Generation of the Germline Peripheral B Cell Repertoire: VH81X-\(\lambda\) B Cells Are Unable to Complete All Developmental Programs

Flavius Martin, Woong-Jai Won and John F. Kearney

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The generation of VH81X heavy chain λ-light chain-expressing B cells (VH81X-λ+ B cells) was studied in VH81X heavy chain transgenic mice as well as in VH81X JH−/− and VH81X JH−/− Ck−/− mice, in which competition resulting from expression of heavy and light chains from the endogenous heavy and κ light chain loci was prevented. We show that although λ light chain gene rearrangements occur normally and give rise to light chains that associate with the transgenic heavy chain to form surface and soluble IgM molecules, further B cell development is almost totally blocked. The few VH81X-λ+ B cells that are generated progress into a mature compartment (expressing surface CD21, CD22, CD23, and low CD24 and having a relatively long life span) but they also have reduced levels of surface Ig receptor and express higher amounts of Fas Ag than VH81X-κ+ B cells. These VH81X-λ+ B cells reach the peripheral lymphoid organs and accumulate in the periarteriolar lymphoid sheath but are unable to generate primary B cell follicles. In other heavy chain transgenic mice (MD2, M167, and M54), λ+ B cells are generated. However, they seem to be preferentially selected in the peripheral repertoire of some transgenic heavy chain mice (M54) but not in others (MD2, M167). These studies show that a crucial selection step is necessary for B cell survival and maintenance in which B cells, similar to T cells, receive signals depending on their clonal receptors. The Journal of Immunology, 1998, 160: 3748–3758.
the immature B cell repertoire (41). This heavy chain has the potential to pair freely with light chains derived from the germline-encoded light chain repertoire, and, in this report, we have studied the development of B cells in which the transgene is associated with λ light chains. By using heavy and κ chain locus knock-out mice, we have restricted the association of the heavy chain transgene to λ light chains, which contain only three Va genes, so that B cells expressing these combinations can be easily followed at both cellular and molecular levels.

Materials and Methods

Animals and cell lines

Eight- to twelve-week-old BALB/c and C57BL/6 mice for breeding were purchased from Charles River Laboratories (Raleigh, NC). The generation of VH81X lines was described previously (39, 40). All other knockout and transgenic mice were generous gifts of Drs. D. Huszar (JH/−/− and JH+/−/−), C. C. Goodnow (C57BL/6 MD2 transgenic mice), J. J. Kenny (C57BL/6M16 transgenic mice), and R. R. Hardy (CB17 M54 transgenic mice). Screening of the VH81X JH/−/− Cκ−/− was made on genomic tail DNA with the following primers for VH81X, JH, Cκ, and Neo: 5′-VH81X, GCCGGGGCCGCGTTGACTGGGAGGAGGCTT; 3′-VH81X, CCCAGATGCAAACTGCTACCTGTACATTG; 5′-JH, GAA CAGAGCCGAGACAGAGCTGCT; 3′-JH, ACCGGGAGGAGT GAACCTGCT; 5′-Cκ, CACTGTCAGTACGTGGGCTGCT; 3′-Cκ, AACGTCAGGACACACTCAGTAC; 5′-Neo, CTGAAAGATGGGGC GCGAT; 3′-Neo, GACTCCCAACACCATCACCAAGAACAGA; and 3′- Cκ−/−, CATGTAGTGGACAGCCAACC. Mice were housed in accordance with institutional policies for animal care and usage. All experiments used 6- to 16-wk-old adults unless otherwise specified. CH12 (42) and J558L, and I558L-μM3 (43) cell lines were previously described. VH81X-A hybridomas were generated from three individual adult male spleens as previously described (39).

