell subset: Involvement in autoimmunity and malignancy

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MOLECULAR CHARACTERIZATION OF THE CD5 B CELL SUBSET INVOLVEMENT IN AUTOIMMUNITY AND MALIGNANCY

by

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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.

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ABSTRACT

MOLECULAR CHARACTERIZATION OF THE CD5 B CELL SUBSET INVOLVEMENT IN AUTOIMMUNITY AND MALIGNANCY

by

Raoul Mayer

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

CD5 is a pan T lymphocyte cytodifferentiation antigen detected on a small but distinct subset of B lymphocytes in both mice and humans. Studies corducted in both species have suggested that the $CD5^+$ B cells are of a "young" phenotype, play a major role in the production of autoantibodies and preferentially undergo transformation events leading to the development of certain kinds of B cell malignancies.

Molecular studies were conducted in the murine hybridoma system which allowed the correlation of the CD5 phenotype ($CD5^+$ or $CD5^-$) with immunoglobulin variable gene utilization and fine antigenic specificity. The transcription of the CD5 gene (Ly-1 in mice) was used for the phenotypic assignment of the hybridomas studied. Among a panel of 140 hybridomas producing autoantibodies, which were obtained from various normal and autoimmune prone mice, the expression of the Ly-1 gene was detected in a large fraction of hybridomas obtained from NZB and viable motheaten mice. It is important to note that both of these strains are autoimmune prone have an increased proportion of Ly-1 B cells as compared to normal

strains. Ly-1 transcripts were also detected in a large fraction of hybridomas producing DNA specific autoantibodies and a smaller fraction of hybridomas producing rheumatoid factors and multispecific autoantibodies. These results firmly establish a major contribution of the Ly-1 B cell subset to the production of DNA specific autoantibodies and a smaller contribution to the production of rheumatoid factors and "natural" multispecific autoantibodies. The transcription of the Ly-1 gene was different in B cells and B cell hybridomas as compared to T cells. Finally, the hybridoma Ly-1 transcripts were proven to be functional in Western blotting and immunofluorescence studies.

The expression of the CD5 gene was also studied in a panel of human EBV transformed lines, chronic lymphocytic leukemias, B cell lymphomas and T cell malignancies. The transcription of the human CD5 gene was proven to be identical in all cases. The study of the utilization of human immunoglobulin V_H and V_κ families was conducted in a large panel of chronic lymphocytic leukemias, $CD5^+$ lymphomas (small lymphocytic lymphomas) and $CD5^-$ lymphomas and was compared to the immunoglobulin variable gene utilization of a panel of human EBV lines. A statistically significant biased usage of VH_4 was detected in chronic lymphocytic leukemias, VH_5 in $CD5^+$ lymphomas and $V_\kappa III$ in both chronic lymphocytic leukemias and $CD5^+$ lymphomas Some differences of V gene utilization were also observed between $CD5^+$ and $CD5^-$ lymphomas.

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. Materials 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

A. General Considerations

V - (D) - J recombination, heavy and light chain association, insertion or deletion of nucleotides at joining regions and somatic mutation are the major molecular events which contribute to the diversity of the immunological repertoire which comprises 10^7 -- 10^9 antigen specificities. The diversity of the Ig receptor occurs before the Ig receptor is expressed on the surface of B cells. Therefore, the antibody specificity arises by a completely random process independent of antigenic or idiotypic stimuli. When investigating the genetic origin of autoantibodies one must keep in mind that the assembly of the Ig receptor, as is the case for any other antigen, occurs independently of the presence or absence of the autoantigen. This concept is supported molecular and cellular data demonstrating rearrangement of Ig genes in pro and pre-B cells lacking Ig receptors. Autoantibodies are detected in low amounts in sera from normal animals and humans (Prabhakar et al., 1984; Dighiero et al., 1982). The mechanism involved in the selective expansion of the autoantibody producing clones remains an open question. Two different possibilities are entertained at the present time.

1. Autoantibody production is a failure of strict regulation

resulting in the breakage of the tolerance to autoantigens instituted at birth. This assumes that autoantibody producing clones are indistinguishable from B cell clones producing antibodies specific for foreign antigens and that their Ig germline organization is the same. Various factors can be envisioned to contribute to the breaking of self tolerance such as cross reactivity between self and foreign antigens (molecular mimicry), polyclonal stimulators of bacterial origin, release of sequestred antigens in circulation,alteration of regulatory T cell subset balance or idiotypic interactions.

2. Autoantibody production occurs due to the selective proliferation of autoantibody producing B cell clones in the absence of any regulatory signals from helper T cells. This hypothesis assumes first of all that autoantibody producing clones are different from B cell clones producing antibodies reacting with non-self antigens due to their ability to spontaneously proliferate in the absence of any external regulatory signals. Indeed there is evidence indicating that the Ly-1 B cell subset in mice is a good candidate for this hypothesis since it appears that these cells can proliferate independently of host environment when transferred to other animals (Forster et al., 1987).

Studies carried out in various laboratories suggested that the production of certain autoantibodies is restricted to a minor subset of B cells which express the CD5 pan T cell cytodifferentiation antigen, Ly-1 in mice and Leu-1 in humans (Plater-Zyberk et al., 1986; Hayakawa et al., 1984; Steele et al., 1978; Casali et al., 1987; Hardy et al., 1987). In addition, it was clearly demonstrated that a great majority of human chronic lymphocytic leukemias of B cell origin (B-CLL) and small lymphocytic lymphoma (SLL) originate from the CD5 subset (Boumsell et al., 1978; Boumsell et al., 1980; Wang et al., 1980; Royston et al., 1980; Kamoun et al., 1981; Knowles et al., 1983). Similar data has been obtained in certain B cell murine malignancies (Lanier et al., 1978; Slavin et al., 1978; Lanier et al., 1981, Haughton et al., 1986).

B. Ig Genes Encoding Autoantibodies.

Our laboratory among several others has concentrated in the last few years on the molecular characterization of the Ig genes encoding autoantibodies by testing the hypothesis that pathogenic autoantibodies are derived from a restricted subset of germline genes. If the pathogenic autoantibodies are highly restricted, they might be generated by the precise association of a few V_H and V_L gene products. Yancopoulos et al have previously demonstrated a 3 preferential V_H gene usage, particularly of the 7183 family in pre-B cells (Yancopoulos

et al., 1984). Results from our laboratory have also shown a 3 preferential V_H gene usage (mostly 7183) in a panel of RF and autoantibodies (Bellon et al., 1987; Monestier et al., 1986; Painter et al., 1988). Since the 7183 V_H family is frequently used early in development, autoimmunity resulting from spontaneous autoantibody production may very well reflect an immature repertoire. Ly-1 B cells as a distinct subset have the characteristics of "young" B lymphocytes, expressing the typical phenotype of high density IgM and little IgD on their surface. Furthermore they are responsible for most of the spontaneous RF production in humans and of mouse autoantibodies directed against bromelein treated RBC, single stranded DNA, and thymocytes (Plater-Zyberk et al., 1986; Hayakawa et al., 1984; Steele et al., 1978; Casali et al., 1987; Hardy et al., 1987).

C. Ly-1 B Cell Subset.

1.The Discovery of Ly-1 and Leu-1 on B Cell Malignancies.

CD5 is a pan T lymphocyte differentiation antigen with a molecular weight of 67 Kd. In 1978 the presence of Leu-1, the human equivalent of Ly-1, was first reported on B cells in a human leukemia study (Bousell et al., 1978). This expression was confined to Ig^+ cells from B-CLL patients as well as SLL patients (Boumsell et al., 1980; Wang et al., 1980; Royston et al., 1980; Kamoun et al., 1981; Knowles et al., 1983). Ig^+ cells from acute leukemias, Burkitt's lymphomas and other B cell proliferative disorders were all negative. In the same

year Lanier and his collaborators reported a high incidence of murine B cell lymphomas in a new strain of mouse called B10-H-2^a H-4^b (2^a4^b) which were induced by hyperimmunization with sheep red blood cells (SRBC) (Lanier et al 1978). Several malignant B lymphoma lines were obtained (CH lines) from these mice. At about the same time, Slavin and Strober (1978) also reported a case of murine B cell leukemia (BCL1) which developed spontaneously in an old BALB/c mouse and was characterized by prominent splenomegaly and a very high lymphocyte count (>200,000/ul) (Slavin et al., 1978). BCL1 has been subsequently used as a model of human BCLL based on phenotypic and morphologic markers. All these mouse B cell malignancies are characterized by variable Ly-1 expression, marked splenomegaly, lymph node enlargement, transplantability using as few as 2 cells and no involvment of the thymus or bone marrow. In particular the CH lines, which were obtained by SRBC hyperimmunization of 2^a4^b mice and BCL1 were shown to express variable levels of the Ly-1 cytodifferentiation antigen (Lanier at al., 1981; Lanier at al., 1982).

2.Characterization of Ly-1 B Cells in Normal Animals.

In the case of mouse and human B cell malignancies expressing Ly-1 on their surface, their large numbers in the spleen and other lymphoid organs made them easily detectable by staining. Since the Ly-1 B cells are extremely rare in normal animals and human subjects their detection proved to be difficult.

With improved immunofluorescence techniques, particularly the development of FACS, the Ly-1 B population in mice was better characterized. The Ly-1 expressing B cells were detected in the spleen and peripheral blood at a very low frequency (1-2%) in most adult mouse strains tested (Hayakawa et al., 1983). A paradoxical enrichment (10-40%) was found among peritoneal B cells (Hayakawa et al., 1986). A large fraction of these B cells were found to produce autoAbs specific foe autologous erythrocytes (Mercolino et al., 1988; Kaushik et al., 1988). Ly-1 B cells are however undetectable in the bone marrow, lymph nodes, Peyer's patches and thymus (Hayakawa et al., 1983; Hayakawa et al., 1986). When young mice (1 week old) were studied, a higher proportion (>30%) of Ly-1 B cells was found among splenic and peritoneal IgM^+ cells (Hayakawa et al., 1983; Hayakawa et al., 1986). This high proportion of IgM^+ Ly-1 B cells is however maintained throughout adulthood only in the peritoneum (Hayakawa et al., 1986). When grown in culture, Ly-1 B cell lines multiply rapidly and show genomic amplification and increased transcription of the c-myc gene (Braun et al., 1983; Citri et al., 1987). While most B cells die after a short time when cultured in vitro, Ly-1 B cells demonstrate prolonged survival and spontaneous IgM secretion (Braun at al., 1983).

3. Ly-1 B Cells as a Separate Lineage. Bone marrow reconstitution experiments showed a dichotomy in the

differentiation of Ly-1$^+$ and Ly-1$^-$ B cells probably originating at the stem cell level. Liver cells from fetal mice (the liver is the site of hematopoiesis in some stages of fetal development) reconstituted both sets, while bone marrow cells originating from adult animals were able to reconstitute only the Ly-1$^-$ B cell population (Hayakawa et al., 1985). As a corollary to these results, it was shown that IgM$^+$ B cells from adult peritoneum or Ig$^+$ B cells from newborn mice were able to reconstitute almost exclusively the Ly-1$^+$ population (Hayakawa et al., 1986). In view of these very significant reconstitution experiments, most researchers today regard the Ly-1 B cells as a distinct subset of B cells which appears early in ontogeny, migrates to the periphery and is maintained independently of the bone marrow. These characteristics as well as the ability to spontaneously secrete Ig when cultured in vitro clearly distinguish the Ly-1$^+$ B cell subset from the conventional, Ly-1$^-$ B cell subset.

4. Studies in Autoimmune Inbred Mouse Strains. The Ly-1 B cell subset was studied in a variety of inbred mouse strains, particularly several immunodefective strains. One of the common features shared by these mice is the spontaneous autoantibody production. At one end of the spectrum, SJL, xid and CBA/N mice show a lower level of expression (Hayakawa et al., 1986; Hayakawa et al., 1984; Hayakawa et al 1987). The proportion of Ly-1 B cells is distinctly higher in NZB mice

(Hayakawa et al., 1983; Manohar et al., 1982). Interestingly, an oligoclonal proliferation of the Ly-1 B subset was observed in NZB mice (Raveche et al., 1981; Raveche et al., 1988; Stall et al., 1988). This spontaneous proliferation is extreme in the peritoneum and later involves the spleen and lymph nodes. The observed splenomegaly was shown to be due mostly to Ly-1 B cells (Raveche et al., 1981; Raveche et al., 1988; Stall et al., 1988). Phenotypical analysis on NZB peritoneal B cells revealed hyperdiploidy which is believed to represent a pre-malignant stage (Raveche et al., 1981; Raveche et al., 1988; Stall et al., 1988). This may very well be due to a selective expansion of the Ly-1 B cells as a functionally distinct subset. A large fraction of the IgM Ig spontaneously secreted by NZB mice are autoantibodies with specificities restricted to Fc gamma, ssDNA and thymocytes (Manny et al., 1979). It therefore appears that Ly-1 B cells in NZB mice are responsible for the spontaneous autoantibody production of the IgM isotype. The viable motheaten $\underline{me}^{v}$ autoimmune strain carries a mutation mapping to chromosome 6 (Green et al., 1975; Shultz et al., 1984). These mice die at a young age, and display severe immunodeficiency and autoimmunity (Shultz et al., 1984). Even though the overall number of B cells is decreased nearly all of them are Ly-1^{+} (Sidman et al., 1986). The high level of spontaneous autoantibody production observed in $\underline{me}^{v}$ mice must also be due to the Ly-1 B cells since this subset is predominant in $\underline{me}^{v}$ mice. Sidman et al showed that

in this strain the increased Ig secretion is promoted by a B-cell derived maturation factor uniquely elevated in me^{v} mice (Sidman et al., 1985). Interestingly enough, when normal mouse strains were examined a close correlation between the Ly-1 B expression and autoantibody secretion was observed. Following in vivo LPS stimulation, a large fraction of Ly-1^{+} sorted B cells were shown to be specific for bromelein treated mouse red blood cells (BrMRBC) (Hayakawa et al., 1984). These cells are consistently enriched in the peritoneal cavity of normal adult mice (Pages et al., 1975; Steele et al., 1978). The same correlation between Ly-1 expression and bromelein treated mouse erythrocytes(BrMRBC) specificity was observed among the CH mouse lymphoma lines; almost all CH murine B lymphoma lines show erythrocyte specificities and Ly-1 expression (Haughton et al., 1986; Pennel et al., 1985; Mercolino et al., 1986). Further, detailed analysis identified the phosphotydil choline determinant on RBC membranes as the epitope recognized by antibodies produced by CH mouse lymphomas which bound BrMRBC (Mercolino et al., 1986).

D. The CD5 B Cell Subset In Humans

1. CD5 Expression In B Cell Malignancies (B-CLL and Small Lymphocytic Lymphoma).

CD5 is a pan T differentiation antigen. Its presence on human B cells was first reported in a human leukemia study by

Boumsell et al (1978). Of all cases tested CD5 expression was restricted to B-CLL and SLL. It has been previously demonstrated that both of these malignancies occur as a result of monoclonal proliferation. Samples obtained from acute lymphocytic leukemias, Burkitt's lymphomas and a variety of other lymphoproliferative disorders were all negative (Boumsell et al., 1978; Bousell et al., 1980; Wang et al., 1980; Royston et al., 1980; Kamoun et al., 1981; Knowles et al., 1983). The expression of a marker of normal thymus derived lymphocytes in B-CLL was initially puzzling since at the time, with the available techniques, all tested B cells from normal individuals appeared negative. With more sensitive techniques however, a small B cell population expressing CD5 was later identified. Repeated studies confirmed these initial observations. Knowles et al (1983) studied the expression of CD5 on peripheral blood and bone marrow lymphocytes obtained from subjects with various lymphoid malignancies with high white blood cell counts (Knowles et al., 1983). In 33 of 36 cases (92%) of CLL most of the cells (often more than 90%) expressed CD5, clearly demonstrating that this cytodifferentiation antigen is expressed by the malignant cells. In 10 of 15 cases (67%) of SLL a large majority of the cells also expressed CD5. A smaller proportion of CD5 positive cases (30-40%) was observed for all other types of lymphoma studied. Among all the cases examined the staining was heterogeneous in different

specimens. CD5 expression was not seen in cases of multiple myeloma and B-acute lymphocytic leukemia (ALL). All T-ALL cases were positive as expected. It is important to emphasize the fact that histopathologically and immunologically small lymphocytic lymphoma is indistinguishable from B-CLL and consequently it is regarded as its tissue analog (Gale et al., 1987). Similar results were obtained in several other studies by Royston et al (1980), Martin et al (1980) and Delia et al (1986). Taken together these results are consistent with the hypothesis that CD5 is a shared antigen (T and B cells), expressed by a minor B cell population from which the majority of cases of B-CLL and some B-cell lymphomas originate (SLL type).

2. CD5 B Cells in Normal Subjects.

Another interesting finding arising from early studies was the observation that most B-CLL lymphocytes form spontaneous rosettes with mouse erythrocytes (MRBC), whereas this phenomenon was only observed for a small subpopulation of normal B cells (Preud'homme et al., 1972). This represents a marker of immature human B cells. Caligaris et al (1982) took advantage of this property and separated normal B cells which formed rosettes with MRBC. The MRBC^{-} B cells were subsequently stained with monoclonal reagents. Using this approach a small population (2-3%) of CD5^{+} B cells was identified in human lymph node and tonsil but not in bone

marrow specimens. These cells showed the phenotypic characteristics of B-CLL cells (Caligaris-Cappio et al., 1982). Subsequent reports by Bofill et al (1985) and Antin et al (1986) identified CD5$^+$ B cells as a major component of the human fetal spleen. Hardy et al (1987) were able to show that a large proportion of human cord blood B cells are CD5$^+$. By using sensitive two and three color immunofluorescence analysis Kipps et al (1987) were able to first detect CD5+ B cells in peripheral blood of normal adults. These sensitive techniques permitted the identification of CD5+ B cells in the majority of normal adults tested. The range of lymphocytes coexpressing CD5 and B cell surface antigens was between .1 and 6%. Despite the heterogeneity seen between individuals, the proportion of circulating lymphocytes that are CD5$^+$ B cells was a relatively constant phenotype for any given subject on repeated analysis (Kipps et al., 1987). Although ordinary siblings had levels of CD5$^+$ B cells often distinct from one another, monozygotic twins and triplets were found to share identical proportions of CD5$^+$ B lymphocytes, implying that genetic factors regulate the level of CD5$^+$ B cells circulating in peripheral blood. In view of the available evidence a definite parallel can be appreciated between mouse and human CD5 B cells. CD5 B cells are considered a minor but distinct B cell subset which appears early in ontogeny when it is more predominant and subsequently migrates to the periphery.

