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Development Of The B Cell Repertoire, A Transgenic And Comparative Study.

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DEVELOPMENT OF THE B CELL REPERTOIRE, A TRANSGENIC AND COMPARATIVE STUDY

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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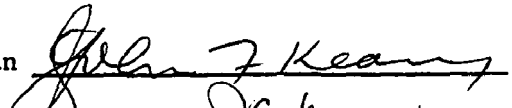
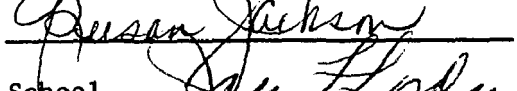
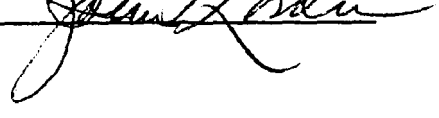
In order to study the significance of developmentally restricted VH gene expression, and natural self-reactive B lymphocytes, VH81X-μ-heavy chain transgenic mice were created where all B cells expressed transgenic heavy chain associated with different κ but not λ light chains. These B cells were predominantly self-reactive and homed normally to peripheral lymphoid organs. When scid mice were reconstituted with both transgenic and littermate mouse-derived bone marrow, transgene-expressing B cells dominated the initial but disappeared from the later repertoire. The majority of B cells in both neonatal transgenic and littermate mice were CD23- and expressed a similar dominant light chain different from that in older transgenic mice. As mice became older, more B cells were recruited into the CD23+/IgMlo B cell pool. In transgenic mice this recruitment was delayed. Among the CD23-/IgMhi immature B cells of transgenic mice, there was a population of clonally unrelated B cells that expressed identical antigen receptors but did not secrete antibody. Continued administration of antibody of the same specificity during postnatal development remarkably reduced generation of these B cells. The CD23-/IgMhi B cells in both transgenic and littermate mice had a shorter half-life than CD23+/IgMlo B cells. The two populations maintained their original phenotype when cultured in the presence of LPS and

proliferated equally well. However, CD23-/IgMhi B cells secreted much more IgM antibody that was more self-reactive than that produced by CD23+/IgMlo cells, which, on the other hand, were more prone to switch to IgG1 isotype than the former in response to LPS plus IL4. Crosslinking sIgM stimulated CD23+/IgMlo rather than CD23-/IgMhi B cells to proliferate. These observations indicate that VH81X-expressing B cells are predominantly self-reactive. They are involved in pioneering the initial repertoire, but later are suppressed and out competed by other B cells. Natural self-reactive B cells are continuously generated due to positive selection by self-antigen, but their maturation is blocked at the CD23-/IgMhi stage. Their failure to enter the long-lived immunocompetent B cell pool may prevent them from receiving T cell help and production of high affinity potentially pathogenic autoreactive IgG antibodies.

Abstract Approved by: Committee Chairman 

Program Director 

Date 7/21/95 Dean of Graduate School 

DEDICATION

I dedicate this dissertation to Hunan Medical University, China, for complete support of my medical training in China and partial support of my graduate study at University of Alabama at Birmingham, and to my parents and my wife for their steadfast encouragement and support.

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INTRODUCTION

Cells of the immune system

The immune system found in its most advanced form in higher vertebrates provides the means to make rapid, highly specific and protective responses against a myriad of potentially pathogenic organisms that inhabit the world in which we live.

The immune system consists of a wide range of distinct cell types, each with an important role to play in this defense process. Lymphocytes occupy central stage since they are the cells that determine the specificity of immunity, and they also orchestrate the effector limbs of the immune system. Other cell types interact with lymphocytes and play critical roles both in presentation of antigen and in mediation of immunologic functions. These cells include macrophages, dendritic cells, and closely related Langerhan's cells, as well as natural killer cells, mast cells and basophils. The cells of the immune system become organized in peripheral lymphoid tissues, such as spleen, lymph nodes, tonsils and Peyer's patches of the intestine, and it is within these tissues that immune response occur. They are also found in the central lymphoid organs, the thymus, and bone marrow, where precursor cells undergo the programmed developmental steps that equip them to mediate the myriad responses of the mature immune system. A very substantial portion of lymphocytes comprise a recirculating pool of cells found in the blood and lymph.

Individual lymphocytes are specialized in that they are committed to respond to a limited set of structurally related antigens. This commitment, which exists prior to the first

contact of the immune system with a given antigen, results from the presence on the lymphocyte plasma membrane of receptors specific for determinants associated with antigens. The ability of an organism to respond to virtually any "non-self" antigen is achieved by the existence of a very large number of different clones of lymphocytes, each bearing receptors specific for a distinct epitope. As a consequence, lymphocytes are an enormously heterogeneous group of cells. Two broad classes of lymphocytes are recognized: B lymphocytes, which are precursor of antibody-secreting cells, and T lymphocytes. T lymphocytes mediate important regulatory functions, such as the ability to help or inhibit the development of specific types of immune response, including antibody production, and increase microbicidal activity of macrophages. Other T lymphocytes are involved in direct effector functions, such as the lysis of virus-infected cells or certain neoplastic cells. The focus of this thesis is the development of the repertoire of antigen specificities of B lymphocytes.

B lymphocytes

Development. B lymphocytes are derived from hematopoietic stem cells by a complex set of differentiation events that are only partially understood. These events begin in the yolk sac then shift to fetal liver and omentum, and spleen (Owen et al., 1975; Gathings et al., 1977; Solvason et al., 1993) and, in adult life, continue principally in the bone marrow (Rosse 1981; Osmond, 1986). Interaction with specialized stromal cells (Kincade et al., 1981) and their products, including cytokines such as interleukin (IL) 7 (Namen et al., 1988), is critical to the normal regulation of these differentiation processes. The initial key events in B cell development occur in cells designated pro-B and/or pre-B cells. They center around the assembly of the genetic elements (Tonegawa, 1983)

encoding the cell surface receptor of B cells, which is an IgM antibody molecule specialized for expression on the cell surface. Membrane IgM receptors are heterodimeric molecules consisting of μ heavy (H) and κ or λ light (L) chains, both of which have regions that contribute to the binding of antigen and that are highly variable from one IgM molecule to another.

Expression of μ heavy chain and an IgM molecule on the cell surface is an essential step for B cell development to occur. Mutant mice that can not rearrange immunoglobulin gene segments, such as scid mice (Bosma and Carroll, 1991) or mice homozygous, for disrupted recombination associated genes (RAG-1 and RAG-2) (Mombaerts et al., 1992; Shinkai et al., 1992), lack mature B and T cells. In addition, targeted disruption of the membrane exon of the IgM μ gene (Kitamura and Rajewsky., 1991) or λ5 gene (Kitamura et al., 1992) was shown to cause a severe deficiency in B cell development. The absolute requirement for the expression of surface μ heavy chain suggests that μ heavy chain, together with surrogate light chain or surface IgM molecules, is involved in transducing signals necessary for development to proceed. The ability of surface μ heavy chain to mediate these effects depends on associated membrane proteins, Igα and Igβ (Hombach et al., 1990; Ishihara et al., 1992), which together with IgM constitute the B cell antigen receptor complex analogous to the T cell receptor complex. The T cell antigen receptors mediate processes that can result in either positive or negative selection of developing T cells in the thymus (Kaye et al., 1989; Murphy et al., 1990). These selective mechanisms result in the generation of immunocompetent T cells that exit the thymus. With respect to B cells, it has been clearly shown that B cell surface IgM antigen receptor (BCR)-mediated negative selection results in the deletion or inactivation of self-reactive B cells

(Goodnow et al., 1988; Nemazee and Burks, 1989; Erikson et al., 1991). Little is known about BCR-mediated "positive" selection with respect to B cell differentiation, the stages of development (pre-B and/or B cell), the anatomical sites where selection may occur and the nature of the selecting ligands. However, with the knowledge that without expression of surface μ chain B cell differentiation does not occur, as discussed above, a positive signal via BCR is likely required for the differentiation of B cells to proceed.

Immunoglobulin gene rearrangement. One of the key molecular events during B cells differentiation is the rearrangement of immunoglobulin genes, in which distantly located immunoglobulin gene segments are juxtaposed to give rise to a complete heavy or light chain immunoglobulin gene (Hozumi and Tonegawa, 1976). This occurs in discrete and ordered steps. The first event brings one diversity (D) segment of the heavy chain gene into proximity with one joining (JH) region segment, and this is followed on the same chromosome by a rearrangement that joins one of many variable (VH) segments to the already rearranged DH-JH segments. The recombination events are directed by consensus signal sequences flanking the gene segments and follow the 12+23 spacer rule (Max et al., 1979; Sakano et al., 1980). Many of the rearrangements do not result in a correct reading frame. For this reason, as many as one out of every three newly formed cells may not able to make functional immunoglobulins (Alt et al., 1986). Synthesis of μ heavy chain begins in those cells where the rearrangement process was successful, yielding pre-B cells, which then proceed to rearrange light-chain genes (VL-JL) and display surface immunoglobulin.

The heavy chain gene locus of mice consists of 100 to 1000 VH gene segments (Brodeur and Riblet, 1984; Livant et al., 1986), about 12 DH gene segments (Kurosawa and Tonegawa, 1982) and 4 JH gene segments (Sakano et al., 1980). The κ light chain

gene locus consists of 100-300 Vκ genes and 4 functional Jκ genes (Sakano et al., 1979). The λ locus has 2 Vλ gene segments and 4 Jλ gene segments, which are organized differently from the heavy chain and κ loci (Blomberg et al., 1981). The variable region genes are classified into families or groups based on their nucleotide sequence similarity. The current scheme allocates two V gene sequences to the same family if they show more than 80% homology and to different families if their sequences are less than 70% identical. So far, 14 VH gene families have been identified in mouse (Brodeur and Riblet, 1984; Dildrop, 1984; Meek et al. 1990; Tutter and Riblet, 1991). The families have been further classified into three "clans" based on sequence conservation in framework I and III regions (Tutter et al., 1989; Kirkham et al., 1992). The complexity of each family varies (Kofler et al., 1992). Several families have only a few members, such as the S107 family, which has only three functional germline members, while VH J558 family may consist of as many as 1000 members in the BALB/c mouse. Members of a given VH family tend to be clustered together (Kemp et al., 1979; Givol et al., 1981), and the families are arrayed in a relative order on the chromosome, although there is some interdigitation among families (Brodeur et al., 1988; Walter et al., 1991). The order of the families closest to Cμ is: S107-Q52-7183-D-J-Cμ.

The 7183 VH gene family belongs to clan III which contains the most evolutionarily conserved VH gene families (Tutter and Riblet, 1989), and has 18 members (Huetz et al., 1993). The most downstream functional VH gene in this family is the VH81X gene. Though a member of the 7183 family, VH81X is the least homologous to all other member of that family (Huetz et al., 1993). The VH81X has a unique framework region one (FR1) with many charged amino acid residues not found in other FR1

(Kirkham et al., 1992). Early during ontogeny, VH genes from the 7183 family, particularly VH81X, are significantly over-represented in the VDJ rearrangements (Yancopoulos et al., 1984; Perlmutter, 1985). The perinatal bias in preferential rearrangement of D-proximal VH genes is retained in adult Abelson virus transformed cell lines (Lawler et al., 1987; Reth et al., 1986). Several genetic mechanisms have been proposed to explain the predominant rearrangements of the VH81X gene. The D-proximity of this gene was taken as evidence favoring a "tracking" model of V gene rearrangement (Malynn et al., 1987). However, D-proximity is apparently not the only reason for over-presentation of VH81X rearrangements, since another gene segment VHE4.Psi, which is a pseudogene, is situated even further downstream to VH81X, but was rarely detected when all non-functional rearrangements involving 7183 family genes were examined (Carlsson et al., 1992; Huetz et al., 1993). The upstream flanking sequences contain transcriptional regulatory elements that may also influence VH gene rearrangement (Chen et al., 1990). The unique 3' flanking recombination signal sequence of VH81X (Schroeder et al., 1989; Yancopoulos et al., 1984) may also contribute to the preferential rearrangement of this gene, since different recombination signal sequences have been shown to give different frequencies of gene rearrangement (Ramsden and Wu, 1991).

Although VH81X is constantly rearranged at a high frequency throughout life, it is predominately expressed in the early but not the adult B cell repertoire. This is reflected by the observation that more than 80% of VH81X rearrangements isolated from neonatal B cells were functional (with open reading frames), whereas almost all VH81X rearrangements present in adult peripheral B cells were non-functional (Decker et al.,

1991; Huetz et al., 1993). The reasons for this extreme in VH81X expression is not completely understood. Based on the presence of short sequence homologies found at the VH81X-D-J junctions isolated from neonates, it was proposed that homology-directed recombination plays a role in facilitating productive rearrangements in newborns but not in adults. In adults, N-sequence addition due to increased terminal deoxynucleotide transferase (TdT) activity makes this mechanisms of rearrangements non-functional (Chukwuocha and Feeney, 1993), suggesting that the developmentally controlled lack of Tdt activity in the early development facilitates VH81X expression. On the other hand, it has also been shown that restricted Ig junctional diversity in neonatal B cells resulted from developmental selection rather than homology-directed V(D)J joining (Pandy and Tjoelker, 1993). In accordance with the selection hypothesis is the finding that the frequency of functional VH81X rearrangements present in pre-B cells from both neonates and adults is similar, i.e. 25-30% (Huetz et al., 1993). This suggests that the predominance of functional VH81X rearrangements in neonatal B cells results from a positive selection for B cells expressing this gene, and absence of these B cells in adults results from negative selection.

However, the questions remain: why are VH81X-expressing B cells positively selected in the early repertoire, but negatively selected in the adult repertoire, and if so, what is the nature of the selecting ligands?

Development of the B cell repertoire and idiotypic networks.

The development of B cells is related to, but should be distinguished from, the acquisition of a diverse B cell repertoire. Similar to other organ systems, a normally developed pre-immune B cell repertoire should have its well-defined structure that is

partially reflected by the composition of B cells in the repertoire. The potential germline V-gene repertoire is estimated to include at least 10^{10} different specificities (Tonegawa, 1983; Berek et al., 1985), whereas the specificity expressed by the available B cell receptor repertoire is estimated to remain well below 10^{8} (Holmberg et al., 1986). The recruitment of specific B cell clones from a vast potential pool to a limited available repertoire is a non-random process, as illustrated by examples of the occurrence of dominant B cell clones. The dominant idiotype in primary antibody response to the hapten NP in different individual C57BL/6 mouse is invariably NP^{b} (Reth et al., 1979), likewise, anti-DEX and anti-PC response in normal BALB/c mice always present stereotype dominant idiotypes (Blomberg et al., 1972; Lieberman et al., 1974), which require specific V(D)J rearrangements (Masmoudi et al., 1990), suggesting that B cell clones bearing the corresponding antigen receptor and idiotype are already present in the pre-immune repertoire. However, these dominant idiotypes could be suppressed or enhanced by perinatal treatment with pertinent anti-idiotypic antibodies (Augustin Cosenza, 1976; Vakil and Kearney, 1986, 1991). These observations suggest that i) the composition and structure of the repertoire is controlled not only at the level of gene rearrangement but is also the result of antibody and/or antigen-mediated interactions and selection, and ii) the B cells present in the repertoire are not simply random survivors of negative selection.

The establishment of the B cell repertoire appears to follow a "programmed" procedure, and there is a hierarchy in the development of the ability to make antibody response to different antigens (Stein, 1992). For example, the T15 idiotype response to phosphorylcholine does not appear until day 4 after birth (Sigal et al., 1976), while the response to α1-3 dextran occurs on day 10 (Stohrer, and Kearney, 1984), and the

responses to inulin and α1-6 dextran occur even later (Bona et al., 1979). As discussed above, the sequential development of the ability to make antibody responses may reflect the temporal process of recruitment of relevant B cell clones from the emergent repertoire into the available repertoire. In an attempt to understand this process, theoretical modeling of idiotype networks has been performed, and it has been proposed that establishment of an immune repertoire occurs through an idiotype network (Boer and Perelson, 1991). In this process, emergent clones that interact with other members are recruited into the network, and consequently the repertoire and network expand. Only those clones with few connections with the network survive, because multi-reactive clones tend to recognize many clones in the network and are suppressed by the large number of interactions. Additionally, as the network develops, its connectivity is reduced because clones tend to occupy similar regions in shape space. Although this theoretical model fits almost perfectly with what has been observed in experimental studies of the immune network, the question remains as to the nature of the first B cells that populate the network and factors that select these first B cells. These unknowns can only be determined by experimentation and empirical observations.

Negative selection and natural self-reactivity.

One of the central problems facing the immune system is that of being able to mount highly effective immune responses to antigens associated with foreign, potentially pathogenic antigens while ignoring antigens associated with the host's own tissues. The mechanisms ensuring this failure to respond pathologically to self-antigen are recognized to be complex and to involve a series of strategies. Chief among them appear to be elimination of cells capable of self-reactivity and the inactivation of cells with this

potential. For B cells, it appears that encounter of immature naive B cells with antigens expressing multiple copies of the same epitope will lead to their elimination, particularly if no T-cell help is provided at the time of the encounter (Bretscher and Cohn, 1970; Metcalf and Klinman, 1976; Morhan et al., 1989; Ales-Martinez et al., 1992). This elimination of potentially self-reactive cells is referred to as clonal deletion (Nemazee and Burki, 1989). However, there are so many self-antigens that are not encountered by the developing B-cell populations or that do not interact appropriately with B-cell receptors to elicit clonal elimination process. There is growing evidence to show that such B cells are nonetheless functionally inactivated, through a process that involves crosslinkage of receptors without the receipt of critical co-stimulatory signals. Such cells are physically present but are unresponsive to antigen and are in a state of anergy (Nossal and Pike, 1980).

Paradoxically, despite the vigorous censoring mechanisms demonstrated experimentally *in vitro*, and *in vivo* in transgenic mouse models, it is clear that the early B cell repertoire is dominantly self-reactive (Dighiero et al., 1985; Levinson et al., 1987; Lehuen et al., 1992; Holmberg and Kearney, 1993). In addition, in normal adult mice or humans, there is a high frequency of self-reactive B lymphocytes (Steele and Cunningham, 1978; Underwood et al., 1985; Souroujon et al., 1988; Rousseau and Mallett, 1989; Schwartz and Stollar, 1985; Cote et al., 1986; Hardy et al., 1987; Kasaian et al., 1992) and there is a large body of data demonstrating the spontaneous presence of self-reactive antibodies in normal sera, referred to as natural antibodies (Karsenti et al., 1971; Guilbert et al., 1982; Nobrega et al., 1993). One of the fundamental assumptions is that natural antibodies result from stimulation of B cells by exogenous antigens. However, it has been shown that hybridomas from fetal and neonatal tissues (Dighiero et al., 1985; Levinson et

al., 1987; Kearney, 1993) or from germ-free and antigen-free mice produce these type of antibodies at similar frequencies (Hooijkaas et al., 1984; Underwood et al., 1985). These naturally occurring self-reactive B cells again are not simply anergic B cells that have survived clonal elimination, because the patterns of self-reactivity appear consistently and are conserved even in phylogenetically distinct species (Underwood et al., 1985; Cote et al., 1986; Souroujon et al., 1988; Martin et al., 1992; Nobrega et al., 1993).

Obviously, several fundamental questions arise here that have yet to be answered. Firstly, how are these self-reactive B cells generated and maintained within the normal immune system, in the face of demonstrated censoring mechanisms that eliminate self-reactive lymphocytes; second, where are these B cells located; and finally, what physiological role do they play in the development and maintenance of the functional immune system?

Hypothesis and the scope of the dissertation.

To further understand the mechanisms that regulate B cell repertoire development, with regard to the questions raised in the previous sections of this dissertation, we propose the following hypotheses:

1. Engagement of the B cell antigen receptor (BCR) with a ligand seems mandatory for developing B cells to proceed to further differentiation. The most abundant antigen epitopes available before birth are expressed on self-antigens, which function as ligands in this B cell receptor engagement, a process analogous to, but different from, positive selection of T cells.

2. The rearranged VH81X genes isolated from fetal B cells encode mostly self-reactive antibodies, even when associated with different light chains. Mechanisms, such as

D-proximity, unique recombination signal sequences, homology-directed recombination, and lack of TdT activity, may ensure over-expression of this VH gene in the initial B cell repertoire. The consequence of these collective mechanisms to limit diversity is that early B cells are guaranteed to have something to recognize, are self-reactive, and over-express VH81X. These self-reactive B cells do not cause auto-immunity because of lack of T cell help, however, they constitute the initial idiotype network, with VH81X-encoded antibodies functioning as self-antigens to present epitopes to emergent B cells and recruit them into the network.

3. As the number of clones in the network that are connected with VH81X-expressing B cells increases, suppression occurs. This suppression plus the advent of TdT activity results in a decrease in VH81X-expressing B cells in the later repertoire. In addition, they are out-competed in the face of other B cells that are more heterogeneous, less self-reactive and relatively long lived.

4. Since self-antigens continually select emergent B cells in adult life, some of these B cells may migrate to the spleen to constitute natural self-reactive B cells. These B cells do not acquire T-cell help, and die before full maturation and entry into the immuno-competent mature B cell pool.

Our approach to this hypothesis is to study the B cell repertoire and its development in VH81X-μ heavy chain transgenic mice and bone marrow chimeric mice, in comparison with that in normal mice. The results, discussion and conclusions derived from the studies are presented in the following three sections.

The first section describes B cell repertoire development in VH81X transgenic and bone marrow chimeric mice. As discussed above, although VH81X is preferentially

utilized in the early repertoire, B cells expressing this gene are rarely found in the adult repertoire. By making VH81X transgenic mice, where all B cells express the transgene due to allelic exclusion, we can directly ask if VH81X-expressing B cells can survive in adult transgenic mice. In addition, we can study the mechanisms that make VH81X-expressing B cells dominate the early but disappear from the adult repertoire.

The second section describes the immunoglobulin light chain repertoire in different sub-populations of B cells in transgenic mice and the origin and specificity of B cells expressing the dominant endogenous light chains and shows evidence indicative of positive selection of natural self-reactive B cells.

The third section describes further evidence that Vk1A5/VH81X-bearing B cells are generated as a result of positive selection, and phenotypic differences between CD23+ and CD23- B cells indicate that the two B cell populations not only represent B cells at distinct differentiation stages but also that each population is intrinsically different in cellular activity.

VH81X TRANSGENIC MICE

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Summary

Immunoglobulin heavy chain variable region gene utilization in the developing B cell repertoire is restricted. In BALB/c mouse, the most D-proximal functional VH gene, VH81X, is preferentially utilized, but, in contrast, is rarely expressed in the adult B cell repertoire. To determine the factors that control the expression of VH genes in general and to determine if B cells expressing the VH81X gene played a role in repertoire development, we developed VH81X-μ heavy chain transgenic mice with a rearranged VH81X gene isolated from fetal liver. In these adult transgenic mice, B cells expressed almost exclusively the VH81X-transgenic heavy chain associated with a variety of κ but not λ light chains and were predominantly self-reactive towards ubiquitous intracellular components. They homed normally to peripheral lymphoid organs, with differentiation markers expressed at the correct developmental stages, indicating that expression of VH81X is not incompatible with B cell longevity. However, when scid mice were reconstituted with bone marrow cells from both transgenic and littermate mice, the transgene-expressing B cells were not found in the spleen at the time when B repertoire development in the recipient was complete. Nevertheless, in spite of their self-reactivity and survival disadvantage in the face of other B cells, transgenic mouse bone marrow-derived B cells contributed significantly to the initial repertoire until several weeks post-reconstitution. Compared with neonates, B cells in adult transgenic mice were more reduced in number. The level of transgene-derived serum IgM was remarkably low. As in normal mice, the majority of the splenic B cells in neonatal transgenic mice were CD23-, with a proportion of them being CD5+, and expressed a dominant κ light chain similar to that seen in the normal neonate, but different from that of adult transgenic mice. As mice

became older, CD5+ B cells disappeared and more B cells were recruited into the CD23+ pool. However, in transgenic mice this recruitment was delayed. Yet, analysis of various B cell differentiation markers revealed that comparable B cell sub-populations were present in both adult mice. The peritoneal cavity B cells in neonatal mice transgenic were dominantly CD5 positive, but they are short-lived. Taken together this study shows that (2) VH81X-expressing B cells tend to be self-reactive and are involved in pioneering the initial B cell repertoire, but as the repertoire expands, they are suppressed and out-competed; and (2) although in transgenic mice B cells express the same heavy chain, B cell repertoire development follows a similar sequential order seen in normal mice, but recruitment of B cells into the bulk CD23+ pool was delayed.

Introduction

B cells have the potential to display an enormous diversity of their antigen receptors (1). Despite the potential to generate a very large number of specificities through immunoglobulin (Ig) gene rearrangement, utilization of this repertoire is restricted in the perinatal B cells, as illustrated by limited heavy chain variable region gene utilization (2-4). Associated with this restricted Ig gene utilization is an early immune system, which is self-reactive (5-7), multi-reactive and connective (8-10), and has a limited capacity to respond to exogenous antigens (11-13).