Abs and flow cytometry analysis

The anti-μ chain allotype Abs anti-IgH6 (RS3.1) and anti-IgH6 (MB86) were described previously (44). Fluorescein-conjugated anti-B220 (RA3-6B2), anti-CD43 (S7), anti-heat-stable Ag (HSA)1 (M1/69), and anti-CD22 (Cy34.1); PE-conjugated anti-B220 (RA3-6B2) and anti-Fas (Jo2); biotin-conjugated anti-IgA (B334), anti-IgM (M1/69), anti-D4 (LT4), and anti-CD86 (53-6-7); anti-CD8 (RA3-6B2) conjugated; H Cyclochrome; and APC and streptavidin (SA)-APC were purchased from PharMingen (San Diego, CA). Fluorescein- and rhodamine-conjugated goat anti-mouse μ, phycoerythrin (PE)-conjugated goat anti-mouse λ and κ, SA-PE, and SA-PECy5 were purchased from Southern Biotechnology, Birmingham, AL. The anti-CD21 (7G6) Ab-secreting hybridoma (45) was obtained from Dr. Michael Holers (University of Colorado Health Science Center, Denver, CO). The purified Ab was biotinylated in our laboratory, and the anti-CD21 PE was conjugated at Southern Biotechnology. Anti-CD19 hybridoma (1D6) was a gift from Dr. Douglas Fearon (University of Cambridge, Cambridge, U.K.). In addition to the preceding, we used biotinylated JCS-1 (rat anti-mouse λ), produced and characterized in our laboratory. Two-, three-, and four-color surface staining was performed as previously described (44). Briefly, 106 cells were first incubated with a mixture of fluorescein-PE-, biotin-, and APC-conjugated Abs, followed by SA-PECy5, 15 min for each incubation. The cells were washed with 1% BSA/PBS between steps. For cytoplasmic staining, cells were first permeabilized with 0.5% paraformaldehyde in PBS for 30 min followed by 0.2% Tween 20 in PBS for 20 min. BrdU incorporation was analyzed in animals fed BrdU in the drinking water, as previously described (46), by staining the spleen cells for B220 and λ expression as well as staining with the anti-BrdU Ab (B4-ITIC, Becton Dickinson, Mountain View, CA). Data from stained cell samples were acquired on a FACScan or FACS Calibur with dead cells excluded using propidium iodide) using Lysis II or CellQuest (Becton Dickinson) and analyzed with WinList 2.01 (Veity Software House, Topsham, Maine) and WinMDI 2.0 (Trotter@scripps.edu) software programs.

Immunofluorescence of tissue sections

Spleens embedded in OCT compound (Lab-Tek Products, Naperville, IL) were flash frozen in liquid nitrogen. Frozen sections were cut, air dried, and fixed in ice cold acetone, blocked with normal horse serum, and stained. The following anti-mouse Abs and secondary reagents were used: biotinylated anti-CD4 (L3T4) and anti-CD8a (53-6-7) (PharMingen); goat anti-mouse μ-RTIC and goat anti-mouse A-FTTC (Southern Biotechnology); and SA7-αmino-4-methylumbelliferon-3-acetic acid (Vector, Burlingame, CA). Sections were washed and mounted in Fluoromount G (Southern Biotechnology) or Gel/Mount (Biomedia, Foster City, CA), then viewed with a Leica Leitz DMRB microscope equipped with appropriate filter cubes (Chromatechnology, Battleboro, VT). Images were acquired with a CS510 series digital color camera (Hamamatsu Photonic System, Bridgewater, NJ) and processed with Adobe Photo Shop and IP LAB Spectrum software (Signal Analytics Software, Vienna, VA).

ELISA

ELISA A/2 plates (Costar, Cambridge, MA) were coated with unlabelled anti-mouse μ Ab at 2 μg/ml in borate buffer (pH 9) and incubated at 4°C overnight, followed by serial dilutions of the transfectant supernatants or of control Ab (B1–8, mouse λκ, starting at 0.5 μg/ml) in 1% PBS-BSA and incubation at 37°C for 2 h. Alkaline phosphatase-conjugated goat-mouse λ Ab was used as the third-layer Ab and incubated for 2 h at 37°C. Between each step, the plate was washed five times with PBS. The plate was developed with alkaline phosphatase substrate (Sigma, St. Louis, MO) at 1 mg/ml in substrate buffer (pH 9.0). The plates were read on a Titerette spectrophotometer at 405 nm, and after 10 min, the OD of each well was plotted as a curve (see Fig. 64).