3. B-CLL and Autoimmunity -- Role of CD5 B Cells.

Epidemiological studies have shown as early as 1976 that B-CLL is often associated with a variety of autoimmune phenomena. Preud'homme et al (1972) observed that the Ig receptor of lymphocytes from B-CLL subjects exhibit RF activity. Autoimmune hemolytic anemia was reported to be associated with B-CLL more frequently than with any other disease (Pinofsky et al., 1976). Between 10 and 20 percent of B-CLL patients eventually develop this autoimmune disorder. Autoimmune thrombocytopenia occurs in about 2% of B-CLL patients. Numerous clinical studies have reported cases in which B-CLL was preceded by autoimmune disorders including autoimmune hemolytic anemia, rheumatoid arthritis, myasthenia gravis, pernicious anemia and thyroid disease. Family studies have also demonstrated that CLL occurs more frequently in relatives of patients with the disease than in the general population. Therefore it appears that the association between autoimmune phenomena and B cell malignancy with familial clustering of cases implies that some individuals may be genetically predisposed to these disorders, although no single autoimmune or lymphoproliferative disease is directly inherited. Conley et al (1980) conducted a study aimed at determining the prevalence of autoimmune diseases and lymphoid neoplasms in 320 relatives of 28 B-CLL patients. The control group consisted of the relatives of 28 patients with other

hematologic disorders. Four patients with B-CLL had one relative with B-CLL (2 in siblings). Eight patients with B-CLL had at least one relative with autoimmune disorders. Overall, autoimmune diseases occurred in 25% of relatives of patients with B-CLL, the most common being rheumatoid arthritis, hyperthyroidism, SLE, and pernicious anemia. Hamblin et al studied the prevalence of autoantibodies in 195 patients with B-CLL (Hamblin et al., 1986). A statistically significant prevalence of RF and antibodies against platelets was found. The implied parallel existing between the mouse Ly-1 subset, which is responsible for a significant proportion of autoantibody production, and the human CD5 B cell counterpart led several groups to investigate the specificity of antibodies secreted by human CD5$^+$ B cells. Preliminary results by Levinson et al (1987) showed that RF represented a larger fraction of the total IgM secreted by cord blood cells stimulated in vitro with Staphylococcus aureus Cowan I (SAC) than SAC stimulated adult peripheral blood mononuclear cells. In view of the fact that cord blood is enriched for CD5$^+$ B cells (75%) and that SAC induced IgM RF production by normal CD5$^+$ B cells this higher proportion of RF producing cells in cord blood can be attributed to CD5$^+$ B cells. In this context, the over-representation of autoreactive clones may be a general feature of the newborn B cell repertoire. More precise studies, using sensitive three color FACS were done by Hardy et al in a panel of patients with autoimmune

diseases (1987). A statistically significant number of patients with RA, Sjorgen syndrome and progressive systemic sclerosis had increased levels of $CD5^+$ B cells (75%) in their blood. Also, Hardy et al, showed an increased level of RF production by sorted $CD5^+$ B cells as compared to $CD5^-$ sorted cells from peripheral blood following in vitro SAC stimulation. Similar results were obtained by Plater-Zyberk (1986) when he analyzed a group of patients with RA. Repeated attempts to culture in vitro sorted $CD5^+$ human B cells, in order to obtain Ig secreting cells, have failed. These findings differ markedly from results obtained with sorted $Ly\text{-}1^+$ mouse B cells which spontaneously proliferate and secrete IgM antibodies in vitro. Casali et al (1987) however, succeeded in culturing $CD5^+$ and $CD5^-$ sorted human B cells subsequent to infection with EBV. Culture supernatants from these transformed lines were analyzed for the presence of autoantibodies and to study their specificities. By using this approach they showed that virtually all lymphocytes producing IgM antibodies to ssDNA and RF segregated with $CD5^+$ B cells. Similarly all IgG antibodies to ssDNA were associated with $CD5^+$ B cells. Lymphocytes producing IgM autoantibodies specific for insulin and thyroglobulin also segregated primarily with $CD5^+$ B cells. In a recent study Sthoeger et al (1989) demonstrated a significant autoAb production by in vitro stimulated B cells obtained from patients with CLL. These results taken together suggest that

the CD5$^+$ human lymphocytes, similarly to the murine Ly-1 B cells in mice, are a distinct lineage which is largely responsible for the production of certain autoantibodies and represent the normal counterpart of B-CLL.

4. Preferential Utilization of a Restricted Set of V genes by B-CLL Cells.

Since studies of V gene utilization by hybridomas producing autoantibodies showed a biased usage of some V_H and V_κ families, several investigators have addressed similar questions concerning the utilization of V genes by human CD5 B cells. Preud'homme and Seligman (1972) observed that the surface Ig of CLL B cells have a high incidence of RF activity. Kipps et al (1987) studied the V kappa (V_κ) genes expressed in 5 cases of B-CLL which reacted with the monoclonal anti-idiotypic Ab 17.109. This Ab was prepared against the RF paraprotein Sie and recognizes crossreactive idiotypes on 48% of human IgM RF but does not react with IgM devoid of RF activity. A large proportion of B-CLL expresses the 17.109 idiotype. Hybridization with short synthetic nucleotides specific for unique framework sequences, revealed that the 17.109 idiotype positive CLL B cells from different individuals expressed highly homologous V_κ light chain mRNAs belonging to the VkIIIb family (Kipps et al., 1987). In subsequent studies Kipps et al (1988) cloned and sequenced the

V_κ genes from two patients with B-CLL which were 17.109 idiotype positive. The results showed a striking nucleic and amino acid sequence homology between the expressed V_κ genes and a V_κ gene previously isolated from placental DNA, designated Humkv325 (99% amino acid homology) the leader sequence being identical. Therefore, the leukemic cells of both patients expressed Humkv325 with little or no somatic mutation. These sequences, when compared to the sequences of several RF paraproteins revealed an amino acid homology of 94 -- 99% to Humkv325. Furthermore two sequences of 17.109 idiotype negative IgM RF showed greater than 95% amino acid homology to Humkv325. Radoux et al (1986) who reported the cloning of Humkv325 found the sequence of 4 IgM RF to be identical to the germline Humkv325 which belongs to the VkIIIB subgroup. A similar restriction of V_κ gene usage among RF in the murine system has been demonstrated by Schlomchik et al (1986) for $V_\kappa 1$, $V_\kappa 8$ and $V_\kappa 19$. Therefore it appears that some human RF autoantibodies can be generated without somatic mutation and genes encoding RF light chains present in normal subjects are expressed preferentially in B-CLL. All these results taken together show that V_κ genes expressed by B-CLL and RF are homologous and are derived from a limited number of conserved germline genes with little if any somatic mutation. Similarly, Meeker et al (1988) has cloned and sequenced the immunoglobulin heavy chain genes from patients with B-CLL and demonstrated the complete absence of somatic

mutation. In conclusion, it appears that malignant CD5$^+$ B cells express a limited subset of germline immunoglobulin genes.

E. Homology of Ly-1 and CD5.

Recently the genes for the Ly-1 and the Leu-1 cytodifferentiation antigens have been cloned (Jones et al., 1986; Huang et al., 1987). Both proteins are 471 amino acids long with 63% overall amino acid homology. Interestingly, there is a gradient of increasing homology. The first amino terminal subregion has 43% identity, the second 58% and the carboxy terminal 90%. Two of the three potential glycosylation sites in the amino terminal and the two at the carboxy terminal are conserved. The sequence is consistent with an extracellular amino-terminal region of 347 amino acids, followed by a hydrophobic transmembrane segment of 31 amino acids and a carboxy-terminal intracellular segment of 93 amino acids. The N-terminal region is relatively rich in cysteine and is separated into 2 homologous domains by a hinge-like region rich in proline and threonine residues. The 23 cysteine residues are present in very similar locations in the 2 sequences. This molecular information represents a strong argument that murine Ly-1 and human CD5 B cell subsets are functionally related.

F. Possible Physiological Role of CD5 and the CD5 B Cell Subset

The physiological function of the vast majority of lymphocyte cytodifferentiation antigens is presently unknown. There are a few examples of CD molecules for which a role in cell-cell interaction (LFA-1, ICAM, CD4 and CD8), intracellular signal transduction (CD3) or ligand binding (CD21,CD23) has been demonstrated. Unfortunately, despite the extensive characterization of the CD5 B cell subset in both mice and humans, its physiological role is poorly understood. In the murine system, Griffith et al (1989) have recently demonstrated the constitutive phosphorylation of CD5 and implicated protein kinase C in this process. While phorbol esters have an enhancing effect on CD5 expression, concavalin A has an opposite action. Furthermore, CD5 was found to be induced upon in vitro activation of both B and T lymphocytes and has been associated with the upregulation of T cell activation when used in conjunction with other T cell stimuli (Hollander et al., 1981; Nishimura et al., 1988). In addition sorted Ly-1$^+$ B cells demonstrated a higher stimulation index of both proliferative and immunoglobulin secretory responses after IL5 treatment as compared to Ly-1$^-$ B cells (Umland et al, 1989). IL-4 on the other hand was found to down regulate the surface expression of CD5 on normal and leukemic B cells (Defrance et al., 1989; Freedman et al., 1989). Finally Ansar-

Ahmed et al (1989) have shown that estrogen increases the net number of CD5$^+$ B cells and augments the autoAb production by sorted CD5$^+$ B cells obtained from autoimmune mice. This finding might explain the effect of sex hormones on the manifestation of autoimmune phenomena.

In a recent report, sorted thymic B cells, which constitutes a small percentage of total thymic lymphocytes were shown to be of the phenotype Ly-1$^+$, Ia+,B220 low and Mac-1$^+$ (Miyama-Inaba, et al, 1988). Therefore it appears that most thymic B cells have the Ly-1$^+$ phenotype.

In conclusion, present data suggests that the expression of CD5 in both T and B lymphocytes is associated with lymphocyte activation. Obviously, this concept applies only to CD5$^+$ B cells and has no bearing on conventional CD5$^-$ B cells. Various soluble mediators (IL5, IL4, estrogen) affect the expression of the CD5 antigen and/or cell activation. Since all available data is based on in vitro experiments, the in vivo role of CD5 in lymphocyte activation remains to be determined possibly by transgenic mouse experiments. The large fraction of Ly-1 B cells detected in the thymus is consistent with a possible regulatory function.

G. Specific Aims

1. To determine the V_H and V_K chain expressed by mouse hybridomas producing autoantibodies.

2. To study the expression of the Ly-1 gene in mouse hybridomas producing autoantibodies with various specificities obtained from murine strains prone to autoimmune diseases and from normal strains. The V_H and V_K families used by Ly-1 expressing hybridomas will also be determined.

3. To study the expression of the CD5 gene in human B-CLL and small lymphocytic lymphoma (SLL). It has been previously demonstrated that more than 80% of human B-CLL cases are stained with the monoclonal anti-CD5 reagent [15].

4. To study the usage of V_H and V_K gene families in $CD5^+$ human lymphomas in comparison with $CD5^+$ B-CLL and $CD5^-$ lymphomas.

II.

THE LY-1 GENE EXPRESSION IN MURINE HYBRIDOMAS PRODUCING AUTOANTIBODIES

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Running Title : Ly-1 B cell Hybridomas

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(Submited for Publication)

A. ABSTRACT

Studies presented here demonstrate the expression of the Ly-1 gene and the detection of the Ly-1 cytodifferentiation antigen in murine hybridomas producing autoantibodies. We examined the transcription of the Ly-1 gene in thymocytes and 140 hybridomas producing autoantibodies of various specificities which were obtained from normal and autoimmune disease prone mouse strains. As previously demonstrated thymocytes stain brightly for Ly-1 by immunofluorescence and express Ly-1 transcripts. In our panel of hybridomas producing autoantibodies Ly-1 transcripts were detected in 31 (45%) out of 69 NZB hybridomas and 7 (88%) out of 8 viable motheaten hybridomas. S1 nuclease protection experiments showed that Ly-1 transcripts detected in thymocytes and B cells are the product of the same gene. The B cell transcripts are functional since immunofluorescence and Western data presented here detected the Ly-1 protein in hybridomas cells which were found to transcribe the Ly-1 gene. Interestingly a polymorphic transcription of the Ly-1 gene was observed in B cells and B cell hybridomas as compared to thymocytes. Our results obtained in the hybridoma system firmly establish a major contribution of the Ly-1 B cell subset to the production of DNA specific autoantibodies and a smaller contribution to the production of rheumatoid factors and "natural", multispecific autoantibodies.

B. INTRODUCTION

Ly-1 (CD5) is a murine lymphocyte differentiation antigen expressed on the majority of T cells and a small percentage of B cells (1-3%). B cells expressing the Ly-1 antigen represent a distinct subset which has been extensively characterized by immunofluorescence and functional studies (Manohar et al., 1982; Hayakawa et al., 1983). This subset represents a larger fraction of total B cells early in ontogeny as compared to the adult (Hayakawa et., al 1985), is significantly enlarged in NZB mice (10-40%) (Hayakawa et al., 1983) and is predominant in viable motheaten (me^v) mice (Sidman et al., 1986). Certain human B cell malignancies (i.e. Chronic Lymphocytic Leukemia) which have a significant association with autoimmune phenomena, were shown to arise almost exclusively from CD5$^+$ B cells (Boumsell et al., 1980). Furthermore, it was suggested that CD5 B cells are involved in humans and autoimmune prone mice in the spontaneous production of rheumatoid factors (RF) and autoantibodies (autoAbs) specific for DNA, thymocytes and bromelein treated mouse red blood cells (Br-MRBC), (Hayakawa et al., 1984; Casali et al., 1987). In view of this data, implicating this distinct B cell subset in certain autoimmune diseases, it was important to firmly establish the contribution of Ly-1 B cells to the production of specific autoAbs. The random immortalization of individual B cells (both Ly-1$^+$ and Ly-1$^-$)

by cell hybridization offers the distinct advantage of permitting the correlation of the fine specificities of the Abs produced and the Ly-1 phenotype. However, these studies were hampered by the difficulties encountered in detecting the surface associated Ly-1 antigen on hybridomas producing autoantibodies.

Since the surface staining of sorted B cells for the Ly-1 antigen was proven consistently weaker as compared to thymocytes, we took advantage of more sensitive molecular techniques. In order to determine the Ly-1 phenotypic origin ($Ly\text{-}1^+$ or $Ly\text{-}1^-$) of hybridomas producing autoAbs, we examined the expression of the Ly-1 gene. In a previous study we demonstrated that while in thymocytes two Ly-1 transcripts of 2.9 and 2.1 kb were detected, Ly-1 transcripts of 2.9 and 1.6kb were detected in a small fraction of MRL/lpr hybridomas producing autoAbs (Bailey et al., 1989). Furthermore, Painter et al (1988) have shown that in <u>me</u>^{v} mice, strain in which nearly all B cells are $Ly\text{-}1^+$, not all (i.e. 60%) of the hybridomas obtained produced self reactive antibodies. In view of these findings the question addressing the contribution of the Ly-1 B cell subset to autoantibody production remained still opened. Consequently, we extended our initial studies to a panel of 140 hybridomas producing autoAbs originating from various normal and autoimmune prone murine strains. The Ly-1 phenotypic assignment of the hybridomas was initially

done by Northern blot analysis. A representative panel of Ly-1$^+$ hybridomas was further studied by immunofluorescence and Western analysis. The questions addressed in the present study were : a) whether the Ly-1 hybridoma transcripts previously detected with the Ly-1 cDNA probe in Northern analysis are indeed derived from the same gene as the corresponding Ly-1 transcripts identified in thymocytes b) whether there is a relationship between the detection of hybridoma Ly-1 gene transcripts and the detection of polypeptides reacting with monoclonal anti - Ly-1 antibodies in immunofluorescence and Western analysis c)whether there is a relationship between the hybridoma Ly-1 phenotype as determined by Northern analysis, the strain of origin, (normal vs autoimmune prone strain) and the specificities of the autoAbs produced by these hybridomas.

Our results indicate that the Ly-1 transcripts detected in hybridomas and thymocytes are the products of the same gene despite the size difference. Two polypeptides of 73 and 67 KD were detected in hybridomas and <u>me</u>^{v} enriched B cells expressing 2.9 and 1.6 Kb Ly-1 transcripts. The frequency of hybridomas expressing the Ly-1 gene is consistently higher in strains prone to autoimmune diseases. A large fraction of hybridomas producing DNA specific autoAbs are of the Ly-1$^+$ phenotype.

C. MATERIALS AND METHODS

1.**Mice**: Me^{v} mice were obtained from Jackson laboratories.

2.**Hybridomas**: Origin of hybridomas and the specificity of antibodies secreted by these hybridomas are described in Table 1.

3.**Enrichment of B Cells**: Spleen B cells harvested from 79 day old me^{v} mice which showed a complete thymic aplasia were enriched. Single cell suspension of 10^{8} splenic cells were incubated for 30 minutes with a cocktail of anti-Thy1, anti-L_3T_4 and anti-Lyt2 antibodies (1:10 dilution) and then one hour with rabbit complement (1:15 dilution). The cells were cultured overnight in RPMI medium supplemented with 5% FCS and then used for RNA extraction, cytoplasmic lysate preparation and immunofluorescence staining. FACS analysis showed that more than 95% of cells stained with anti-IgM were also stained with anti-Ly1 mAb.

4.**Ly-1 Probe** The preparation of Ly-1 probes was carried out as previously described (Huang et al., 1987).

5.**RNA Extraction and Northern Blotting**

RNA was extracted from 3-5X10^{7} cells using the guanidinium-thiocyanate method (Maniatis et al., 1982). Northern blotting was performed by electrophoretically fractionating the RNA on a 0.8% agarose formaldehyde gel. The gel was blotted overnight onto nitrocellulose using 20XSSC. Blots were baked in a vacuum oven for 2 hours at 80°C. Prehybridization was carried out at 42°C in 50% formamide,

TABLE 1.

ORIGIN AND SPECIFICITY OF HYBRIDOMAS

MRL/lpr	MRL5-51,MRL55-23MRL50-8,MRL55-18,GP75-9(RF) M56 (Sm) FM35-4,63-99-7(DNA) GP88, GP138-10,GP99-5,GP75-9,GP133, GS13-1,GS11-1,GS4-1, H45-5,H16-5,H17-1,H4-2,H91-12,63-86-7(Multispecific)
DBA/1J	D3m, E10, C1, C1D2, A12, B1, D3 (CII)
B6-me^v	UN32-15(IF) UN17-19(RF) UN42-5,UN34-11(Thy) UN55-5(NA) UN59-9,UN37-5,UN40-9(Multispecific)
C3H/HeJ	CH44-7,CH113-1,CH55-8(DNA) CH154-1,CH24-10CGS21-17(Multispecific)
BALB/c	HB2 (DNA) HB8, HB9 (Microfibrils) CP8ED2 (Br-MRBC) NL103-3,NL119-3,NL121-6,NL127-2,NL104-1, NL105-10,NL124-1, NL126-4,NL112-9,NL113-6,NL122-5,NL110-6 (Polysaccharides) F4F5, F9, A7, F1B4 (Multispecific)
(CBA/N x BALB/c) F1	M15-1(Myosin) M40-1,M34-5,M25-8,M25-9,F5-2,F30-4(Multispecific) M16-3(Unknown)
NZB 6d-old	A2F9 (Actin) A1C10, A4G8, B15D3 (TNP) B8H5, B6F2 (DNA) B1E2, A9A7, A3D2, B13D4, B1F5, B10B9 (Multispecific)
NZB 1mo-old	Z51, Z317 (TG) Z41, Z218 ZK9A4-10(DNA) Z49, Z39 (AchR) ZK4G3-8(Br-MRBC) Z121, ZX25, Z14, ZK2H5-3,ZK2A7-3,ZK2A8-11, ZK5F11-3(Multispecific) Z250, ZK244, ZK2A11-1,ZK9D9-1,Z239, ZK2H6-2,ZK9B9-8,ZK3G11-1,ZK8E8-1(Unknown)
NZB 5mo-old	N8G2 (Myosin) N5C5, N8F6, N10E9, Z8E5 (Multispecific)
NZB 16mo-old	ZL154-1(H2b) ZL177-5(RF) ZL173-6,ZL179-6,ZA1C7-5,ZA2B12-10,ZA3E3-12,ZA4A10-13, ZA4B7-2,ZA2G8-3,ZA3E5-12(DNA) ZL37-9(AchR, DNA) ZA4B8-2,ZA1B8-1,ZA1C7-12,ZO121-12,ZO55-3,ZO120-6(Coombs) ZA2C2-6,ZL34-8,ZL37-14,ZA4C3-1 (Multispecific) ZA5C2-5,ZA5E4-8,ZA4A7-1,ZA1B8-1,ZA1E3-1, ZA3C4-2(Unknown)
Abbreviations:	RF:rheumatoid factor, Sm:Smith antigen, CII:collagen type II, IF:intrinsic factor, Thy:thymocytes, NA:nuclear antigen, Br-MRBC:bromelain treated mouse red blood cells, AchR: acetyl choline receptor, H2b:histone 2b, TG:thyroglobulin

5XSSPE, 5X Denhardt's, 50 μg/ml sheared and denatured salmon sperm DNA. Probe was added at 2X10⁶ cpm/ml in hybridization solution (50% formamide, 5XSSPE, 1X Denhardt's, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate). Blots were hybridized overnight at 42°C, then washed two times for 15 minutes in 2XSSC, 0.1% SDS at 65°C then two times in 0.1SSC.01% SDS for 15 minutes at 65°C. Finally the blots were autoradiographed on Kodak XAR film with intensifying screens at -70°C for a few days.

6. **S1 Nuclease Analysis**.