In mouse, the fetal and neonatal repertoire preferentially express VH genes from the 7183 and Q52 family, with the most DH-proximal functional VH gene, VH81X, predominating (2, 3, 14, 15). This preference for certain VH genes stands in contrast to the "normalized" adult mouse repertoire where B cells express VH genes at a frequency proportional to the complexity of the particular VH family, and where, surprisingly,

VH81X-expressing B cells are rarely found (15, 16, 17). The regulatory mechanisms responsible for these two extremes in VH81X gene expression by B cells do not appear to occur at a molecular level where regulatory mechanisms might favor rearrangements involving this particular VH gene, since it has been demonstrated that this VH gene segment is rearranged constantly at a high frequency not only in the early repertoire, but throughout life (14, 17). The main difference with respect to VH81X expression and the age of mice is that the VH81X rearrangements identified in the early repertoire were predominantly functional, while those analyzed in the adult B cell repertoire were virtually all non-functional (16, 17). These observations suggest that in the early repertoire there is positive selection for B or pre-B cells that express VH81X, but that similar B cells are negatively selected in the adult repertoire (17).

To obtain insight into the significance of restricted diversity in the early repertoire, the function of these cells, and the mechanisms which favor expression of VH81X early but not late, we produced VH81X-μ-heavy chain transgenic mice with a rearranged VH81X gene isolated from fetal liver.

Materials and Methods

Generation of Transgenic Mice. To make the transgene construct, genomic DNA was isolated from the 15-d fetal liver-derived hybridoma BC2; rearranged VH81X was PCR amplified from the DNA with two primers that anneal to the VH81X promoter region and the JH2-3 intron. The sequences of the primers are: 5'-CTCAGATGGAGGC-CACTGAGGGAC-3' (anneals to the promoter) and 5'-GGGGATCCCTTATATCTATG-ATGATCACTGG3' (anneals to JH2 intron). The PCR amplified fragment was cloned into pUC119 at EcoR1 and BamH1; two restriction sites originally existed in the VH81X

gene and JH2 intron. The cloned DNA fragment was first subjected to nucleotide sequence analysis to ensure that there were no PCR-introduced mutations. The rearranged V-D-J DNA was then joined to its original 5' non-translated DNA (the 3.5-kb DNA fragment was a gift from Dr. F. Alt), at the EcoR1 site. This promoter-V-D-J DNA was recloned into a eukaryotic expression vector pSVG-gpt and JH3-4, and Cμ sequences (gift from Dr. C. Goodnow) were inserted downstream of the VDJ, resulting in the entire 18-kb transgene (Fig. 1). The transgene was tested for in vitro expression in heavy chain-loss light chain-producing B cell lines. For microinjection, the plasmid DNA was removed at Sal1 sites that flank the transgene. Transgenic mice were generated by microinjecting the gene construct into C57BL/6 / DBA F2 fertilized eggs. The founder mice were backcrossed to BALB/c and C57BL/6 strains mice for six generations. Transgenic mice were identified by PCR and Southern blot analysis of mouse tail DNA. The primers for PCR were designed in such a way that only the transgenic but not endogenous VH81X was amplified: 5'-CGCGCGGCCGCGTGGAGTCTGGGGGAGGCT-3' to anneal to framework I of VH81X and 5'-CCCAGACATCGAAGTACCAGCTACTACCATG-3' to anneal to the CDR3 of the transgene.

Flow Cytometry And Immunofluorescence Cytological Analysis. The antibodies used for flow cytometry are: fluorescein-conjugated goat anti-mouse μ, κ, B220, CD72, HSA and CD38, and PE-conjugated secondary reagents (Southern Biotech, Birmingham, AL); biotin-conjugated rat anti-mouse IgD (11-26c; a gift from D. Bole); B220/14.8 (18); CD21, CD22, CD23 (B3B4; a gift from M. Kehry and D. Conrad), IgH6a/RS3.1 (19) and IgH6b/MB8.6 (20); I-A^d class II MHC (MK-D6). Two-color surface staining was performed as previously described (21). Briefly 5 x 10^5 cells were first incubated with

goat anti-mouse IgM, then with either anti-B220, IgD, CD23, or class II MHC, followed by SA-PE (Birmingham Southern Biotech), 20-min for each incubation. The cells were washed with 1% BSA/PBS twice between each step. For cytoplasmic staining, Ag8 cells were fixed with 70% ice-cold ethanol for 30 m and then washed twice with BSA/PBS before antibody staining. Transgenic B cell hybridoma-derived antibodies were used at 10 μg/ml and followed by FITC-conjugated goat anti-mouse IgM-FITC. Negative control antibody, #15, is a transgenic B cell hybridoma-derived antibody containing the VH81X heavy chain and light chain, Vκ4/5. Stained cell samples were analyzed using the FACScan Consort 30 program (Becton-Dickinson).

ELISA. E.I.A A/2 plates (Costar) were coated with unlabeled goat anti-mouse antibody (anti-IgM, or other isotype) or RS3.1 or MB8.6 at 2 μg/ml in borate buffer (pH 9), incubated at 4°C overnight, followed by the test antibody (serum at 1 to 1000 dilution in 1% PBS-BSA and incubated at 37°C for 2 hr. Alkaline phosphatase-conjugated goat-mouse IgM antibody was used as the third layer antibody, and incubated for 2 hr at 37°C. Between each step, the plate was washed five times with PBS. The plate was developed with alkaline phosphatase substrate 1 mg/ml in substrate buffer (pH 9). For quantitative ELISA, mouse antibody of known concentration was included in each plate, and the plate was read by a spectrophotometer at 405 nm, and antibody concentration was determined using an ELISALITE program.

Construction of Bone Marrow Chimeric Mice. Bone marrow cells were harvested from the femur of donor mice and washed with cold 1% FCS/PBS. *scid* mice were irradiated with 300 rads and then injected intravenously with 5 x 10^6 total bone marrow cells from either transgenic or littermate mice or both.

Southern blot analysis. To isolate genomic DNA, mouse tissues (including tail, spleen, sorted B cells, LPS cultured cells and hybridomas) were digested with digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS, 10 μg/ml RNase A and 200 μg/ml proteinase K) at 55°C overnight, followed by phenol and chloroform extraction and ethanol precipitation. Southern blots were performed according to standard procedures. Briefly, 10 μg of DNA were digested at 37°C overnight to completion with restriction enzymes (either HindIII or BamH1, from BRL), electrophoresed through 0.8% agarose and transferred to a nylon membrane (BRL). Membranes were cross-linked with a DNA Cross Linker (Stratagene). Hybridization with ^{32}P-labeled VH81X or Jκ1-5 DNA probes was carried out at 65°C. All final washes were conducted under high stringency conditions.

DNA Sequencing Analysis. Vκ gene sequencing was carried out from either cDNA or genomic DNA. To make cDNA, total RNA was isolated from hybridomas using guanidinium thiocyanate-phenol-chloroform extraction (22). The cDNA was synthesized using a Cμ primer: 5'-GAAGCTTATACACAGTTGGTGCAGCATCAGCC-3' followed by PCR using the same Cμ primer as 3' primer and a degenerate Vκ primer as 5' primer: 5'-GCCATGGAPRTQLWLMTSACCCAGTCTCCA-3'. When genomic DNA was used for sequence analysis, the Jκ5 primer was utilized as a 3' primer. The PCR amplified Vκ DNA was cloned into pUC119 and subjected to sequencing using Sequenase Kit (Stratagene). The DNA sequence was analyzed using DNAstar program to determine the origin of Vκ, Jκ, N-region addition and somatic mutation.

Results

Generation of Transgenic Mice Expressing A fetal B Cell-Derived IgM Heavy Chain Gene. Hybridoma BC2 was derived from an 18-d BALB/c fetal liver and expressed VH81X-IgM heavy chain in association with a κ light chain. The VH81X, which rearranged to germline DFL16.1 and JH1 and had no nucleotide additions at V-D and D-J joins, was cloned from this hybridoma (Fig. 1A). The rearranged gene was placed downstream of the original VH81X gene promoter contained in a 3.6-kb DNA fragment of untranslated sequence. The heavy chain enhancer was ligated down stream of JH1, followed by BALB/c derived genomic μ heavy chain DNA to make the transgeneconstruct (Fig. 1B). The gene construct was micro-injected into fertilized eggs from DBA/J x C57BL/6 F2 mice to generate transgenic mice that were identified by PCR and Southern blot analyses. From the first round of micro-injection, we obtained two transgenic mice carrying 1 or 12 copies of the transgene, respectively. The founder mice were backcrossed onto BALB/c and C57BL/6 background for six generations for the following study. We conducted most of our analysis using the single-copy transgenic mouse.

B Cells In Transgenic Mice Express Almost Exclusively The Transgene. To study transgene expression, we utilized two anti-allotypic antibodies, RS3.1 and MB8.6, which recognize IgH6a and Igh6b, respectively. B cells in adult transgenic mice on the C57BL/6 background were found to express predominantly the IgH6a positive transgenic heavy chain, and only a few B cells (about 5% by 6 m of age) expressed endogenous IgM heavy chains of IgH6b allotype (Fig. 2A). Similar observations were made in transgenic mice on a BALB/c background by using the lack of IgD expression as a marker for transgene expressing B cells (Fig. 2B). These results showed that the transgenic VH81X

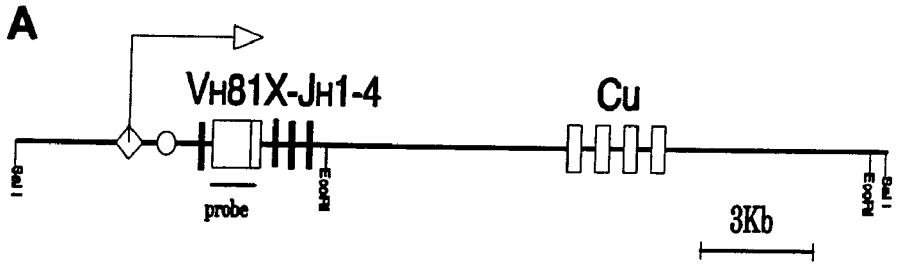

Figure 1. Transgene construction and sequence of its variable region. (A) The rearranged VH81X gene was cloned from a fetal liver-derived B cell hybridoma BC2. A functional transgene was made with the VH81X gene joined to 3.6-kb DNA fragment originally 5' to VH81X gene, which contains promoter and other regulatory sequences (a gift from Dr. F. Alt). The IgH enhancer contained in a 1.9-kb BamH I and EcoR I fragment is followed by the Cμ region, which is BALB/c derived and is contained in a 12-kb EcoR1-EcoR1 fragment. For micro-injection, this whole 18-kb transgene was cut out of the cloning vector at the flanking SalI sites. (B) The complete sequence of the variable region of the BC2 derived transgenic VH81X heavy chain. The rearranged VH81X is germline and rearranged to germline DFL16.1 and JH1, without nucleotide addition at either D-J or V-D joins.

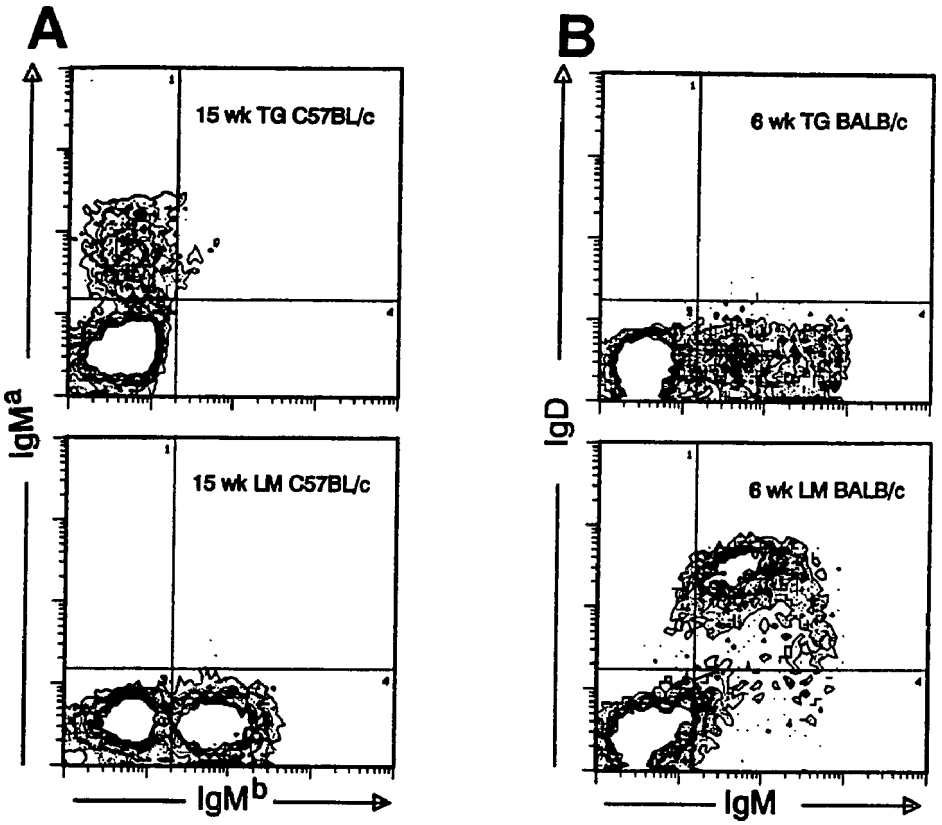

Figure 2. FACS analysis of transgene expression on spleen B cells. (A) Spleen cells from transgenic C57BL/6 and littermates were stained with RS3.1 and MB8.6 which recognize transgene-derived Igh6a and endogenous Igh6b respectively, and analyzed by flow cytometry. B cells in transgenic mice were Igh6a positive, and in the littermate Igh6b positive. (B). Spleen cells were stained with anti-IgM and anti-IgD antibodies. Littermate B cells were IgD positive but B cells in transgenic mice were IgD negative.

encoded heavy chain exerted strong allelic exclusion of the endogenous heavy chain genes.

Immunoglobulin light chain expression was determined by using anti-mouse λ or κ antibodies and FACS analysis. The splenic B cells in transgenic mice expressed almost exclusively κ light chains, with λ positive B cells accounting for only 1.4% of all B cells, while in the littermate mice λ^+ B cells accounted for 7.8±1.9% of total spleen B cells (Fig. 3). Although λ-positive B cells were few in the spleen of transgenic mice, there were many (20-50% of total B cells) in the peritoneal cavity. There they were found to be associated with endogenous rather than transgenic μ heavy chains when analyzed in combination with anti-allotypic antibodies, RS3.1 or MB8.6. Analysis of 74 hybridomas made from transgenic mice also showed that none expressed the VH81X transgene with λ light chains unless they also co-expressed an endogenous μ chain.

B cells in adult transgenic mice expressed the B cell markers CD23, B220, HSA, S7, CD72, CD38, CD21, CD22 and surface IgM at the correct developmental stages. The composition of spleen B cell sub-populations was also similar to that of normal mice (Fig. 4). Histologically, the spleens of transgenic mice had an overall normal architecture and B cell follicles developed in correct relationship to T cells (Fig. 5). The observations indicate that VH81X-expressing B cells are not incompatible with longevity, and that lack of B cells expressing VH81X previously observed in normal adult mice is not due to the inability of such B cells to be generated or because they cannot survive in the environment of the adult mouse lymphoid tissues.

However, despite the overall similarity, some subtle but consistent phenotypic differences between B cells of transgenic and littermate mice could be detected. As shown in Fig. 4, the bulk of B cells in transgenic mice expressed slightly lower levels of HSA,

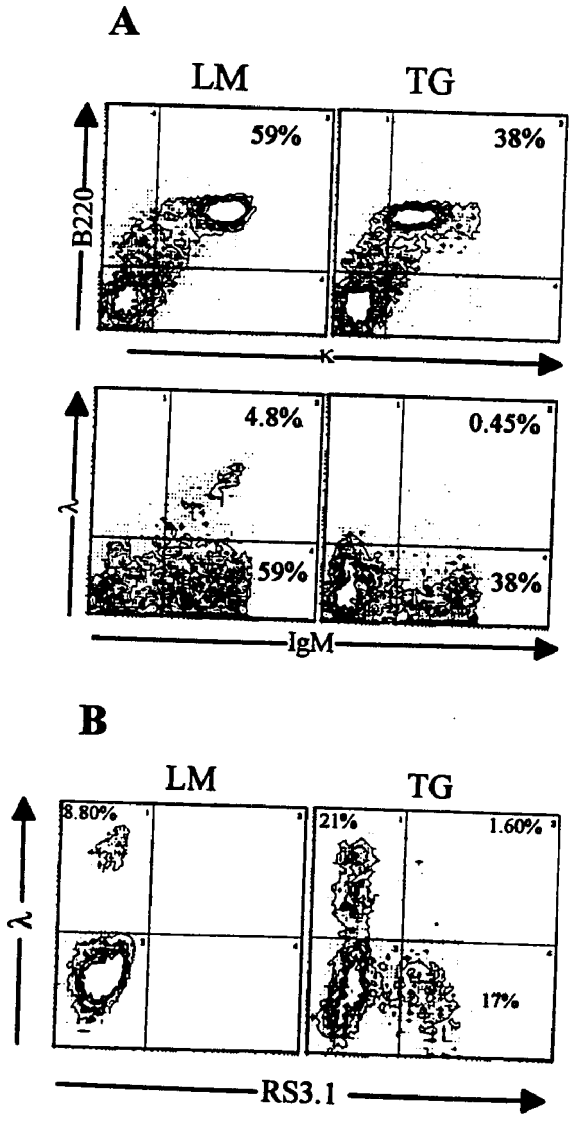

Figure 3. FACS analysis of light chain expression in spleen B cells. (A) Spleen cells were double stained with anti-B220 and anti-κ, or with anti-μ and λ. The λ positive B cells were few in transgenic mouse spleen. (B) In the peritoneal cavity of transgenic mice, there were more λ positive B cells, but they expressed the endogenous heavy chain, which is RS3.1 negative, in C57BL/6 transgenic mice.

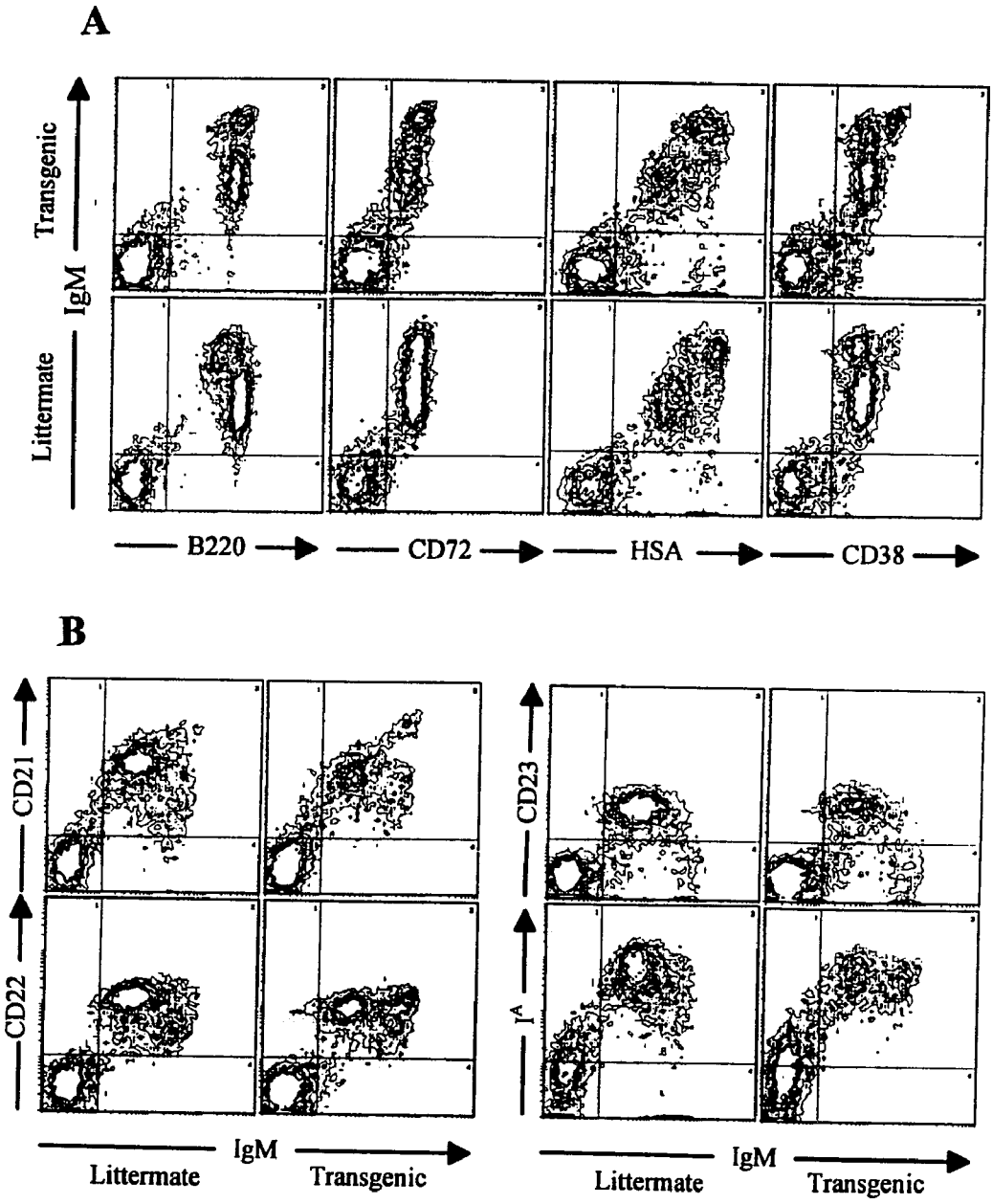

Figure 4. FACS analysis of B cell surface marker expression. Spleen cells from 2 m old mice were stained with (A) PE-conjugated anti-IgM and FITC-conjugated anti-B220, CD72, HSA and CD38; or (B) FITC-conjugated anti-IgM and biotin-conjugated anti-CD21, CD22, CD23 and class II MHC I-A. Comparable sub-populations of B cells could be identified in both transgenic and littermate mice, with B cells in transgenic mouse spleen expressing slightly higher levels of CD23 and CD38 but lower levels of other cell markers.

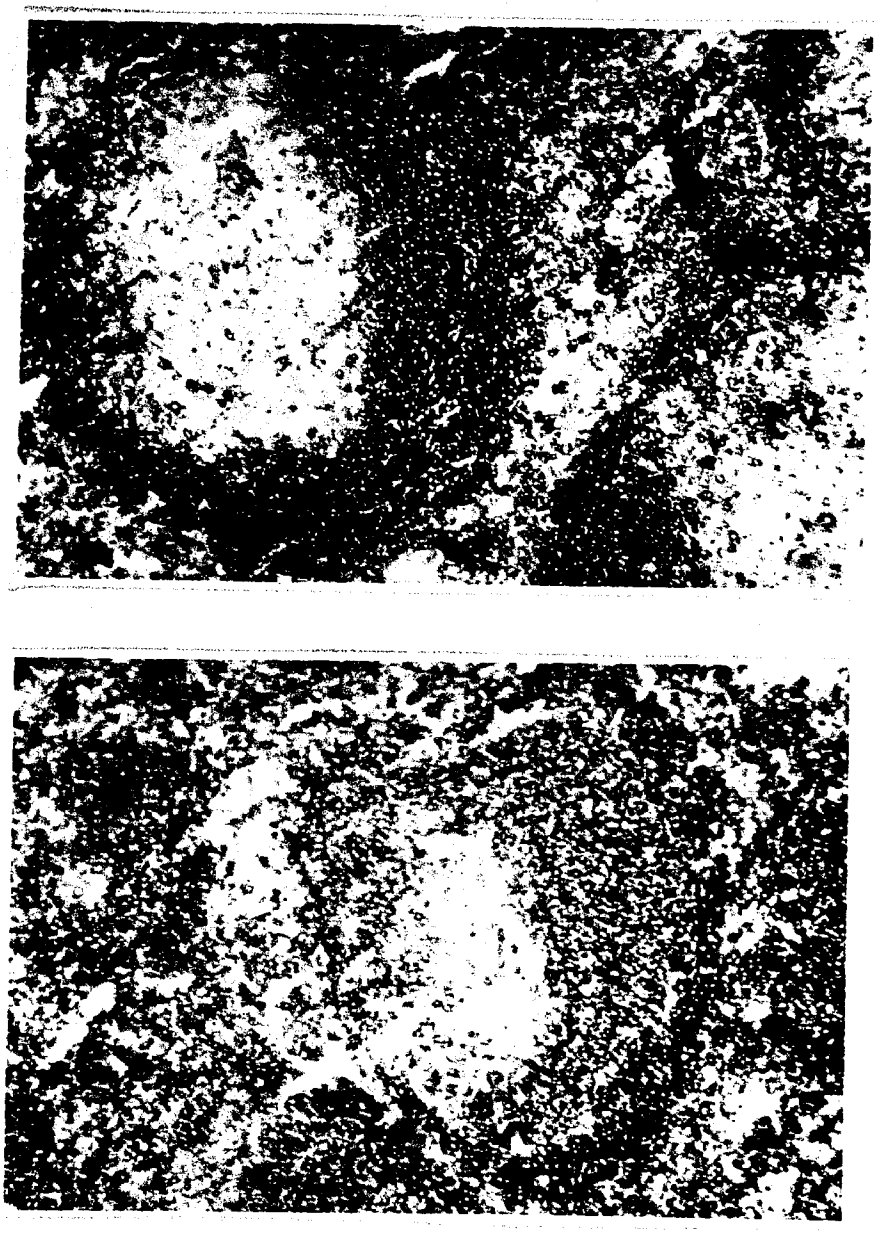

Figure 5. Histological structure of the spleen. Frozen sections of the spleens were stained with alkaline phosphatase-conjugated anti-IgM (purple) and peroxidase-conjugated anti-CD3 (yellow) antibodies. The B cell areas in the lymphoid follicles in transgenic mouse spleen (top) were smaller than that of littermate (bottom).