RT-PCR and sequence analysis of λ gene expression

RBC-depleted bone marrow or spleen cells (3–5 × 106) were used to extract RNA. B lineage cells (104–5 × 106 λ or λκ) were directly sorted into reaction tubes on a FACStar™plus (Becton Dickinson). Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and similar amounts of RNA were reverse-transcribed with 500 ng oligo(dT), dNTPs (5 mM), 20 U of RNAsin (Promega, Madison, WI), and 100 U of AMV reverse transcriptase (Life Technologies, Gaithersburg, MD) in a total buffer volume of 25 μl. PCR was performed using primers and conditions for β-actin, mb-1, and different λ rearrangements as previously described (47, 48). The samples were electrophoresed on a 0.8% agarose gel and blotted onto Nitran membranes (Schleicher & Schuell, Keene, NH). The membranes were prehybridized at 42°C (6× SSC, 0.25% SDS, 2× Denhardt’s solution, and 100 μg/ml denatured salmon sperm DNA), then hybridized using 3P end-labeled internal probes for MB-1 (5′-CATGGTGGTGACCCAGTGCTC-3′), λ (48), and β-actin as described (47). Blots were washed in 6× SSC and 0.1% SDS, then scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to detect signal intensity, and finally exposed to photographic film. In the case of sorted cells, if sequence analysis of λ rearrangements was desired, a nested amplification was performed using the 5′ VA1 and VA12 (48). For sequence analysis of λ junctions, PCR products were cloned using a TA cloning kit (Invitrogen, San Diego, CA), and sequencing was performed in the laboratory of the University of Alabama at Birmingham automated sequencing facility.

Transfection of J558L cell line

The same VH81X construct used to generate transgenic mice was cotransfected with a Neo construct (gift of Charles Mashburn, Howard Hughes Medical Institute, Birmingham, AL) into J558L as previously described (43). As control, we used a human VH4-Neo (gift of Dr. Thomas Kipps, University of California San Diego, CA). Subsequently, an mb-1-hyogromicin B construct (kind gift of Dr. Michael Reth, Max Planck Institute, Freiburg, Germany) was introduced in stable transficients selected in the first round. Selection was conducted in 1 mg/ml neomycin (Sigma) and/or 1.2 mg/ml hygromycin B (Calbiochem, San Diego, CA).

Results

VH81X transgenic heavy chains in association with λ light chains do not promote efficient B cell development

Splenic λκ- B cells in VH81X transgenic mice were fewer in number compared with nontransgenic littermates (1.3 ± 0.5% vs 4.3 ± 1.2% of the lymphoid gate, n = 12) (Fig. 1A). By analyzing B220+ cells in transgenic and littermate mice, a small but consistent population of λκ- B cells was easily detectable (3.1 ± 0.8% vs 8.5 ± 1.5% of the B cell gate, n = 12) (Fig. 1B). This phenotype was seen in two lines of VH81X transgenic mice, one with a single copy (Fig. 1A) and the other with approximately 12 copies (data

1 Abbreviations used in this paper: HSA, heat-stable Ag; SA, streptavidin; PE, phycoerythrin; PALS, periaorterolar lymphoid sheath.
transgene and chain rearrangements have rescued B cells that express the are not allelically excluded, suggesting that endogenous heavy chains develop in JH \( -/- \) transgenic mice, a clear population of B cells are displayed. \( \lambda^+ \) B cells are entirely missing in VH81X JH \( -/- \) transgenic mice (TG H \( -/- \)).

The absence of VH81X-\( \lambda \) B cells is not due to their inability to compete with VH81X-\( \kappa \) B cells