Two hundred ng of the Ly-1 double stranded cDNA insert were co-precipitated with 10 μg total RNA (large probe excess conditions) using tRNA as carrier. The pellet was resuspended in 10 μl of S1 hybridization mix (80% formamide, 40mM Pipes, .04M NaCl, 1mM EDTA, pH 6.4), heated to 85°C for 15 minutes and hybridized overnight at 52°C. Under these hybridization conditions the formation of RNA-DNA hybrids is favored over the DNA-DNA reassociation. The RNA-DNA hybrids were treated with 1000 units S1 in 300 μl S1 mix for 1 hours at 37°C. S1 mix is 0.28MNaCl, 4.5MZnSO_4, 50mM NaOAc, pH4.5. The reaction was stopped after 1 hour by adding 75 μl termination buffer (2.5 M NH_4OAc, 50mM EDTA). Samples were phenol and chloroform extracted twice and precipitated four times (tRNA was used as carrier). Following the last precipitation the samples were resuspended in TE and loaded on a 1% horizontal agarose gel

(3V/cm). The gel was soaked in denaturation buffer (0.2N NaOH, 0.5M NaCl) for 1 hour and then neutralized for 1 hour in 0.5M NH_4OAc, 0.5M NaCl. Subsequently the gel was blotted onto nitrocellulose in 20xSSC and baked in vacuum oven for 2 hours at 80°C. The filters were subsequently prehybridized, hybridized with random primed probes and autoradiographed as described for Northern blots.

7. **Immunofluorescence staining**

Immunofluorescence was carried out using the FACSAN instrument (Becton Dickinson). Hybridoma cells (viability greater than 95%) were stained with optimal amounts of biotin labeled 53-7.5 (anti - Ly-1) or isotype matched 53-6.7 (anti-Lyt2) followed by the addition of PE-avidin. For cytoplasmic staining the cells were fixed for 15 minutes at 4°C in 50% ethanol prior to staining. Both profiles are shown on the same figure.

8. **Western blotting**

The identification of Ly-1 gene products was carried out by Western blotting of cell lysates. 10^7 cells were centrifuged for 10 minutes at 1500g and resuspended in 15 μl NTE buffer (20mM Tris-HCl, 150mM NaCL, 0.1mM EDTA, 0.2mM PMSF and 7mM 2.ME). After a 5 minute incubation on ice, 15 μl of 2% triton X 100 in NTE buffer were added and incubated for an additional 5 minutes on ice. The cells were centrifuged for 5 minutes at 12,000g, the supernatant harvested and frozen at -70°. Thirty μl supernatant corresponding to 10^7 cells were

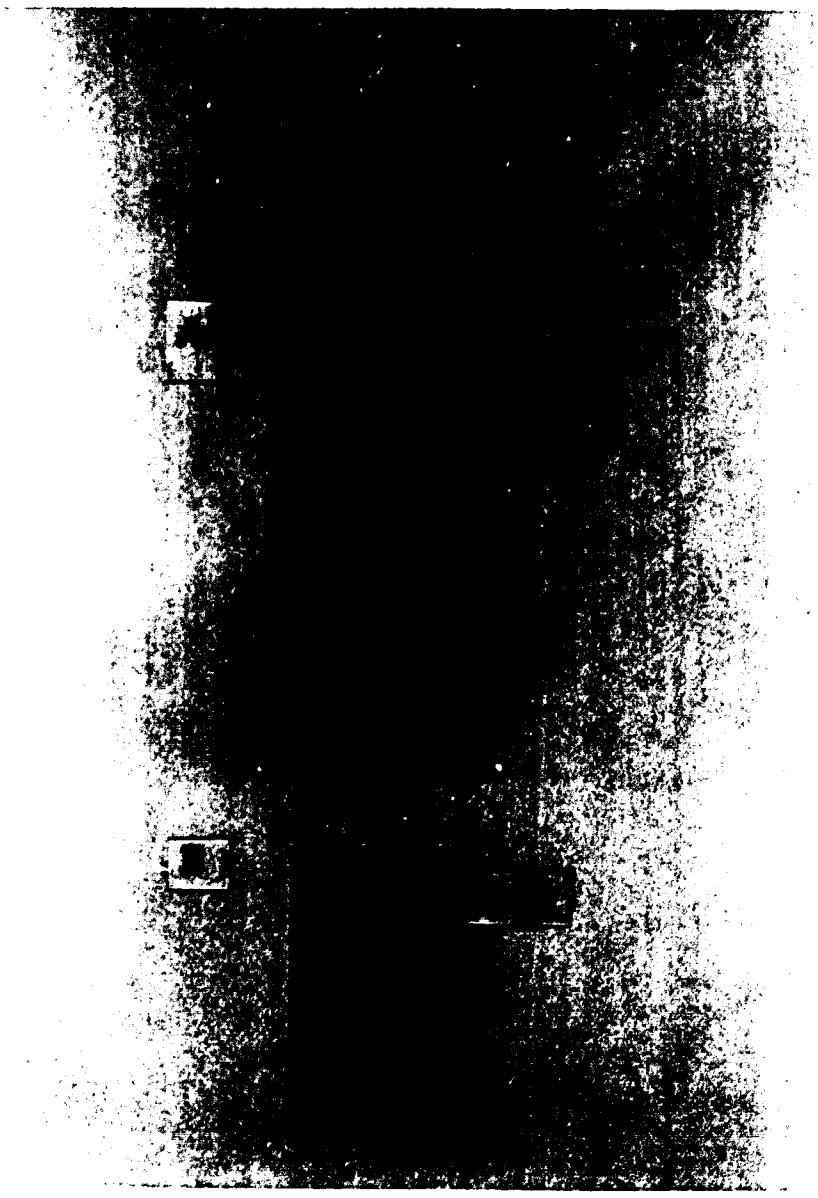
mixed with 30 µl of loading buffer (20% glycerol, 10% 2-ME, 4.6% SDS, 125mM Tris-HCl pH6.8) and electrophoresed on a 10% polyacrylamide - SDS gel and then electrotransferred onto a DEAE membrane. The membrane was saturated overnight with 5% BSA-PBS and then incubated for 2 hours at RT in a solution containing rat anti-Ly-1 mAb (53-7.5) (1µg/ml). After extensive washing in a solution of 0.05M Tris-Hcl pH8.2, 0.15M NaCL and 2% Tween 20, the membrane was incubated for 30 minutes with a solution containing ^{125}I-goat anti-rat IgG (10^6 cpm/ml) 0.05M Tris-HCL pH8.2, 0.15 M NaCL, 20% goat Ig and 5% BSA. After incubation the membrane was washed extensively, dried and autoradiographed.

D. RESULTS

1. Expression of the Ly-1 Gene in Thymocytes, Motheaten Enriched B Cells and Hybridomas

The study of Ly-1 gene expression in total, nuclear and cytoplasmic RNA extracted from thymus, demonstrates two transcripts of 2.9 and 2.1 kb respectively, the shorter one being significantly more abundant (Figure 1A). Both transcripts were clearly detected in total and nuclear thymic RNA. The less abundant 2.9 kb Ly-1 transcript however was detected mostly in the nuclear thymic RNA, suggesting that it probably represents a nuclear precursor transcript. As control for the separation of nuclear and cytoplasmic RNA,

Figure 1. Northern blot of nuclear and cytoplasmic thymic RNA hybridized with the Ly-1 cDNA probe (panel A) and glyceraldehyde phosphate dehydrogenase (panel B).



the same blot was hybridized with the glyceraldehyde phosphate dehydrogenase (GADPH) cDNA probe, since GADPH transcripts are detected primarily in the cytoplasm (Fig. 1B). In contrast, total RNA extracted from highly purified $\underline{me}^{v}$ splenic B cells as well as from $\underline{me}^{v}$ and NZB hybridomas, (UN17-19, Z49 and Z51) all showed Ly-1 transcripts of 2.9 and 1.6 kb. It is significant to note that the 2.9 kb Ly-1 hybridoma transcript corresponds to the longer, less abundant thymic Ly-1 transcript (Fig. 2). The shorter, 1.6 kb Ly-1 transcript identified in $\underline{me}^{v}$ enriched B cells and hybridomas can be explained by : a) cross hybridization of Ly-1 cDNA probe, which was obtained from a thymic library, with transcripts of a homologous gene, specifically expressed in B cells; b) aberrant transcription or; c) alternate splicing of a primary Ly-1 transcript or different transcription initiation/ termination of the Ly-1 gene transcript related to either cell type (B versus T) or fusion event. These possibilities were investigated in further experiments.

2. S1 Nuclease Protection

RNA obtained from thymus, CH31LX, a B cell lymphoma line which stains brightly for Ly-1, two $Ly\text{-}1^{+}$ hybridomas (Z41 and UN40-9), and SP2/0 myeloma cells was analyzed in S1 nuclease protection experiments. As depicted in Fig. 3 a protected fragment of 1.6 kb was observed for both thymic and CH31LX RNA, while in the case of both hybridomas analyzed, a Figure

Figure 2. Northern blot of total RNA extracted from thymocytes, $\underline{me}^{v}$ enriched B cells and Z51, Z49 (NZB) and UN17-19 ($\underline{me}^{v}$) hybridomas hybridized with the Ly-1 cDNA probe.

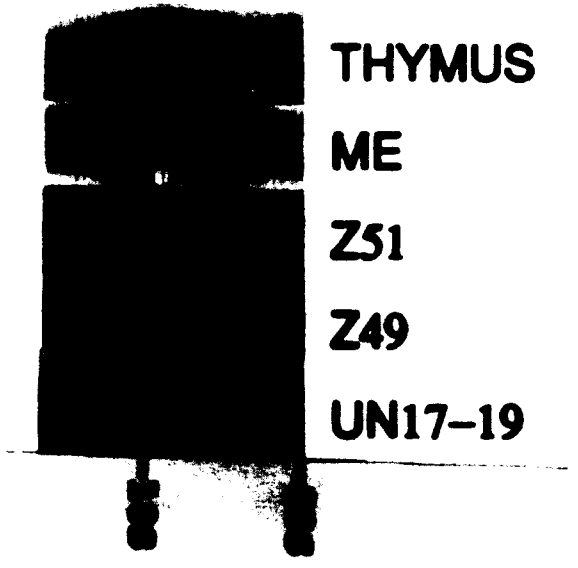

THYMUS
ME
Z51
Z49
UN17–19

Figure 3. S1 nuclease protection. Two hundred ng Ly-1 cDNA insert was hybridized with 10 μg total RNA obtained from thymocytes, CH31LX lymphoma cells and Z41 and UN17-19 hybridomas. The DNA-RNA heteroduplex was S1 digested, electrophoresed, transferred to nitrocellulose and hybridized with the Ly-1 cDNA probe.


THYMUS
CH31
Z41
UN17-19
1.6kb -
1.2kb -
.8kb -

A protected fragment of 0.8kb was observed. An additional protected fragment of 1.2 kb was observed in thymic RNA. These results clearly demonstrate that the Ly-1 transcripts previously detected in Northern analysis in thymus, CH31LX and the 2 hybridomas tested are the products of the same gene. No protected fragments were observed with SP2/0 RNA (data not shown).

3. Correlation of Ly-1 Gene Transcription and the Detection of Ly-1 Polypeptide in Hybridomas.

In order to investigate the functionality of the Ly-1 hybridoma transcripts detected by Northern and S1 nuclease protection experiments, we carried out Western blotting and immunofluorescence staining in a representative number of hybridomas expressing Ly-1 transcripts and highly purified B cells isolated from me^{v} splenocytes. The panel of hybridomas consisted of two hybridomas from me^{v} mice (UN17-19 and UN40-9), one from C_3H/HeJ mice (CH113-1) and four from NZB mice (Z239, Z19, Z41 and ZL176-6).

The Western data presented in Figure 4 show that in the thymic lysate three bands of 67, 72 and 130 kd were observed and in me^{v} B cell lysate only the bands of 67 and 72 kd reacted with anti-Ly-1 mAb. These two bands were also detected in all hybridomas studied. The band of 130kd observed in thymus, was also detected in some hybridomas. Duplicate blots incubated with an isotype matched mAb (anti-FcR) did not reveal any of these bands (data not shown). The

Figure 4. Western blot of cellular lysate obtained from SP2/0 myeloma cells, thymocytes, $\underline{me}^{v}$ enriched B cells and UN17-19, UN40-9 ($\underline{me}^{v}$), CH113-1 (C,H/HeJ), Z239, Z41, Z49 and ZL176-9 (NZB) hybridomas. Cytoplasmic lysates were obtained under reducing conditions from 10^{7} cells, electrophoresed under reducing conditions transferred to DEAE membrane and incubated with ^{125}I labelled anti - Ly-1 monoclonal antibodies.

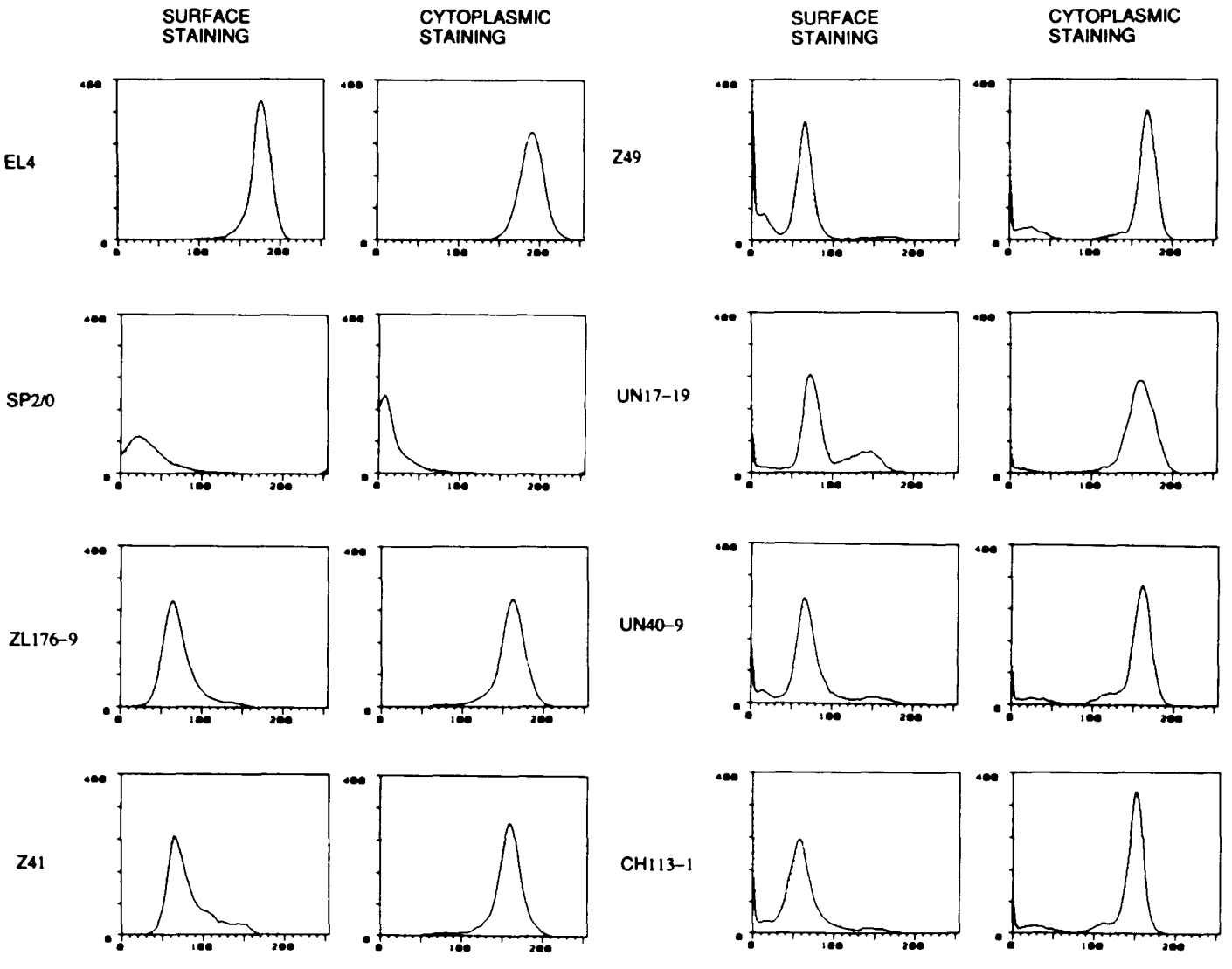
130 Kd -
75 Kd -
SP2/0
THYMUS
ME
UN17-19
UN40-9
CH113-1
Z239
Z41
Z49
ZL176-9
72 Kd -
67 Kd -

67 and 72 Kd bands detected in our Western blotting analysis correlate with the previously described labeled material obtained from NP-40 extracts of murine thymocytes precipitated with specific anti - Ly-1 polyclonal anti-serum (Durda et al., 1978). The 67 Kd band detected in both Western and immunoprecipitation experiments corresponds to the mature Ly-1 gene product made up of 471 amino acid residues (Huang et al., 1987). The 72 kd band detected in our Western analysis and the 80 kd band previously detected by immunoprecipitation in thymocytes probably represent glycosilated forms or partially processed gene products.

The significance of the 130 kd band, which was detected in thymus, some of the hybridomas as well as in SP2/0 lysates is not clear. The presence of reducing agents in both the lysing and loading buffers formally excludes the possibility that the 130 kd band is a dimer of the 67 kd protein. Since this band was also observed in SP2/0 lysates, a myeloma line in which no Ly-1 transcripts were detected, and which is clearly negative by immunofluorescence, we conclude that the 130 kd polypeptide probably bears a cross reactive epitope interacting with the rat anti - Ly-1 mAb.

Immunofluorescence staining with anti - Ly-1 and anti - Lyt2 mAbs was also carried out on this group of $Ly\text{-}1^{+}$ hybridomas. The data illustrated in Figure 5 show a strong intracytoplasmic staining of all six hybridomas studied.

Figure 5. FACSCAN contours of surface (left panel) and cytoplasmic (right panel) immunofluorescence staining of EL4 (T cell control), SP2/0 and UN17-19, UN40-9 (me^v), CH113-1 (C,H/HeJ), Z41, Z49 and ZL176-9 (NZB) hybridomas. The solid line represents the staining profile with 53-7.5, the anti-Ly-1 reagent. The dotted line represents the staining profile with the isotype matched 53-6.7 anti-Lyt2 reagent.


SURFACE STAINING
CYTOPLASMIC STAINING
SURFACE STAINING
CYTOPLASMIC STAINING
EL4
Z49
SP2/0
UN17-19
ZL176-9
UN40-9
Z41
CH113-1

Three of these six Ly-1$^+$ hybridomas studied exhibited a weak surface staining (ZL176-9, UN17-19 and UN40-9). The staining profile pattern of surface and intracytoplasmic immunofluorescence is identical to previous results obtained with NZB splenocytes (Manohar et al., 1982). In order to determine the relationship between the Ly-1 transcripts and the 72 and 67 kd proteins, studies in progress in our laboratory are aimed to synthesize full length cDNAs from thymic nuclear (2.9 kb), cytoplasmic (2.1 kb) and Z239 hybridoma (1.6 kb) RNA. These cDNA will be expressed and the protein product will be subsequently studied.

4. Expression of the Ly-1 Gene in 140 Hybridomas Producing Autoantibodies

Since we established a good correlation between the detection of hybridoma Ly-1 transcripts and polypeptides we used Northern blot analysis in order to determine the frequency of hybridomas of the Ly-1$^+$ phenotype (Ly-1$^+$ hybridomas) in a large panel of 140 hybridomas producing autoantibodies derived from normal and autoimmune prone murine strains. Table 2 illustrates the strain of origin and the specificities of the autoAbs produced. A large fraction of the hybridomas were obtained from strains prone to autoimmune disease such as NZB, me^v and MRL/lpr. The hybridomas specific for collagen II (CII) were obtained from DBA/1J mice injected with CII whereas those obtained from

TABLE 2.

LY-1 GENE EXPRESSION IN HYBRIDOMAS OBTAINED FROM VARIOUS MURINE STRAINS

	NZB	MRL/ lpr	DBA	ME^{v}	C3H/ HeJ	BALB	CBAN	Total
Ly-1^{+}	31	6	0	7	1	2	1	48
Ly-1^{-}	38	16	7	1	5	6	7	80
Total	69	22	7	8	6	8	8	128

Abbreviations: DBA: DBA/1J, BALB: BALB/c,
CBAN: (CBAN x BALB/c)F1

C,H/HeJ originated from animals injected with anti-idiotypic antibodies (Monestier et al., 1983; Bailey et al., 1989). The BALB/c NL series of hybridomas were obtained from 1 month old LPS-stimulated lymphocytes and were selected by screening with the V_HX24 probe (Bonilla et al., manuscript in preparation). These hybridomas bind to polysaccharides such as levan, ACHO and galactan and were used as controls. Ly-1 transcripts were detected in 48 out of 140 hybridomas tested. No Ly-1 transcripts were detected in the BALB/c NL series of hybridomas. Figure 5 illustrates a Northern blot carried out with RNA extracted from a representative group of hybridomas obtained from adult BALB/c and NZB mice of various ages.