CD21, CD22, CD45 and CD72, but higher levels of CD23 and CD38, than B cells in normal mice. In addition, the percentage of B cells in each sub-population was different. These subtle but significant phenotypic differences were associated with a reduction in B cell numbers, as reflected by overall smaller B cell area in lymphoid follicle (Fig. 5), and a low level of transgene-encoded serum IgM antibody in the transgenic mice (discussed below).

B Cells Were Decreased In Number In Adult But Not Neonatal Transgenic Mice. The average spleen weight was 88 mg vs. 116 mg, respectively, for 2 to 3 m old transgenic versus littermate mice. The decreased spleen size was primarily due to a decrease in B cell number, as manifested by smaller lymphoid follicles (Fig. 5). By flow cytometric analysis, B cells in the spleen of transgenic mice accounted for 38% of total lymphoid cells, while in normal littermate mice, this number was 59%. The decreased B cell number in the spleen was associated with a decrease in B cell number in the total lymphocyte gate in the bone marrow from 34% in the littermates to 19% in transgenic mice. The reduction in B cell number in Ig heavy chain transgenic mice was attributable to a more rapid transition of B cells through the pro-B cell stages because of the presence of rearranged heavy chain leading to a reduction in a number of obligatory cell divisions necessary to move through subsequent stages of differentiation (23). However, when B lymphocytes in neonatal transgenic mice were examined, the reduction was not observed. As shown in Fig. 6, although pre-B cells were decreased, B cell numbers in either liver or spleen of 1-d-old transgenic mice were not decreased and averaged 27.7% and 27% in the liver, and 28.1% and 29.8% in the spleen, of transgenic versus littermate mice, respectively. Clearly the reduction of B cells in adult bone marrow can not be simply explained by the accelerated

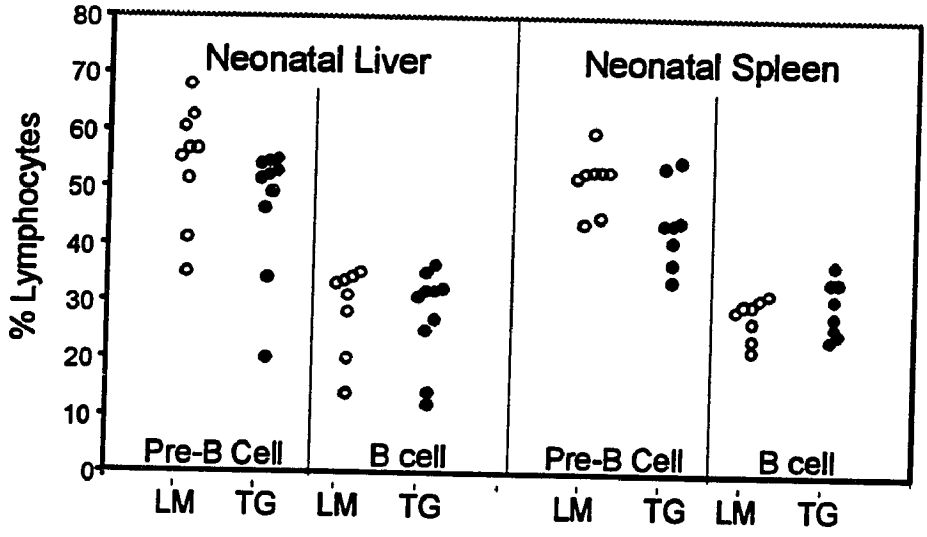

Figure 6. Percentage of Pre-B and B cells in the neonatal liver and spleen. "Pre-B" cells included all the B220+/sIgM- cells, while B cells were sIgM+ cells.

differentiation of the B cell lineage due to the presence of a rearranged transgene. The difference between newborn and adult transgenic mice in the number B cells generated in bone marrow and resident in peripheral lymphoid organs suggests the presence of micro-environmental influences that regulate B lymphopoiesis differently in neonatal versus adult life.

VH81X-Expressing B Cells Pioneered The Initial B Cell Repertoire In Bone Marrow-Reconstituted scid Mice. To understand the reason for the discrepancy that VH81X-expressing B cells are absent in normal but present in transgenic adult mice, we constructed bone marrow chimeric mice using bone marrow cells from either transgenic or littermate mice, or both sources transplanted together into adult severe combined immunodeficiency (*scid)* mice. Using IgD as a marker, the B cell development was followed by FACS analysis of B cell composition in blood, spleen and bone marrow at different time points after reconstitution. As shown above, while B cells from normal mice were IgD+, transgene-expressing B cells were IgD negative (IgD-). Although newly generated B cells can appear similarly in this respect to transgene-expressing B cells, they are usually IgDlo and IgMhi instead of IgD-, whereas, transgene-expressing/IgD- B cells can be either IgMlo (CD23+) or IgMhi (CD23-). These phenotypic features make transgene-expressing and normal B cells easily distinguishable. As shown in Figure 7, 3 w after transplantation about 45% of B cells in the blood of recipients of mixed bone marrow were IgD negative. Five weeks later, IgD- B cells in the spleen remained about 33% (with IgD- B cells in the bone marrow of the same mice being about 50%, data not shown). However, 3 m later IgD- B cells had almost disappeared from the spleen, with all the B cells there being IgD+, while about 35% B cells in the bone marrow of the same mice

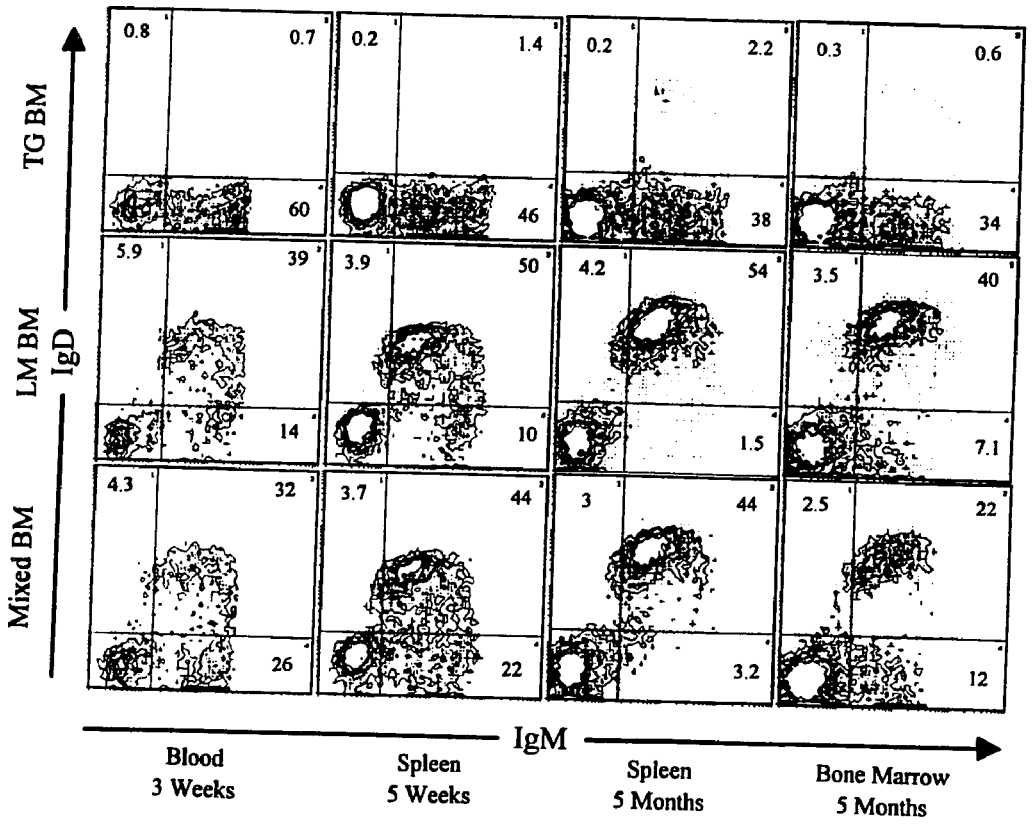

Figure 7. FACS analysis of B cell composition in bone marrow-reconstituted *scid* mice. Peripheral blood, spleen and bone marrow lymphoid cells were double stained with anti-IgM and IgD. Transgene-expressing B cells were completely IgD negative, whereas newly generated, normal mouse bone marrow-derived B cells were IgDlo and IgMhi, but not IgD negative. In bone marrow chimeric mice, IgD negative B cells were scarce five month after reconstitution, but account for ~45% PBL B cells three weeks after reconstitution. The ratio of transgene-expressing vs. normal B cells declined with time after reconstitution. However, this ratio was always higher in the bone marrow than in the spleen of the same recipients (data not shown).

were IgD-. Throughout the course of follow up, B cells in the recipient of transgenic mouse bone marrow alone were IgD-. These observations indicate that, as in the transgenic mice, VH81X-expressing B cells could survive by themselves in the recipient, but disappeared in the presence of simultaneously generated normal mouse bone marrow-derived B cells when the repertoire development was completed or space was "filled up." These results suggest that the presence of suppression of, and competition for survival with, other B cells is responsible for the absence of VH81X bearing B cells. Despite their disadvantaged survival during the repertoire development, VH81X-expressing B cells were still generated, and a proportion of them, like other B cells, migrated to the spleen to contribute to the peripheral B cell pool. We have not determined the heavy chain repertoire of the normal mouse bone marrow-derived B cells simultaneously generated shortly after reconstitution, but the dynamic changes of VH81X-transgene-expressing B cells in the reconstituted mice is reminiscent of normal repertoire development, where VH81X-expressing B cells dominated the early repertoire, suggesting their role in establishing a normal B cell repertoire.

B Cells In Transgenic Mice Are Predominantly Self-Reactive Toward Constitutively Expressed Intracellular Antigens But Do Not Secrete Antibody. To further understand the factors responsible for the kinetics of B cell numbers in adult transgenic and bone marrow chimeric mice, we analyzed the reactivity of B cells in transgenic mice in comparison to littermate mice. Hybridomas were made from spleen B cells of adult transgenic and littermate mice. The antibodies produced by B cell hybridomas from both sources were tested for self-reactivity by staining of a mouse myeloma cell line, Ag8.653, and various mouse tissue cells including bone marrow, spleen and thymus. Staining was examined by

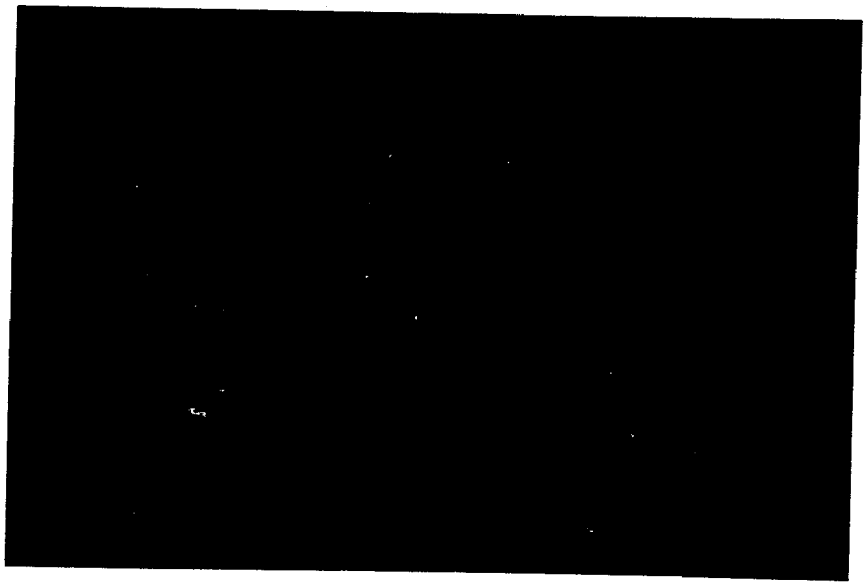

Figure 8. Immunocytological analysis with transgenic mouse B cell hybridoma-derived antibodies. Ag8.653-plasmacytoma cells were fixed with 70% ethanol in water and stained with transgenic mouse B cell hybridoma-derived antibodies. The staining pattern (top) is representative of other reactive antibodies composed of the transgenic heavy chain in association with different light chains. The negative control (bottom) is another transgenic mouse B cell hybridoma-derived antibody.

fluorescence microscopy or FACS analysis. As shown in Figure 8, the 60/68 hybridoma antibodies reacted with the cytoplasm of fixed Ag8.653-plasmacytoma cells. In contrast, only 3/12 of littermate B cell-derived hybridomas produced antibodies that were reactive and these stained less brightly. These self-reactive transgenic mouse B cell hybridoma-derived antibodies were composed of the transgenic heavy chain with a variety of different κ light chains that, as determined by DNA sequence analysis, belong to at least 11 different groups. When a particular antibody was reactive, it stained all cells derived from all tissues of all mouse strains tested. These results suggest that: (1) the VH81X transgene has an intrinsic propensity to encode self-reactive antibodies although expression of self-reactivity is not dictated by the transgenic heavy chain alone but can also be influenced by the light chain since not all transgene containing hybridomas produced self-reactive antibodies, and (2) the self-antigens with which the antibodies react are ubiquitous and present for the most part in many different tissues.

As demonstrated in previous transgenic studies, self-reactive B cells are either deleted or rendered anergic (24, 25, 26). Anergic B cells are short lived and do not secrete antibodies in the serum (27). The serum IgM was quantitatively analyzed by ELISA; among transgenic mice, serum IgM was lower than in the littermates (Fig. 9). At 8 to 12 w of age, the total serum IgM in transgenic mice was less than half of that of littermates, with only a small fraction of it derived from the transgene. As the mice age, transgene derived serum IgM, which is of IgH6a allotype, tended to increase slightly, but a proportion of this IgM was found to consist of IgM molecules containing of both transgenic and endogenous μ heavy chains (data not shown).

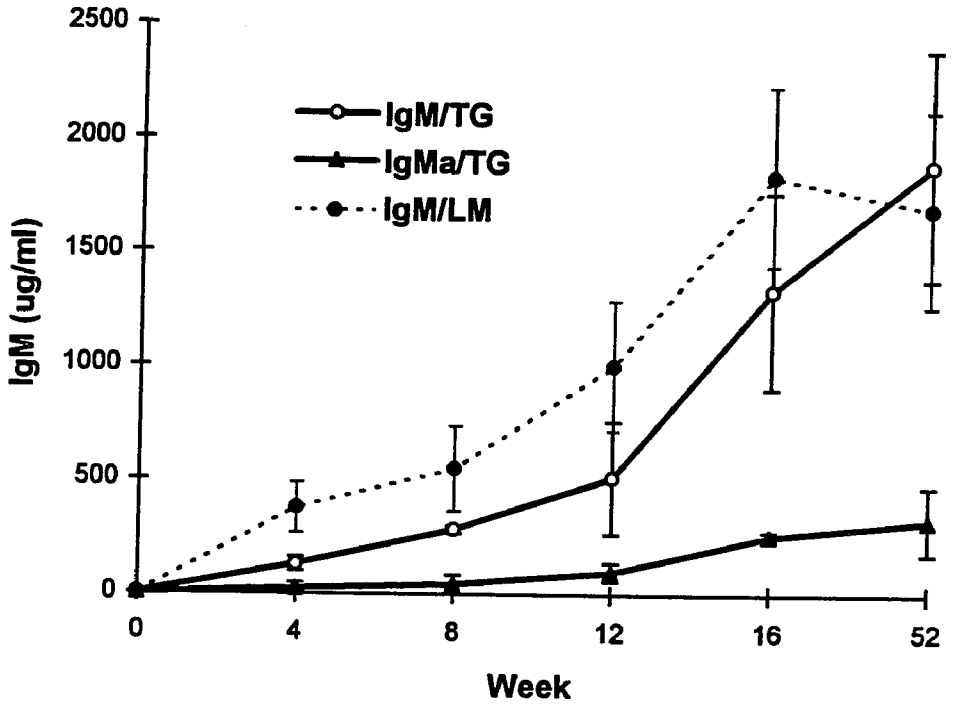

Figure 9. Quantitative ELISA of serum IgM. The sera tested were from C57BL/6 transgenic and littermate mice. Three to five mice were included in each age group. IgM = total serum IgM, IgMa = transgene derived serum IgM, which is not present in the littermates. The total serum IgM levels in BALB/c transgenic was similar. TG = transgenic mice, LM = littermates.

B Cell Repertoire Development Follows A Similar Dynamic Change Seen In A Normal Mice. By using B cell differentiation makers, changes in composition of the B cell population during ontogeny could be followed. As shown in Figure 10, in both neonatal transgenic and littermate mice, the majority of the splenic B cells were CD23-, and a proportion of them were CD5+. As mice became older, CD5+ B cells disappeared and more B cells were recruited into the CD23+ pool, but in transgenic mice this recruitment was delayed as reflected by the delayed appearance of $CD23^+$ B cells during ontogeny and the lower CD23+/CD23-B cell ratio in transgenic mice versus non-transgenic littermates at each age point. At 3 m of age, the transgenic mice have slightly more $CD23^-/IgM^{high}$ ($18\pm3.7\%$) than the littermates (13.4 ± 4.26), but the number of $CD23^+/IgM^{low}$ B cells in transgenic mice is about half that of littermate mice ($21\pm3.5\%$ in TG vs. $42.8\pm9.8\%$ in LM). This observation indicates that the reduced peripheral B cell number in transgenic mice is primarily due to a decrease in $CD23^+/IgM^{low}$ B cells. When additional B cell markers were utilized, more comparable B cell sub-populations could be identified in both adult mice, though the frequencies of B cells in each sub-population were different between transgenic and littermate mice.

By Southern blot analysis, dynamic changes in Vκ gene expression during ontogeny were detected. When Jκ gene segments are rearranged to different Vκ gene segments, DNA fragments of different lengths are generated after restriction enzyme digestion. Upon hybridization with a Jκ1-5 DNA probe, these rearrangements, when present in sufficient quantity, gave rise to a correspondingly heterogeneous pattern of restriction fragment lengths. The spleen DNA of transgenic and littermate mice was digested with Hind III and probed with Jκ1-5 DNA. As shown in Figure 11, although the

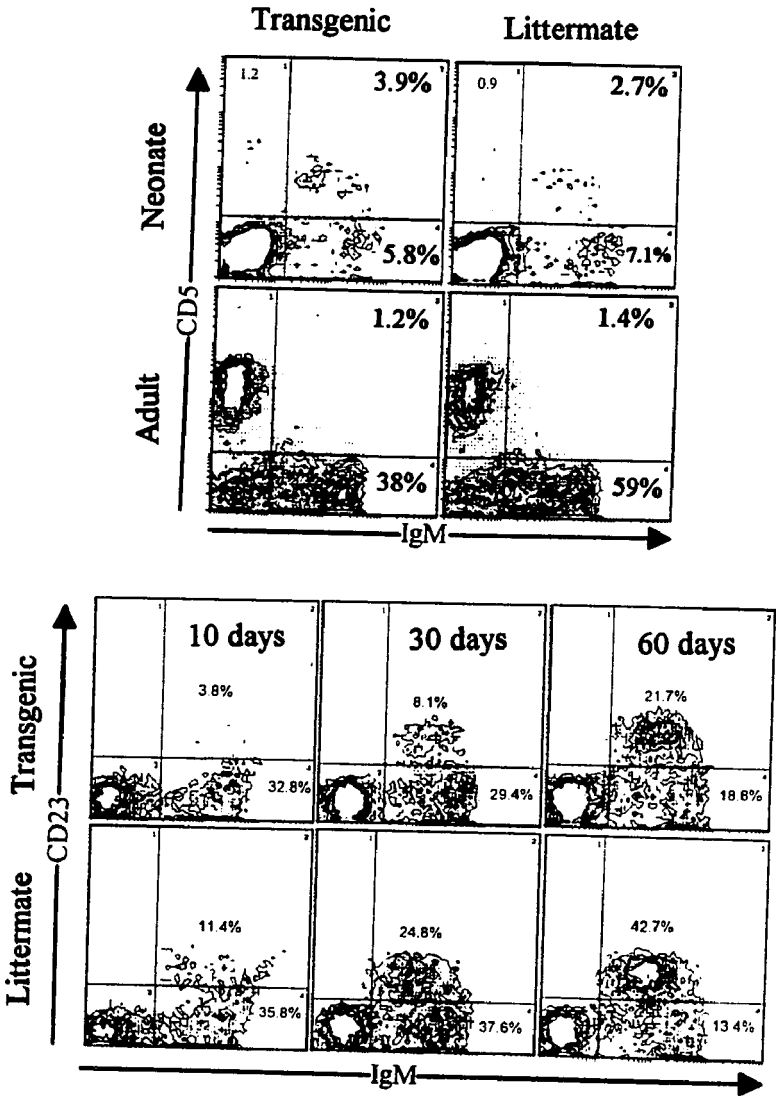

Figure 10. FACS analysis of dynamic changes in spleen B cell population during ontogeny. Spleen cells were stained with anti-IgM plus CD5 or CD23. CD5+ B cells were present in the neonatal but not adult spleen. The ratio of CD23+/CD23- B cells is consistently lower at each age point in the spleen of transgenic mice than that of littermate mice.

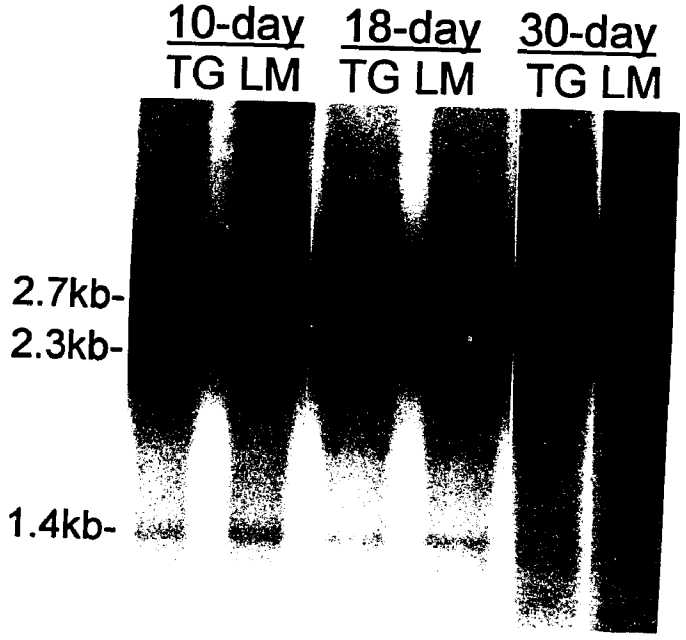

Figure 11. Southern blot analysis of Vκ gene rearrangement in spleen B cells at different ages. Hind III-digested spleen DNA was hybridized with Jκ1-5 probe. Transgenic and littermate B cells show some common dominant rearrangements as represented by 4.5-kb and 3.7-kb bands, which did not change with age. However, a 1.4-kb Jκ-containing band was detected in both transgenic and littermate mice at day ten and day eighteen, but disappeared at day 30. The 2.3-kb rearrangement is not detected in transgenic spleen at day 10 (in three experiments), but become detectable at day 18 when the 1.4-kb rearrangement is still present. The changes in dominant κ rearrangements reflects changes in κ light chain composition with age in both transgenic mice and the littermates. (TG = transgenic; LM = littermate.)

number and size as of visible bands were heterogeneous and similar between transgenic mice and the littermates, a dominant kappa rearrangement represented by a 1.4-kb band, which was present in the spleens of 10- to 18-d transgenic and littermate mice, disappeared when the mice became older. Instead, a 2.3-kb rearrangement appeared in transgenic mice. These observations indicate that although in transgenic mice, where the B cells expressed the same transgenic heavy chain, the B cell repertoire development tended to follow a similar dynamic order to normal mice. Such dynamic change was associated with changes in light chain expression and possibly in antibody specificity.