To determine whether any VH81X-\( \lambda^+ \) B cells could be formed, we generated VH81X JH \( -/- \) Ck \( -/- \) mice (VH81X kHD). In this case, the entire B cell receptor repertoire is formed by the association of the transgenic heavy chain with light chains derived from the remaining functional endogenous \( \lambda \) locus. In VH81X kHD mice, the VH81X transgene promotes the transition of large B220\(^{+}\)CD43\(^{+}\) pro-B cells into small B220\(^{+}\)CD43\(^{-}\) pre-B cells (Fig. 2A). Although small CD43\(^{-}\) pre-B cells are generated in comparable numbers in the bone marrow of VH81X kHD and VH81X JH \( +/- \) Ck \( +/- \) (Fig. 2A) mice, only the latter develop a substantial \( \lambda^+ \) B cell population in the bone marrow (not shown), spleen (10.5 \( \pm \) 2% of the lymphoid gate, \( n = 6 \)) and peritoneal cavity (38 \( \pm \) 9% of the lymphoid gate, \( n = 6 \)) (Fig. 2B). In the VH81X kHD mice, in contrast to the JH \( +/- \) Ck \( +/- \) (kHD) mice in which no \( \lambda^+ \) cells are found, a small but detectable population of \( \lambda^+ \) cells is found in both bone marrow (data not shown) and spleen (0.25 \( \pm \) 0.05% of the lymphoid gate, \( n = 6 \)). However, most of these B cells express 10- to 20-fold lower levels of surface \( \lambda \) light chains than normal B cells, as determined by flow cytometry. Although it is clear that the activity of a single intact endogenous heavy chain locus together with the transgenic heavy chain permits \( \lambda^+ \) B cells to develop, B cell development is abnormal, since a significant population of B220\(^{-}\)\( \lambda^+ \) pro-/pre-B cells are present in the spleens of adult VH81X JH \( +/- \) Ck \( +/- \) mice (Fig. 2A).
FIGURE 2. Development of VH81X-λ⁺ B cells is blocked in VH81X JH−/− k−/− transgenic mice (VH81X kHD). A, Bone marrow cells were stained with anti-B220, anti-CD43, and anti-IgM. The surface IgM-negative lymphoid cells were gated and their B220/CD43 profiles displayed. The VH81X transgene can promote normal development of pre-B cells (B220⁺ CD43⁻) in the absence of endogenous H and κ loci. B, Spleen and peritoneal cavity (PEC) cells are stained with anti-B220 and anti-λ. Small amounts of λlow B cells can be detected in the spleen but not in the peritoneal cavity of VH81X kHD mice.

2B). The low level-expressing λ⁺ cells were below detection limits in the peritoneum of the VH81X kHD mice (Fig. 2B). These data confirm the observations made in VH81X and VH81X JH−/− mice and show conclusively that there is a block in the development of VH81X-λ⁺ B cells.

VH81X-λ⁺ B cells are lost after the pre-B/B cell interface

Although a normal-sized small pre-B cell compartment is present in the bone marrow of the VH81X kHD mice (Fig. 2A), only a very small number of λ⁺ B cells develop, suggesting that there is a block in B cell progression at an immature B cell stage.

Immunohistologic analysis of spleen sections (Fig. 3, A–D) shows that pre-B/B cells from the VH81X kHD mice that have either migrated from the bone marrow or developed in the spleen appear to be localized in the T cell-rich areas around the arteriolar vessels (periarteriolar lymphoid sheath (PALS)) (Fig. 3, B and D). However, in contrast to B cells from VH81X JH−/+ κ−/− (Fig. 3A) they do not accumulate further outside the distal PALS to form primary B cell follicles. Both cytoplasmic μ only pre-B cells and λ⁺ B cells are found in the PALS, albeit at a low frequency (Fig. 3, D1 and D2). Most of the B cells in VH81X kHD mice probably die in situ at this stage since there is no appreciable accumulation of surface IgM/A high B cells elsewhere in lymphoid organs of these mice (Figs. 2B and 3B).

Most of the λ⁺ B cells detected in VH81X kHD mice have a mature phenotype

We next determined if the small numbers of λ⁺ B cells that are generated, accumulate to greater numbers in the spleens of old VH81X kHD mice. Mice up to 12 mo of age were analyzed and as seen in Figure 4A, a large normal B cell population never develops. The splenic B cell compartment, determined by flow cytometry as the proportion of B220⁺ cells within the lymphoid gate, is similar between 12-wk-old (4.13 ± 0.5%, n = 6) and 12-mo-old (4.33 ± 0.8%, n = 3) mice. Since B lymphopoiesis is considerably diminished in old mice, pre-B cells decline in numbers, so that in a B cell compartment of similar size, there are proportionally more λlow B cells (6.05 ± 0.9% at 12 wk vs 43.5 ± 5.1% at 12 mo). Surprisingly, phenotypic analysis of these λlow B cells revealed that most of them express several surface markers typical of mature B cells. Although surface IgM and λ are very low on these cells, they express CD21, CD22, CD23 (the majority), and CD24 (HSA) at levels similar to mature follicular B cells (Fig. 4B). In addition, by measuring their BrdU incorporation in vivo over a period of 7 and 14 days, only 20 and 33%, respectively, were labeled (Fig. 4B), in contrast to bone marrow-derived newly formed B cells that are all labeled within 3 to 4 days (50). To gain insight into the mechanisms that remove these cells that have a phenotype and life span more similar to long-lived recirculating B cells than to newly formed B cells, we analyzed their expression of Fas Ag. As seen in Figure 4C, VH81X-λ⁺ B cells (TG H−/− κ−/−), which express CD23, have higher levels of Fas than VH81X-κ⁺ B cells (TG H−/− κ⁺/−), providing a possible explanation for their disappearance.