The data presented in Table 2 show the strain distribution of the $Ly\text{-}1^+$ and $Ly\text{-}1^-$ hybridomas. Approximatively one half of NZB and 7 of 8 $\underline{me}^v$ hybridomas express the Ly-1 gene, data in agreement with the high proportion of Ly-1 B cells previously reported in these strains (Hayakawa et al., 1983; Sidman et al., 1986). Only 6 out of 22 hybridomas obtained from 1-month old MRL/lpr mice, two out of 19 obtained from BALB/c mice and 1 out of 6 obtained from C,H/HeJ mice were found to express the Ly-1 gene. Interestingly one out of eight (CBA/N x BALB/c) F_1 hybridomas was found to express the Ly-1 gene. Prior studies have reported an inability to detect Ly-1 B cells in this strain by conventional surface immunofluorescence techniques.

The data depicted in Table 3 summatizes the Ly-1

Figure 6. Northern blot of RNA extracted from representative BALB/c and NZB mice of various ages (6-day, 1, 5 and 16 month old). The blots were hybridized with the Ly-1 cDNA probe.

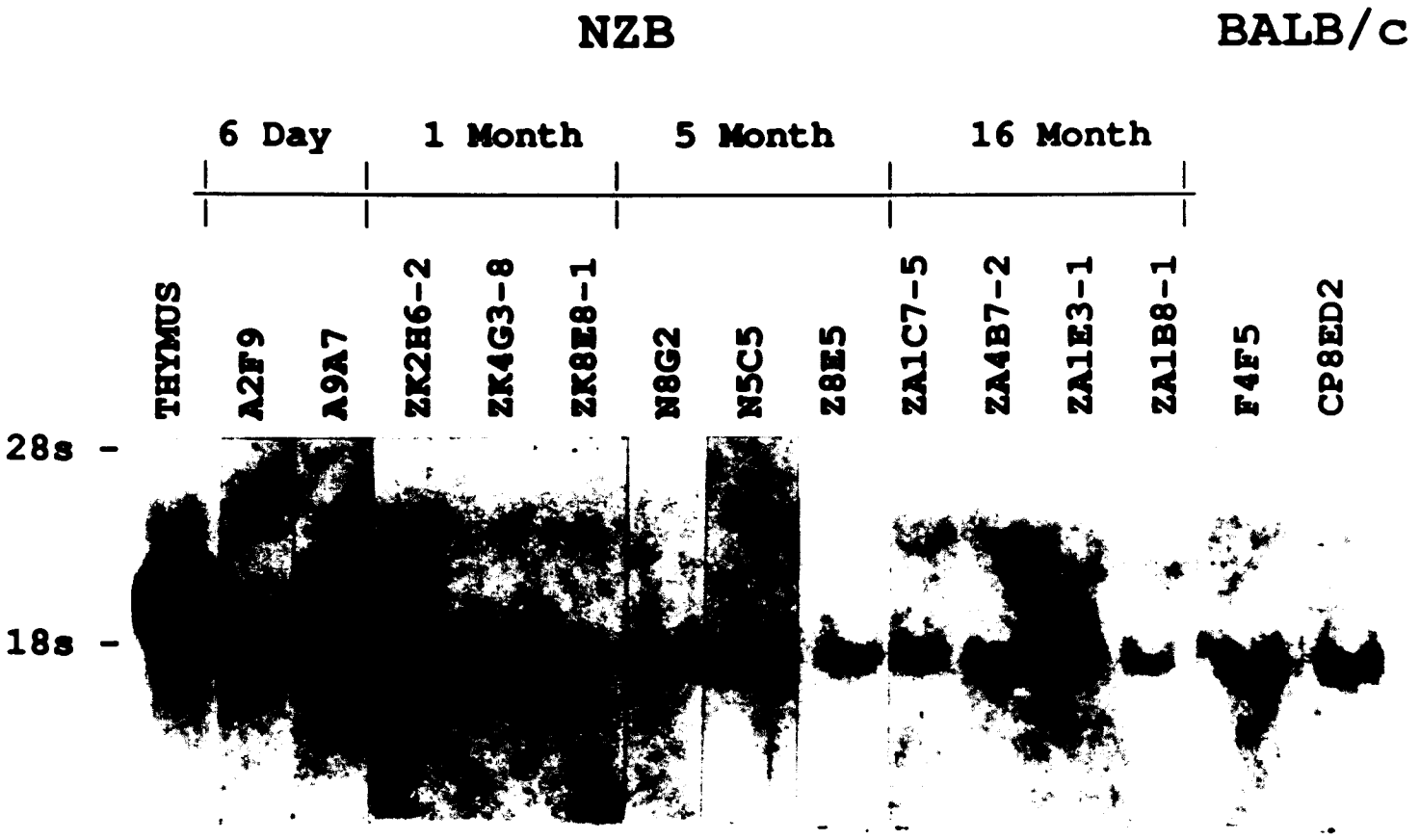

NZB
BALB/c
6 Day
1 Month
5 Month
16 Month
THYMUS
A2F9
A9A7
ZK2H6-2
ZK4G3-8
ZK8E8-1
N8G2
N5C5
Z8E5
ZA1C7-5
ZA4B7-2
ZA1E3-1
ZA1B8-1
F4F5
CP8ED2
28s
18s

TABLE 3.

EXPRESSION OF LY-1 GENE IN HYBRIDOMAS PRODUCING AUTOANTIBODIES OF VARIOUS SPECIFICITIES

	LY-1$^+$	LY-1$^-$	TOTAL
DNA	11	9	20
RF	2	5	7
Sm	0	1	1
H2b	1	0	1
CII	0	7	7
IF	1	0	1
TG	1	1	2
NA	0	1	1
Br-MRBC	2	0	2
AchR	2	0	2
Microfibrils	0	2	2
Myosin	1	1	2
Actin	1	1	1
Thy	2	0	2
RBC(Coombs)	1	5	6
TNP	1	2	3
Polysaccharides	0	12	12
Multispecific	13	38	51
Total	39	83	

Abbreviations:

RF: rheumatoid factor, Sm: Smith antigen,H2b: histone 2b, CII: collagen type II, IF: intrinsic factor, TG: thyroglobulin, NA: nuclear antigen, Br-MRBC: bromelein treated mouse red blood cells, AchR:acetyl choline receptor, Thy:thymocytes

and the fine specificities of the autoAbs produced in our panel of hybridomas. The most significant findings arethat the majority of DNA specific autoAbs are produced by hybridomas of the Ly-1$^+$ phenotype, whereas the majority of hybridomas producing RF and multispecific autoAbs are produced mostly by hybridomas of the Ly-1$^-$ phenotype. Despite the small sample size it should also be noted that hybridomas producing autoAbs specific for H,b, IF, AchR, actin, thymocytes and Br-MRBC all express the Ly-1 gene. On the other hand none of the DBA/1J hybridomas producing CII specific autoAbs and only 1 out of 6 NZB hybridomas producing Coombs autoAbs originate from the Ly-1$^+$ phenotypic B cell subset. These two types of autoAbs are considered to be pathogenic.

E. DISCUSSION

Prior data have shown that the Ly-1 B cell subset is enlarged in certain strains prone to autoimmune disease such as NZB and <u>me</u>^{v} (Haykawa et al., 1983; Sidman et al., 1986) and appears to play an important role in the production of RF and certain autoAbs such as DNA, RF, Br-MRBC and thymocyte specific (Hayakawa et al., 1984). The hybridoma technology permitted the immortalization of autoimmune B cells from normal and autoimmune animals as well as human subjects.

However, since the Ly-1 antigen is weakly expressed on the membrane of B cells it was difficult to directly correlate the Ly-1 phenotype with the production of certain types of autoAbs. All prior work on the contribution of the Ly-1 B cell subset to autoAb production was done solely by surface staining and was based on the indirect association, seen in a limited number of murine strains, between an increased proportion of Ly-1 B cells and a high frequency of autoAb production. Using more sensitive molecular techniques in a previous study we were able to identify Ly-1 transcripts in autoAb producing hybridomas obtained from MRL/lpr mice and therefore make a Ly-1 phenotypic assignment based on the detection of gene expression (Bailey et al., 1989). Consequently, we extended our study to a large panel of 140 autoAb producing hybridomas obtained from normal and autoimmune prone murine strains.

The initial analysis of thymic RNA revealed two Ly-1 transcripts of 2.1 and 2.9kb respectively. The longer, less abundant, 2.9 kb thymic transcript is probably a precursor since it was found mostly in nuclear RNA. In Northern experiments Ly-1 B cell transcripts of 2.9 kb (corresponding to the less abundant thymic Ly-1 transcript) and 1.6 kb were demonstrated. In view of this apparent polymorphic transcription of the Ly-1 gene in B cells as compared to thymocytes, we carried out S1 nuclease protection experiments. Our results clearly demonstrated that the Ly-1

transcripts detected in B cells are the product of the same gene which is expressed in thymocytes.

It is important to note that analysis of highly purified B cells from $\underline{me}^{v}$ mice, a strain in which nearly all B cells bear the Ly-1 antigen on their membranes (Sidman et al., 1986), and a representative group of Ly-1^{+} hybridomas, all show the same 2.9 and 1.6 kb Ly-1 transcripts in Northern blots, the same 73 and 67 kd bands in Western blots and all demonstrate clear intracytoplasmic staining. The correlation between the detection of Ly-1 transcripts and intracytoplasmic proteins, which interact with monoclonal anti-Ly-1 antibody in both Western blots and immunofluorescence, demonstrate that these B cell Ly-1 transcripts are functional.

In preliminary experiments we ruled out the effect of cell fusion on the expression of the Ly-1 gene. Three quadromas obtained by the fusion of the CH12LX lymphoma line, which stains brightly for Ly-1, with SP2/0 all have the same pattern of gene transcription and surface immunofluorescence staining as the parent line. Taken together our data strongly suggest that the Ly-1 gene product in B cells is different from that in T cells. In view of the surface versus intracytoplasmic immunofluorescence results it is also apparent that in hybridomas, and probably B cells in general, the Ly-1 protein accumulates in the cytoplasm where it can be

readily detected. Only a small fraction of the Ly-1 protein however, probably becomes anchored on the membrane, which explains the characteristic "dull" surface staining seen in hybridomas and sorted B cells. A very likely explanation of this phenomena is the generation of isoforms at the carboxy terminus in B and T cells, since the C-terminal region of the Ly-1 protein is involved in membrane anchoring. This process has recently been described for the human dystrophin gene (Feener et al., 1989). There are other examples indicating that the cytodifferentiation antigens identified with the same monoclonal antibodies can differ in T and B cells. Thus, while T200 antigen is of 200 kd in T cells, in B cells it has a size of 180 kd (Trowbridge et al., 1975). The different size of Ly1 transcript seen in B cells and hybridomas is probably due to alternate splicing or different initiation/termination of transcription. Alternative splicing of the mRNA coding for glucose-6-phosphate dehydrogenase in different tissues was described in human (Hirono et al 1989) and it was demonstrated that the secreted form of CD8 also arises from alternative splicing (Giblin et al., 1989).

Our data on Ly-1 hybridoma gene transcripts and the corresponding proteins detected with monoclonal anti - Ly-1 antibodies show that the identification of Ly-1 gene transcript is a valuable marker for the Ly-1 phenotypic assignment, taking into account the low density or even

absence of the Ly-1 antigen on the surface of B cells. Using this criterion we have shown that a significant proportion of autoAb producing hybridomas are of the Ly-1$^+$ phenotype. This proportion is particularly high in hybridomas obtained from NZB and <u>me</u>^{v} mice, which is in agreement with prior data showing an increased proportion of this B cell subset in these autoimmune murine strains. Our data clearly demonstrates that a high proportion of autoAbs specific for DNA, BR-MRBC, and thymocytes are produced by Ly-1 B cells and a smaller fraction of hybridomas producing RF and multispecific autoAbs expressed the Ly-1 gene. This is surprising since the clones producing multispecific autoAbs, which represent a large fraction of the pre-immune repertoire (Avrameas et al., 1988), were considered to belong to the Ly-1 B cell subset (Kearney and Vakil 1986). It is important to note that two types of pathogenic autoAbs, one group specific for CII and the second group represented by NZB hybridomas producing Coombs antibodies are not produced by Ly-1$^+$ hybridomas.

Collectively, these results strongly suggest that non organ specific antibodies are produced by both Ly1$^+$ and Ly1$^-$ B cells and that at least some pathogenic autoantibodies such as those specific for collagen II and erythrocytes are produced by Ly1$^-$ B cells.

III.

CD5 AND IMMUNOGLOBULIN V GENE EXPRESSION IN B CELL LYMPHOMAS AND CHRONIC LYMPHOCYTIC LEUKEMIA

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A. ABSTRACT

We have studied the expression of CD5 and immunoglobulin variable gene families in a panel of monoclonal EBV transformed lines, chronic lymphocytic leukemias and CD5$^+$ and CD5$^-$ B cell lymphomas. The CD5 gene expression was in all cases identical to that of T cell malignancies. The utilization of the various V_H and V_κ gene families was roughly proportional to the estimated gene family size in EBV lines obtained from adult healthy subjects. In contrast we found a statistically significant biased usage of V_H6 in CLL and V_H5 in CD5$^+$ lymphomas as compared to EBV lines and of VKIII in both CLL and CD5$^+$ lymphomas as compared to EBV lines. Some differences in the variable gene usage were also noted when comparing CD5$^+$ and CD5$^-$ lymphomas. These findings are analyzed in the context of possible mechanisms involved in the malignant transformation of CD5$^+$ B cells.

B. INTRODUCTION

Chronic lymphocytic leukemia (CLL) represents a malignancy of a discrete subset of B lymphocyte that express the CD5 antigen (Bousell et al., 1980; Kamoun et al., 1981). This T cell cytodifferentiation antigen is expressed on 20% of B cells in adult human blood and spleen, on 10% of B cells in tonsil and in more than half of fetal spleen and cord blood B cells (Antin at al., 1986). The CD5 antigen is found however on more than 90% of CLL cases (Boumsell et al., 1980; Kamoun et al., 1981). $CD5^{+}$ CLL B lymphocytes also coexpress CD19 or CD20. Also about 60% of small lymphocytic lymphomas express CD5. Only 25% of follicular lymphomas and a small percentage of non-Hodgkin's lymphomas express CD5 (Knowles et al., 1983). This antigen was not detected on the surface of malignant plasma cells, acute lymphocytic leukemia cells, and Burkitt lymphomas cells (Knowles et al., 1983). Malignant B cells, like normal B cells express Ig receptors, encoded by rearranged V (D)J gene segments (Tonegawa et al., 1983). In humans there are approximatively 70-100 germline V_H gene genes, organized in 6 families (V_H1-V_H6) as derived by nucleotide sequence homology. The complexity of these families varies from 1 in the case of V_H6 to 20-30 for V_H1 and V_H3 (Kodaira et al., 1986; Lee et al., 1987; Matthyssens et al, 1980; Berman et al., 1988). Similar to the V_H locus, the human V_κ locus contains 30-40 germline V_κ gene segments, which have been classified into 4 families based both on serological

reactivity with anti-V_κ subgroup antibodies and on nucleic acid sequence (Kunkel et al., 1974; Bentley et al., 1981). The complexity of these families varies from 1 for V_κ IV (Klobeck et al., 1985; Marsh et al., 1985) to 15-20 for V_κ1 (Bentley et al., 1981; Jaenichen et al., 1984). Studies of the utilization of V_H gene families in CLL and acute lymphocytic leukemia (ALL) demonstrated a high usage of V_H5 in 30% of patients with CLL and ALL (Humphries et al., 1988). Similarly the V_κIIIb family has been found to be highly utilized in a small panel of CLL samples selected for the expression of a cross reactive idiotype that was originally defined on a significant proportion of rheumatoid factor (RF) paraproteins (Kipps et al., 1987; Kipps et al 1988). The first aim of our study was to investigate whether the expression of the CD5 gene in CLL is identical as that found in T cells. The study of the expression of CD5 gene in human B cell malignancies as compared to T cells is important since we have previously determined that in the murine system, multiple Ly-1 (CD5) transcripts are detected and the expression of the Ly-1 gene differs in T and B cells (Bailey et al., 1989). A second goal of our study was to determine the expression of various V_H and V_κ gene families in a large randomly selected panel of CLL and B cell lymphomas (BCL). Our results demonstrate that two CD5 transcripts of 3.6 and 2.7kb are found in both T cells, B-CLL and BCL. We also demonstrate a biased usage of V_H5, V_H6 and V_κIII in CLL and CD5$^+$

B cell lymphomas (BCL), the latter of which are classified as small lymphocytic lymphomas.

C. MATERIALS AND METHODS

1. Patients and Cell Lines A) **Lymphoma samples** - Lymph node tissue was obtained from biopsy specimens of patients undergoing diagnostic testing. The lymphoma panel consisted of 10 samples obtained from patients with $CD5^+$ BCL (small lymphocytic lymphomas) and 8 samples obtained from patients with $CD5^-$ Non-Hodgkin's lymphomas. Sections were used for conventional histological studies, cell marker analysis and RNA extraction. Fig 7 shows a typical histopathological lymph node section from a patient with malignant small lymphocytic lymphoma (panel A). The vast majority of lymphocytes show positive staining for CD5 (panel B). Table 4 illustrates the histological type and cell marker analysis.