VH81X-Expressing B Cells Are Either CD5+ or CD5-. It has been demonstrated that B cells in the early repertoire are predominantly of CD5 (B1) phenotype (28, 29, 30) and that certain natural self-reactive B cell specificities are sequestered into the population (31, 21, 32).

In transgenic mice, CD5 B cells were in a state of flux. As shown in Figure 10, in neonatal transgenic mice, a proportion of spleen B cells, as in normal mice, were CD5+. As mice became older, CD5+ B cell numbers in the spleen declined. At day 20, no CD5+ B cells could be detected in the spleen. The spleen B cells could be clearly divided into two populations: IgMhi/CD23- and IgMlo/CD23+. IgMhi and CD23- are phenotypic markers of B2 cells, but the IgMhi/CD23- B cells in spleen did not appear to be B1 cells in nature or origin because their surface IgM was not as high as B cells in the peritoneal cavity of the same mouse, and in addition, they were Mac1 negative (data not shown). However, the majority of B cells in the peritoneal cavity of these same twenty day old mice were CD5 positive. As in the spleen, CD5+ B cell numbers in the peritoneal cavity of transgenic mice also decreased, as manifested by a decreasing ratio of CD5+/CD5- B cells

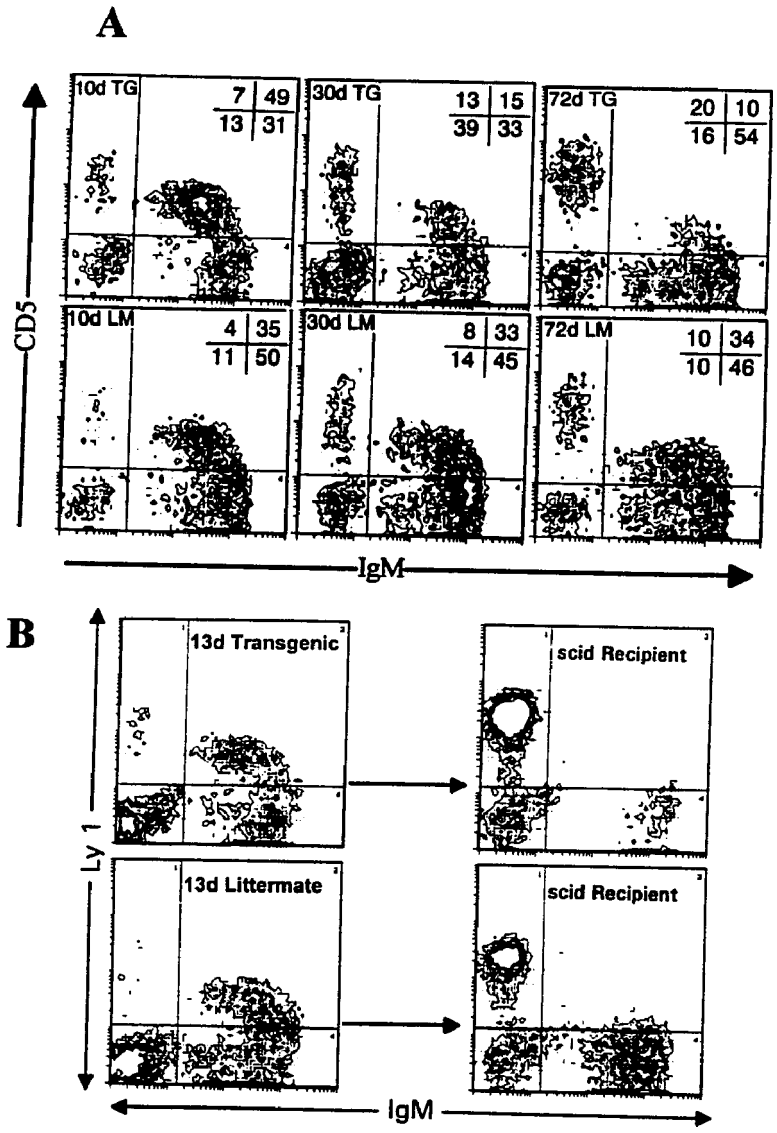

Figure 12. FACS analysis of peritoneal cavity B cells. (A) Peritoneal cavity cells were stained with anti-IgM and CD5. (B) Peritoneal cavity cells from 10 day-old mice were transplanted into the peritoneal cavity of scid mice. FACS analysis was conducted on donor cells and cells harvested from the recipient peritoneal cavity one month after transplantation.

with increasing age (Fig. 12A). In adult transgenic mice, the majority of peritoneal cavity B cells were CD5-, but these CD5- B cells had the IgMhi, Mac1+ and CD23- B1b phenotype (data not shown).

To determine if the diminished number of CD5+ B cells is due to loss of CD5 expression or disappearance of these cells, we transplanted peritoneal cavity cells from day 10 transgenic mice, where 80% of B cells were CD5+ (Fig. 12B), into the same anatomic site of the severe combined immunodeficiency (*scid*) mice. At 1 and 3 m post-transplantation, the cells were harvested and analyzed. As shown in Figure. 9C, B cells from littermate mice had expanded five-fold in the recipient scid in 1 m, and then remained constant up to 3 m. In contrast, B cells from transgenic mice almost completely disappeared, while T cells from the graft expanded. These results suggest that peritoneal cavity B cells from normal mice were indeed long lived and capable of self-renewal, while the equivalent B cells from transgenic mice were not, even though they were CD5+. Therefore, the diminishment of CD5 B cells in the peritoneal cavity of transgenic mice is likely due to death of these cells and replacement by CD5- B cells. The reason for the shortened life span is probably related to the expression of VH81X heavy chain.

Interestingly, although B cell from littermate mice and T cells from both sources expanded in peritoneal cavities of recipient *scid* mice and were present in large numbers, the equivalent cells were not found in the spleen of the same recipient mice.

These results indicate that the expression of VH81X gene neither dictates nor is exclusive of B1 phenotype, but rather the age and anatomical site, i.e., the spleen vs. peritoneal cavity, is more closely related to the B2 or B1 phenotypes, since B cells in both sites express the same transgenic μ heavy chain.

Discussion

Why Is VH81X Preferentially Expressed In The Early Repertoire? The normal function of the immune system depends on its capacity to respond to a enormous variety of exogenous antigens, and this is made possible by a large repertoire of germline Ig genes, which can be utilized in a variety of possible combinations. However, the early repertoire does not utilize all of the potential mechanisms for the generation of diversity, which is, on the surface, disadvantageous to the newborn and therefore must have some other significance. Several mechanisms may be involved in preferential utilization of VH81X. The D-proximity of this gene and its unique recognition signal and recombinogenic sequences may cause this gene segment to be a hotspot of VH gene rearrangement, a phenomenon that is maintained at a high frequency through life (33, 34, 35). Homology-directed recombination was proposed to account for predominantly productive rearrangements of VH81X in neonates but not in adults (36). On the other hand, it has also been shown that restricted Ig junctional diversity in neonatal B cells resulted from developmental selection rather than homology-directed V(D)J joining (37). All of the above proposed mechanisms, including lack of TdT activity early development, seem to function to ensure overexpression of the VH81X gene. But why is VH81X over-expressed in the early repertoire?

In the analysis of VH81X transgenic mice, we have shown that this gene has a propensity to encode self-reactive antibodies even when associated with a variety of κ light chains. In fact, the antibody produced by BALB/c fetal liver derived hybridoma BC2, from which the transgene is isolated, is also self-reactive. This self-reactivity is not defined by the CDR3 present in the VH81X-transgene-encoded antibodies. We have characterized

other VH81X-encoded antibodies, which differed in their CDR3's but still encoded self-reactive antibodies (data not shown). Therefore, the observed intrinsic property of VH81X to encoded self-reactive antibodies and the fact that this gene is preferentially expressed in the perinatal repertoire suggest that genetically programmed mechanisms are responsible for the active rearrangement of VH81X to ensure an early repertoire that is self-reactive.

The need for self-reactivity of an early repertoire is probably derived from the inherent property of developing B cells. That failure to express Ig on the cell surfaces causing a severe deficiency in B cell development (38, 39) indicates that developing B cells must receive positive signal(s), through their μ heavy chain together with surrogate light chain or through surface IgM molecules, necessary for development to proceed. The ligand or the antigen involved in delivering such signals is not known, but before birth, the only antigen presented to the immune system is self-antigen. Consequently, only those B cells that recognize self-antigen would be selected into the initial repertoire. Active rearrangement of VH81X obviously ensures availability of self-reactive B cells. The idea of positive selection for such self-reactive B cells is consistent with our findings that, although in adult, B cells were markedly reduced, the newborn transgenic mice have normal numbers of B cells but decreased pre-B cells, which may result from the expansion of B cells or more accelerated transition from pre-B to B cells. These self- and multi-reactive B cells probably helped to prime the development of a normal B cell repertoire (40). In theoretical modeling studies of idiotype networks, it has been proposed that establishment of an immune repertoire occurs through a idiotype network. In this process, emergent clones that interact with member clones in the networks are recruited into the network (41), thus the later mature B cell repertoire is built upon a more self-reactive

initial repertoire. However, it remains unclear what the first B cells that initiate the network are, and what selects the first B cells. Based on our observations, we postulate that VH81X-expressing B cells function to initiate the first network. The multireactivity and connectivity of germline VH81X gene-encoded antibodies (8, 10) also endow these B cells with this function. This idea is supported by our study of B cell repertoire development in adult *scid* mice reconstituted with bone marrow cells from both transgenic and normal mice. In these recipients, although VH81X B cells are self-reactive and thus disadvantaged for survival (42), and eventually disappeared, they did pioneer the initial repertoire, indicating that their generation is favored at the early stage of repertoire development.

That a more mature repertoire is built upon a more self-reactive immature one can also be perceived by following B cell repertoire development in transgenic mice. Although all B cells express the same heavy chain, B cell repertoire development follows a hierarchy, with initial B cells being CD5+ and CD23-. A more mature repertoire is established by recruiting cells into the CD23+ B cell pool. It is likely that the specificities of the early B cells are different from that of adult, because they express different light chains and are more self-reactive. Similarly, in the adult, the CD23- B cells may be more self-reactive than the CD23+ ones. Therefore, the sub-populations detected by using different B cell markers seen in both transgenic and normal mice may represent B cells not only at different differentiation stage but of different specificity, with immature B cells being generally more self-reactive and functionally located in a more inner layer of the network than more mature B cells.

Apparently, the sacrifice in this process of repertoire development is a limited capacity of the early repertoire to respond to foreign antigen, but a repertoire developed this way may be most effective and meanwhile not self-reactive.

Lack of B cells that express the transgene in association with λ light chains suggest that the VH81X encoded heavy chain may be unable to associate with λ light chains. This observation is similar to recent reports that VH81X containing μ heavy chains cannot associate with λ5, which is homologous to λ light chain (43). Successful generation of B cells in transgenic mice suggests that pre-B cells expressing VH81X heavy chains may not need to go through an obligatory developmental stage in which the surrogate light chain is associated with μ heavy chains. The exemption from such a requirement may also contribute to the dominance of VH81X-expressing cells in the early repertoire.

Why Is VH81X Not Expressed By Normal Adult B Cells? In adult transgenic mice, VH81X-expressing B cells are formed and, though decreased in number, populate lymphoid organs, indicating that these B cells can be generated and survive in the adult environment. This difference between adult transgenic and normal mice, where VH81X-expressing B cells were rarely found, suggests that the lack of these B cells in normal mice is due to influences from other B cells. This idea was tested by bone marrow transplantation experiment. Although VH81X-expressing B cells were generated normally in *scid* mice that had received only transgenic mouse-derived bone marrow, they disappeared in the *scid* reconstituted with mixed bone marrow cells, suggesting they are inhibited by other B cells. This again suggests that VH81X B cells function to initiate the network and recruit emergent B cells they react with into the network. According to the

theoretical model of idiotype network, these B cells are suppressed by large number of interactions the network expands (41).

The dynamic changes in B cell number with age in transgenic mice recapitulated the pattern of expression of the VH81X in the normal situation. This suggests that in adult transgenic mice there is a similar idiotypic network suppression that caused reduction in B cell number. Such suppression may result from increasing amounts of endogenously-derived immunoglobulins and development of a more mature B cell repertoire signified by CD23+ B cells. On the other hand, the recruitment of B cells into the CD23+ pool was delayed in transgenic mice, probably because the specificity they can display is limited. This delay can aggravate B cells' reduction, since CD23+ B cells constitute the bulk B cell pool in adults.

Additionally, although the B cells in the transgenic mice express almost exclusively the transgene, very little transgene-derived antibodies were detected in the serum. The lack of serum antibodies could be due to the consumption of transgenic IgM by complexing with self-antigen *in vivo*. However, it is more likely that the transgene B cells were inactivated and are anergic because of their self-reactivity, as has been demonstrated in other transgenic studies (24, 44). Absence of RS3.1 positive plasma cells in the spleen of C57BL/6 mice is also indicative of a block in B cell maturation prior to reaching antibody-secreting cell stage. Anergic B cells are short-lived (45, 26).

These observations together with results of previous studies suggest that lack of VH81X-expressing B cells in adult mice is due to (1) idiotypic network suppression, (2) delayed recruitment of B cells into the long-lived pool, and (3) a competition for survival (Cyster et al., 1993) between B cell that express VH81X and those that have the potential

to rearrange other VH genes. B cells expressing VH81X are self-reactive, anergic and short-lived, and therefore do not live as long as B cells expressing other VH genes. This collection of negative selection mechanisms functioning with other developmentally controlled functions, such as increased TdT activity, results in a reduction in VH81X-expressing B cells in adult mice.

The CD5 Phenotype Is Not Dictated By VH Gene Usage. The relationship between the CD5 phenotype and VH gene utilization is not yet completely clear. It has been shown that CD5 B cells express some VH genes at a relatively high frequency (46, 47). Transgenic mice expressing the VH12 gene were found to generate a high proportion of CD5 B cells (48). In this study, we found the VH gene expression and the acquisition of CD5 phenotype of B cells is neither exclusive nor inclusive. B cells expressing VH81X could be either CD5+ or CD5-. In transgenic mice, a normal proportion of B cells in the neonatal spleen were CD5+, but disappeared in adult spleen. Similar but delayed dynamic changes of CD5+ B cells occurred in the peritoneal cavity where CD5+ B cells were found primarily in young mice, indicating that expression of CD5 is related more to the age and anatomical location of a B cell population than to the expression of a particular VH gene. Interestingly, even at the same anatomical site, the number of CD5 B cells declined with increasing age. The reason for this dynamic change in peritoneal cavity B cells composition is not understood. However, it is possible that the CD5+ B cells present in young transgenic mice expressed light chains different from that of CD5- B cells present in older mice. Those CD5+ B cells may be more self-reactive than CD5- B cells and had less survival advantage than the latter. By Southern blot analysis we have found that light chain

composition in spleen B cells of young transgenic mice was different from that of old mice.

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SELECTION OF NATURAL SELF-REACTIVE B CELLS

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Summary

Natural antibody self-reactivity is a hallmark of a normal immune system. The mechanisms that generate and maintain self-reactive B cells are not well understood. We have studied the complexity of natural self-reactivity and the mechanisms that shape the B cell repertoire by comparing B cell development in transgenic mice that express the most D-proximal VH gene, VH81X, as a μ-heavy chain, with normal littermate mice. In VH81X transgenic mice, B cells express almost exclusively the transgenic heavy chain in association with a variety of κ light chains, but with Vκ1A5 as a dominant light chain. B cells that express the Vκ1A5 light chain gene are generated from clonally independent precursors, and, surprisingly, all the rearrangements of Vκ1A5 involved Jκ5, with identical Vκ-Jκ joins, resulting in the generation of the same antibody specificity. These B cells were more highly self-reactive than B cells expressing VH81X with other light chains and recognized a ubiquitous epitope(s) on proteins from different tissues. In the spleen, their maturation ceases at the CD23-IgMhi stage. No detectable serum IgM was secreted by these B cells even though other transgene-encoded IgM was detected in the serum. Instead, the natural serum IgM in transgenic mice has similar self-reactive patterns to the serum IgM of the normal littermates. In neonatal transgenic mice, the B cells did not express Vκ1A5-Jκ5 as a dominant light chain, but another transient light chain dominated in both transgenic and the neonatal littermates. These observations suggest that in transgenic mice, as well as in normal mice, there is a continuous generation of self-reactive B cells through positive selection by self-antigen. These B cells migrate to the periphery where they are kept functionally silent and are probably short-lived.

Introduction

Multiple regulatory mechanisms have been proposed to prevent pathological immunity by counteracting the functional activation of self-reactive B cells. Initially, it was hypothesized that self-reactive lymphocytes were physically eliminated from the mature repertoire by a process termed clonal deletion or clonal abortion (1, 2, 3). Later studies suggest that cells could also be diverted into a disarmed state termed clonal anergy (3), or could be constantly kept in check by suppressor T lymphocytes (4) or by immunoregulatory networks (5). More recently it was shown in transgenic mice that self-reactive B cells resulting from an initial series of heavy and light chain rearrangements may be diverted from self-reactivity by receptor editing, involving a further round of receptor rearrangements (6, 7). Paradoxically, despite the vigorous censoring mechanisms demonstrated experimentally *in vitro* and *in vivo* in transgenic mouse models, it is clear that the early B cell repertoire is dominantly self-reactive (8-11). In addition, there is a high frequency of self-reactive B lymphocytes in normal adult mice or humans (12-19) and there is a large body of data demonstrating the spontaneous presence of self-reactive antibodies in normal sera, referred to as natural antibodies (20-22). One of the fundamental assumptions is that natural antibodies result from stimulation of B cells by exogenous antigens. However, it has been shown that hybridomas from fetal and neonatal tissues (8, 9, 11) or from germ-free and antigen-free mice produce these type of antibodies at similar frequencies (13, 23). Taken together, these observations indicate that self-reactivity occurs not only in the potential and available V gene repertoire, but also in the actual B cell receptor and secreted antibody repertoire.

In an attempt to explain elements of this paradox, it has been suggested that self-reactive B cells exist in a state of clonal ignorance, because of the failure of B cells with low receptor affinity for self-antigen or with specificity for self-antigens present at low concentrations to be deleted or anergized (24, 25). However, the presence of detectable natural serum autoantibodies with their characteristic specificities do not support such a concept. The binding activities of natural self-reactive antibodies are frequently directed towards intracellular structures (13, 17, 26) and epitopes on homologous regions of highly conserved molecules in phylogenetically distinct cells (27, 28, 29, 30). It has also been shown recently that the natural serum IgM antibodies of mice preserve a strain-characteristic fingerprint of self-reactivities, some of which are common to different strains (22). These observations have been taken to suggest that germ-line V genes encoding such antibody specificities and the mechanisms involved in generating fixed patterns of self-reactivity are conserved in evolution, and that auto-reactive B cells and natural auto-antibodies play a role in normal immune functions.

Because of existing evidence that self-reactive B cells are not purged from the immune system during ontogeny, we have asked two questions: first, how are these self-reactive B cells generated and maintained under normal conditions; and secondly, what physiological role do they play in the development of the immune system? To understand these issues, we have produced transgenic mice with a rearranged VH81X-μ heavy chain gene isolated from a BALB/c fetal liver-derived hybridoma. This hybridoma produced a multireactive IgM antibody representative of those found at high frequencies in fetal and neonatal life. The rearranged VH gene, VH81X, is the most D-proximal functional VH gene in BALB/c mice and is expressed at a high frequency in mouse fetal but not adult B

cell repertoires (31-33). These features make this transgenic mouse distinct from other available transgenic mice designed to study immune tolerance and autoimmunity, which generally express mutated transgenes encoding high affinity antibody for particular self-antigens or transgene-encoded antigens (34-37). By following the development of B cells expressing this germ-line VDJ-μ heavy chain gene isolated from a spontaneously derived autoreactive fetal B cell, we have obtained insight into the complexity of spontaneous autoimmunity and the shaping of the B cell repertoire.

Materials and Methods

Generation of Transgenic Mice. To make the transgene construct, rearranged VH81X genomic DNA was isolated from a 15-day fetal liver-derived hybridoma BC2 and joined to its original 5' non-translated DNA (the 3.5kb DNA fragment was a gift from Dr. F. Alt), at the EcoR1 site. This promoter-V-D-J DNA was recloned into a eukaryotic expression vector pSVG-gpt and JH3-4 and Cμ sequences (gift from Dr. C. Goodnow) were downstream of the VDJ. Transgenic mice were generated by microinjecting the gene construct into C57BL/6 x DBA F2 fertilized eggs. The founder mice were backcrossed to BALB/c and C57BL/6 for six generations. Transgenic mice were identified by PCR and Southern blot analysis of mouse tail DNA.

ELISA. E.I.A A/2 plates (Costar) were coated with unlabeled goat anti-mouse antibody (anti-IgM, or other isotype) or RS3.1 or MB8.6 at 2μg/ml in borate buffer (pH 9), incubated at 4°C overnight, followed by the test antibody (serum at 1 : 1000 dilution in 1% PBS-BSA, or supernatant at 1 : 100 dilution) and incubated at 37°C for 2 hours. Alkaline phosphatase-conjugated goat-mouse IgM antibody was used as the third layer

antibody, and incubated for 2 hours at 37°C. Between each step, the plate was washed five times with PBS. The plate was developed with alkaline phosphatase substrate 1mg/ml in substrate buffer (pH 9). For quantitative ELISA, mouse antibody of known concentration was included in each plate, and the plate was read by a spectrophotometer at 405 nm and antibody concentration was determined using an ELISALITE program.

Southern blot analysis. To isolate genomic DNA, mouse tissues (including tail, spleen, sorted B cells, LPS cultured cells and hybridomas) were digested with digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS, 10μg/ml RNase A and 200μg/ml proteinase K) at 55°C overnight, followed by phenol and chloroform extraction and ethanol precipitation. Southern blots were performed conducted according to standard procedures (38). Briefly, 10μg of DNA were digested at 37°C overnight to completion with restriction enzymes (either HindIII or BamH1, Gibco BRL), electrophoresed through 0.8% agarose and transferred to a nylon membrane (BRL). Membrane was cross-linked by UV Cross Linker. Hybridization with ^{32}P-labeled VH81X or Jκ1-5 DNA probes was carried out at 65°C. All final washes were conducted under high stringency conditions.

Flow Cytometry Analysis. The antibodies used for flow cytometry are: fluorescein-conjugated goat anti-mouse μ, κ and PE-conjugated secondary reagents (Southern Biotech, Birmingham, AL); biotin-conjugated rat anti-mouse IgD (11-26c; a gift from D. Bole); B220 (39); CD23 (B3B4; a gift from M. Kehry and D. Conrad); IgH6a (40); IgH6b (41) and I-A^d class II MHC (42). Two color surface staining was performed as previously described (43). Briefly 5 x 10^5 cells were first incubated with goat anti-mouse IgM, then with either anti-B220, IgD, CD23 or class II MHC, followed by SA-PE (Birmingham

Southern Biotech), 20' for each incubation. The cells were washed with 1% PBS-BSA twice between each step. For cytoplasmic staining, BALB/c or C57BL/6 cell suspensions were fixed with 70% ice-cold ethanol for 30' and then washed twice with PBS-BSA before antibody staining. Transgenic B cell hybridoma-derived antibodies were used at 10 μg/ml. In direct staining, these monoclonal antibodies were biotin-conjugated and SA-PE was used. In indirect staining, the unlabeled antibodies were used followed by FITC-conjugated goat anti-mouse IgM or RS3.1-FITC. Three IgM antibodies were used as negative controls for FACS analysis, and they were equally non-self reactive. These antibodies were: antibody #15, 26.30 and 81.13.46. #15 is a transgenic B cell hybridoma-derived antibody containing the VH81X heavy chain and light chain, Vκ4/5; 26.30 is a fetal liver hybridoma-derived μ/λ antibody; and 81.13.46 is an omentum hybridoma-derived μ/κ antibody. They were used at 10- or 20- μg/ml IgM in 1% PBS-BSA. The mouse sera used for staining were first quantitated for IgM concentration by quantitative ELISA, then diluted to 20 μg IgM/ml with 1% PBS-BSA. Cells were stained with diluted sera followed by RS3.1-FITC or goat anti-mouse IgM-FITC. Stained cell samples were analyzed using the FACScan Consort 30 program (Becton-Dickinson).

Western blot analysis. Tissue cell suspensions were made from mouse bone marrow, thymus, spleen and heart. Peripheral blood nucleated cells were prepared by centrifugation of heparinized and PBS-diluted BALB/c or C57BL/6 blood on Lympholyte separation medium. The cells were washed twice with 1% PBS and lysed using SDS lysis buffer (50% glycerol, 2% SDS, 0.5M Tris-HCl, pH 6.8) and 3 ml lysis buffer per 10^8 cells. The lysate was boiled for 15 minutes and kept at -70°C for use. Protein concentration in lysate

was quantitated using a Pierce protein assay system. 100 microgram of protein were loaded onto each lane of 7.5%, 140 x 140 x 1.5 mm SDS polyacrylamide gel and eletrophoresed (44). After separation through SDS PAGE, cellular proteins were transferred onto a nitrocellulose membrane. The filter was first blocked with 3% BSA in TBS for 2 hours and then incubated at 4°C overnight with primary antibodies or mouse sera diluted at 10 μg/ml in 3% BSA-TBS. The second antibody was either goat anti-mouse IgM-AP, or RS3.1-biotin followed by SA-AP. Between each step, the membrane was washed with TBS three times, 10' each. The membrane was developed with AP-substrate. The negative control antibodies were the same as described above in flow cytometry analysis.