All of the above data show that there is a major block in further development of the VH81X-λ⁺ B cells after the pre-B/B cell interface. Few pre-B cells develop into B cells, which express low levels of surface IgM, fail to organize into B cell follicles, and fail to generate a numerically normal B cell compartment. However, the maturation program of these B cells seems partially intact, since they express surface markers characteristic of normal mature B cells, but they also express higher levels of Fas.

The developmental block in VH81X-λ⁺ B cells is not due to abnormal λ rearrangements or failure of heavy chain-light chain pairing

Because of the genetic features of our transgenic model, we next determined whether the λ rearrangements giving rise to λ⁺ B cells are representative of the normal λ repertoire. Robust rearrangements at the λ locus involving all VA segments can be detected in the bone marrow of the VH81X kHD mice (Fig. 5). Such rearrangements are not seen at this level of sensitivity in kHD non-transgenic littermates. We also sequenced a panel of Vλ-Jλ-Cλ splenic rearrangements from mice with the genotypes described in Figure 5. From the heterogeneity observed at the Vλ-Jλ junctions, it is clear that these rearrangements are derived from a polyclonal set of B cells (Table I). The most frequent junctions (A, B, and C) in all of the experimental mice are the same as previously reported in normal BALB/c mice (48, 51, 52). These experiments show that abnormal rearrangement or transcription of λ light chain genes does not account for the observed developmental block in the VH81X kHD mice. In addition, since low levels of λ protein
are detected both on the cell surface (Figs. 2B and 4A) and in cytoplasm (Fig. 3D1), these mice can also translate the \( \lambda \) message into protein.

A second possibility for the observed block in development may be the impaired ability of the VH81X heavy chain polypeptide to pair with \( \lambda \) light chains. To study this possibility, we transfected the VH81X heavy chain into the J558L plasmacytoma variant, which has a commonly expressed functional VA1-JA1-CA1 rearrangement (type A in Table I) but no heavy chain (53). VH81X-\( \lambda \) protein secreted by these transfectants was detected in supernatant (Fig. 6A) by a standard capture ELISA (captured with anti-\( \mu \) and developed with anti-\( \lambda \) Abs). The VH81X-\( \lambda \) IgM Ab was also purified on an anti-IgH6a Sepharose column, and when analyzed on reducing and nonreducing SDS-PAGE gels, appeared to be intact and of the appropriate size (data not shown). In parallel, we generated five VH81X-\( \lambda \) hybridomas from three different VH81X kHD mice, and all were able to secrete VH81X-\( \lambda \) Abs. Although these experiments prove that the VH81X heavy chain can associate with \( \lambda \) as a secreted Ab, it was essential to determine the ability of the two proteins to form a surface receptor. Due to the lack of Ig-\( \alpha \) protein, J558L-VH81X stable transfectants do not express a surface IgM receptor (Fig. 6B), as described previously for other heavy chains (53). However, after transfection of the mb-l cDNA into J558L-VH81X cells, surface expression of IgM receptors was detected in several independent lines at levels comparable with J558L cells expressing a VH4 human \( \mu \) chain used as control (Fig. 6B) and with the J558L-\( \mu \)M3 previously described (43). From this in vitro system, we conclude that surface expression of the VH81X heavy chain-\( \lambda \) light chain complex can occur and that failure of surface receptor expression is unlikely to be the cause of the block in VH81X-\( \lambda \) B cell development. Since \( \lambda \) represents the majority of \( \lambda \) rearrangements (=70%), and the junction A (Table I; used in the J558L \( \lambda \) chain and repeatedly isolated in the analysis of our mice) is the most commonly used junction in all Abs (48), these data provide evidence for the ability of the VH81X \( \mu \) chain to pair with the available \( \lambda \) light chains in vivo.