B) **CLL samples** - Peripheral blood was obtained from patients with CLL. Lymphocytes were separated by Ficoll-hypaque gradient centrifugation. The cells were used for lymphocyte marker analysis and RNA extraction. In selected instances separated mononuclear cells were depleted of T cells forming E-rosettes with VCN treated sheep erythrocytes as previously described (Knowles et al., 1983). Table 5 illustrates the stage of the disease, total leukocyte number, and lymphocytic markers. C) **EBV lines** - 97 EBV lines obtained from adult PBL

TABLE 4

HISTOPATHOLOGICAL CHARACTERISTICS OF B CELL LYMPHOMAS

Patient	Histopathological type				CD5
	Rappaport	Working formulation	Surface Ig H	Surface Ig L	
629	WDL	SL	μ	λ	+
643	WDL	SL	ND		+
749	WDL	SL	μ	λ	+
768	WDL	SL	μ	λ	+
792	WDL	SL	μ	λ	+
859	WDL	SL	μ	κ	+
863	WDL	SL	μ	κ	+
976	WDL	SL	μ	κ	+
997	WDL	SL	μ	κ	+
998	WDL	SL	μ	κ	+
852	NML	FM	μ	λ	-
857	DPDL	DSC	μ	κ	-
859	DHL	DLC	ND		-
887	DHL	DLC	μ	κ	-
888	NPDL	FSC	μ	λ	-
897	UNB	SNC	ND		-
912	DHL	DLC	ND		-
880	NML	FM	μ	κ	-

LEGEND

Rappaport:
- WDL = well differentiated lymphocytic lymphomas
- DPDL = diffuse poorly differentiated lymphocytic lymphomas
- NPDL = Nodular poorly differentiated lymphomas
- DHL = diffuse histiocytic lymphomas
- UNB = undifferentiated non Burkitt lymphomas

Working formulation
- SL = small lymphocytic
- FM = follicular
- DSC = diffuse small cleared cell
- FSC = full small cleared cell
- FM = follicular mixed small clear and large cell
- DLC = diffuse large cell
- SNL = small non cleared cell

- ND = not determined
- H = heavy chain class
- L = light chain type (κ or λ)
- μ = IgM
- λ = lambda
- κ = kappa

TABLE 5

CLINICAL CHARACTERISTICS OF CLL PATIENTS STUDIED

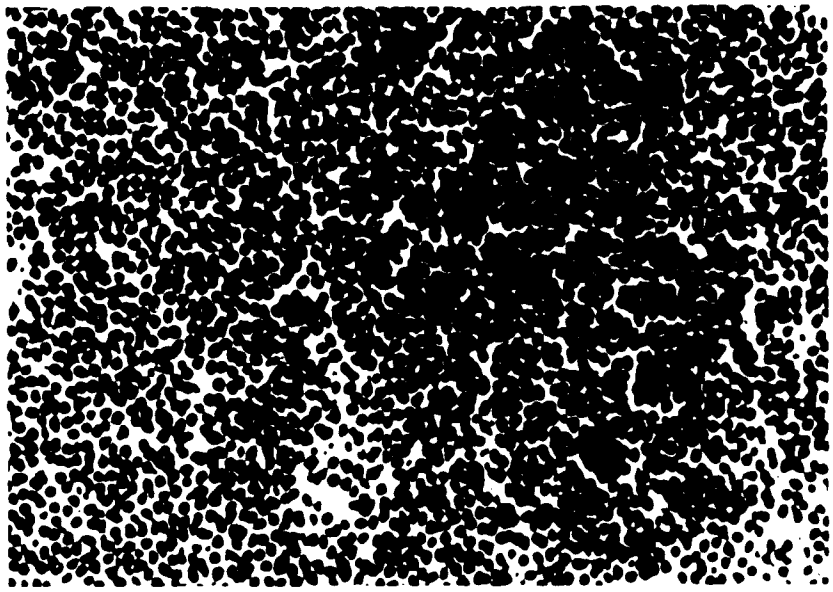
PN	Stage	WBC	%lymph.	Light chain type	%$CD5^+$ $CD19^+$	% T cells
1	A	70.0	99	κ	85	0.3
2	A	22.2	72	κ	55	26.0
3	A	12.0	71	λ	75	10.0
4	A	60.0	78	κ	90	6.0
5	A	21.0	78	κ	91	4.0
6	A	64.1	90	κ	82	5.0
7	A	11.9	80	κ	45	22.0
8	A	28.7	81	κ	78	ND
9	A	44.0	83	κ	79	8.0
10	A	13.2	78	λ	74	16.0
11	A	60.0	82	κ	79	8.0
12	A	41.0	76	κ	64	6.0
13	B	45.3	80	κ	75	6.0
14	B	56.4	85	λ	68	8.0
15	B	50.2	76	κ	63	10.0
16	C	43.0	100	κ	96	2.0
17	C	88.0	84	λ	94	5.0
18	0	25.0	54	κ	38	26.0
19	II	87.0	90	κ	91	5.0
20	II	56.3	79	κ	83	11.0
21	II	35.0	86	κ	79	11.0
22	II	36.0	80	κ	92	5.0
23	II	25.0	80	κ	90	5.0
24	II	24.0	64	κ	32	21.0
25	III	15.4	85	λ	93	4.0
26	III	25.0	80	λ	80	10.0
27	III	40.0	87	κ	88	6.0
28	IV	23.9	72	κ	93	7.0
29	IV	66.0	98	κ	62	0.0
30	IV	82.0	64	κ	32	21.0
31	IV	80.0	92	κ	84	6.0
32	IV	29.0	78	λ	70	13.0
33	IV	45.0	98	κ	91	6.0
34	IV	31.4	90	κ	74	11.0
35	IV	67.5	56	κ	31	5.0
36	IV	71.0	95	κ	90	1.0
37	IV	29.5	88	κ	70	11.0
38	IV	29.4	87	κ	27	9.0
39	IV	66.0	91	κ	94	3.0
40	IV	106.0	89	κ	70	5.0

Legend

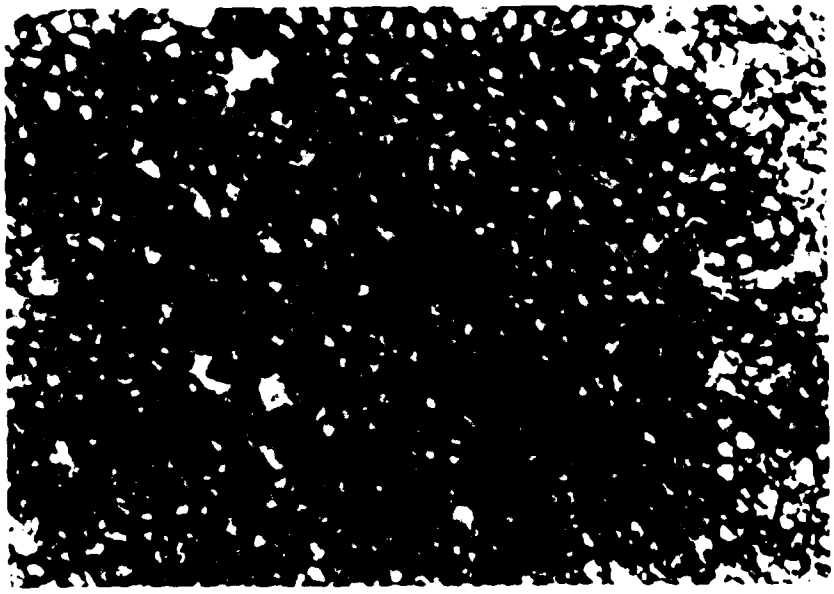
λ	=	lambda
κ	=	kappa
ND	=	not determined
PN	=	Patient number
Stage	=	Patients CLL1-CLL17 were classified according to Binet et al (1981) whereas patients CLL18-CLL40 were classified according to Rai et al (1975).
WBC	=	leukocytes/μl x 10^3

Figure 7. Histopathological lymph node section from a patient with small lymphocytic lymphoma. Morphology (panel A) and CD5 immunochemical staining (panel B).

A



B



were previously assayed for V_H utilization (Logtenberg et al., 1989). This data were used in this study as control group. In addition, 11 EBV lines producing anti-DNA antibodies (Abs), obtained from SLE patients were used for the study of the CD5 gene expression. This panel of 11 EBV lines was not subsequently used in our analysis of the V gene expression to avoid a possible bias related to antigen selection.

2. Cell markers: Surface Ig phenotype and expression of CD5, CD3, CD19, CD4, CD8 antigens was determined by FACS analysis using fluoresceinated antibodies. In the case of B lymphomas the surface Ig phenotype and lymphocyte markers were determined by immunochemical staining of acetone fixed frozen sections by the avidin-biotin-peroxidase technique (Kamoun et al., 1981; Antin et al., 1986).

3. CD5 probe is a 990 bp EcoRI fragment cloned into the pT1-1 plasmid (pUC13) kindly donated by Dr. Jack Strominger, (Harvard University, Cambridge, MA).

4. CTα probe is a 390 bp HpaII fragment of plasmid PY14 and was kindly donated by Dr. T. Mak (Toronto University, Canada)

5. Human V_κ gene family probes: The V_κ1 probe is a 320 bp RsI/PstI fragment cloned into M13mp19 (Jaenichen et al., 1984). The V_κII is a 290 bp BamHI/SphI fragment cloned into M13mp19 (Klobeck et al., 1984). The V_κIII probe is a 5.3 kb EcoRI fragment of the germline Humkv325 gene (Klobeck et al., 1984). The V_κIV probe is a 550 bp BamHI/SphI fragment cloned

into M13mp18 (Marsh et al., 1985). The V_{κ}I,II and IV probes were kindly provided by Dr. H. Zachau (University Munchen, FRG) and the V_{κ}III probe was provided by Dr. P. Chen (Scripps Clinic, La Jolla, CA).

6. Human V_H probes: The V_H1 probe is a 550 bp Nco/EcoRI fragment in pUC13 (Berman et al., 1988). The V_H2 probe is a 369 ScaI/HhaI insert in PuC18 (Berman et al., 1988). The V_H3 probe is a 550 bp Nco/PstI fragment in pUC13 (berman et al., 1988). The V_H4 probe is a 330 bp PstI fragment in PuC 13 (Berman et al., 1988). The V_H5 is a 380 bp HincII/PstI fragment in pUC13 (berman et al., 1988). The V_H6 is a 300 bp EcoRI/StuI fragment in pUC13 (Berman et al., 1988). The purification of inserts was done by electroelution according to standard techniques (Maniatis et al., 1982).

7. Northern blotting analysis: Total RNA was prepared using the guanidinium thiocyanate method as previously described (Maniatis et al., 1988). Ten micrograms of total RNA was electrophoresed on a .8% denaturing formaldehyde gel for 14 - 16 hours at 1.5 V/cm. After brief staining the gel was blotted onto nylon reinforced nitrocellulose in 20 x SSC. The nitrocellulose filter was baked for 2 hours at 80°C, prehybridized for 6 - 8 hours and hybridized for 16 - 20 hours with random primed probes at 1 - 2 x 10^6 cpm/ml. The prehybridization and the hybridization solutions contained 50% formamide, 5 x SSPE, 5 x Denhardt's solution and .1 mg/ml sonicated salmon sperm DNA. The hybridization solution

contained 10% dextran sulfate. The filters were washed two times in 2 x SSC, .1% SDS at room temperature for 15 minutes and two times in .1 x SSC, .1% SDS at 65°C for 15 minutes. Finally the blots were autoradiographed on Kodak XAR - 5 film at -70°C using Kronex intensifying screens for 2 - 3 days. The same filters were repeatedly stripped and rehybridized with all V_H and V_κ probes.

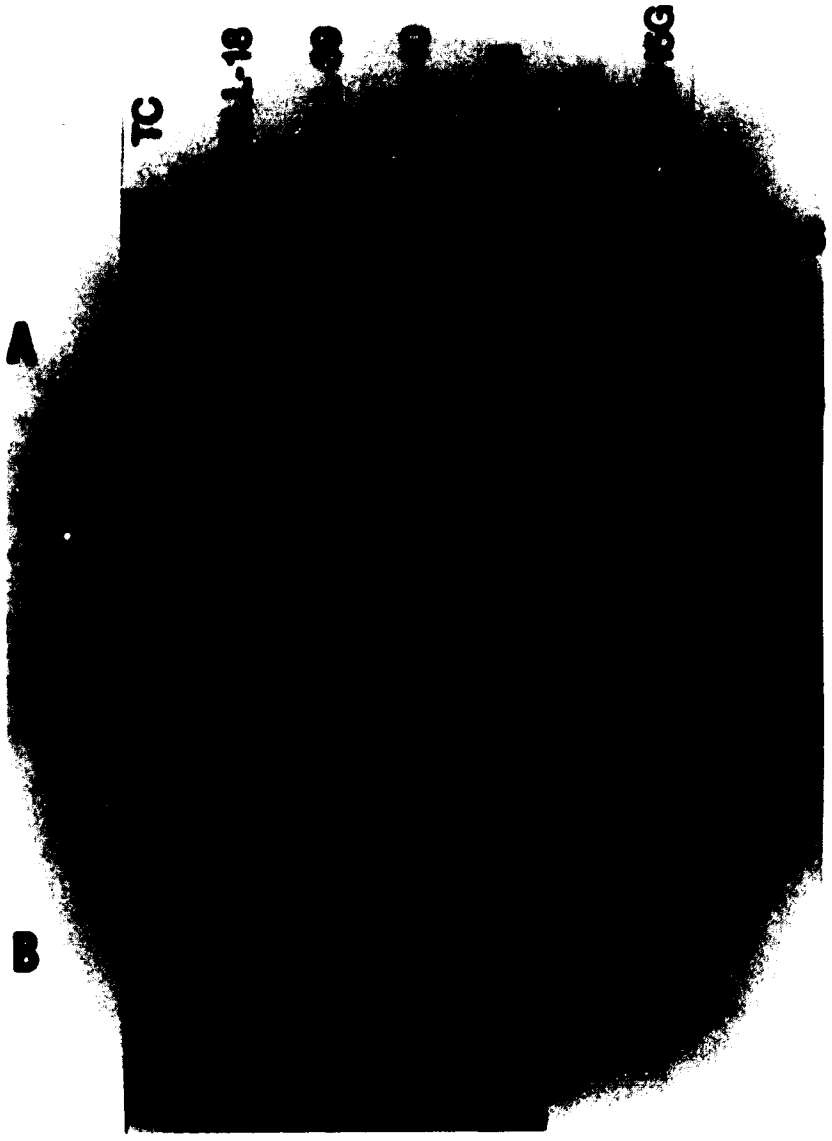
8. Statistical analysis The distribution of the various VH and VK gene families in our panel of CLL, CD5+ lymphomas, CD5- lymphomas and EBV transformed lines was analyzed using the chi-square test. The distribution of V_H and V_κ gene families in our panel of randomly selected CLL and $CD5^+$ lymphoma samples was compared to that of 97 EBV lines. Also, the distribution of V_H and V_κ gene families in the panel of $CD5^+$ BCL was compared to that of the $CD5^-$ BCL. Only the samples for which a unique gene family was assigned were used in the statistical analysis (samples crosshybridizing with several gene family probes were excluded from the test). The p value was determined from the standard chi-square tables. Statistical significance was considered for p values of 0.05 or smaller. When a statistically significant p value for the overall distributions was obtained, the largest contributor for the total chi-square value was subtracted and the remainder was tested for statistical significance using the same criterion (p smaller than 0.05).

D. RESULTS

1. CD5 gene expression: Northern blotting analysis was carried out on RNA extracted from a T cell CLL (TC), a panel of B cell CLLs and B lymphomas and 11 EBV transformed lines. The RNA extracted from the T cell leukemia shows two CD5 transcripts of 3.6 and 2.7kb identical in size to those previously identified in RNA extracted from a human ALL line (Jones et al., 1986). Identically sized transcripts were detected for all CLL samples, BCL samples and for 4 out of 11 EBV lines studied. Data presented in figure 8A illustrates Northern blotting analysis of RNA obtained from representative CLL samples depleted of T cells which were hybridized with the CD5 cDNA probe. Panel 8B shows that the CD5 transcripts are not due to contaminating T cells since no hybridization with CTα probe was observed. These results strongly suggests that the CD5 gene is expressed identically way in B and T cells.

2. V_H gene family utilization: Data depicted in Table 6 shows the utilization of V_H families in a panel of 40 CLL as compared to a group of 97 EBV clones obtained from adult human peripheral blood lymphocytes. The EBV lines show a V_H gene utilization roughly proportional to the estimated family size (Logtenberg et al., 1989). The analysis of the CLL panel shows a statistically significant ($p < 0.05$) biased usage of V_H6, which is a family composed of a single V gene

Figure 8. Northern blot analysis of RNA extracted from a T cell CLL (TC), 4 representative CLL samples (CLL-18, CLL-39, CLL-40 and CLL-2) and 2 EBV lines (H3A and R35H5G). Blots were hybridized with the CD5 cDNA probe (panel A)and CTα (panel B).


TC
A
B

and is the most D proximal. The frequency of V_H gene utilization in our panel of 40 CLL is similar to that described by Logtenberg et al in a panel of 24 CLL samples (Logtenberg et al., 1989). The expression of the V_H6 gene family on the other hand, was not detected in our panel of 10 small lymphocytic lymphomas ($CD5^+$ lymphomas), probably a consequence of the small sample size. A statistically significant biased usage ($p < 0.05$) of the V_H5 family however, was found in this group as compared to the EBV panel. No statistically significant difference was detected in the V_H gene expression of the $CD5^+$ lymphomas as compared to the $CD5^-$ lymphomas. Figure 9 illustrates selected examples of Northern blot analysis with various V_H probes.

3. V_K gene family utilization: The utilization of V_K gene families was also determined by Northern blotting analysis (Table 7). In our panel of 40 CLL samples we found statistically significant biased usage of the V_KIII. Similarly in our panel of $CD5^+$ lymphomas we found an over expression of V_KIII ($p < .05$). Figure 10 illustrates representative Northern analysis of V_K gene families. It is important to mention that RNA extracted from 5 CLL and one lymphoma sample hybridized with more than 1 probe. This might be due to bi-clonal origin of B cell tumors. However it should be mentioned that in two cases the RNA hybridized with one V_H probe and two V_K probes (patients CLL-12 and CLL-13) and on the other hand the RNA from the patient CLL-35

TABLE 6

FREQUENCY OF EXPRESSION OF V_H FAMILIES IN CLL, B LYMPHOMAS AND EBV LINES

	Total tested	V_H1	V_H2	V_H3	V_H4	V_H5	V_H6	cross hybridization (1,5),(4,6),(1,3)
CD5⁺ lymphomas	10	0	1	2	4	3	0	
CD5⁻ lymphomas	7	0	3	2	1	1	0	
CD5⁺ CLL	40	6	3	13	6	3	5	2 1 1
PBL-EBV lines	97	13	1	57	19	5	2	
Complexity		20-25	5-10	25-30	6-10	2-3	1	

Legend

PBL-EBV = Peripheral blood lymphocyte EBV lines.
Complexity = Gene family size as reflected by the number of bands detected in Southern blot analysis.

Figure 9. Northern blot analysis of RNA extracted from representative CLL and BCL samples. Blots were hybridized with V_H family gene probes.

CLL-17
BCL-666
CLL-7
CLL-7
CLL-30
CLL-36
CLL-14
CLL-15
BCL-666
BCL-629
CLL-5

hybridized with one V_κ and two V_H probes. This type of hybridization suggests cross hybridization rather than bi-clonality. Indeed, the studies of Jaenichen et al (1984) clearly show cross hybridization between $V_\kappa I$ and $V_\kappa III$ or $V_\kappa III$ and $V_\kappa IV$ which probably is due to the conservation of the third framework by $V_\kappa I$, $V_\kappa III$ and $V_\kappa IV$ genes.

E. DISCUSSION

The CD5 antigen is expressed on a high proportion of malignant B cells obtained from CLL and small lymphocytic lymphoma samples. We detected identical CD5 transcripts of 3.6 and 2.7kb in B-CLL and lymphoma specimens, a T cell CLL and several EBV transformed lines. This constitutes molecular evidence suggesting that the expression of the CD5 gene is identical in human B and T cells. It is important to note that we studied the expression of the CD5 gene in 11 EBV lines which do not express the CD5 antigen on their surface, and in 4 of them we have demonstrated the transcription of the CD5 gene in Northern analysis. This situation is reminiscent of the mouse system, in which Ly-1 (CD5) transcripts and corresponding polypeptides were demonstrated by Northern and Western analysis in hybridomas which were negative by surface staining (Mayer et al., submitted). Therefore these findings imply that the transcription of the CD5 gene can serve as a valuable marker for the detection of B cell malignancies arising from this

TABLE 7

FREQUENCY OF THE EXPRESSION OF V_κ FAMILIES IN CLL, B LYMPHOMAS AND EBV LINES

	Total	V_KI	V_KII	V_KIII	V_KIV	cross hyb. III,IV	cross hyb. III,I	λ	NI
CD5$^+$ lymph.	10	1	0	6	0			3	
CD5$^-$ lymph.	8	1	0	3	1		1	2	
CD5$^+$ CLL	40	10	1	16	2	2	2	6	1
PBL-EBV	42	21	8	9	4				
Complexity		15-20	10	8-10	1				

Legend

PBL-EBV	=	Peripheral blood lymphocyte EBV lines.
Complexity	=	Gene family size as reflected by the number of bands detected in Southern blot analysis.
λ	=	Lambda light chain.
NI	=	Not identified.
hyb.	=	hybridization.

Figure 10. Northern blot analysis of RNA extracted from representative CLL and BCL samples. Blots were hybridized with V_κ family gene probes.

VκIII
CLL-17
CLL-28
CLL-37
CLL-25
BCL-680
BCL-643
BCL-749
CLL-29
-18S
-28S

VκI
CLL-34
CLL-30
BCL-659
BCL-667
BCL-697
-18S
-28S

VκII
CLL-16
CLL-25
-18S
-28S

VκIV
CLL-3
BCL-697
BCL-687
-18S
-28S

small but distinct B cell subset as well as for the determination of the CD5 phenotypic origin of EBV lines.

In view of previous data demonstrating that this B cell subset represents a selected target for malignant events leading to CLL and CD5$^+$ BCL, it was important to determine whether malignant CD5$^+$ B cells preferentially rearrange particular V gene families. If proven correct, these findings would imply, a similarity between malignant B cell clones arising in different individuals. Previous data obtained in a small group of CLL samples sharing a cross reactive idiotype, demonstrated a high nucleic acid sequence homology of immunoglobulin kappa genes for several patients. Our study was aimed at analyzing the immunoglobulin gene expression of all known V_H and V_κ families in a large randomly selected sample of B-CLLs and BCLs. Our data indicated that a statistically significant biased V_H and V_κ repertoire is present in CLL as compared to EBV lines obtained from adult healthy subjects and in CD5+ BCL compared to EBV lines. The apparent bias of V_H4 (4 versus 1) V_H5 (3 versus 1) and $V_\kappa III$ (6 versus 3) seen in CD5$^+$ BCL as compared to CD5$^-$ BCL did not however reach statistical significance. This may be due to the small sample size of BCLs. Although EBV transformed cells may entail a selective bias, they represent the best control available since human B cell lines are not readily available. Furthermore recent studies have demonstrated that the V gene usage of EBV lines is proportional to the respective gene

complexity for each family as determined from Southern blot analysis (Logtenberg et al., 1989). Our molecular data indicating a similar pattern of biased V gene family usage seen in both CLL and CD5+ BCL is suggestive of the fact that these two clinical and histopathological entities are very closely related and that the CD5+ lymphoma represents the tissue equivalent of CLL.