DNA Sequencing Analysis. Kappa light chain sequencing was carried out from either cDNA or genomic DNA. To make cDNA, total RNA was isolated from hybridomas using guanidinium thiocyanate-phenol-chloroform extraction as previously described (45). cDNA was synthesized using a Cκ primer: 5'-GAAGCTTATACACAGTTGGTGCAGC-ATCAGCC-3', followed by PCR using the same Cκ primer as 3' primer and a degenerate Vκ primer as 5' primer: 5'-GCCATGGAPRTQLWLMTSACCCAGTCTCCA-3'. When genomic DNA was used for sequence analysis, the Jκ5 primer was utilized as a 3' primer. The PCR amplified Vκ DNA was cloned into pUC119 and subjected to sequencing using STRATAGENE Sequenase kit. The DNA sequences were analyzed using DNAstar program to determine the origin of Vκ, Jκ, N-region addition and somatic mutation.

Results

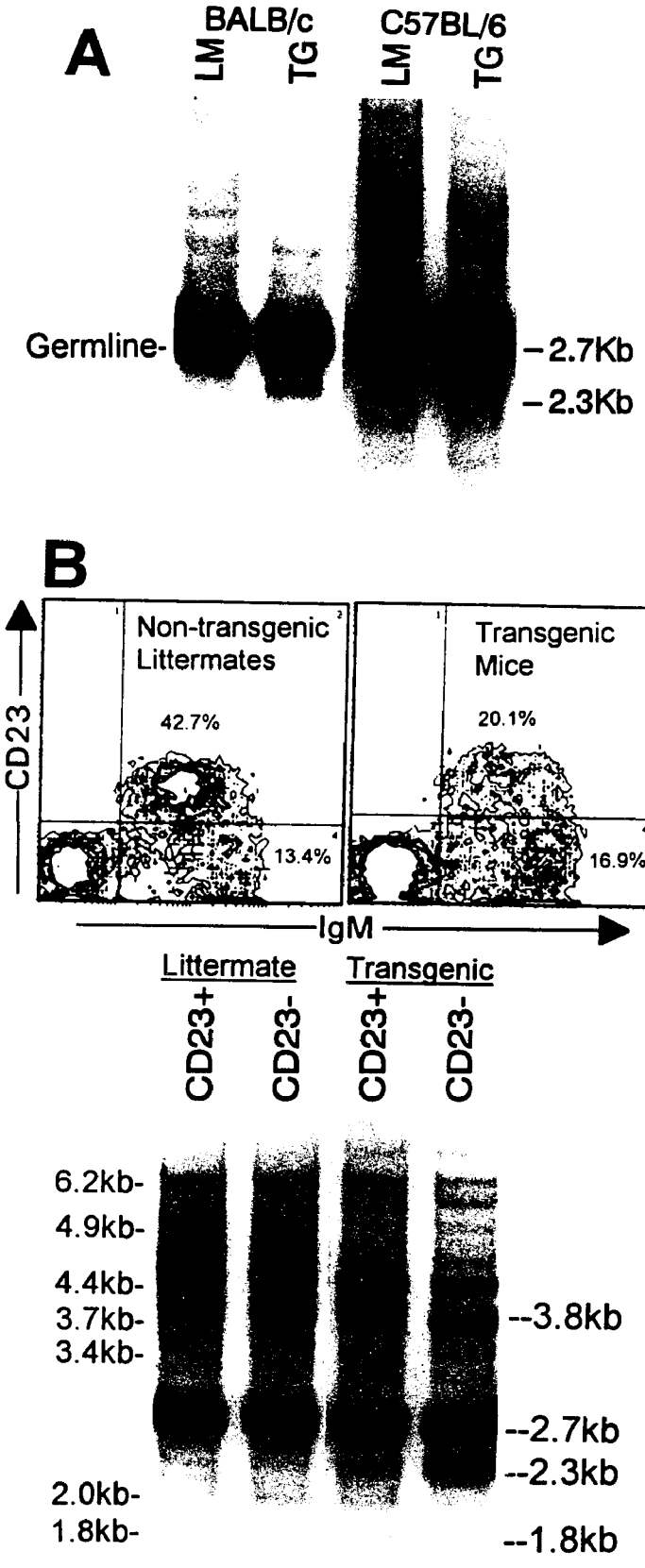
B Cells Express Almost Exclusively VH81X Transgenic Heavy Chains With Heterogeneous but Biased κ Light Chains. Transgenic founder mice were generated by

micro-injecting the gene construct into fertilized eggs of DBA/J x C57BL/6 F2 mouse, and the founder mice were back crossed into BALB/c and C57BL/6 for six generations for the following study. By using anti-allotypic and anti-κ or λ antibodies in FACS analysis, B cells in transgenic mice were found to express almost exclusively transgenic μ heavy associated with κ light chains.

The κ light chain heterogeneity was studied by DNA sequencing analysis of light chains in transgenic mouse-derived hybridomas. Vκ genes from at least 11 different families were identified. This κ light chain heterogeneity could also be detected by Southern blot analysis. As shown in Figures 1A and 1C, when spleen DNA of transgenic and littermate mice was digested with Hind III and probed with Jκ1-5 probe, the number and size of visible bands that hybridized to the probe were heterogeneous in both transgenic and littermate mice and, in addition, were remarkably similar between transgenic mice and the non-transgenic littermates, indicating that the VH81X transgenic heavy chain can associate with a variety of κ light chains.

Despite the general similarity in overall patterns of κ rearrangements detected in the transgenic and littermate splenic B cells, there are also some notable differences in the representation of Vκ genes. One of the striking differences is that transgenic B cells present a dominant 2.3-kb band about one third of the intensity of the 2.7-kb germline Jκ band. This 2.3-kb band is not present in B cells from the littermates. (Fig. 1A and C). Taken together, the Southern blot analysis of total spleen DNA, supplemented with our previous sequence studies, suggests that: although Vκ gene utilization in transgenic mice is heterogeneous, there is a predominant Vκ rearrangement represented by the 2.3-kb Jκ-containing band that is present in the spleen of transgenic but absent in the littermate mice.

Figure 1. Southern blot analysis of V-κ gene rearrangements in transgenic mouse and littermate spleen. (A) Total spleen DNA from transgenic and littermate F5 mice of either BALB/c or C57BL/6 background was digested with Hind III, hybridized with a Jκ1-5 probe and washed under high stringency. The 2.7-kb band represents the unrearranged germline Jκ gene that is present in some B cells which have rearranged only one of their κ alleles, and in all other cells that do not rearrange Ig light chain genes, and thus give rise to an intensive germline band. A 2.3-kb Jκ-containing band was detected in transgenic mice of either BALB/c or C57BL/6 background, but not in the littermates. (B) Spleen cells were stained with goat anti-mouse IgM and anti-CD23 antibodies. By flow cytometry, B lymphocytes can be separated in two populations: $CD23^{+}/IgM^{low}$ versus $CD23^{-}/IgM^{high}$. Both populations of B lymphocytes were present in the spleen of transgenic mice, but there were fewer $CD23^{+}/IgM^{low}$ B cells than in the littermates. DNA was isolated from sorted $CD23^{+}/IgM^{low}$ or $CD23^{-}/IgM^{high}$ spleen B cells and analyzed as in (A). Both transgenic and littermate B cells showed an array of similar dominant κ light chain gene rearrangements represented by 6.2-kb, 4.9-kb, 4.4-kb, 3.7-kb, 3.4-kb, 2.0-kb and 1.8-kb visible bands. The 2.3-kb rearrangement is predominantly present only in the $CD23^{-}/IgM^{high}$ B cells of transgenic mouse. Some other rearrangements, such as 1.8-kb and 3.8-kb bands, which are present in $CD23^{+}/IgM^{low}$ B cells, are missing in $CD23^{-}/IgM^{high}$ population. The CD23+ and CD23- B cells from the littermate contain essentially identical dominant rearrangements. The differential presence of the visible bands in different B cell population suggests Vκ-Jκ rearrangements represented by these bands are productive since non-productive rearrangements should be non-differentially distributed because they are not under selection. The multiple bands detected here did not result from non-specific hybridization of the Jk1-5 probe because such rearrangements are not seen in non-B cells (see Figure 6). (The result is consistent in six repeated experiment ; TG = transgenic; LM = littermate.)


A
BALB/c
C57BL/6
LM
TG
LM
TG
Germline-
-2.7Kb
-2.3Kb
B
Non-transgenic
Littermates
Transgenic
Mice
42.7%
20.1%
13.4%
16.9%
CD23
IgM
Littermate
Transgenic
CD23+
CD23-
CD23+
CD23-
6.2kb-
4.9kb-
4.4kb-
3.7kb-
3.4kb-
2.0kb-
1.8kb-
--3.8kb
--2.7kb
--2.3kb
--1.8kb

Clonally Independent B Cells Contain the Same κ Rearrangement. To determine if the κ rearrangement represented by the 2.3-kb fragment was the result of expansion of B cells containing a particular rearranged Vκ gene segment, we studied Vκ gene rearrangements in individual hybridomas isolated from transgenic and non-transgenic littermate mice. Southern blot analysis of DNA, from randomly chosen splenic B cell hybridomas generated in independent fusions of spleens from two BALB/c and two C57BL/6 transgenic mice, showed that 27/68 contained the 2.3kb fragment (Fig. 2), while 0/18 hybridomas made from non-transgenic littermates contained this rearrangement. The results of hybridoma analysis concurred with the dominance of the 2.3-kb Vκ band detected in Southern blot analysis of total transgenic spleen DNA.

Nine of the 27 hybridoma clones carrying the dominant 2.3-kb fragment rearranged only one κ allele, with the other allele in germ-line configuration. Eighteen out of 27 clones had rearranged both Vκ alleles, generating one 2.3-kb fragment and a second Jκ-containing fragment that differed in size among individual hybridomas. The single allele-rearranged hybridomas could not be used to determine clonality since they may have been derived either from independent B cell precursors that rearranged only one κ allele, or from clonal expansion of the same precursors. However, hybridomas that had rearranged both alleles can be analyzed in this way to determine their clonal origin: if both κ alleles are rearranged identically, the hybridomas are likely to be derived from the same precursors, if one of the alleles is rearranged identically to the other members of the panel but the other allele rearranged differently, then it is likely to be derived from different precursors. From this analysis, it clear that the remaining 18 hybridomas, with both κ alleles rearranged, are of independent origin. There have been reports that B cells may

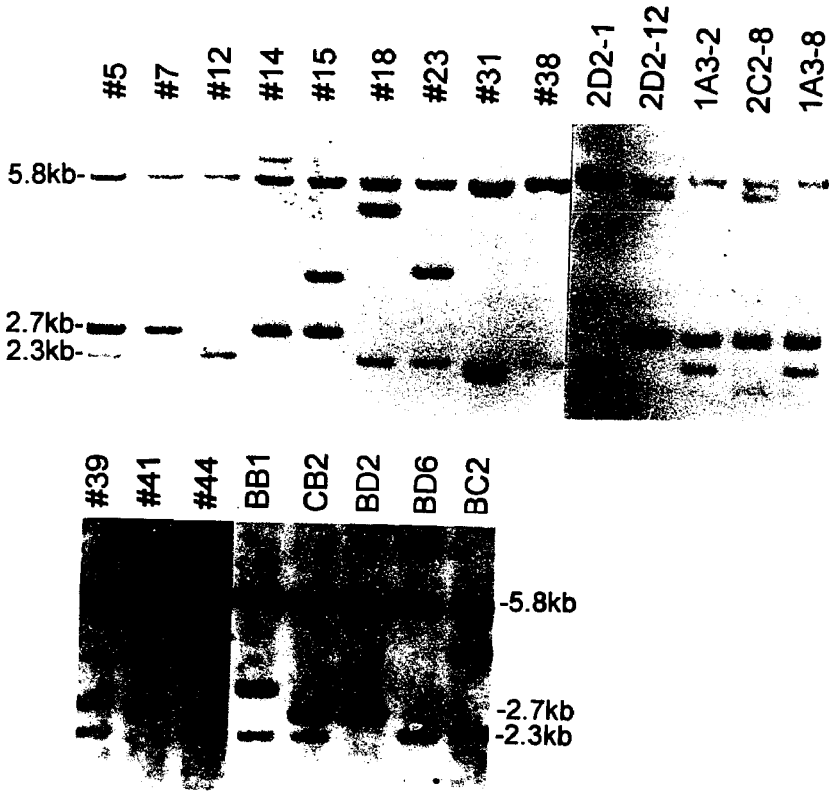


Figure 2. Southern blot analysis of Vκ gene rearrangement in transgenic B cell-derived hybridomas. Transgenic B cell hybridoma-derived DNA was digested with Hind III and hybridized with a Jκ1-5 DNA probe. The 5.8-kb band present in all lanes is a non-functional Vκ rearrangement derived from the plasmacytoma fusion partner Ag8.653. Hybridomas #39, CB2, #5, #7, 1A3-2 and 1A3-8 have only one of the κ alleles rearranged, producing a 2.3-kb rearrangement with the other Jκ allele remaining in germline configuration and represented as a 2.7-kb band. The intensity of all 2.3-kb bands are only half that of the 2.7-kb bands. Hybridoma #44, BB1, BD6, BC2, #18, #23, and #31 have both κ alleles rearranged, producing a common 2.3-kb band and other bands that all differ in size from each other. Hybridoma #12 and 2D2-1 exhibit only one 2.3-kb rearrangement and must have lost the other κ allele.

undergo further κ light chain gene rearrangement in the presence of a functionally rearranged κ light chain (46), but such events are too infrequent (47) to account for the strikingly high frequency of B cells that exhibit the 2.3-kb Vκ rearrangement bands and, at the same time, contain a unique κ rearrangement at the other allele. These results show that: (1) κ light chain gene rearrangements in transgenic B cells are similar to those in B cells in normal mice with respect to the number of alleles rearranged, with approximately 30% of B cells rearranging only one and 60% rearranging both alleles; (2) B cells containing the frequent 2.3-kb κ rearrangement arise independently in different transgenic mice, of different background strains, from clonally independent precursors, and (3) after their generation, these B cells do not appear to proliferate extensively *in vivo* since we did not detect, within this panel, sibling B cell hybridomas that had rearranged both κ alleles identically.

Independently Generated 2.3-Kb κ Gene Rearrangements Have Identical CDR3 Regions. We next analyzed the Vκ gene segments involved in the 2.3kb Jκ rearrangements by DNA sequencing of mRNA or genomic DNA derived from eight independently cloned hybridomas from three individual mice. The nucleotide sequences of the eight randomly chosen rearrangements were found to be identical. They consisted of a germ-line Vκ1A5 gene rearranged to a germ-line Jκ5 gene without nucleotide addition or deletion at the Vκ-Jκ joins (Fig. 3). Therefore the 2.3-kb fragments in these B cells and hybridomas encode identical κ light chains. The Vκ1A5-Jκ5-expressing B cells, which give rise to these hybridomas, all express the transgenic κ heavy chain, and therefore must have identical antibody specificity.

	FR3				CDR3									FR4				
#12	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
#23	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
BB1	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
CB2	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
BC2	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
BD6	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
3BB4	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
1BB6	GTT	TAT	TAC	TGC	TTT	CAA	GGT	TCA	CAT	GTT	CCG	CTC	ACG	TTC	GGT	GCT		
					Vk1A5							Jk5						

Figure 3. Sequence analysis of 2.3kb κ rearrangements from independently derived hybridomas. The eight hybridomas are from four different transgenic mice, two from each. The rearranged Vκ genes were cloned by PCR of either DNA or cDNA. The entire variable regions were sequenced, and all eight hybridomas were shown to have germline Vκ1A5 rearranged to germline Jκ5 with identical joins.

In addition, we sequenced κ light chains from another 21 hybridomas that did not contain the 2.3-kb rearrangement, and of these, 20 complete sequences were found to represent functional rearrangements. Vκ gene segments of these 21 light chains belong to 11 different Vκ groups joined to all possible Jκ segments. However Vκ1A5 was not detected in this group. These results show that: (1) the high frequency of Vκ1A5-Jκ rearrangements is not due simply to preferential Vκ rearrangements involving this particular Vκ gene segment, since the Vκ1A5 gene segment was not found rearranged to other Jκ segments in this panel; and (2) it appears obligatory for Vκ1A5 to be rearranged to Jκ5 to result in the generation of antibodies with a defined specificity.

The dominant Vκ1A5-Jκ5 Rearrangement is Confined to the CD23- B Cell Population.

B cell maturation is normally associated with the acquisition of CD23 (48, 49). In addition, mature $CD23^+$ B cells also express lower levels of surface IgM than immature CD23-B cells. In VH81X transgenic mice, though B cells expressed the same heavy chain, the repertoire development was similar to that of normal mice, with the majority of B cells in neonatal mice being CD23-. As mice became older, more cells were recruited into the CD23+ B cell pool. However, this recruitment in transgenic was delayed as reflected by the delayed developmental appearance of $CD23^+$ B cells and the lower CD23+/CD23-B cell ratio in transgenic mice versus non-transgenic littermates at each point during ontogeny. When these $CD23^+/IgM^{low}$ and $CD23^-/IgM^{high}$ populations of B cells were sorted and characterized for kappa light chain expression by Southern blot analysis, Vκ1A5-Jκ5 light chain expression was found to be principally confined to the $CD23^-/IgM^{high}$ B cell population in transgenic mice (Fig. 1C). Although a very faint 2.3-kb band

could be detected in the CD23+/IgM^{low} population, the predominance of Vκ1A5-Jκ5 light chain expression in the CD23-/IgM^{high} population was a consistent finding in all six independent experiments involving eight different transgenic mice. The remainder of the visible κ light chain rearrangement bands detected by Southern blot appeared to be similar between the two populations except for the 1.8-Kb and 3.8-Kb bands that were present in CD23+/ IgM^{low} cells but absent in CD23-/IgM^{high} B cells. In the non-transgenic littermates, however, the Vκ rearrangements within these two B cell populations appear to be identical (Fig. 1C). The discrepancy in light chain expression between CD23- and CD23+ B cells in the transgenic mice is not due to differences in heavy chain expression because both populations express the μ transgene almost exclusively. Similarly, the preferential expression of the Vκ1A5-Jκ5 gene in CD23-/IgM^{high} B cells was not due to pairing with a mutated VH81X transgene, nor were mutant transgenes present in the CD23+/IgM^{low} population. We cloned and sequenced VH81X transgenes in 20 clones, 10 from each of the CD23^{-} and CD23^{+} B cell populations, and found no nucleotide. The striking confinement of Vκ1A5-Jκ gene expression to the CD23-/IgM^{high} B cell population apparently indicates that expression of the predominant Vκ1A5-Jκ gene in the transgenic mice is not due to preferential physicochemical pairing of this particular light chain with the VH81X heavy chain, nor is it the result of a generalized preferential κ rearrangement, because over expression of this particular light chain was not found in the mature CD23+/IgM^{low} B cells.

Vκ1A5-Jκ5 Rearrangements Are Not Increased In Neonatal Spleen B Cells and Adult Bone Marrow. To determine where the Vκ1A5-Jκ gene expression become predominant

during development, we compared Vκ rearrangements between the bone marrow and spleen B cells of transgenic mice by Southern blot analysis. As illustrated in Figure 4, both spleen and bone marrow B cells have some common Jκ rearrangements, however only in spleen was Vκ1A5-Jκ5 detected as a predominant rearrangement. This finding suggests Vκ expression in the nascent B cell population in the bone marrow is more heterogeneous than in the spleen, and, as a consequence, the Vκ1A5-Jκ5 was not detected as a dominantrearrangement in bone marrow.

We have previously studied κ light chain expression in splenic B cells at different time points after birth. The Vκ1A5-Jκ5 rearrangement was not detected as a dominant light chain in the neonatal transgenic spleen. Instead, another dominant κ rearrangement was present in both the transgenic mouse and littermate spleens. This rearrangement remained dominant on 20 days of age when the Vκ1A5-Jκ5 rearrangement became visible in transgenic mice. Later, it faded. It has been shown previously that at about 10 days after birth, B cell lymphopoiesis begins to decline in spleen and liver and shifts to bone marrow (50). Therefore, the majority of B cells at 10 days of age should not be bone marrow derived. The flux of dominant Vκ rearrangements implies that: (1) in both transgenic and littermate mice, the κ light chain repertoire changes with age, at least as detected by dominant κ rearrangements; (2) the κ light chain expression is more similar between transgenics and littermates in young rather than older mice; and (3) Vκ1A5-Jκ5-expressing B cells may be bone marrow-derived.

VH81X-Vκ1A5-Jκ5-Encoded Antibody is Self-reactive Towards Ubiquitous Intracellular Components. To understand the potential reactivity of VH81X/Vκ1A5

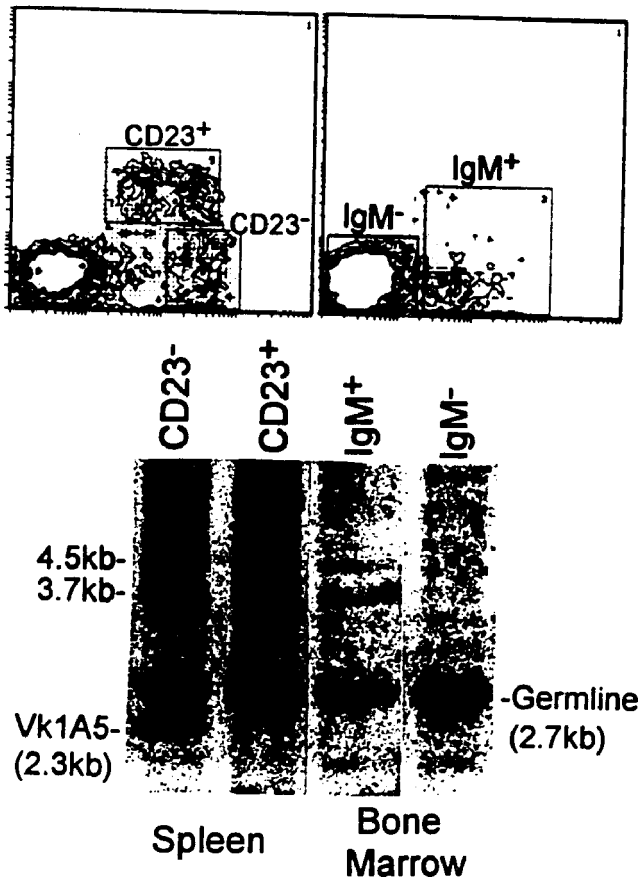


Figure 4. Southern blot analysis of V-κ gene rearrangement in the bone marrow and spleen B cells of transgenic mice. DNA was isolated from the sorted CD23+ and CD23- spleen B cells, or total sIgM+ bone marrow B cells (because in this tissue CD23^{-} B cells are few) and surface IgM negative bone marrow lymphoid cells of the same mouse, and hybridized with Jκ1-5 probe. While the 2.3-kb Vκ1A5-Jκ5 rearrangement is dominant in CD23^{-} spleen B cells, it is not visible in bone marrow B cells, although some other rearrangements, as represented by 4.5-kb and 3.7-kb bands, were detected in B cells of either source. No Jκ hybridizations other than to the germline fragment is detected in IgM negative bone marrow lymphoid cells. This indicates that those rearrangements detected in B cells are not the result of non-specific hybridization of the Jκ1-5 probe.

Jκ5 antibodies, we examined the ability of this and other antibodies to bind to self antigens. Using 68 hybridoma antibodies, surface or cytoplasmic immunofluorescence staining of cells from BALB/c or C57BL/6 bone marrow, spleen and thymus was analyzed by flow cytometry. From this panel, only two antibodies were found to stain the cell surfaces, but 60/68 (27 of which expressed Vκ1A5-Jκ5) were found to be self-reactive by staining the cytoplasm of cells. As shown in Figure 5, there is a broad spectrum of reactivity as judged by the intensity of staining by individual antibodies expressing various light chains. When the reactivity was arbitrarily divided into seven grades of fluorescence intensity, Vκ1A5-Jκ5-encoded antibody was second- to third- most highly reactive. These results suggest that: (1) the VH81X transgene frequently encodes antibodies that are self-reactive, (2) expression of self-reactivity is dictated not only by the transgenic heavy chain alone but is also influenced by the light chain, (3) the self-antigens with which the antibodies react are present for the most part in many different tissues, and iv) the dominance of Vκ1A5-Jκ5-expressing B cells does not result from mechanisms operative to escape from self-reactivity.