A final and most likely explanation for the developmental block is that the VH81X-\( \lambda \) B cell receptor delivers a negative or inappropriate (lacking positive selection) signal to the emerging B cell, preventing further maturation. We next screened the VH81X-\( \lambda \) IgM Abs from a transfecctoma and hybridomas on different mouse tissues and cell lines by methods used in our laboratory to identify self-reactive Abs, but no self-reactive Abs were found (Fig. 6C). Although the VH81X-\( \lambda \) Ab has multireactive specificities as assayed by Western blots of mouse tissue lysates (P. Zimmer and J.F.K., unpublished results), the pattern is similar to other VH81X-\( \kappa \) control Abs isolated from hybridomas derived from mature splenic B cells in VH81X transgenic mice. Furthermore, the VH81X-\( \lambda \) repertoire is polyclonal (VA1-JA1-CA1, VA1-JA3-CA3, VA2-JA2-CA2, VAx-JA2-CA2 each with junctional diversity), and
it seems unlikely that all possible VH81X-\(\lambda^\circ\) combinations form self-reactive receptors that ensure deletion of all B cells expressing these receptors. As a way of assaying at a molecular level whether there is selection of the array of potential \(\lambda^\circ\) chains between the pre-B compartment in which the \(\lambda^\circ\) light chain genes rearrange and the more mature B cells, we sorted B220\(^{+}\) and B220\(^{-}\) low cells from the VH81X kHD spleens and compared the rearrangements found in these two populations. A similar polyclonal repertoire containing \(\lambda^1, \lambda^2, \lambda^3,\) and \(\lambda x\) was found in both populations (data not shown), and sequence analysis of the \(\lambda 1\) rearrangements revealed the same major hierarchical representation of junctions in both populations (Table I, last two columns). These results collectively argue against a large scale negative selection step between the pre-B cell compartment, which is normal in size, and the drastically reduced in size B cell compartment, suggesting that the entire polyclonal VH81X-\(\lambda^\circ\) repertoire is blocked in development at this point.

Is the VH81X-\(\lambda^\circ\) developmental block unique for the VH81X transgene?

To compare the ability of Ig heavy chain transgenes to generate \(\lambda^\circ\) B cells, we studied other independently derived \(\kappa\) transgenic mice. Although it is known that several \(\mu\) transgenes—MD2, 3H9, anti-H2K, and M54—generate mature \(\lambda^\circ\) B cells, a comparative analysis between these individual transgenic mice has not been performed (12, 37, 54–56). We compared the abundance of \(\lambda^\circ\) B cells within the splenic B220\(^{+}\) compartment of VH81X, M167 (57), MD2 (6), and M54 (58) transgenic mice (Fig. 7). In the VH81X, MD2, and M167 lines, 20 to 55% fewer \(\lambda^\circ\) B cells are present in the transgenic vs the littermate mice (Fig. 7). As shown above, in the case of VH81X, all of these \(\lambda^\circ\) B cells are rescued by an endogenous heavy chain rearrangement. Both the anti-HEL heavy chain (MD2 transgenic mice) and the M167 heavy chain (M167 transgenic mice) can generate transgene-\(\lambda^\circ\) B cells (37, 59). The reduction in the number of \(\lambda^\circ\) B cells in the VH81X, MD2, and M167 transgenic mice compared with littermates (Fig. 7) suggests that in the establishment of the mature repertoire, transgenic B cells expressing \(\kappa\) light chains are at an advantage. An opposite situation is apparent in the CB17 M54 mice, in which more \(\lambda^\circ\) B cells are present in the transgenic (10.3 ± 1.5%) than littermate (4.2 ± 0.9%) mice, suggesting that in this case the M54 heavy chain \(\lambda^\circ\) B cells have a selective advantage. Interestingly, the original hybridomas from which the transgenic heavy chains were derived are all \(\kappa\) with the exception of M54, which was derived from a \(\lambda\)-producing anti-nitrophenyl-acetyl (NP) hybridoma (58).
VH81X-κ+ B CELLS

These data suggest that B cells expressing individual heavy-light chain pairs are subject to a selection process at the level of the immature B cell stage before they reach the mature B cell compartment. For the particular case of λ, VH81X is at one extreme (totally unable to generate λ B cells), M54 is at the other extreme (having a predilection for λ B cells), while M167 and MD2 are intermediate in their ability to produce λ+ B cells.