Several possibilities can be envisioned to explain the biased usage of V gene families in CLL and CD5' lymphomas. Because the CD5' cells represent the majority of fetal spleen lymphocytes (Antin et al., 1986) they may represent the phenotype of "young" lymphocytes". It is well known that in the murine system that 3' V_H gene families and certain V_K families, located in the middle of the locus are frequently used in neonatal mice (Yancopoulos et al., 1984; Malynn et al., 1987; Kaushik et al., 1989). Similarly the biased usage of the V_H families 5 and 6 (3' families) in CLL and CD5+ BCL may represent an inherent predisposition of rearranging 3' V_H genes in normal young lymphocytes undergoing transformation. Secondly, the biased V gene usage may be related to antigen selection particularly by autoantigens. It is plausible that immune activation is part of the transformation process and therefore self-reactive clones could be transformed subsequent to antigenic stimulation. This hypothesis is supported by data which shows that a significant fraction of CLL exhibit RF binding activity (Preud'homme et al; 1972) and

recent data that shows that lymphocytes of CLL patients following in vitro polyclonal stimulation secrete anti-DNA Abs and RFs (Sthoeger et al., 1989).

Taken collectively our data demonstrate that the CD5 gene is expressed identically in T cells, as well as in malignant CD5$^+$ B cells and CD5$^-$ B cells producing autoantibodies. A biased usage of 3' V_H families and of V_κIII family was observed in CD5$^+$ CLL and BCL. If we consider the CLL and BCL B cells as the malignant counterparts of normal CD5$^+$ B cells, this biased V gene usage may reflect the repertoire of the normal CD5$^+$ B cell population that for some unknown reason preferentially undergoes these types of malignant transformation. In view of the correlation seen between V gene usage and antigenic specificity, the role of repeated antigenic stimulation in the development or maintenance of the malignant phenotype needs to be investigated. Finally, more detailed molecular studies aimed at characterizing this distinct B cell subset and at determining the possible physiological significance of the CD5 antigen in lymphocyte activation, might provide an explanation for the striking preferential transformation of CD5$^+$ B cells.

IV

AUTOANTIBODIES, LY-1 AND IMMUNOGLOBULIN V GENE EXPRESSION IN HYBRIDOMAS OBTAINED FROM YOUNG AND OLD NZB MICE

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A. ABSTRACT

NZB mice are prone to develop autoimmune diseases such as systemic lupus and autoimmune hemolytic anemia. A large number of hybridomas were obtained from 1 and 16 month-old NZB mice in order to study the fine specificities of the autoantibodies produced as well as the expression of Ly-1 and immunoglobulin V gene families.

Analysis of the autoantibody specificities showed that they can be classified into two major categories: antibodies specific for a single autoantigen and antibodies exhibiting multispecific binding. Among multispecific antibodies two categories were distinguished: antigen inhibitable and antigen non-inhibitable. While the usage of V_H families is stoichiometric with respect to the number of germline V_H genes contained in various families, a biased usage of $V_\kappa 1$, $V_\kappa 8$ and $V_\kappa 9$ gene families was observed among autoantibody kappa chains. Autoantibodies are produced by both Ly-1$^+$ and Ly-1$^-$ B cell subsets.

B. INTRODUCTION

NZB mice, prone to a lupus-like syndrome and autoimmune hemolytic anemia, represent an excellent animal model for studying the immunochemical and molecular characteristics of both organ specific and non-organ specific autoantibodies (autoAbs). Whereas Coombs anti-RBC autoantibodies represent typical organ-specific pathogenic (autoAbs) (Warner et al., 1973), anti-DNA, Sm, histones and rheumatoid factor (RF) autoAbs represent non-organ specific autoAbs associated with various systemic autoimmune diseases (Andrews et al., 1978). There is suggestive evidence that a discrete subset of B cells bearing the CD5 antigen (Ly-1 in mice and Leu1 in humans) is the major contributor for the production of certain autoAbs (Hayakawa et al., 1984; Hardy et al., 1986). NZB mice are a good model for studying the cellular origin of autoAbs since it was shown that the Ly-1 B cell subset is enlarged in this strain (Hayakawa et al., 1983; Manohar et al., 1982) and that aged NZB mice show an unusual clonal expansion of Ly-1 B cells exhibiting premalignant properties such as autonomous proliferation resistant to immunoregulatory signals (Seldin et al., 1987; Tarlington et al., 1988).

During the past decades, the autoreactivity of antibodies was defined by the ability of a heterogenous population of immunoglobulin molecules, isolated from sera of patients afflicted with autoimmune diseases, or animals prone to autoimmune diseases, to bind autoantigens. The development

of hybridoma technology and of sensitive cellular immunological techniques permitted a detailed analysis of self reactivity of homogenous antibodies. Utilization of such techniques showed that an important fraction of antibodies are multispecific (Dighiero et al., 1985; Dighiero et al., 1982) and that they have a moderate affinity (10^{-5}-10^{-6} gr/l) for self and foreign antigens (Monestier et al., 1987). Consequently studies of immunochemical and molecular properties of monoclonal antibodies can provide important information on the origin of autoAbs, namely, on the fraction of clones producing antibodies with a single specificity and of multispecific antibodies which probably play a physiological role in the maintenance of macromolecular homeostasis.

Based on these considerations we addressed three questions in this study:

1) what is the fraction of organ and non-organ specific autoantibodies?

2) what fraction of autoantibodies are produced by cells expressing the Ly-1 gene

3) are certain V_H and V_κ gene families preferentially used by NZB autoAbs?

We considered that more accurate information can be obtained by studying B cells immortalized as hybridomas rather than other methods such as in situ hybridization or splenic fragment culture, since clonality of antibodies and hybridomas is required for detailed molecular studies.

C. MATERIALS AND METHODS

1. **Mice** 1 and 16 month-old NZB/J mice were purchased from the Jackson Laboratory, Bar Harbor, ME.

2. **Antigens** Autoantigens used in this study are presented in Table 8.

3. **Preparation of hybridomas and purification of mAbs**

Hybridomas were prepared by fusion of splenic lymphocytes with SP2/0 cells according to a previously described technique (Bellon et al., 1987). The 1 month-old NZB hybridomas were obtained by fusion of LPS stimulated spleen cells, whereas the 16 month old NZB hybridomas were obtained from non-stimulated lymphocytes. Hybridoma supernatants were initially tested for binding to dsDNA, histones and Br-RBC with labeled anti-K and lambda antibodies according to a previously described technique (Bellon et al., 1987). Twenty hybridomas exhibiting self reactivity in the initial screening and 20 without self binding properties were cloned under stringent limiting dilution conditions (0.5 cells/well) and expanded in culture. Antibodies from culture supernatant were purified on a rat anti-murine K-Sepharose 4B column.

We included in this study a group of hybridomas (Z series) obtained from 1 month old NZB mice selected with the V_H 7183 probe which have been previously described (Bellon et al., 1987).

4. **Antigen binding** RIA was used to detect self reactive antibodies. Microtiter plates were coated with 50μl of

TABLE 8.

ANTIGENS USED IN THIS STUDY

Antigen	Origin	amount used to coat plates
DNA	salmon/phage	10µg
H1	rat thymus	5µg
H2a	-"-	-"-
H2B	-"-	-"-
H4	-"-	-"-
Sm	murine	1µg
Collagen I	rat	10µg
Collagen II	-"-	-"-
Collagen III	-"-	-"-
Collagen IV	-"-	-"-
HOPC1 (G2a)	murine	-"-
MBP	-"-	3µg
IF	bovine	1µg
TG	-"-	10µg
TSH R	human	-"-
Ach R	fish	5µg
Transferrin	murine	10µg
(Jo)	human	-"-
Br-MRBC	murine	5µg
Topo I	human	10µg

DNA: salmon or phage (T4)
H1: histone H1
H2a: histone H2a
H2b: histone H2b
H4: histone H4
MBP: myelin basic protein
IF: intrinsic factor
TG: thyroglobulin
TSH R: TSH receptor
Ach R: acetyl choline receptor
Jo: hystydil t-RNA synthetase
Topo I: topoisomerase I (Scl 70)

soluble antigen in carbonate buffer pH9.0. After overnight incubation at 4°C the plates were saturated for 30 minutes at RT with 1% PBS-BSA and then extensively washed. Fifty µl per well of various dilutions (0.1,0.3,1,3 and 10µg/ml) of affinity chromatographically purified antibody was added for 3 hours at RT; plates were then washed and incubated for 2 hours with ^{125}I-rat anti-murine kappa mAb (50,000 cpm/well) After extensive washing the radioactivity was measured on a gamma-spectrometer counter.

Specificity of the binding was confirmed by using a competitive inhibition RIA. This technique was carried out in two steps. a) in liquid phase, 0.5 µg of antibodies were incubated with various amounts of antigen (0.1-10 µg) for 2 hours at room temperature in microtubes coated previously with BSA and b) 50 µl of this mixture was transferred to microplates previously coated with antigens as described above (Monestier et al., 1987).

5. **Immunofluorescence staining** was carried out using the FACSCAN instrument (Becton Dickinson). Hybridoma cells (viability greater than 95%) were stained with optimal amounts of biotin labeled 53-7.5 (anti-Ly-1) or 53-6.7 (anti-Lyt2 isotyped matched reagent for 53-7.5) followed by the addition of PE-avidin. For cytoplasmic staining the cells were fixed for 10 minutes at 4°C in 50% ethanol prior to staining. Both profiles are shown on the same figure.

6. **Western blotting**

The identification of Ly-1 gene products was carried out by Western blotting of hybridoma lysates. 10^7 cells were centrifuged for 10 minutes at 1500g and resuspended in 15 μl NTE buffer (20 mM Tris-HCl, 150 mM NaCL, 0 1 mM EDTA, 0.2 mM PMSF and 7 mM 2.ME). After a 5 minute incubation on ice 15 μl of 2% triton X 100 in NTE buffer were added and incubated for an additional 5 minutes on ice. The cells were centrifuged for 5 minutes at 12,000g, the supernatant harvested and frozen at -70°C. Thirty μl supernatant corresponding to 10^7 cells were mixed with 30 μl of loading buffer (20% glycerol, 10% 2-ME, 4.6% SDS, 125 mM Tris- HCl pH6.8), electrophoresed on a 10% polyacrylamide - SDS gel and then electrotransferred onto a DEAE membrane. The membrane was saturated overnight with 5% BSA-PBS and then incubated for 2 hours at RT in a solution containing rat anti-Ly-1 mAb (53-7.5) (1μg/ml). After extensive washing in a solution of 0.15M NaCL and 2% Tween 20, the membrane was incubated for 30 minutes with a solution containing ^{125}I-goat anti-rat IgG (10^6 cpm/ml), 0.05M Tris-HCL pH8.2, 0.15 M NaCL, 20% goat Ig and 5% BSA. After incubation the membrane was washed extensively, dried and autoradiographed.

7. **RNA Extraction and Northern blotting**

RNA was extracted from 3-5X10^7 cells using the guanidinium-thiocyanate method (Maniatis et al., 1982). Northern blotting was performed by electrophoretically

fractionating the RNA on a 0.8% agarose formaldehyde gel. The gel was blotted overnight onto nitrocellulose using 20XSSC. Blots were baked in a vacuum oven for 2 hours at 80°C. Prehybridization was carried out at 42°C in 50% formamide, 5XSSPE, 5X Denhardt's, 50 μg/ml sheared and denatured salmon sperm DNA. Probe was added at 2X10^6 cpm/ml in hybridization solution (50% formamide, 5XSSPE, 1X Denhardt's, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate). Blots were hybridized overnight at 42°C, then washed two times for 15 minutes in 2XSSC, 0.1% SDS at 65°C then two times in 0.1SSC, 01% SDS for 15 minutes at 65°C and autoradiographed on Kodak XAR film with intensifying screens at -70°C for a few days. Background was reduced by rewashing the blots with proteinase K in buffer.

8. **Ly-1 and V gene probes**

The V_H gene family probes (V_HX24, V_H36-60, V_H36-09, V_HJ606, V_H558, V_HGAM, V_H11, V_HS107, V_HQPC52 and V_H7183) and V_κ gene family probes ($V_\kappa1$, $V_\kappa2$, $V_\kappa4$, $V_\kappa8$, $V_\kappa9$, $V_\kappa10$, $V_\kappa19$, $V_\kappa21$, $V_\kappa22$, $V_\kappa23$, $V_\kappa24$, and $V_\kappa28$) were prepared as described elsewhere (Painter et al., 1988; D'Hoostelaere., 1988). The preparation of the Ly-1 probe was carried out as previously described (Huang et al., 1987).

D. RESULTS

1. Preparation of hybridomas and initial analysis

In order to investigate the phenotypic and molecular characteristics of cells producing autoAbs we prepared a large

library of hybridomas from 1 and 16 month-old NZB mice. One may argue that because of the frequency of the cell fusion event (10^{-4}-10^{-5}) the utilization of hybridoma technology may introduce a clonal bias in the analysis. However, this technique remains the most adequate for phenotypic and molecular studies as compared to other techniques in which either the molecular or phenotypic analysis can be carried out, but not both simultaneously on a large number of progeny of the same clone. While the hybridomas obtained from 1 month-old NZB mice were prepared from LPS-stimulated lymphocytes those from 16 month-old mice were from unstimulated cells. LPS was used in the case of 1 month-old splenocytes in order to increase the fusion frequency. The hybridomas were tested for the binding to dsDNA, histones, Br-RBC, RF, and Coombs antibody activity.

The data presented in Table 9 show that among 828 hybridomas obtained from 1 month-old mice, 251 exhibited binding to self antigens and only two to three exhibited binding to the foreign antigens (bacterial levan, Providencia stuarti and Neisseria lactamica polysaccharides) used in this study. Among 672 hybridomas obtained from 16 month-old NZB mice, 288 exhibited self reactivity and 5 bound to foreign antigens.

Fifteen hybridomas from 1 month-old (mice ZK series) and 30 from 16 month old mice (ZL, ZA and ZO) series were randomly chosen for cloning and further studies. Half of each group

TABLE 9

FREQUENCY OF AUTOREACTIVE HYBRIDOMAS AND DESIGNATION OF MONOCLONAL ANTIBODIES

Origin	NT	REACTIVITY Self x	Foreign xx	Unknown	Designation
1 mo-old	828	251	4	576	ZK9B9-8,ZK3G11-1 ZK2A11-1,ZK9D9-1 ZK2H6-2,ZK8E8-1 ZK9A4-10,ZK3H6-26 ZK2A7-3;ZK2A8-11 ZK4G3-8;ZK2F2-1 ZK3G11-1,ZK2H6-2 ZK8E8-1;ZK2H5-3 ZK9E1-1,ZK5F11-3
1 mo-old	356				Z41;Z218,Z49;39 Z317,Z26,Z118;Z121 Z14,Z113,ZX25;Z250 Z244,Z239
12 mo-old	672	288	5	379	ZL173-6;ZL179-6; ZL154-1;ZL37-8; ZL37-9;ZL37-14; ZL177-5;ZA1B8-1, ZAIC7-12,ZA2C2-6; ZA4C3-1,ZA1C7-5; ZA2B2-10;ZA3E5-12, ZA4A10-13,ZA3C4-6, ZA4B7-2,ZA2G8-3; ZA4H6-11,ZA4H10-6, ZA4A7-1,ZA464-2, ZA1B8-1;ZA1E3-12, ZA4E12-6,ZA5C2-5, ZA5E4-8,ZA4B8-2; ZA3C4-2,Z0103-12; Z0121-12,Z055-3; Z0120-6

x DNA, histones, RBC and IgG_{2a} were used for selection of self reactive antibodies

xx Providentia stuarti, Neisseria lactamica and bacterial levan were used for selection of self reactive antibodies

NT number tested

had self reactivity, the other had unknown antigen binding.

After stringent limiting dilution cloning and retesting, one clone from each hybrid was grown, isotyped and the antibodies were chromatographically purified on a rat anti-murine kappa mAb-Sepharose column. For comparison in some molecular and immunochemical studies we included a group of 14 hybridomas obtained from 1 month-old NZB LPS stimulated splenic lymphocytes which were selected for the expression of the V_H7183 gene family (Bellon et al., 1987).

2. Self reactivity of monoclonal antibodies

Prior to the study of fine specificity of monoclonal antibodies in competition inhibition assay, two pilot experiments were initially carried out. In the first, we studied the self reactivity by measuring in RIA the binding of each mAb to the 21 antigens listed in Table 1. In the second experiment, we studied the dose-effect binding of a given mAb for each reactive antigen using 0.1-10μg of chromatographically purified antibody (data not shown). The antibodies exhibiting dose-dependent binding to a given antigen were subsequently used in competition inhibition studies.

The data depicted in Table 10 show the binding properties of mAbs exhibiting a single specificity with a panel of 21 autoantigens. As can be seen, 7 mAbs from 1 month-old (Z and ZK series) and 8 from 16 month-old (ZL and ZA series) strongly bound to procaryotic or eukaryotic dsDNA.

TABLE 10

MONOSPECIFIC MONOCLONAL ANTIBODIES

A. DNA

	BINDING TO DNA		
	procaryotic	eukaryotic	μg giving 50% inhibition
			DNA
Z41	1,951±135[x]	2,349±139	3.0
Z218	1,098±36	2,098±71	4.8
ZK9A4-10	2,132±50	1,283±14	3.4
ZK9E1-1	3,815±429	6,648±317	1.8
ZK3H6-26	2,602±155	5,115±266	0.48
ZK5F11-3	6,916±255	6,478±587	10.9
ZK2A7-3	20,006±409	14,760±315	2.4
ZL173-6	6,574±598	11,483±1.11	1.2
ZL179-6	7,868±130	9,069±958	7.4
ZA1C7-5	ND	2,109±303	>10
ZA2B12-10	4,365±145	5,194±603	2.0
ZA3E5-12	7,685±193	14,585±909	2.6
ZA4A10-3	1,198±30	1,239±43	0.92
ZA4B7-2	679±12	2,058±14	5.9
ZA2G8-3	1,741±46	1,603±121	1.4

B. RF	binding to IgG_{2a}	IgG_{2a}
ZL177-5	1,031±265	3.5

C. H2b	binding to H_2b	H2b
ZL154-1	14,707±823	3.7

D. AchR	binding to AchR	AchR
Z49	2,675±100	5.0
Z39	2,120±6	6.8

E. TG	binding to TG	TG
Z317	2,887±178	3.2

F. Sm	binding to Sm	Sm
Z26	10,107±1,100	2.5
Z118	2,183±304	2.9

Legend

x cpm = average ± SD of triplicates
ND= not done
RF= rheumatoid factor
H2b= histone H2b
AchR= acetyl choline receptor
TG= thyroglobulin
Sm= Smith antigen

antigen inhibition assay showed that some antibodies required as little as 0.4 μg/ml of dsDNA to reach 50% inhibition and other required up to 10.8 μl/ml. This indicates that there are differences in the affinity of various monoclonal antibodies for dsDNA.

Five antibodies from 1 month-old NZB mice (Z series) showed binding to AchR, thyroglobulin or Sm and two from 12 month-old (ZL series) were specific for H,b or exhibited RF activity.

All mAbs reacting with RBC were monospecific. Among 9 mAbs, two bound only to Br-RBC and were all obtained from 1 month-old mice and seven which exhibited Coombs activity were obtained from old mice. As can be seen from Table 11, all Coombs antibodies did not bind to Br-RBC membranes. This indicates that the antigenic determinants unmasked by enzymatic treatment are different from those recognized by Coombs antibodies.

The second group of antibodies exhibited multiple binding specificities. This panel of antibodies can be grouped into three categories:

a) bispecific antibodies. The data depicted in Table 12 show the binding specificity of five antibodies: two bind to DNA and H1, two to AchR and histidyl-tRNA transferase and one to AchR and DNA. The binding affinity of Z113 and ZL37-9 for both self antigens probably was low as illustrated by

TABLE 11.