To characterize further the specificity of Vκ1A5-Jκ5 bearing antibodies, we performed Western blot analysis on a variety of tissue extracts. As shown in Figure 6, the antibodies are "multi-reactive", and bound dominantly to 72-kd, 50-kd and 46-kd bands in the bone marrow, and 48-kd, 40-kd, 33-kd and 30-kd bands in heart. This "multi-reactivity" is, however, selective, considering the thousands of different protein molecules that are present on the membrane. Since the protein molecules on the membrane are essentially completely denatured, the epitopes detected by antibody in this assay are probably linear rather than conformational structures. Furthermore, FACS analysis did not

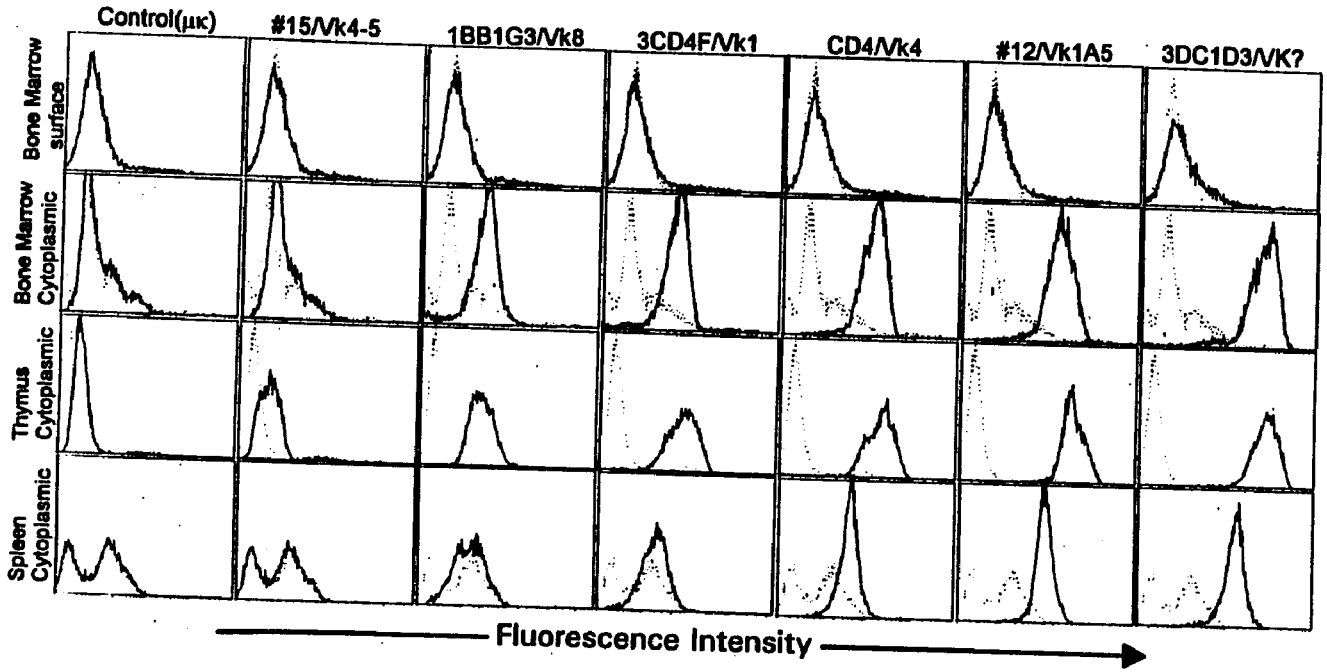


Figure 5. FACS analysis of the reactivity of transgenic B cell hybridoma-derived antibodies towards mouse tissue cells. Mouse tissue cell suspensions were made and stained with different transgenic B cell hybridoma-derived antibodies for either cell surface or cytoplasmic components, followed by flow cytometry analysis. The name and the light chain group of the antibodies are given on the top of the FACS profile. For cytoplasmic staining, the cells were pre-fixed with 70% ethanol in water to permeablize the cell membrane. When unlabeled antibodies were used for the first step staining, a second fluorescent antibody, either RS3.1, which recognize Igh6a, or a goat anti-mouse IgM antibody was used. All cells were gated except for red blood cells and dead cell debris. These isotype-matched μκ antibodies were used as control antibodies, at the same concentration as the test antibodies (10 μg/ml). The top panel shows surface staining of bone marrow cells. The surface staining of thymus and spleen cells were similar to bone marrow and is not shown.

detect reactivity of the antibody towards cell surface components indicating that the antigens revealed by Western blot are intracellular. These epitopes exist in all tissues tested, but are present in proteins of different molecular weight in different tissues. This may be due tothe crossreactive nature of these antibodies, the presence of commonconserved epitopes on these different proteins, or both.

VκlA5-Jκ5-Encoded Antibody Is not Detected In The Serum. Because multiple VH81X/Vκ1A5-Jκ5-expressing B cells are preferentially generated from independent precursors, apparently as a result of selection, and constitute a relatively large number of splenic B cells in transgenic mice, we wished to determine how much transgene-encoded serum IgM is derived from these cells. We took advantage of the findings that reactivity to various tissues by VH81X-Vκ1A5 antibodies provided a fingerprint characteristic for those antibodies. We therefore compared the specificity and self-reactivity of total transgenic serum IgM versus VH81X/Vκ1A5-Jκ5-encoded monoclonal antibody by western blot analysis of different mouse tissue lysates. As detected by ELISA using anti-IgM-allotypic antibodies, there are increasing amounts of transgene-encoded as well as endogenously-derived IgM antibodies as the mice age. By 3 months of age, there are 123±42 μg/ml of Igh6a (transgene-derived) and 496±102 μg/ml of Igh6b (endogenously-derived) IgM in the serum. However, when these sera were tested by Western blot analysis for Vκ1A5-Jκ5 antibody specificity, the characteristic specificity of these antibodies was not detected as indicated by the lack of Vκ1A5-Jκ5 antibody staining patterns on heart, bone marrow (Fig. 6) and other tissue lysates. Although some identical protein bands were stained by both Vκ1A5-Jκ5 and serum IgM antibodies, the patterns resulted from individually distinct antibodies, because the serum IgM failed to stain all of

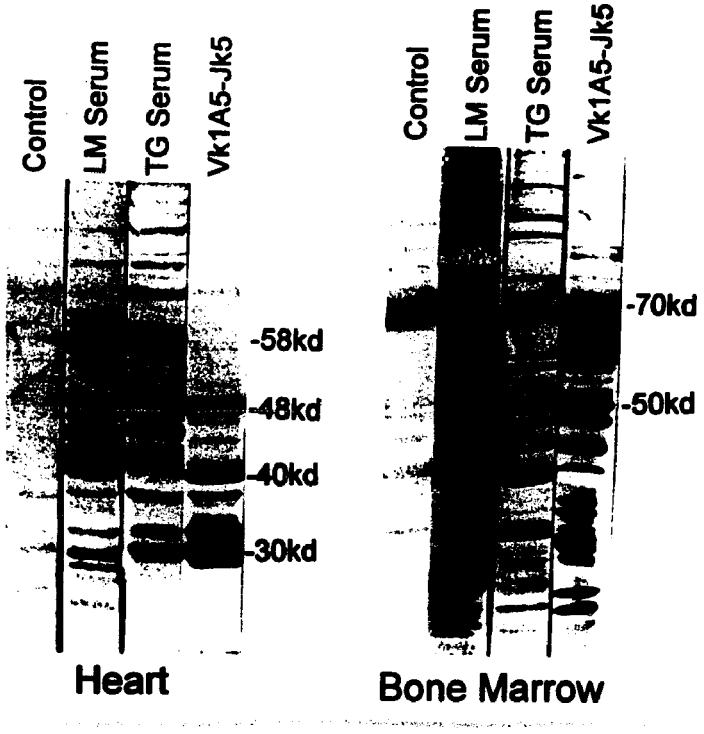


Figure 6. Western blot analysis of natural serum IgM vs Vk1A5 antibody reactivity towards mouse tissue extracts. Tissue extracts were made from BALB/c mice. The total IgM concentration in mouse sera and VH81X/Vκ1A5-Jκ5-encoded antibody was adjusted to 10 μg/ml, and membranes were developed with alkaline phosphatase-conjugated goat anti-mouse IgM antibody. The 48-kd and 30-kd proteins recognized by both the serum IgM antibody and VH81X/Vκ1A5-Jκ5-encoded antibody in heart extract may be the same (or different), but the reactive antibodies in the sera are different from the VH81X/Vκ1A5-Jκ5-encoded antibody because they did not react with the 40-kd protein which VH81X/Vκ1A5-Jκ5-encoded did. Likewise, there was no detectable reactivity in the serum toward the 70-kd protein that was recognized as a dominant protein in bone marrow. (LM = littermate, TG = transgenic, LPS = LPS-conditioned culture-derived supernatant.)

the bands that reacted with Vκ1A5-Jκ5 antibody, which is monoclonal. By using purified VH81X-Vκ1A5 antibody, we have shown that the limit of detection of this specificity by antibodies in this Western blot assay is <10 ng/ml. Therefore, lack of Vκ1A5 specificity in the serum suggests that Vκ1A5-Jκ5-expressing B cells in the spleen do not secrete detectable amounts of antibodies, implying that they are functionally inactivated or anergic (34, 36). However, upon stimulation with LPS *in vitro*, they did secrete (data not shown.)

It is of interest that the natural serum IgM of transgenic mice and non-transgenic littermates show some similar self-reactivity patterns as indicated by the presence of common 58-Kd, 48-Kd and 45-Kd bands on Western blot of heart extract and similar reactivity patterns on bone marrow lysate (Fig. 6). The serum IgM antibodies in transgenic mice that react with the same protein bands may be different from that of the littermates, but they tend to have similar reactivities toward self-antigens.

Similar self-reactivities of serum IgM in transgenic mice and the littermates can also be demonstrated by FACS analysis of mouse tissues. Mouse cell surface components from different sources, including bone marrow, thymus and spleen, were not stained with the serum IgM from either transgenic or littermate mice. However, when the serum IgM were tested on intracellular components, both serum IgM were equally reactive as judged by immunofluorescence intensity (Fig. 7). Only occasionally, did transgenic mouse sera stain brighter than sera from the littermates. Both sera reacted with all cells of all tissue sources tested, suggesting the reactive antigens recognized by these "natural" antibodies are ubiquitous and intracellular. However, this reactivity toward intracellular components is not due to non-specific "stickiness" of the serum IgM antibodies, because such reactivity is not demonstrable toward cell surface components.

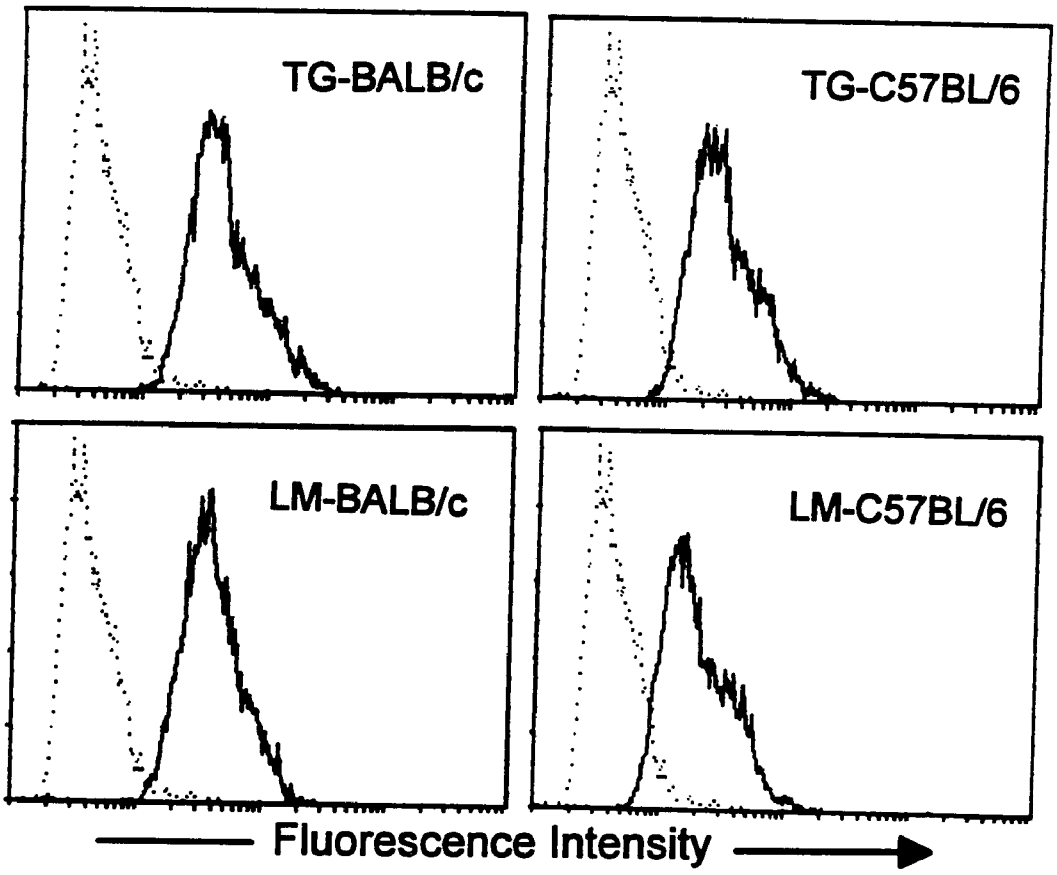


Figure 7. FACS analysis of self-reactivity of natural serum IgM in transgenic and litter mate mice. Mouse thymocytes were pre-fixed with 70% ethanol in water to permeablize the cytoplasmic membrane and stained with the diluted serum of either transgenic or littermate mice, followed by fluorescein goat anti-mouse IgM (or RS3.1 in different experiment). Antibody #15, a IgM (μ/κ) mAb derived from VH81X transgenic mouse, was used as a control at the same concentration as the serum IgM (20 μg/ml). In some other experiments, a μ/λ mAb was used as control antibody, and the results were similar. The surface staining of thymocytes with serum IgM was negative (not shown). The cytoplasmic staining of bone and spleen cells was similar to thymocytes, and is not shown.

Discussion

Dominant Vκ1A5 Expression Arises by Positive Selection. A observation in our transgenic mice is the predominant expression of the Vκ1A5 light chains. The over expression of this particular light chain is clearly not due to the inability of VH81X transgene to associate with other κ light chains, nor is there a preferential association of the VH81X μ chain with the Vκ1A5 because this Vκ gene is not expressed as a dominant light chain by CD23+/IgM^{low} B cells. Furthermore, the dominance of Vκ1A5-expressing B cells does not appear to result from mechanisms operative to escape from self-reactivity, since the VH81X/Vκ1A5-Jκ5-encoded antibody was not less but rather more self-reactive than the majority of the other Vκ gene-encoded antibodies in the panel of 68 transgenic mouse-derived hybridoma-produced antibodies. Additionally, these B cells are not generated through a clonal expansion of common B cell precursors. Taken together, these findings suggest that some process favors the *de novo* generation and/or selection of Vκ1A5-Jκ5-expressing B cells. A finding that supports the idea of "positive selection" is the detection of identical Vκ-Jκ joins involving this Vκ gene in clonally unrelated hybridomas. Identical Vκ-Jκ joins create identical light chains, which associate with the same transgenic heavy chain to create antibodies of identical specificity. This indicates that VH81X/Vκ1A5-Jκ5-encoded antibody is generated to react with an antigen rather than to avoid some antigens. A high frequency of Vκ1A5 expression has been previously described in peritoneal B cell-derived hybridomas, where this Vκ gene segment was identified in 5 out of 12 clonally independent hybridomas, 4 in association with the same and 1 with different heavy chains (51). Although no selecting ligand was demonstrated,

the unusually high frequency of expression of this Vκ gene was considered to result from a selection of B cells expressing this light chain. We have recently injected VH81X/Vκ1A5-Jκ5-encoded monoclonal antibody into neonatal transgenic mice, aiming to block engagement of B cells bearing the corresponding IgM receptor with the postulated selecting ligand. Such treatment substantially reduced the frequency of Vκ1A5-Jκ5-expressing B cells in adult mice (unpublished data). We have not yet determined the nature of the antigen responsible for the selection, but our available data suggest that this antigen is more likely a self antigen than an exogenous antigen: (1) the VH81X/Vκ1A5-Jκ5-encoded antibody is remarkably reactive against ubiquitous intracellular self-antigens (Such self-reactivity can be demonstrated either by FACS or Western blot analysis. The intracellular antigens must be released during continuous cell turn over, perhaps in analogy to DNA that is released and bound to anti-DNA antibody in SLE; and (2) Vκ1A5-Jκ5-expressing B cells are anergic (as discussed below), implying they are not generated in response to an exogenous antigen.

Our data also reveal some unexpected similarities between the serum IgM antibody of transgenic mice and non-transgenic littermates with respect to self-reactivity toward self-antigens that are ubiquitous but exist as intracellular components. These observations suggest that even in transgenic mice the natural IgM antibody repertoire has been strongly selected so that it tends to be the same as in normal mice. Transgenic and normal mice probably have similar dynamic changes in light chain composition early during the development, as detected by Southern blot analysis in spleen B cells at day 10. It has been shown that in fetal and neonatal BALB/c mice, B cells preferentially express D-proximal VH genes of the VH7183 family, especially VH81X (32). Heavy chain restriction of this

kind in the 10-day-old littermate mice may result in an accompanying dominant κ chain expression in both transgenic and littermate young mice.

However, in our transgenic mice, due to allelic exclusion, B cells express almost exclusively the transgene throughout life. This results in a restricted IgM repertoire even though the transgenic heavy chain can associate with various light chains, and consequently transgenic B cells may react only with limited self-antigens. This limitation provides a chance to appreciate B cell "positive selection," which is more difficult to detect in an unmanipulated normal mouse.

VκlA5 Expressing B cells are Functionally Silenced. An intriguing observation is the apparent absence of the Vκ1A5-Jκ5 band, as detected by Southern blot analysis, in the bone marrow when compared to the spleen of the same individual mice. The absence of this band, however, does not exclude the possibility that these B cells are produced in the bone marrow, because the nascent B cell population in bone marrow may be so heterogeneous that Southern blot analysis may not detect Vκ1A5-Jκ5-expressing B cells because of its limited sensitivity. It has been shown that B lymphocytes are continually generated at a rate of approximately 5×10^7 per day, yet less than 25% survive to enter the periphery (52, 53). In transgenic mice, the percentage of the survivors is probably even less, due to the B cell self-reactivity and the more vigorous subsequent selection process. Perhaps it is only among this small number of bone marrow "emigrants" that Vκ1A5-Jκ5-expressing B cells become dominant and detectable in the spleen by Southern blot analysis. That only 2 out of 68 VH81X transgenic mouse-derived hybridomas produced cell surface reactive antibody is in contrast to our previous *in vitro* study, where the transgene, when transfected into a set of light chain only plasmacytomas or hybridomas, gave rise to the

frequent production of surface reactive antibodies (unpublished data). These findings suggest that vigorous negative selection *in vivo* deletes B cells expressing receptors equivalent to these artificially created antibodies. These results are similar to the observations made in anti-DNA transgenic mice, in which anti-dsDNA B cells were not detected even though the transgene has a high propensity to encode such antibodies (36).

Another striking finding with respect to Vκ1A5-Jκ5 light chain is its restriction to the $CD23^-/IgM^{high}$ immature B cell population. These $CD23^-/IgM^{high}$ B cells are not B-1 cells or of B-1 origin since they were Mac-1 and CD5 negative. In addition, we have recently analyzed light chain gene rearrangements in 14 transgenic mouse peritoneal cavity B cell-derived hybridomas, and did not detect Vκ1A5-Jκ5 rearrangement (data not shown). Our interpretation for this restriction is that maturation of these CD23-/IgM^{high} B cells is arrested before the CD23+ stage because they are selected by ubiquitous self antigens and are rendered anergic in the spleen by these or other related antigens. As a consequence, they are not detected in mature CD23+/IgM^{low} B cell pool and probably die shortly after migration to spleen. The anergic state of these B cells is consistent with lack of detectable Vκ1A5-Jκ5-encoded antibody in the serum. This interpretation is consistent with the kinetics of B cell generation in normal mice, where bone marrow continuously produces $>5 \times 10^7$ B cells/day, but only the short-lived rather than the long-lived B cell pool is constantly being replenished at a high rate (54-56). Although it is not known why and how these short-lived B cells are generated, it is possible that many are self-reactive. It has also been shown in other transgenic studies that anergic B cells are short-lived (57), especially when they have to compete with a diversity of other B cells for

survival in the periphery (58). Therefore, the predominance of anergic Vκ1A5-Jκ5-expressing B cells should result from continuous generation rather than a long term accumulation of these B cells.

Clearly, a paradox is raised: why should self-reactive B lymphocytes be selected and continuously generated, but are subsequently inactivated and eliminated? The reason for this may be two-fold: (1) B cell receptor engagement is mandatory for differentiation to proceed, and the most abundant antigens involved in this engagement are self-antigens; and ii) these self-reactive B cells may constitute the initial idiotype network early during development and function to select and maintain a normal B cell repertoire in adult, or to present self-antigens for the maintenance of peripheral T cell tolerance, as suggested in the studies of rheumatoid factor transgenic mice (59).

In conclusion, we would like to propose that the occurrence of natural self-reactive B lymphocytes and antibodies in a normal immune system results from continuous generation of these cells as a result of selection by self-antigens.

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GENERATION AND INACTIVATION OF NATURAL SELF-REACTIVE B LYMPHOCYTES

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Summary

Self-reactive B lymphocytes dominate the early repertoire and continue to be part of normal repertoire in the adult. To study the generation, function and fate of natural self-reactive B cells we studied B cell repertoire development in VH81X transgenic mice. In the spleen of these mice there was a population of clonally unrelated self-reactive B cells confined to the CD23⁻/IgMhi B cell pool, which expressed identical antigen receptors encoded by Vκ1A5-Jκ5 light chains in association with the transgenic heavy chain, suggesting that B cells expressing this specificity were positively selected. When purified antibody of the same specificity was continually administered into transgenic mice to block the engagement of this antigen receptor with the corresponding selecting ligands, it substantially reduced the generation of Vκ1A5-Jκ5-bearing B cells. This antibody had a half-life of only 8 hours *in vivo*, while the isotype-matched control antibody BH8 had a half-life of about 3 days. To understand the reason for the confinement of these B cells to the CD23⁻/IgMhi pool, we compared the life spans of CD23-/IgMhi vs CD23+/IgMlo cell by BrdU labeling and found that CD23-/IgMhi B cells in transgenic and normal mice have a shorter half life than CD23+/IgMlo B cells. When cultured in the presence of LPS, the two populations of B cells proliferated equally well and tended to retain their original phenotype. However, CD23-/IgMhi B cells produced 20 time mores IgM antibody than the CD23+/IgMlo B cells, which, on the other hand, were more prone to undergo an IgG1 isotype switch than the former in the presence of IL-4. The IgM antibodies secreted by the CD23-/IgMhi B cells were more self-reactive than that produced by IgMlo/CD23+ B cells. In addition, crosslinking of surface IgM stimulated the CD23+/IgMlo rather than CD23-/IgMhi B cells to proliferate. These observations suggest that natural self-reactive B cells appear in the

spleen as a result of continuous generation in bone marrow through positive selection by self-antigens, and that maturation of these B cells is arrested at the CD23-/IgMhi stage. Such a block is important since these B cells not only represent cells at different differentiation stages but are intrinsically different from CD23+/IgMlo B cells in that they are not ready to respond to T-cell derived cytokines.

Introduction

Mutant mice that can not rearrange Ig gene segments, such as severe combined immunodeficiency (*scid*) mice (1) or mice homozygous for disrupted RAG-1 and RAG-2 gene (2, 3), lack mature B and T cells. In addition, targeted disruption of the membrane exon of the IgM μ gene (4) or the λ5 gene (5) causes a severe deficiency in B cell development. The absolute requirement for the expression of surface μ heavy chain suggests that engagement of μ heavy chain or surface IgM molecules with a putative ligand is important to transduce positive intracellular signals necessary for the B cell development process to continue. However, it is not yet clear at what stages, pre-B or B or both, the positive signals are required, if it is a variable region-directed process, and what the ligands are. That a primary B cell repertoire develops in fetal life prior to exposure to exogenous antigens suggests that self-antigens are involved in delivering positive signals, and that they play a role in selection of the pre-immune repertoire. The predominant self-reactivity of the fetal and neonatal B cell repertoire (6-9), and the presence in the immune system of normal adults of natural self-reactive B cells (10, 11), support this concept. How self-reactive lymphocyte are maintained in the face of vigorous mechanisms that induce self-tolerance by eliminating or inactivating auto-reactive cells (12, 13,), where these cells are located, and the nature of their phenotype are not

understood.