Discussion

We investigated the generation of the mature primary B cell repertoire using transgenic mice as a model in which a limited population of VH81X-λ+ B cells were generated alone or in conjunction with a diverse population of VH81X-κ+ B cells.

We showed previously that a largely polyclonal κ repertoire develops in VH81X transgenic mice with VH81X-Vκ pairs being preferentially selected into distinct peripheral B cell subsets (39, 60). However, VH81X-λ+ B cells, which suffer a different fate, are missing in the mature B cell subsets. The VH81X-λ+ cells are not generated either in the presence of a heterogeneous VH81X-kappa repertoire or in the absence of competition with Vκ-expressing B cells in VH81X kHD mice. Genomic λ rearrangements and λ polypeptide synthesis appear normal, as does the capacity of the transgenic VH81X heavy chain to pair with λ light chains in vitro. The possibility remains that in pre-B cells, in contrast to plasma-cytoxins, there are subtle imperfections in molecular mechanisms of heavy-light chain pairing or in the folding and transport of the assembled receptors to the surface. It is also clear that pairing of the same heavy chain transgene with a wide variety of κ light chains can occur to form functional B cell receptors. In a parallel experiment using >100 transgenic hybridomas generated from VH81X spleen cells, all but two of the hybridomas had functional κ light chains belonging to 11 different families (39). The two exceptions, which expressed λ light chains, had also productive endogenous IgH6b gene rearrangements (J.F.K., unpublished observation).

The block in the development of VH81X-λ+ B cells occurs after the pre-B/B cell transition, the point at which it has previously been reported that half of the loss of VH81X-expressing B cells occurs in normal BALB/c mice (61). A few of the B cells that are generated move through immature stages and reach a longer-lived compartment in which they display low levels of clonal receptors and are then removed. Although at present we cannot completely disprove the explanation that negative selection resulting in B cell deletion is the cause of the developmental block in our mice, the only self Ags for which this has been shown are dsDNA or membrane-bound HEL and H-2K. Recent studies have shown that even very low affinity membrane expressed self Ag are able to induce central tolerance by deletion (38). Our attempts to screen for potential deleting Ags using VH81X-λ Abs failed to reveal any specific self-reactivity to the mouse tissues and cell lines that we tested. In addition, the λ repertoire is quite diverse, with four possible Vλ-Jλ-CA combinations and superimposed junctional diversity. It has also been shown that in cases of self-reactive Abs, such as in the 3H9 heavy chain transgenic mice, only 3H9-A1 reacts with dsDNA while the other λ combinations that are generated do not (55).

The presence of elevated Fas expression on the VH81X λ+ B cells also argues against a central deletion mechanism (62, 63) as the cause of the cell loss and suggests the possibility that a peripheral B-T cell interaction is involved in the homeostatic maintenance of the B cell compartment as described for foreign antigenic stimulation (30, 64). The level of Fas expression on VH81X λ+ B cells is similar to that expressed by tolerant B cells that were

Table I. The λ1 junction of splenic B lineage cells from VH81 × kHD (H –/+ , k –/- , TG+) and littermates as well as from VH81 × kHD-sorted B220+λ− and B220+λlow populations

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* Nomenclature of individual rearrangements - as described in Reference 48.
subsequently reexposed to Ag and is lower than on foreign Ag-stimulated B cells (30). These observations might explain the topographical location of these unusual cells and their failure to develop B cell follicles. From our data, we favor the explanation that the block is most likely due to a failure to receive the proper balance of signals (positive and negative) inducing further development or maintenance.