MONOCLONAL ANTI-ERYTHROCYTE ANTIBODIES

Antibodies	Coombs HA (log2)	Binding to Br-MRBC (cpm)
UN40-9	0	2,997±279
ZK4G3-8	0	2,361±106
ZK2F2-1	0	1,420±37
ZA1B8-1	4	42±3
ZA1C7-12	4	243±5
ZA4B8-2	3	25±2
ZO103-17	4	121±12
ZO121-12	3	29±11
ZO55-3	3	47±6
ZO120-6	1	53±11

UN40-9 is a monoclonal antibody from me^{v} mice and was used as a positive control.

TABLE 12

MONOCLONAL ANTIBODIES EXHIBITING DOUBLE SPECIFICITY

	binding to		μg/ml giving 50% inhibition	
			DNA	H1
ZK2A8-1	DNA	5,725±2,052	1.0	2.8
	H1	1,379±60	2.2	5.5
Z121	DNA	10,683±283	6	0.88
	H1	3,952±72	2.2	4.0
			AchR	JO
Z14	AchR	7,653±23	0.13	ND
	JO	1,972±41	0.92	8.6
			AchR	JO
Z113	AchR	7,284±36	10.3	10
	JO	6,985±14	8.6	9.8
			AchR	DNA
ZL37-9	AchR	4,440±294	9.3	2.3
	DNA	12,343±1,562	>10	9.8

ND = not done

the high concentration of antigen required to reach 50% inhibition

b) antigen-inhibitable multispecific antibodies. The data depicted in Table 13 show the binding activity of two antibodies: ZA2C2-6 reactive with DNA, H1 and H_1b and ZK2H5-3 reactive with H1, H2a, H2b, H4, AchR and TG. The binding to these antigens was reciprocally antigen inhibitable with the exception of the binding of ZK2H5-3 to thyroglobulin

c) antigen non-inhibitable multispecific antibodies. The data presented in Table 14 show the binding activity of four mAbs to a variety of antigens. In competition assay, 50% inhibition was never achieved using 10-20 μg/ml of various antigens. However these antibodies cannot be considered "sticky" antibodies since while they bind strongly to certain self antigens, they did not bind to others.

3. V gene usage in NZB hybridomas

Study of the V_H genes expressed in hybridomas producing monoclonal autoAbs showed that, like in neonatal B cells, there is a biased usage of 3' VH gene families (Bona et al., 1988). In contrast, analysis of V gene expression by in situ hybridization of individual plasma cells showed an increased frequency of expression of V_H J558 in old MRL mice and that this increased utilization of V_H J558 frequency correlates well with the severity of the disease (Komisar et al., 1989).

TABLE 13

MONOCLONAL ANTIBODIES EXHIBITING MULTIPLE SPECIFICITIES

binding to		μg/ml giving 50% inhibition		
ZA2C2-6				
		DNA	H1	H2b
DNA	11,697±480	1.8	>10	1.6
H1	1,907±10	4.5	9.8	6.4
H2b	4,696±34	1.1	10	0.88

ZK2H5-3							
		H1	H2a	H2b	H4	AchR	TG
H1	3,046±112	10.3	9.8	6.8	10	>10	>10
H2a	3,707±153	4.0	0.9	5.7	0.9	8.6	>10
H2b	2,171±23	2.6	3.1	0.9	0.8	>10	>10
H4	2,310±49	2.4	3.0	0.8	1.0	4.8	>10
AchR	11,119±59	>10	>10	7.0	>10	3.2	>10
TG	8,382±80	>10	>10	>10	>10	3.6	9.6

TABLE 14

MONOCLONAL ANTIBODIES EXHIBITING NON RECIPROCAL INHIBITABLE ANTIGEN BINDING ACTIVITIES

Antigen	Monoclonal antibodies			
	ZX25	ZL37-8	ZL37-14	ZA4C3-1
PDNA	9,804±107	8,903±48	ND	8,685±393
EDNA	10,988±698	12,343±147	6,327±163	10,169±289
H1	3,801±205	3,470±60	1,946±166	3,797±11
H2a	1,401±65	1,825±178	990	802
H2b	8,994±356	4,142±130	2,708±66	1,031±1
H4	1,943±33	9,738±941	3,124±448	179
Sm	5,011±332	323	542	1,388±18
IgG,a	3,504±13	877	653	2,540±65
AchR	15,709±991	4,466±884	101	7,286±190
TSHR	386	72	277	135
JO	20,212±137	2,429±218	233	4,455±200
IF	347	81	370	124
CI	1,726±54	368	1,177±31	432
CII	1,308±18	217	886	135
CIII	624	73	591	337
TR	578	76	210	47
TG	11,067±75	778	780	11,163±851
MBP	264	322	371	395

PDNA = prokaryotic DNA
EDNA = eukaryotic DNA

Initial studies of usage of V_κ families in hybridomas producing autoAbs showed a high frequency of utilization of $V_\kappa 1$, VK_4, $V_\kappa 10$, and $V_\kappa 19$ (Kasturi et al., 1988). Later it was shown that in contrast to the stoichiometric usage of V_H gene families (reflecting the number of germline genes in a particular V_H gene family) the usage of V_κ families is not stoichiometric and some families such as $V_\kappa 1$, $V_\kappa 9$ and $V_\kappa 8$ are frequently used in both young and adult mice (Kaushik et al., 1989). In view of these findings, it was important to study the V_H and V_κ gene family utilization in our panel of NZB hybridomas.

Figures 11 and 12 illustrate examples of this analysis. The data presented in Table 15 show that there is no preferential usage of V_H gene families in our panel of hybridomas. Furthermore, no differences in the VH usage was observed between the group of hybridomas producing autoAbs and those which do not bind to our panel of autoantigens. It should be pointed out that this conclusion is based on results which exclude the Z series of hybridomas which were selected with a VH 7183 probe.

In the case of V_κ gene families, as expected from previously reported data, $V_\kappa 1$ is highly used in both young and adult animals (Kaushik et al., 1989). The $V_\kappa 9$ and $V_\kappa 10$ families were also frequently expressed by autoAbs. Five hybridomas producing autoaAbs expressed $V_\kappa 9$ and $V_\kappa 10$ but none of the hybridomas producing antibodies devoid of self

Figure 11. Total cytoplasmic RNA hybridized with indicated V_H probes. MOPC460 was used as negative control except for the blots hybridized with 36-60 in which AL42-7 (V_HX24) was used. Positive controls used: MOPC 460 for V_H36-60; 129-48 for V_H7183 and IDA16 for V_HQPC52.

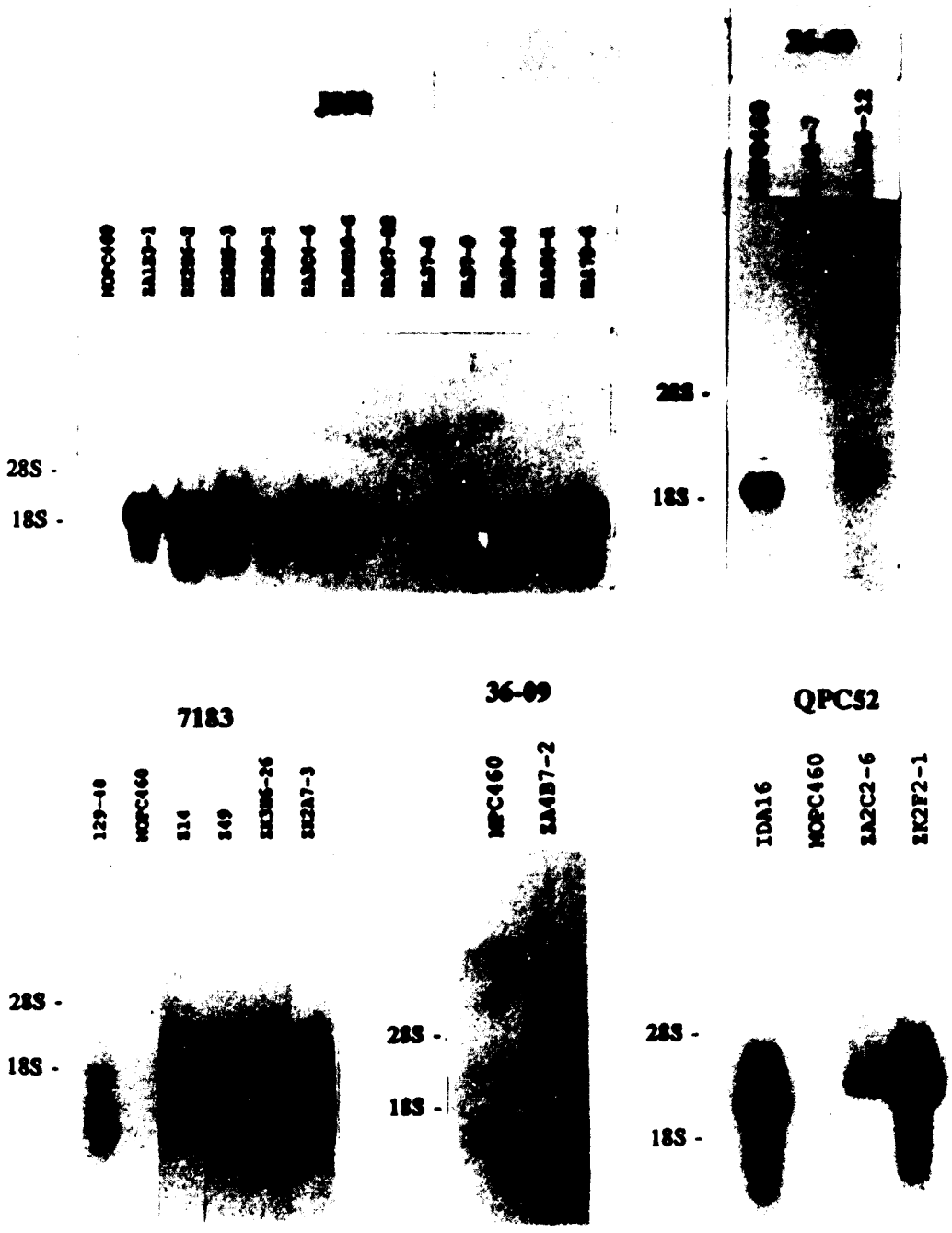

28S -
18S -
28S -
18S -
7183
129-48
MOPC460
814
849
28S -
18S -
36-09
MPC460
28S -
18S -
QPC52
IDA16
MOPC460
28S -
18S -

Figure 12. Total RNA hybridized with indicated V_κ probes. AL42-7 was used as negative control except for the blot hybridized with $V_\kappa 23$ in which Z0121-12 ($V_\kappa 28$) was used. Positive controls used: M56 for $V_\kappa 8$ CP5 for $V_\kappa 9$ IRE121-4 for $V_\kappa 10$ NF8 for V_κ 19 H17-1 for $V_\kappa 22$ and AL42-7 for $V_\kappa 23$.

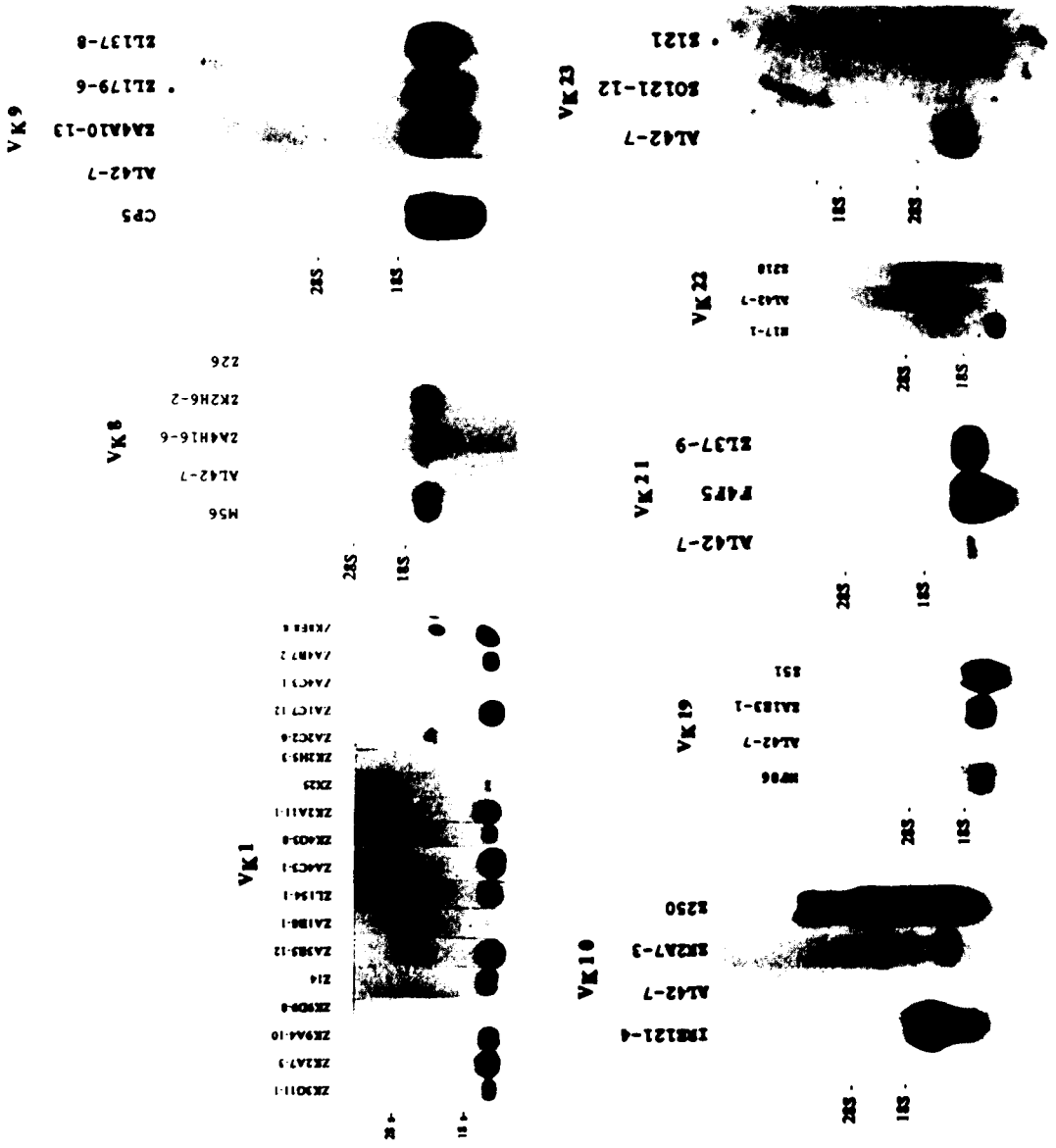

Vκ1
Vκ2
Vκ3
Vκ4
Vκ5
Vκ6
Vκ7
Vκ8
Vκ9
Vκ10
Vκ19
Vκ21
Vκ22
Vκ23
28S
18S

TABLE 15

V_H AND V_K FAMILIES EXPRESSED IN NZB HYBRIDOMAS

Monoclonal antibodies exhibiting autoreactivity

1 month-old

	V_H	V_K
Z41	7183	4.
Z218	7183	22.
Z49	7183	NI.
Z39.	7183	1.
Z317.	7183	NI.
Z26.	7183	8.
Z118.	7183	NI.
Z121.	7183	23.
Z14.	7183	1.
Z113.	7183	NI.
ZX25.	7183	1.
ZK9A4-10.	NI	10.
ZK3H6-26.	7183	NI.
ZK2A7-3.	7183	10
ZK2A8-1.	J558	1.
ZK4G3-8.	J558	1.
ZK2F2-1.	J558	1

16 month-old

	V_H	V_K
ZL173-6.	7183	NI.
ZL179-6.	J558	9.
ZL154-1.	J558	1
ZL37-8.	3660	21
ZL37-9.	J558	9.
ZL37-14.	J558	21.
ZA1B8-1.	NI	1.
ZA1C7-12.	J558	1.
ZA4C3-1.	3660	1.
ZA1C7-5.	S107	NI
ZA2B12-10	S107	28.
ZA3E5-12.	3660	1.
ZA4A10-1.	3660	9.
ZA4B7-2.	3609	1.
ZA2G8-3.	J558	22.

Monoclonal antibodies with unknown specificity

	V_H	V_K
Z250	7183	NI.
Z244	7183	22.
Z239	7183	NI.
ZK9B9-8	7183	1.
ZK3G11-1	Q52	1.
ZK2A11-1	NI	1.
ZK9D9-1	VGAM	19.
ZK2H6-2	J558	8.
ZK8E8-1	NI	1.

	V_H	V_K
ZA4H10-6	J558	8.
ZA4A7-1	3660	NI.
ZA1B8-1	NI	1.
ZA1E3-12	J558	21.
ZA5C2-5	X24	19.
ZA5E4-8	NI	8.
ZA3C4-2	J558	19.
ZA2C2-6.	Q52	1.

NI = Not identified
Q52= V_HQPC52
3660= V_H36-60
3609= V_H36-09

reactivity expressed these gene families. Otherwise, no important differences were observed in the utilization of other families V_κ between two groups of hybridomas.

These results show: a) a stoichiometric utilization of V_H gene families; b) no differences in the usage of V_H or V_κ between the hybridomas obtained from 1 and 16 month-old NZB and c) no differences in V gene usage between hybridomas producing autoAbs and those producing antibodies devoid of self reactivity.

4. Ly-1 gene expression

It is generally accepted that a discrete subset of B lymphocytes bearing Ly-1 antigen is mainly involved in self reactivity (Hardy et al., 1986). The study of Ly-1 expression by hybridomas producing autoAbs was hampered by the difficulties in detecting the Ly-1 antigen by conventional IF techniques since on Ly-1$^+$ B cells the antigen is expressed on the membrane at a low density.

In a previous work we have shown that thymocytes express two transcripts (2.9 and 2.1 kb) whereas 5 of 20 MRL/lpr hybridomas producing autoAbs express a less abundant 2.9 kb transcript which corresponds to the thymic Ly-1 transcript, and a more abundant 1.6 kb Ly-1 transcript (Bailey et al., 1989). These results suggested that the detection of Ly-1 gene transcription could serve as a method for assigning the Ly-1 origin of hybridomas.

The data illustrated in Figure 13 show an example of

Figure 13 Total cytoplasmic RNA hybridized with the Ly-1 probe. Blot was exposed for 5 days. An abundant 1.6 kb transcript is observed in NZB hybridomas.

28s -
18s -
THYMUS
Z239
ZK2H6-2
ZK4G3-8
ZK8E8-1
ZA1C7-5
ZA4B7-2
ZA1E3-1
ZA1B8-1
ZL179-6
ZA4A10-13
ZK3H6-26

eleven NZB hybridomas from our panel which have a weak 2.9 kb transcript but a more intense 1.6 kb Ly-1 transcript.

If 2.9 kb transcript which was found in nuclear but not in cytoplasmic thymic RNA appears to be the precursor of 2.1 kb functional transcript found in thymic cytoplasmic RNA there is no data to indicate that the detection of 1.6 kb transcript in hybridomas relates to a functional product. Actually one may argue that 1.6 kb transcript is an aberrant transcript.

In order to test this hypothesis we carried out IF staining and Western blot analysis on four NZB hybridomas in which the 1.6 kb Ly-1 transcript was detected. The data illustrated in Figure 14 show that all four hybridomas show a strong intracytoplasmic staining with anti-Ly-1 mAb and two of them weak, but significant surface staining.

Western blots show three bands of 130, 73 and 67 Kb which react with anti-Ly-1. mAb (Figure 15). While in the thymus and Z239 hybridomas all three bands are detected, in three remaining hybridomas only 73 and 67 Kd bands are detected. The 67 Kb band corresponds to Ly-1 antigen, associated with thymic membranes (Huang et al., 1987) while 130 Kd protein can be dimeric form of 67 Kd gene product, 73 Kd band either represent an unprocessed Ly-1 gene product or a hyperglycosilated protein. These data are in agreement with earlier studies aimed to identify Ly-1 antigen. In these studies two bands of 67 and about 80 Kd were identified

Figure 14 Hybridomas cells stained with fluoresceinated anti-Ly-1 and Lyt2 monoclonal antibodies. Left panel surface staining, right panel cytoplasmic staining. Dotted line indicates the staining pattern with anti-Lyt2, while continuous line staining pattern with anti-Ly-1 mAbs.