We have previously shown that B cells in VH81X transgenic mice expressed various κ light chains in association with the transgenic VH81X-μ heavy chain. In the spleen of the mice, however, there was a population of clonally unrelated self-reactive B cells confined to the CD23⁻/IgMhi B cell pool which utilized identical Vκ1A5-Jκ5 light chains and expressed identical antigen receptors. The experimental data indicated that there is a B cell antigen receptor (BCR)-mediated selection mechanism that favored the generation or selection of B cells that bear this specificity. Here we describe further studies to determine the nature of the selective mechanisms, the phenotype and the fate of self-reactive B cells in transgenic as well as normal mice.

Materials and Method

Southern Blot analysis. To isolate genomic DNA, sorted B cells were digested with digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS, 10 mg/ml RNase A and 200 mg/ml proteinase K) at 55°C overnight, followed by phenol and chloroform extraction and ethanol precipitation. Southern blots performed conducted according to standard procedures (14). Briefly, 10 mg of DNA were digested at 37°C overnight to completion with restriction enzyme, HindIII, electrophoresed through 0.8% agarose and transferred to a nylon membrane (BRL). Membrane was cross-linked by UV Cross Linker. Hybridization with Jκ1-5 DNA probes ^{32}P-labeled by random priming was carried out at 65°C. All final washes were conducted under high stringency conditions.

ELISA. E.I.A A/2 plates (Costar) were coated with unlabeled goat anti-mouse antibody (anti-IgM), or IgH6a (15) 2 μg/ml in borate buffer (pH 9), incubated at 4°C

overnight, followed by the test antibody (serum at 1 to 100 or 1000 dilution in 1% PBS-BSA, or supernatant at 1 to 100 dilution) and incubated at 37°C for 2 hours. Alkaline phosphatase-conjugated goat-mouse IgM antibody was used as the third layer antibody, and incubated for 2 hours at 37°C. Between each step, the plate was washed five times with PBS. The plate was developed with alkaline phosphatase substrate 1 mg/ml in substrate buffer (pH 9). For quantitative ELISA, mouse antibody of known concentration was included in each plate, and the plate was read by a spectrophotometer at 405 nm and antibody concentration was determined using an ELISALITE program.

In Vivo BrdU Labeling And Staining Of Labeled B Cells. About 2 month-old transgenic and littermate mice were fed with drinking that contained 1 mg/ml of BrdU and 2% glucose to improve the taste of water. One week later, mice were sacrificed and spleen cells were double stained with anti-IgM and anti-CD23. CD23-/IgMhi vs. CD23+/IgMlo B cells were sorted out and then centrifuged onto glass slide. A modified method was used to stain labeled cells for incorporated BrdU. Briefly, cytospin preparation of sorted cells was fixed in 3% paraformadehyde for 30 minutes. DNA was denatured with 3M HCl for 15 minutes at 37°C, followed by neutralization in borate, pH 8, for 15,' twice. The cells then were stained with rat anti-BrdU antibody, followed with alkaline phosphatase (AP) conjugated goat anti-rat antibody, 20' at each step. Between each step, cells were washed with 1% PBS/BSA. Slides were developed with AP substrate NDB for microscopy. Percentages of BrdU positive cells were calculated by dividing number of positive cells with total cells counted. The formula used to calculate half life of a population is $C=C_0 \times (0.5)^{t/T1/2}$, where C = cell number at a given time *t*, C_0 = initial cell number, T/2 = half life.

Flow Cytometry Analysis. The antibodies used for flow cytometry are: fluorescein-conjugated goat anti-mouse μ, and γ1 (Southern Biotech, Birmingham, AL); biotin-conjugated rat anti-CD23 (B3B4; a gift from M. Kehry and D. Conrad.) Two-color surface staining was performed as previously described (16). Briefly 5 x 10^5 cells were first incubated with goat anti-mouse IgM, then with CD23, followed by SA-PE (Birmingham Southern Biotech), 20' for each incubation. The cells were washed with 1% PBS-BSA twice between each step. For cytoplasmic staining, BALB/c or C57BL/6 cell suspensions were fixed with 70% ice-cold ethanol for 30' and then washed twice with PBS-BSA before antibody staining. The mouse IgM-containing sera and supernatant derived from B cell culture used for staining were first quantitated for IgM concentration by quantitative ELISA, then diluted to 20 μg IgM/ml with 1% PBS-BSA. Transgenic B cell hybridoma-derived antibodies were used at 10 μg/ml. Three IgM antibodies were used as negative controls for FACS analysis, and they were equally non-self reactive. These antibodies were: antibody #15, 26.30 and 81.13.46. #15 is a transgenic B cell hybridoma-derived antibody containing the VH81X heavy chain and light chain, Vκ4/5; 26.30 is a fetal liver hybridoma-derived μ/λ antibody; and 81.13.46 is an omentum hybridoma-derived μ/κ antibody. Staining was followed by RS3.1-FITC or goat anti-mouse IgM-FITC and analyzed using the FACScan Consort 30 program (Becton-Dickinson).

Western Blot Analysis. Tissue cell lysates were made from mouse heart and bone marrow. The cells were washed twice with 1% PBS and lysed using SDS lysis buffer (50% glycerol, 2% SDS, 0.5M Tris-HCl, pH 6.8), 3 ml lysis buffer per 10^8 cells. The lysate was boiled for 15 minutes and kept at -70°C for use. Protein concentration in lysate

was quantitated using a Pierce Protein Assay System. 100 microgram of protein were loaded onto each lane of 7.5%, 140 x 140 x 1.5mm SDS polyacrylamide gel and eletrophoresed (17). After separation through SDS PAGE, cellular proteins were transferred onto nitrocellulose membrane. The filter was first blocked with 3% BSA in TBS for 2 hours and then incubated at 4°C overnight with primary antibodies or serum or supernatant diluted to 10 μg/ml of IgM in 3% BSA-TBS. The second antibody was goat anti-mouse IgM-AP. Between each step, the membrane was washed with TBS three times, 10' each. The membrane was developed with AP-substrate NDB. The negative control antibodies were the same as described above in flow cytometry analysis.

In Vitro B Cell Culture And Proliferation Assay. Spleen cells or sorted B cells were washed with 1% PBS/BSA and cultured in RPMI medium with 2 x 10^5 cell/well. Other reagents used were at following concentration: LPS, 30 μg/ml and goat anti-mouse IgM antibody Fab2, 50 mg/μl. For proliferation assay, 1μCi of ^{3}H-thymidine was added per well 8 hours before culture termination.

Results

Administration of Vκ1A5/VH81X-Encoded Antibody Substantially Reduced Frequency Of B Cells Expressing This Combination Of Heavy And Light Chains. Because our previous studies of B cell development in transgenic mice suggested that the Vκ1A5/VH81X-expressing B cells present in the IgM^{hi}/CD23- compartment were generated through positive selection, we wanted to determine if this selection is directed specifically through the B cell receptor. In transgenic mice, although Vκ1A5/VH81X-bearing B cells were present at a relatively high frequency, no corresponding IgM antibody was detected by Western blot analysis, which, in our assay system, could detect at least 20

ng/ml of this antibody if present in the serum at this concentration. If generation of Vκ1A5/VH81X-bearing B cells requires that their IgM receptor engage a corresponding ligand, then the administered monoclonal antibody of the same specificity will compete for the ligand and diminish the chance that the B cell will engage the putative ligand. To this end, we continually administered purified Vκ1A5/VH81X-encoded monoclonal antibody (#32) into neonatal transgenic mice. Transgenic mice were divided into three groups and given either the test antibody, control antibody or PBS by the intra-peritoneal route. In the first experiment a fetal-liver derived μ/κ antibody, MD6, was used as a control antibody and injections were started two weeks after birth and continued for 2 weeks, administering doses of 100 μg/mouse/day for the first and 200 μg/mouse/day for the second week. In the second experiment, injections were begun on the day of birth and were continued for 3 weeks using similar protocol. In this case, the control antibody used was the T15 idiotype bearing BALB/c derived hybridoma antibody BH8 (μ/κ). All mice were sacrificed on the day after the last injection. Spleen B cells were sorted into IgMhi/CD23- or IgMlo/CD23+ populations, and the κ gene rearrangements were detected by Southern blot analysis of genomic DNA isolated from the sorted cells. As shown in Figure 1, the results of the two experiments were similar. The 2.7 kb band represents the un-rearranged germline Jκ gene, and the 2.3-kb band represents the Vκ1A5-Jκ5 rearrangement. In antibody #32 treated mice, the intensity of the 2.3-kb band was weaker than corresponding bands present either in the control antibody or PBS-treated mice, indicating that the generation of B cells expressing the Vκ1A5-Jκ5 light chain was severely affected in the treated mice. The total number of B cells in the spleens of #32 antibody-treated mice was, however, not decreased, indicating that there must be a compensatory increase in B cells expressing

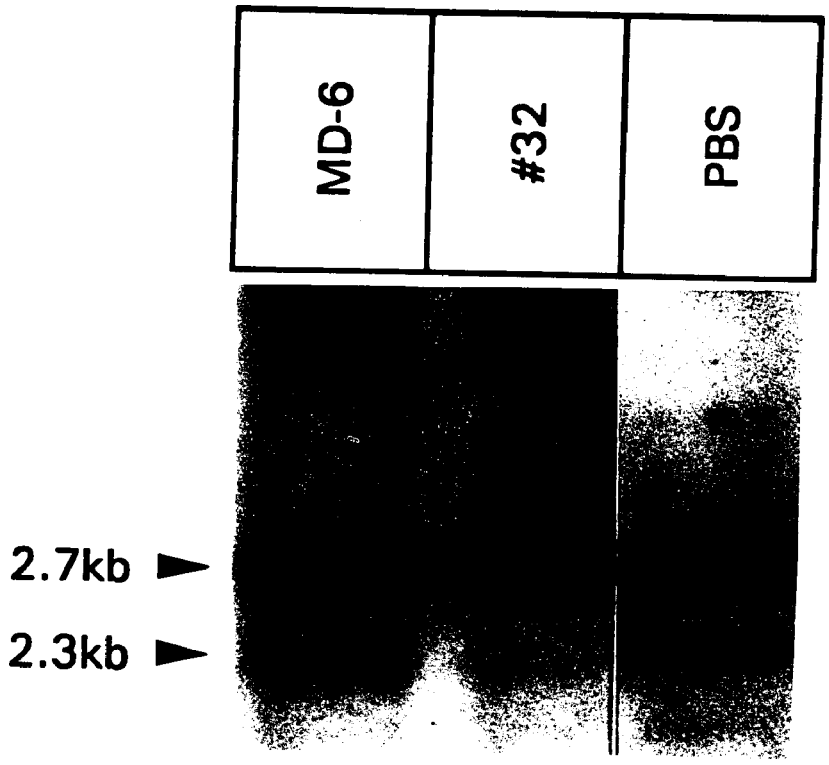


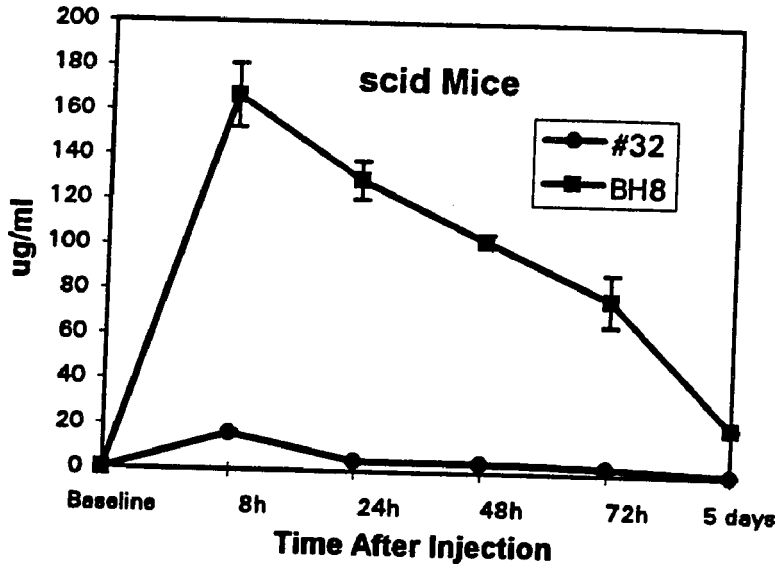
Figure 1. Southern blot analysis of Vκ gene rearrangements in CD23-/IgMhi B cells of antibody treated transgenic mice. Genomic DNA from sorted B cell was digested with Hind III, hybridized with a Jκ1-5 probe and washed under high stringency. The 2.7-kb band represents the un-rearranged germline Jκ gene that is present in some B cells, which have rearranged only one of their κ alleles. The 2.3-kb band represents Vκ1A5-Jκ5 rearrangement, whose intensity is lowest in the mouse that received monoclonal antibody #32. The intensity of the germline Jκ bands in three lanes are similar, indicating the amount loaded is comparable.

other light chains.

An additional observation made in #32 antibody-treated transgenic mice was that the B cells in the peritoneal cavity almost completely disappeared if the treatment started at 20 days of age. If the treatment was started at 8 days of age, the peritoneal B cells appeared normal in number.

Vκ1A5/VH81X-Encoded Antibody Had A Short Half-Life. We next compared the half lives of Ab #32 and control antibodies following injection. *scid* and normal C57BL/6 mice were given a single dose of 400 μg of IgM antibody i.p., and were bled at intervals for 4 days. Serum IgH6a was quantitated by ELISA (Fig. 2). In both *scid* and C57BL/6 mice each antibody exhibited a similar half life with #32 antibody having a half life of only 8 hours versus 2 days for BH8. Since the two antibodies are of the same isotype, the difference in their half lives *in vivo* most likely results from the different specificities of the antibodies. We have demonstrated previously that antibody #32 reacted with ubiquitous intracellular antigens that are present in all cells tested, while BH8 reacts with phosphorylcholine. The short life of the antibody may then be attributed to the formation of immune complexes and accelerated antibody clearance. These results also indicate that antibody #32 has access in the intact animal to the self-antigens that appear to be involved in positive selection as well as inactivation of Vκ1A5/VH81X-bearing B cells.

CD23-/IgM^{hi} B Cells Have A Shorter Half Life Than CD23^{+}/IgM^{lo} B Cells. In a previous study, we found that Vκ1A5/VH81X-bearing B cells were largely confined to the CD23-/IgMhi compartment. We next compared the *in vivo* life span of this population with that of the CD23+/IgMlo B cells by the 5'-bromo-'deoxyuridine (BrdU) labeling assay. Two-month-old adult mice were given BrdU in their drinking water continuously



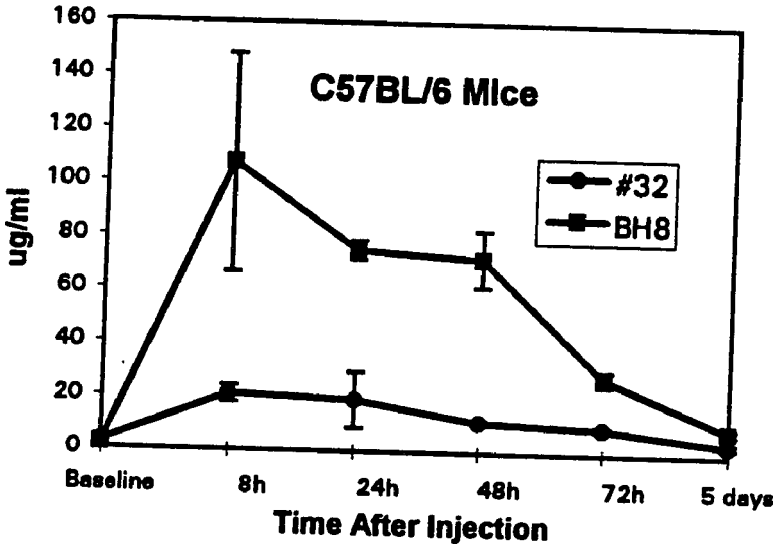
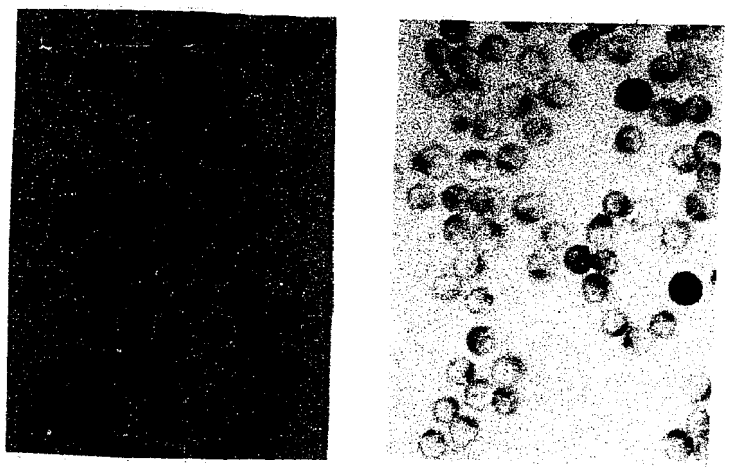


Figure 2. Serum IgMa level at different time points after antibody administration. Test antibodies were injected i.p. into *scid* or C57BL/6 mice, which were bled at the indicated intervals indicated. The antibody utilized in ELISA was RS3.1, which only recognizes the allotype of the administered antibodies #32 and BH8, which are IgH6a, but not endogenously-derived IgM, which is IgH6b. The peak level of antibody #32 was significantly low, and kinetics of the two administered antibodies were similar in both mice.

for a week, and B cells were sorted based on their sIgM and CD23 expression into CD23-/IgMhi and CD23+/IgMlo populations. Since BrdU is a thymidine analog it is incorporated into the DNA of the cells that have divided during the period of administration. Cytocentrifuge smears were made of each population and stained for BrdU. As shown in Figure 3, about 50% of CD23+/IgMhi B cells from both transgenic and normal mice were BrdU-labeled, in contrast to only about 10% of the CD23+/IgMlo B cell populations. With these variables, it can be calculated that the half life of CD23-/IgMhi cells is about 1 week, whereas the half life of CD23+/IgMlo B cells is about 7 weeks. This finding is consistent with previous observations that the bulk of the peripheral B cell pool is stable with a half life of about several weeks (17), while most newly formed B lymphocytes that arrive in the spleen are short lived (18). It also concurs with our hypothesis that Vκ1A5 bearing B cells represent short-lived continually renewed B cells, which fail to be recruited into the bulk long lived CD23+/IgM low B cell pool. In normal mice the difference in life span between the CD23-/IgMlo and CD23+/IgMlo B cells indicates that the two populations of B cells are different, probably with different specificity so that only a small proportion of newly generated B cells mature to become CD23+. Although about half the CD23-/IgMhi B cells were labeled with BrdU, this did not result in an expansion of this B cell population, since we have previously demonstrated that CD23-/IgMhi B cell pool becomes smaller as the mice get older, while the CD23+ pool becomes larger. In lymph nodes, CD23- B cells are infrequent, suggesting they do not recirculate to other lymphoid organs. Therefore, an un- expanded B cell pool with half of the cells being newly generated indicates that about an equal number of B cells in this pool died through apoptosis. Apoptotic cells are rapidly removed by scavenger cells. When the spleens of



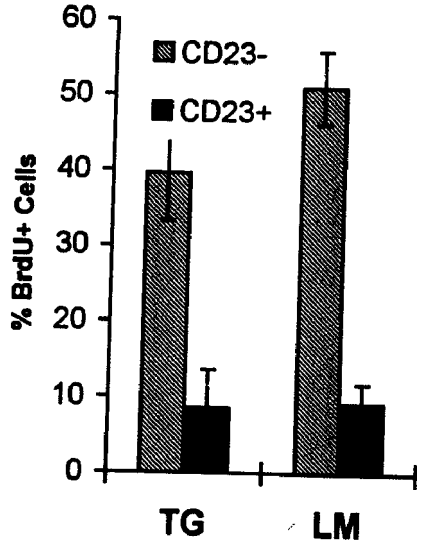


Figure 3. BrdU labeled cells in CD23+/IgMlo vs. CD23-/IgMhi populations. Spleen B cells were sorted as CD23-/IgMhi and CD23+/IgMlo to make cytosmears, which were stained with anti-BrdU antibody, followed by alkaline phosphatase (AP)-conjugated second antibody and developed with AP substrate NDB. Positive cells are dark purple. The smears shown are of sorted B cells from a normal mouse. The frequency of BrdU positive cells are summarized in the graph (the value represents average from five pair of mice).

both transgenic and littermate mice were sectioned and analyzed for B cell apoptosis, using terminal deoxylnucleotide transferase, TdT, which labels apoptotic cells by adding biotin-conjugated nucleotides to broken DNA ends that is present specifically in apoptotic cells, apoptotic cells were found in the B cell areas of the spleens from both sources (Fig. 4). However, there were about as twice as many in the transgenic mouse spleen. Although we have not determined the nature of the apoptotic cells, this result indicates that B cell apoptosis in the spleen is not uncommon and is consistent with the result derived from the BrdU labeling experiment that showed CD23-/IgMhi B cells in both transgenic and littermate mice underwent rapid turnover.

CD23- B Cell Secrete More IgM Antibodies Than CD23+ B Cell In Responce To LPS. Since CD23-/IgMhi B cells turned over more rapidly than the CD23+/IgMlo B cells, and a majority of them were not recruited into the CD23+/IgMlo B pool, we determined if there are further characteristics that distinguish the two populations with respect to this transition. The spleen B cells from either transgenic or littermate mice were sorted based on CD23-/IgMhi and CD23+/IgMlo phenotypes and cultured *in vitro* in the presence of the B cell mitogen, LPS. Both populations of B cells from either transgenic or littermate mice proliferated almost equally well in response to LPS, as judged by ^{3}H-thymidine incorporation. Each cell population tended to maintain its original phenotype. Most of the CD23- B cells remained CD23- and expressed higher levels of surface IgM than did CD23+ B cells, which up-regulated their CD23+ expression to even higher levels, with some B cells losing CD23 expression (Fig. 5a). However, when these two populations of B were cultured in presence of anti-mouse IgM antibody (Fab_2)', only CD23+ but not CD23- B cells proliferated extensively. However, lack of extensive proliferation of CD23-

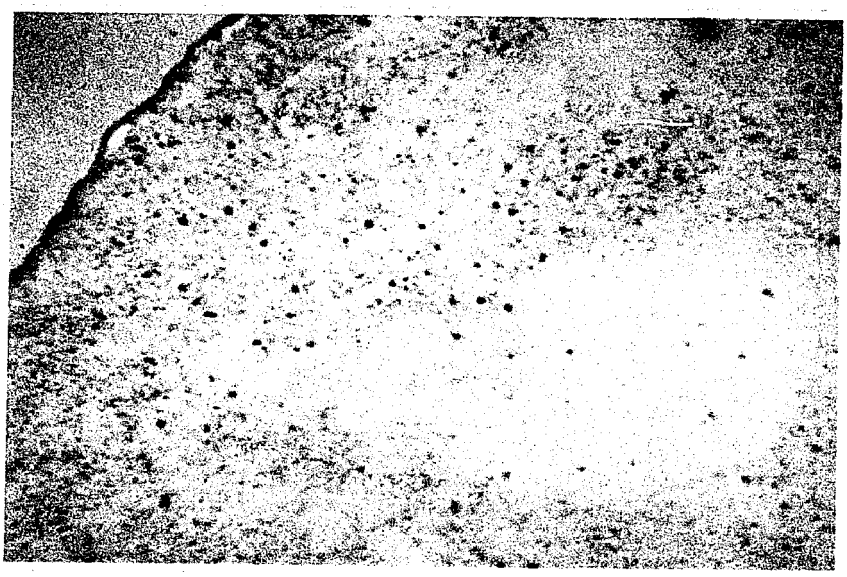


Figure 4. Apoptotic cells in the spleen. Frozen section of spleen were stained with biotin-conjugated RS3.1 antibody to show B cell areas. Apoptotic cells were stained by TUNNEL method and can be seen as dark-purple stained cells that are scattered throughout B cell areas with more located near the spleen capsule. Shown is spleen of transgenic mouse. There are about as half as many apoptotic cells in normal mouse spleen.