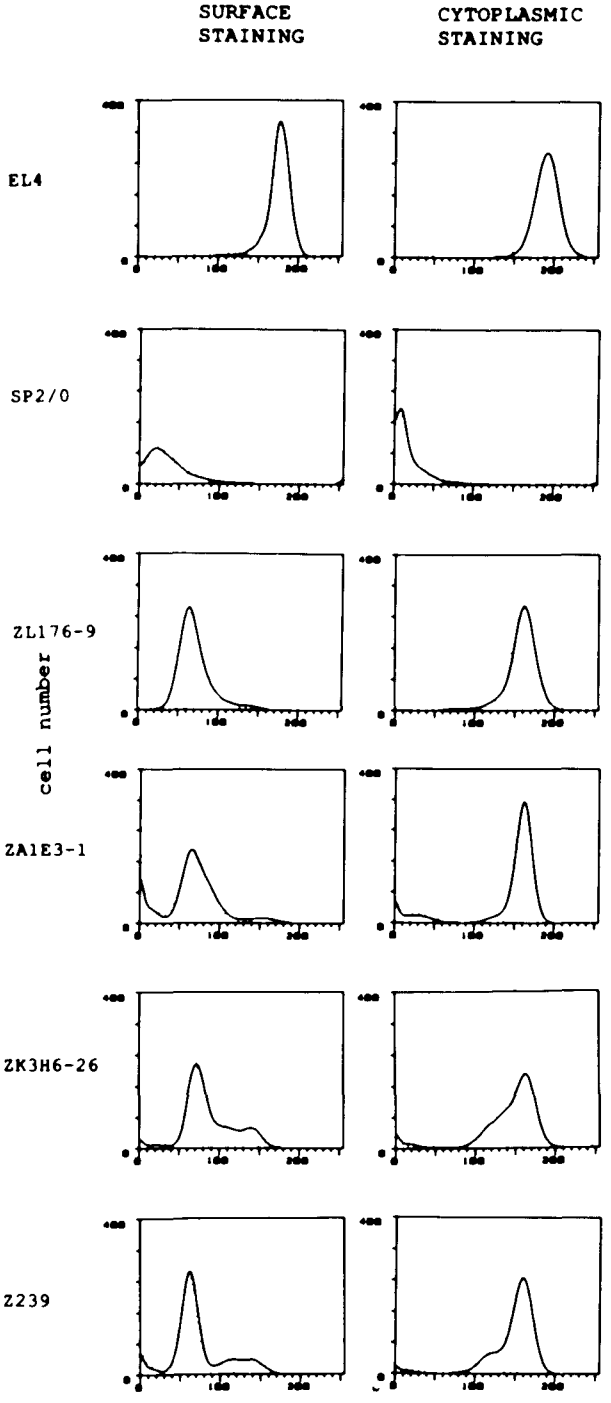
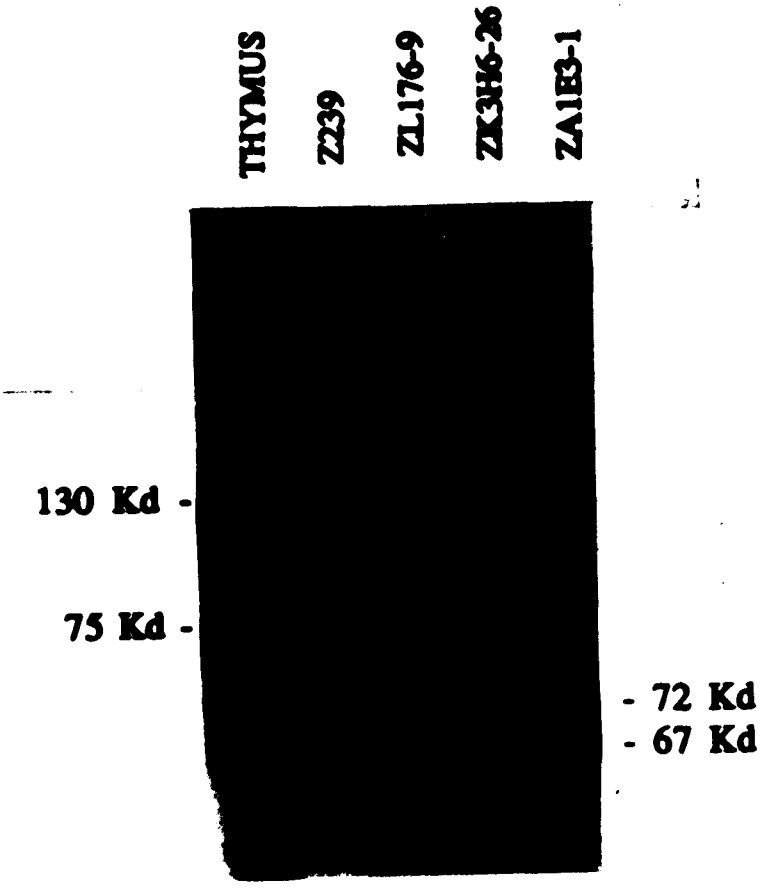

SURFACE STAINING
CYTOPLASMIC STAINING
EL4
SP2/0
ZL176-9
ZA1E3-1
ZK3H6-26
Z239
cell number

Figure 15 Western blotting of lysates of ZL176-9, ZA1E3-1, ZK3H6-26 and Z239 hybridomas. The autoradiograph shows electrophoretically separated proteins incubated with rat anti-Ly-1 mAb followed by incubation with ^{125}I-goat anti-rat purified antibodies.


THYMUS
Z239
ZL176-9
ZK3H6-26
ZA1E3-1
130 Kd -
75 Kd -
- 72 Kd
- 67 Kd

(Durda et al., 1978). cDNA cloning and nucleotide sequencing of the 2.9, 2.1 and 1.6 Kb Ly-1 transcripts from thymocytes and NZB hybridomas will allow us to determine their relationships to one another and provide new information concerning expression of the Ly-1 gene.

The data summarized in Table 16 show that 7 of 15 anti-DNA, 2 of 2 anti-AchR, 1 of 2 anti-TG, 1 of 5 Coombs antibodies and 2 of 11 multispecific antibodies were produced by hybridomas expressing an Ly-1 gene. Among 19 antibodies devoid of binding activity to our panel of 21 antigens, 8 expressed Ly-1 transcripts.

These results indicate that in NZB mice, autoAbs can be produced by Ly-1$^+$ as well as Ly-1$^-$ B cells.

E. DISCUSSION

There are two major models to explain the appearance of non-organ specific autoAbs in systemic autoimmune diseases including lupus erythematosus. One model proposes that polyclonally activated B cells produce antibodies specific for a single antigen and the second model proposes that the activated B cells produce multispecific antibodies (Dighiero et al., 1987; Klinman et al., 1987).

NZB mice represent an excellent model to shed light on this controversy. This strain develops not only a lupus like syndrome associated with non-organ specific antibodies but also an autoimmune hemolytic anemia mediated by organ

TABLE 16

RELATIONSHIP BETWEEN ANTIGEN BINDING SPECIFICITY AND THE EXPRESSION OF THE LY-1 GENE

Specificity	age (month)	Ly1$^+$	Ly1$^-$	Total studied
DNA	1	1	6	7
	12	6	2	8
AchR	1	2	0	2
	12	0	0	0
TG	1	1	1	2
	12	0	0	0
BrRBC	1	1	0	1
	12	0	0	0
Coombs	1	0	0	0
	12	1	4	5
Multispecific	1	0	6	6
	12	2	3	5
Unknown specificity	1	5	5	10
	12	3	6	9

specific pathogenic autoAbs.

Several studies concerning the frequency of B cells producing autoAbs were carried out in NZB mice. Dighiero et al (1987) have shown that while 49% of a panel of hybridomas obtained from young NZB mice produce multispecific antibodies, the percentage decreases to 24% and the frequency of monospecific B cell clones became higher in hybridomas obtained from 5-6 month old mice. Klinman et al (1987) using the splenic fragment culture assay showed that the frequency of multispecific antibodies is similar in NZB mice and DBA/2 mice used as controls for a strain not prone to autoimmune disease. Unfortunately these studies did not report data concerning Coombs antibodies which play an important role in the occurrence of autoimmune hemolytic anemia (Warner et al., 1973).

The results presented in this communication indicate that an important fraction of the B cell repertoire in 1 and 16 month-old NZB is self reactive. It appears that the high frequency of clones producing non-organ specific and multispecific autoAbs is not related to occurrence of disease, since these are found in both young and old NZB mice. By contrast, we have found clones producing Coombs antibodies only in old NZB mice.

Study of the specificity of mAbs produced by hybridomas obtained from 1 and 16 month-old mice indicate that the precursors specific for DNA are more frequent than for

histones or RFs. The former bind strongly to both procaryotic and eukaryotic DNA like human anti-DNA antibodies from patients with SLE (Karounos et al., 1988). A high frequency of clones producing antibodies specific for DNA was also found in strains not prone to autoimmune diseases (Avrameas et al.,1988; Konger et al., 1987). The finding that monospecific anti-DNA clones are equally frequent in young and old NZB mice, suggests that the expansion of DNA reactive clones is not related to antigen-driven clonal selection. It is possible that such clones have been evolutionary selected and play a role in the clearing of antigens liberated by aged or destroyed cells. The second possibility is that such clones can be easily detected since antibodies with low affinity can bind to DNA bearing repetitive epitopes.

Among the panel of 1 month-old hybridomas we detected clones producing anti-AchR, thyroglobulin and Br-RBC monoclonal antibodies. This can be due to LPS stimulation which was used to increase the fusion efficiency in the case of hybridomas obtained from 1 month-old mice. These results clearly show that precursors producing autoAbs against self antigens which are not target of autoimmune phenomena occurring in a given strain, are a normal component of B cell repertoire independent of the predisposition for autoimmune disease.

It is important to note that Coombs antibodies were

found only in old mice. This is in agreement with other observations indicating that the development of autoimmune hemolytic anemia is age related (Warner et al., 1973).

Clones producing multispecific antibodies were found in both young and old animals. These clones are heterogenous and can be classified into three categories a) bispecific exhibiting a moderate binding to autoantigens; b) multispecific exhibiting a low to a moderate binding to autoantigens and; c) multispecific which are non-antigen inhibitable and exhibit a very low affinity. However it should be pointed out that these clones do not produce "sticky" antibodies since while they bind strongly to some self antigens they do not bind to others.

The clones producing multispecific antibodies probably represent an important component of the B cell repertoire of animals from various species and their functional role remains to be elucidated.

Study of the expression of V_H genes in hybridomas producing autoAbs suggested a preferential usage of 3' V_H families (Bona et al., 1988) like in neonatal preimmune repertoire (Conger et al., 1987).

Studies of a limited number of hybridomas obtained from NZB mice showed that for both self reactive and mAbs devoid of self reactivity there is a stoichiometric usage of V_H families. These data are in agreement with previous reports which did not find a biased usage of 3' V_H families in murine

strains prone to lupus (Kofler et al., 1987).

In the case of V_κ families we observed a high usage of $V_\kappa 1$, $V_\kappa 8$ and $V_\kappa 9$. However, this frequency does not differ from normal adult mice. Actually Kaushik et al (1989) recently showed that the expression of V_κ families in both neonate and adult mice is not stoichiometric since some families clustered in the middle of the V_κ locus are highly represented, whereas other families located 3' or 5' are poorly expressed.

There are numerous studies indicating that self reactivity in the B cell repertoire is related both in mice and humans to a discrete subset of B cells bearing the CD5 antigen (Hayakawa et al., 1984; Hardy et al., 1986). While this antigen is expressed at high density on T cells, it is expressed at low density on B cells (Hayakawa et al., 1984; Hardy et al., 1986). Furthermore, it is rarely detected on the surface of B cell hybridomas. Since we have recently shown that some hybridomas obtained from MRL/lpr mice also have transcripts detected with Ly-1 probe (Bailey et al., 1989) we used a molecular approach to assess expression of Ly-1 in immortalized NZB B cells producing autoAbs.

Our data showed that a high frequency of hybridomas having Ly-1 transcripts was detected in NZB mice compared to other strains such as MRL/lpr (Bailey et al., 1989). IF staining and Western blotting analysis carried out on four hybridomas expressing Ly-1 kb transcripts suggest that this

transcript is functional in hybridomas.

Furthermore, our data showed that anti-DNA and multispecific antibodies can be produced by both Ly-1$^+$ and Ly-1$^-$ hybridomas. All hybridomas producing AchR specific antibodies belong to Ly-1$^+$ subset but our panel is too small to draw definitive conclusions. This contrast with other pathogenic antibodies such as Coombs antibodies which were produced mainly by Ly-1$^-$ B cells.

It is important to mention that clones producing antibodies devoid of self reactivity as assessed by binding to 21 autoantigens are of the Ly-1$^+$ and Ly-1$^-$ B phenotype.

Taken collectively our data showed that clones producing non-organ monospecific autoAbs are present in both 1 and 16 month-old NZB while those producing Coombs antibodies were identified only in old mice. Clones producing multispecific antibodies are found in NZB mice like in normal strains. No significant differences were found in the utilization of V gene families among hybridomas producing autoAbs and those producing antibodies devoid of self reactivities. Both type of antibodies are produced by hybridomas expressing or not the Ly-1 gene.

V. DISCUSSION

A. Expression of the Ly-1 Gene

Prior work, which was based solely on immunofluorescence data, demonstrated a very high density of the Ly-1 antigen on the membranes of conventional T lymphocytes and a very sparse distribution on the membranes of sorted B cells. This observation suggested a difference in the expression of the Ly-1 gene and/or peptide processing in B versus T lymphocytes. Therefore the initial experiments were aimed at studying the transcription of the Ly-1 gene in different cell types. Northern blot analysis of thymic RNA revealed two distinct Ly-1 thymic transcripts of 2.1 and 2.9kb respectively. The longer, less abundant 2.9 kb Ly-1 transcript is probably a precursor since it was detected mostly in nuclear RNA. Subsequently RNA analysis was conducted using T cell depleted B cells obtained from *me*^{v} mice, since in this strain the proportion of Ly-1 B cells is close to 100%. Northern analysis revealed Ly-1 B cell transcripts of 2.9kb, corresponding to the longer thymic transcript, and 1.6kb respectively. Identical sized Ly-1 transcripts were also seen in B cell hybridomas. Furthermore S1 nuclease analysis revealed Ly-1 protected fragments of different size in T cells versus B cell hybridomas. The

sparse distribution of surface Ly-1 on B cell membranes was reflected by the lower intensity of Ly-1 transcripts detected. Taken together these results suggest that the Ly-1 gene expression differs in T and B lymphocytes.

B. Correlation of Ly-1 Gene Expression and Detection of Ly-1 Polypeptides in Purified B cells and B cell hybridomas.

The functionality of Ly-1 B cell transcripts detected by both Northern and S1 nuclease protection analysis was tested in Western and immunofluorescence experiments carried out in a representative number of hybridomas expressing the Ly-1 gene and highly purified <u>me</u>^{v} B cells. Western data demonstrated Ly-1 polypeptides of 67 and 72 kd in all samples. The 130kd band seen in some of the hybridomas as well as in Sp2/0 lysates probably represents a peptide bearing a cross reactive epitope, since other monoclonal anti - Ly-1 reagents fail to detect it (Herzenberg unpublished results). The use of reducing agents in both the lysing and loading buffers formally excludes the possibility that the 130kd band represents a dimer. The 67kd band detected in Western analysis was also previously demonstrated by immunoprecipitation and probably corresponds to the mature 471 amino acid protein. The 72kd band detected in Western analysis and the 80kd band previously reported by immunoprecipitation probably represent glycosylated forms.

Immunofluorescence data shows a strong intracytoplasmic staining of all 6 hybridomas studied and weak surface staining in 3 out of 6. Taken together these results suggest that in B cells the Ly-1 protein accumulates in the cytoplasm where it can readily be detected. It appears however, that only a small fraction of the total cytoplasmic Ly-1 protein becomes anchored on the membrane, therefore explaining the "dull" surface staining seen in hybridomas and purified B cells. The most likely explanation of this phenomena is the generation of isoforms at the carboxy terminus in B and T cells, since the C-terminal region is involved in membrane anchoring. The RNA data is clearly consistent with this hypothesis.

C. Contribution of the Ly-1 B cell Subset to Autoantibody Production.

The involvement of the Ly-1 B cell subset in the production of AutoAbs was suggested by the indirect association, seen in a few autoimmune prone mouse strains, between an increased fraction of Ly-1 B cells and the characteristic AutoAb production. The hybridoma technology on the other hand permits the random immortalization of autoimmune B cells from normal and autoimmune prone mice. The sparse distribution of the Ly-1 antigen on B cell

membranes however, previously hampered the attempts of assigning the hybridoma Ly-1 phenotype.

The demonstration of transcription of the Ly-1 locus and functionality of these transcripts in hybridomas, enabled an efficient screening modality of a large panel of hybridomas producing AutoAbs obtained from autoimmune prone as well as normal mice. Consequently the correlation of the Ly-1 phenotypic assignment with the fine specificities of antibodies produced was made possible.

A high proportion (32%) of the hybridomas of the Ly-1 phenotype produce AutoAbs, finding which clearly bears statistical significance. This proportion is especially high in NZB (45%) and $\underline{me}^{v}$ (88%) mice, data in agreement with prior results. The fine specificities of the Abs produced was carried out by competition RIA. The majority of hybridomas producing DNA specific Abs and a smaller but nevertheless statistically significant fraction of hybridomas producing RF and multispecific AutoAbs are of the Ly-1 phenotype. In conclusion these results clearly demonstrate that this small B cell subset (1-3%) plays a significant role in the production of AutoAbs in general and that this role is especially significant for certain specificities (DNA, RF, multispecific).

D. Immunoglobulin Variable Gene Utilisation in CLL and CD5⁺ Lymphomas.

A large fraction of CLL and SLL cases arise by the preferential malignant transformation of CD5⁺ B cells in humans. Taking into account the small fraction of CD5+ B cells detected in the blood of normal individuals, this preferential transformation is most striking. Prior studies have demonstrated a high degree of amino and nucleic acid homology of V_κ chains in a small group of CLL cases selected on the basis of a cross reactive idiotype. The study of immunoglobulin variable gene expression in these types of malignancies offers a modality of determining the relatedness of the cells undergoing malignant transformation in different individuals. A restricted utilization of particular immunoglobulin variable gene families supports this hypothesis, suggesting a similarity between malignant CD5⁺ B cells in different individuals.

The utilization of all known human V_H and V_κ gene families was studied in a large panel of randomly selected CLLs, CD5⁺ lymphomas and CD5⁻ lymphomas (all lymphomas were of the non-Hodgkin's type). A group a EBV transformed lines obtained from normal individuals was used as control since it was previously demonstrated that the V gene utilization of EBV lines is roughly proportional to the family size. A statistically significant biased usage of V_H6, the most D proximal family composed of only one member, was observed in

CLLs as compared to EBV lines. Similarly, V_H5 was overexpressed in CD5+ lymphomas. It is important to point out that V_H5 is also a small family (2-3 members) and is located D proximal. A statistically significant biased usage of $V_\kappa III$ was demonstrated for both CLL and CD5+ lymphomas when compared to the V gene utilization of EBV lines.

This 3' biased V_H gene usage and the bias in the utilization of $V_\kappa III$ in CD5+ malignancies is reminiscent of the V gene utilization of "young lymphocytes" (pre-B cells) in mice. When $CD5^+$ and $CD5^-$ lymphomas were compared an apparent bias of VH4 (4 versus 1), VH5 (3 versus 1) and VKIII (6 versus 3) was seen. These differences however did not reach statistical significance, which is probably due to small sample size of lymphomas used in the study. The biased V gene usage of malignant CD5+ B cells may be the consequence of a restricted repertoire of normal CD5+ B cells which represent the normal counterpart. Alternatively, this type of immunoglobulin V gene usage might be the consequence of a "young phenotype". Taking into account the correlation seen between antigenic specificity and V gene usage, the possible role of repeated antigenic or autoantigenic stimulation needs to be investigated. Autoimmune phenomena have been demonstrated in CLL and in the murine system, analogous lymphomas were obtained from mice hyperimmunized with RBC. Also the study of the physiological role of CD5 and its

possible involvement in lymphocyte activation might provide an explanation for the striking preferential transformation of $CD5^{+}$ B cells.

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