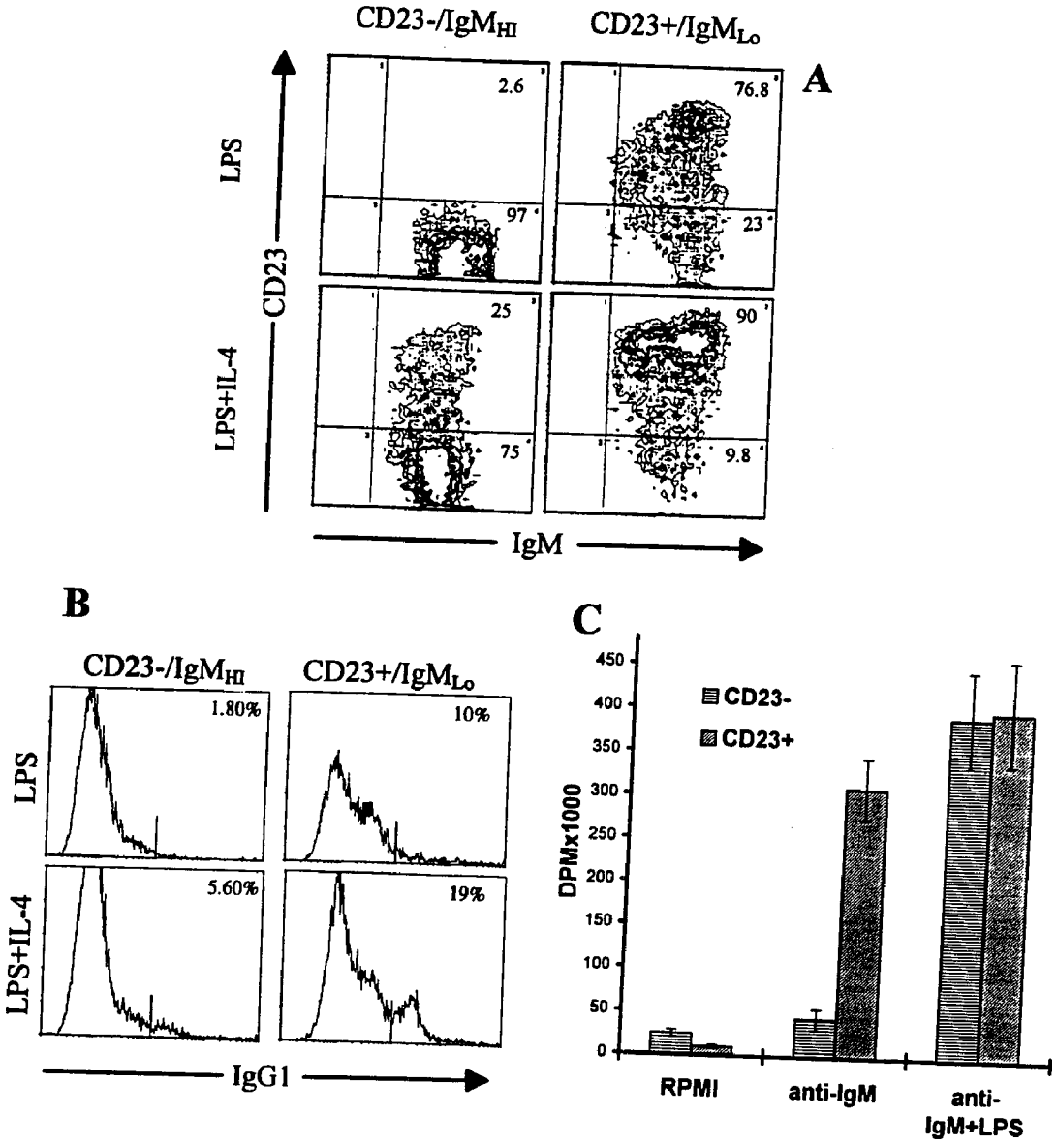


Figure 5. Phenotypic feature of CD23/IgMhi vs. CD23+/IgMlo B cells after *in vitro* culture with LPS and IL4. B cells were derived from normal mouse spleen. (A) Sorted B cells cultured for 3 days in presence of LPS or LPS plus IL4 are double stained with anti-μ and anti-CD23 antibodies, or (B) stained with anti-IgG1. (C) Sorted B cells are cultured in presence of anti-μ antibody Fab2 alone or plus LPS. Their proliferation was analyzed by a 3H -thymidine assay.

B cell induced by crosslinking surface IgM could be overcome by addition of LPS into culture (Fig. 5c). These results again indicate that CD23-/IgMhi B cells represent more immature cells because they tend to be inhibited more easily by sIgM-crosslinking (20-22), and LPS can reverse B cell tolerance (23, 24). On the other hand, for CD23+/IgMlo B cells, crosslinking sIgM is stimulatory.

In response to LPS, $CD23^-$ B cells secreted much more IgM antibodies (248 ± 63 μg/ml) in the supernatant than the CD23+ cells (16 ± 5 μg/ml). When the IgM antibodies secreted in the supernatants were tested on mouse tissues or cell lines and analyzed by FACS, the antibodies secreted by the CD23- B cells from transgenic mice were more self-reactive, as judged by fluorescence intensity, than IgM produced by $CD23^+$ B cells of the same source, with respect to their reactivity toward cell surface components and intracellular antigens (Fig. 6a). Such difference in reactivity was not demonstrated with B cells from normal mice, probably because the B cell specificities are too heterogeneous.

We have previously shown that VH81X/Vκ1A5-Jκ5-expressing B cells did not secrete antibody in serum. When transgenic spleen cells were cultured with LPS, and IgM antibodies secreted in the supernatant were tested by Western blot analysis, the reactivity characteristic of Vκ1A5-Jκ5 antibody was clearly apparent in the supernatant. In additionto the appearance of Vκ1A5-Jκ5 associated specificities, many other new reactivities absent in the serum were revealed as shown by the increased complexity of stained bands on Western blots (Fig. 6B). Interestingly, the littermate spleen cell culture supernatants gave some similar, though somewhat different, staining patterns, and surprisingly, VH81X/Vκ1A5-Jκ5-encoded antibody-like specificity is also detectable in the non-transgenic-derived supernatant, though to a lesser extent than the transgenic mice

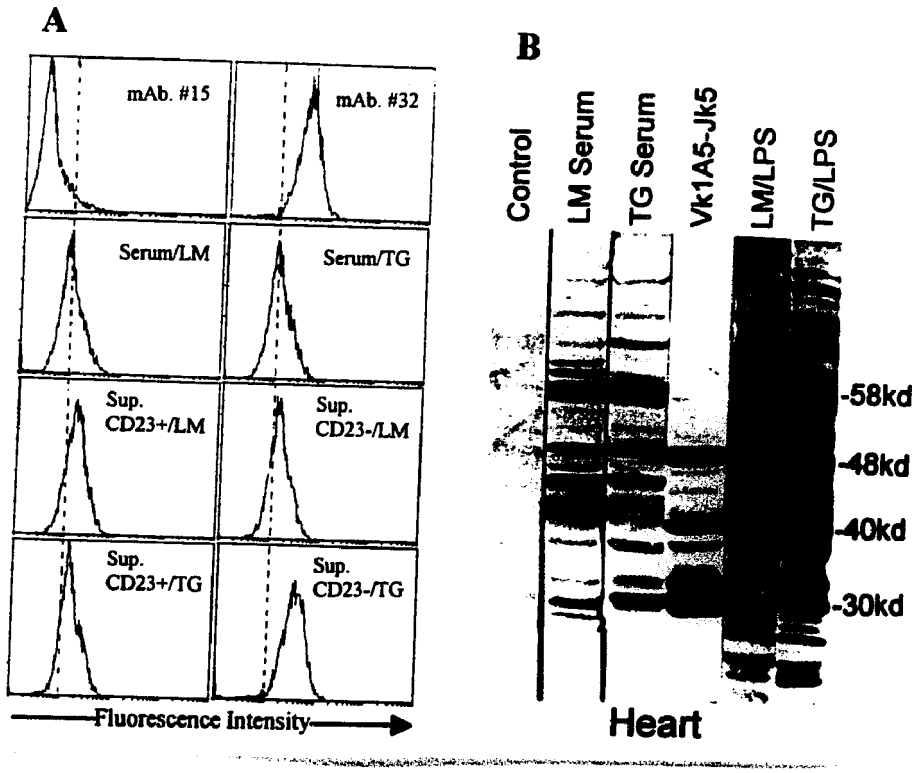


Figure 6. Self-reactivity of IgM antibodies secreted by CD23-/IgMhi vs. CD23+/IgMlo B cells. (A) FACS analysis of antibody reactivity: Single cell suspensions made of BALB/c thymus were fixed with ethanol and stained with monoclonal antibody #32 or IgM antibodies present in mouse sera or supernatant derived from *in vitro* LPS stimulated cultures of sorted B cells from both transgenic and littermate mouse. Antibody #32 is encoded by the VH81X transgenic heavy chain with Vk1A5-Vκ5 light chain. Control antibody #15 is another transgene-encoded antibody with a different light chain, Vκ4.

(B) Western blot analysis of antibody reactivity: Tissue lysate made of BALB/c heart was boiled and stained with antibody #32 or with IgM antibodies present in the supernatant derived from *in vitro* LPS stimulated cultures of spleen cells from either transgenic or littermate mice and developed with alkaline phosphatase-conjugated goat anti-mouse IgM antibody. The control antibody was the same as is (A) The 48-kd and 30-kd proteins recognized by both the serum IgM antibody and mAb #32 in heart extract may be the same (or different), but the reactive antibodies in the sera are different from the #32 antibody because they did not react with the 40-kd protein, which #32 antibody did. The VH81X/Vκ1A5 characteristic fingerprint becomes apparent when the membrane was stained with LPS-stimulated culture-derived supernatant. (LM = littermate, TG = transgenic, Supt. = LPS-conditioned culture-derived supernatant.)

B cell-derived supernatant. This VH81X/Vκ1A5-Jκ5-encoded antibody-like reactivity present in normal mice is probably also derived from CD23-/IgMhi B cells although it is clearly not encoded by this heavy and light chain, because we did not detect Vκ1A5-Jκ5 rearrangement in this B cell pool of normal mice. Therefore, these observations suggest that: (1) Vκ1A5-Jκ5 expressing B cells have the potential to secrete antibody but their progression to antibody secreting cells is normally blocked; (2) CD23-/IgMhi rather than CD23+/IgMlo B cells in both transgenic mice and littermate spleens are capable of secreting large amount IgM antibody in response to LPS; (3) most self-reactive antibodies are not normally in the serum (previous data); and iv) the non-secreting self-reactive B cells are selected in such a way that they display similar anti-self reactivity regardless of whether they are from transgenic or littermate mice, even though the former express only a single transgenic heavy chain. Included in this array of antibody self-reactivities is a specificity similar to that encoded by the VH81X/Vκ1A5-Jκ5 heavy and light chain genes, which may be positively selected in the non-transgenic littermate mice as well. However, the composition of heavy and light chains used in these serum antibodies is not known.

CD23+ B Cells Are More Prone To Isotype Switch. Antibody specificity is primarily determined by amino acid sequences of heavy and light chain variable regions, but the specific effector mechanisms that can be elicited by antibody, on the other hand, are determined by the class of heavy chain expressed by the antibody molecule. Natural self-reactive antibodies are characteristically of the IgM isotype and encoded by un-mutated V genes. To understand the mechanism that controls the isotype of these antibodies, we compared the ability of CD23-/IgMhi and C23+/IgMlo B cells to switch to IgG1 isotype in response to LPS and IL-4. IL-4 primes mouse B cells for switching to IgG1 (24), but

the actual switching requires additional stimuli such as LPS (25). B cells utilized in this study were from normal mice, since B cells in transgenic only express the transgenic μ heavy chain as a result of allelic exclusion and thus are unable to undergo isotype switching. The spleen B cells were sorted based on $CD23^-/IgM^{hi}$ and $CD23^+/IgM^{lo}$ phenotypes, and cultured *in vitro* in the presence of LPS alone or LPS plus IL-4. After culture for 3 days, cells were analyzed for surface expression of IgG1. As shown in Figure 5B, in the CD23+/IgMlo LPS B cell culture, three populations of B cells could be identified with respect to the intensity of IgG1 expression. They were IgG1 negative(67%), dim (24%) and bright (19%). In contrast, in the CD23-/IgMlo B cell cultures, the IgG1 dim and bright cells were fewer and accounted for 5.3% and 5.6%, respectively. In the presence of LPS alone, the difference between the two populations was similar, but IgG1 positive B cells were fewer. This small fraction of originally CD23-/IgM B cells that switched to IgG1 may represent those B cells that have the potential to become CD23 *in vivo*, as we have demonstrated above in the BrdU labeling experiment that about 10% of cells in the CD23+ B cells pool were newly recruited. Indeed, a proportion of CD23- B cells become CD23+ in response to IL-4.

Discussion

BCR-mediated B Cell Selection Is Specifically V-region Directed. Our studies on B cell repertoire development in VH81X transgenic mice have provided insight into the issue of positive selection B cells by self-antigens. This transgenic mouse has several unique advantages over other transgenic models to study the development of the pre-immune B cell repertoire. First, the VH81X transgenic heavy chain that we selected is preferentially expressed in the fetus (26, 27) prior to exposure to exogenous antigens, so

that if B cells show evidence for positive selection, the ligand involved is likely to be self-antigen. Secondly, the heavy chain uses V, D and J segments that are germline and non-mutated with a short CDR3 because of the lack of N regions and has the propensity to encode antibodies with low affinity, poly-reactivity and connectivity characteristic of natural self-reactive antibodies (11, 28, 29). Third, the range of specificities expressed by B cells generated in this mouse is limited because of the presence of the transgenic heavy chain that inhibits endogenous heavy chain expression, and consequently changes in the antigen receptor repertoire dependent on the light chain composition of B cell receptors is exaggerated.

In the previous analysis of light chain expression in the VH81X heavy chain transgenic mice, we proposed that the occurrence of a clonally independent heterogeneous population of B cells expressing identical antigen receptors encoded by Vκ1A5-Jκ5 light chain in association with the transgenic heavy chain was the result of positive selection. Our ability to block this B cell antigen receptor engagement with the corresponding ligand and prevent the appearance of B cells displaying this specificity by administration of purified monoclonal IgM antibody encoded by the same gene and of the same specificity further strengthens this hypothesis. The inhibitory effect of the antibody is specific. Only B cells expressing the same VH81X-Vκ1A5-Jκ5 were reduced, and the generation of B cells bearing other light chains was not disturbed, since the total number of B cells was not notably diminished. These results indicate that the depletion of the VH81X-Vκ1A5-Jκ5 bearing B cells is V-region-directed, similar to previous results where we have suppressed the expression of normally dominant idiotypes by passive administration of immunoglobulins bearing the same idiotype. Antibody administration, which was started

late (at 2 weeks after birth), was equally as effective in preventing the appearance of these clones as antibody administered from 1 day of age, indicating that the predominant of the Vk1A5-Jk5-expressing B cell clones is not the result of long-term accumulation of these B cells.

We have demonstrated previously that the antigens recognized by Vk1A5-Jk5/transgene encoded antibody are primarily located in intracellular sites. This raised the question of how intracellular antigens can be accessible to the antigen receptor in order to function as a selective ligand. The effective blockade by the antibody of generation of corresponding B cells indicates the antigen is accessible. In anti-DNA transgenic mice, B cells were also anergic but with a large proportion of B cells reactive against ssDNA, suggesting that DNA is available at sites that permit selective and anergy-inducing effects through the B cell receptor (30). In addition, the rapid clearance of antibody #32 when injected into mice, compared to the isotype-matched control antibody, is most likely due to the formation of antibody-antigen complexes. Intracellular antigens can be released into the internal environment during continuous cell renewal to function as a selection ligand and also to induce B cell anergy. It has been shown that the self-antigens recognized by natural self-reactive B cells are usually constitutively-expressed conserved intracellular components (31, 32, 11). Based on our observation, these characteristics of natural self reactivity appear to result from positive selection of B cells by these antigens rather than solely to represent non-specific polyreactivity presented by B cells surviving from negative selection.

B Cells Acquire IgMlo/CD23+ Phenotype For Longevity. In another transgenic mouse model, it has been demonstrated that self-reactive (anti-HEL) B cells died soon after being

adoptively transferred into normal mice, because of a failure, in the presence of the "self-antigen" HEL, to compete with normal B cells and enter lymphoid follicular niches (33). However, it is not clear yet what phenotypic changes and/or differentiation steps are necessary for the B cells to enter lymphoid follicles, where they become long-lived members of the B cell pool. Here we have demonstrated that self-reactive B cells, which are continuously generated in the same host, are short-lived because they failed to enter the CD23+/IgM^{lo} B cell pool. Taking together these two observations in parallel, we postulate that the lymphoid follicle is probably the site where newly generated B cells acquire the IgM^{lo}/CD23+ phenotype and become long-lived B cells. This postulate is consistent with histological observations that CD23+ B cells are found within the follicle and the CD23- B cells are located outside. The phenotypic dissection of these two B cell populations will help to understand mechanisms of self-tolerance as well as the physiological functions of the two B cell subsets. It has been proposed that CD23+ B cells need T cell help in order to take part in antigen responses while CD23- B cells do not. In accordance with this, we found that polyclonal B cell mitogen LPS could induce extensive CD23^{+} B cell proliferation, but had little effect on inducing IgM secretion, suggesting that these B cells may have to a large extent lost the ability to secrete IgM antibodies but are prime for isotype switching if T cell help or cytokines are provided for this event. In this study, we showed that a large proportion of CD23+ could switch to IgG1 in response to IL4.

Because CD23-/IgMhi B cells upon activation with LPS produce large amounts of IgM antibodies that are more self-reactive than the CD23^{+} B cells derived antibodies, it would be imperative that CD23-/IgMhi B cells be excluded from the long-lived CD23^{+} B

cell pool and from sites where they may receive T cell help, in order to decrease the possibility that hypermutation and class switching events may lead to the production of high affinity IgG autoantibodies. In addition, since IgM antibodies have limited ability to diffuse rapidly from sites of production to distant sites (34) and have much shorter half life than IgG (35), a self-reactive antibody of IgM isotype would be less harmful than IgG isotype. Unresponsiveness of CD23-/IgMhi B cells to sIgM cross-linking also suggests that these B cells are immuno-incompetent, and unlike CD23+ B cells, when they encounter antigens through their antigen receptor, they would not respond. This is apparently advantageous to the organism because most of these B cells are self-reactive.

The self-reactivity of natural antibodies has been thought to contribute partially to their IgM isotype. In our experiments, we found that after LPS activation, CD23- B cells remained CD23- and produced large amounts of IgM antibodies, but showed little tendency for isotype switch even in the presence of IL-4. These results indicate that the dominant IgM isotype of natural self-reactive antibody may result from the differentiation arrest and property intrinsic of the B cells that produce antibodies of IgM isotype.

Taken together the observations made in this model, we conclude that because B cell antigen receptor engagement is mandatory for differentiation to proceed, most newly generated B cells are self-reactive, as a result of positive selection by self-antigen. During ontogeny, these self-reactive B cells function to initiate the early repertoire, and in the adult function to facilitate and maintain a possibly diversified pre-immune repertoire. In addition, the continued generation and export of self-reactive B cells in T cell areas may be necessary to present self-antigen peptides in a way that leads to regulation or tolerance of potentially self-reactive peripheral T cells. These B cells are, however, prevented from

entering the long-lived and immuno-competent pool of B cells residing in the follicular areas. In this way the potential of these B cells to produce pathological autoimmunity is minimized.

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GENERAL DISCUSSION AND CONCLUSION

B cells in adult transgenic mice express almost exclusively the transgene, indicating that VH81X-expressing B cells are not incompatible to adult life. However, when compared with neonates, B cells are reduced in number in adult transgenic mice. When *scid* mice are reconstituted with bone marrow from both transgenic and littermate mice, transgenic mouse bone marrow-derived B cells dominate the recipient bone marrow and account for one third of B cells in the spleen 4 weeks post-transplantation. However, later, when the B cell repertoire in the recipient mice is fully developed, transgene-expressing B cells become few in the bone marrow and undetectable in the spleen of the recipient scid mice, while the same B cells are present normally in the scid recipient transplanted with only transgenic mouse-derived bone marrow. Such dynamic changes apparently fit our major hypothesis that the role of VH81X-expressing B cells is to pioneer the initial repertoire to recruit emergent B cells into the network. As the repertoire expands, these B cells are suppressed by other B cells in the repertoire.

Most antibodies derived from B cell hybridomas of adult transgenic mice were self-reactive towards ubiquitous self-intracellular components, even when they were encoded by different light chains, demonstrating that an intrinsic propensity of this VH gene is to encode self-reactive antibodies. This property ensures, as we have hypothesized, that the "first" B cells expressing VH81X will be guided to proceed further along a differentiation pathway. It is

perhaps also due to this property that in the adult life these B cells fail to compete for survival with non-reactive or less self-reactive B cells, which further contribute to the distinct lack of VH81X-expressing B cells in the mature B cell repertoire.

B cell repertoire development in transgenic mice also followed a sequential order similar to that seen in a normal mouse. The dominant light chains expressed in neonatal transgenic and normal mice were similar but different from that of older transgenic mice. The majority of the splenic B cells in both neonatal transgenic and littermate mice were CD23-, and a proportion of them were CD5+. As mice became older, CD5+ B cells disappeared and more B cells were recruited into the CD23+ pool. However, in transgenic mice this recruitment was delayed. Nonetheless, by using different B cell markers, comparable B cell sub-populations in the pre-immune repertoire could be identified in both adult mice. The sub-populations may represent B cells not only at different differentiation stages but may also have different specificity. The immature B cells are generally more self-reactive and functionally located around the core of the idiotype network than more mature B cells that are selected by self-reactive immature B cells.

In transgenic mice, there is a dynamic change in B cell composition when dissected based on expression of B cell surface maker during ontogeny and the presence of B cell sub-populations in adult transgenic mouse. The presence of different B cell sub-population suggested differences in light chain expression. To elucidate this issue, we dissected these B cell populations and analyzed their light chain expression.

The dominant light chains expressed in the splenic B cells of neonatal transgenic mice are different from that of older transgenic mice, but are similar to that of normal neonatal mice, suggesting the early repertoire is more similar between transgenic and normal mice than the

adult repertoire, and consistent with the previous observations that VH81X is preferentially expressed in the early repertoire of normal BALB/c mice.

The splenic B cells in both transgenic and littermate mice can be divided into IgMhi/CD23+ and IgMlo/CD23- populations and undergo similar dynamic changes. In neonatal mice, the majority of B cells are CD23-; and when mice become older, more B cells are recruited into the CD23+/IgMlo B cell pool. In transgenic mice this recruitment is delayed. When these two population were analyzed for light chain expression, it was found that CD23-/IgMhi B cells in transgenic mice express a dominant light chain, Vκ1A5, that was not observed in the CD23+/IgMlo population as judged by Southern blot analysis. The light repertoire in the CD23+/IgMlo B cells of transgenic mice is similar to that of the littermate mice. B cells that express the Vκ1A5 light chain gene were generated from clonally independent precursors, and surprisingly all the rearrangements of Vκ1A5 involved Jκ5, with identical Vκ-Jκ joins, resulting in the generation of the same antibody specificity. These B cells were more highly self-reactive than B cells expressing VH81X with other light chains and recognized an ubiquitous epitope(s) on proteins from different tissues. These B cells were generated in the bone marrow and migrate to the spleen where they failed to proliferate, and their maturation ceased at the CD23-/IgMhi stage. No detectable serum IgM was secreted by these B cells even though other transgene-encoded IgM was detected at low levels. Although there can be several interpretations for these observations, the possibility that explains all the observations is that there is a positive selection for B cells expressing this light chain. We did not identify the ligand involved in this selection, but all the related data suggest it is a self-antigen.

The purified Vκ1A5/VH81X-encoded monoclonal antibody (#32) was continually administered into transgenic mice. If generation of Vκ1A5/VH81X-bearing B cells required that their IgM receptor engage with a corresponding ligand, then presence of administered monoclonal antibody of the same specificity may compete for the ligand so as to diminish the chance of the B cell engagement with the ligand. This treatment remarkably reduced the frequency of these B cells. The half life of antibody #32 was about nine-fold shorter than the isotype-matched control antibody, suggesting it was cleared rapidly from serum, probably as immune complex.

Since the Vκ1A5/VH81X-bearing B cells were primarily confined to the CD23-/IgMhi B cell population, we compare the life span of these B cells with that of CD23+/IgMlo B cells. In both transgenic and littermate mice, the half life of the CD23-/IgMhi B cells was about 1 week, whereas the half life of CD23+/IgMlo B cells was longer than several weeks. The two populations tend to keep their original phenotype when cultured in the presence LPS and IL4. LPS stimulated the CD23-/IgMhi B cells to proliferate more promptly and secreted more IgM antibodies than the CD23+/IgMlo counterparts, which, on the other hand, were more prone to undergo IgG1 isotype switch and produce more such antibodies than the former in the presence of IL4. The IgM antibodies secreted by the CD23-/IgMhi B cells are more self-reactive than that produced by CD23+/IgMlo B cells. In addition, crosslinking surface IgM stimulate the CD23+/IgMlo rather than CD23-/IgMhi B cells to proliferate.

These observations indicate that the presence of natural self-reactive B cells result from continuous generation of these cells whose maturation is blocked at the CD23-/IgMhi B cells stage and which survive only for a short time and function to maintain the immune

network. The CD23-/IgMhi and IgMlo/CD23+ B cells not only represent B cells of different maturation stage but are intrinsically different in life span and immune competency.

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DISSERTATION APPROVAL FORM

Name of Candidate Xinjian Chen

Major Subject Microbiology

Title of Dissertation Development of the B cell repertoire,
A transgenic and comparative study

Dissertation Committee:

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