Evidence is accumulating for positive selection steps involving surface Ig signaling at the immature B cell stage that is necessary for further development. In the soluble HEL/anti-HEL system, by manipulating the accessory molecule CD45, which is involved in the transduction of sIgM mediated signals, it was shown that B cells lacking CD45 entered the mature repertoire much more efficiently when soluble HEL was present (23). In addition, the increased size of some B cell compartments from CD45+/− mice might be due to positive selection events (65). Another genetic manipulation of signaling through the sIgM receptor in syk-derivative signal was found to be necessary for further B cell selection and/or expansion but not for negative selection (66). In all of these cases, the signal was manipulated indirectly through accessory molecules, while in our system the block appears to be due to the composition of surface IgM receptor itself. Although we cannot estimate how frequently rearranged germline V<sub>H</sub> genes fail to match with appropriate light chains to permit delivery of a functional signal at this crucial selection point, this case is unlikely to be restricted to VH81X-<sub>A</sub> receptors. It appears that VH12 heavy chain may not generate B cells in association with A light chains (S. Clarke, unpublished observations). In addition, it is possible that B cells are not generated in a VH1-V<sub>K</sub>22 (T15) “monoclonal” mouse due to a similar phenomena (F. Alt, unpublished observations). In the sp6-rag−/− transgenic mice (67), a similar B cell block occurs, which is partially released by the i.v. injection of trinitrophenyl Ag. Since, in this case, reactivity of the transgene-encoded receptor with dsDNA can also be demonstrated, a deletional mechanism was proposed as being responsible for the observed block. A very recent report investigates the development of different A types in the M54 and H3 heavy chain transgenic mice and concludes that positive selection favors different transgenic A B cell clones in the two models (68).

We have compared and summarized the observations made in the generation of VH81X-<sup>A</sup> cells and B cell development in current transgenic and gene deletion mice (Fig. 8). The B cell developmental program is considered from four independent aspects: 1) initial clonal receptor development, 2) appearance of maturation markers, 3) subsequent migration patterns in vivo, and 4) clonal life span. Normal mice as well as nonautoimmune transgenic mice have all four programs intact (6, 7, 40, 68), while a first group of genetic defects including scid and rag−/− μTG (65, 69) have cells that are blocked completely at the pre-B stage in vivo. Under certain conditions of in vitro growth or activation, portions of these programs can be resurrected (70) but give rise to cells that do not have in vivo counterparts in intact mice. A second group of defects occur in the deletional models of B cell self-reactivity (6, 8, 71) in which these four programs are blocked at an immature B cell stage. Introduction of a bcl-2 transgene into mice of the first group releases the block in maturation markers and migration pattern of pre-B cells that now express mature markers and can form (pre-B) follicles in the spleen (72, 73). However, if a bcl-2 transgene is introduced into mice of the second group, as represented by the mHEL/anti-HEL deletional system, the maturation program is only partially released (50); there have been no reports, however, on the topographical migration pattern of B cells in peripheral lymphoid organs of these mice. Our VH81X-<sub>A</sub> transgenic model resembles the deletional transgenic mouse models with a block in clonal receptor selection and further migration; but in contrast to...
other models, they appear to have at least part of the maturation marker program intact. A possibility exists that very small numbers of B cells with a mature phenotype also develop in the above-mentioned deletional models, but these have not yet been described. Each of the maturation markers is up-regulated by signals from the environment through which the maturing B cell moves. Therefore, the chronologic period from the generation of B cells until their removal by death might be different in each of these transgenic models depending on the form, location, and affinity of the specific self Ag. The subsequent maturation marker profiles identified on the B cells that develop in each system may reflect the intrinsically different characteristics of each mouse. In turn, the fate of B cells in these systems viewed collectively reflects the normal fate of heterogeneous populations of cells expressing receptors to Ags of different characteristics and presentation modes.

In conclusion, we have investigated at a molecular and cellular level the inability of a polyclonal germline-encoded receptor-expressing B cell population (VH81X-\(\lambda\)) to develop in the peripheral repertoire and have defined precisely the site of the block. Abs containing the VH81X-\(\lambda\) chains do not seem to be highly self-reactive, suggesting that the lack of positive selection is the operative mechanism in this system and that it prevents further maturation and expansion of a normal peripheral B cell population. In addition, by comparing the \(\lambda\) compartments in other well-characterized heavy chain transgenic mice, we show that there is a hierarchy in the frequency of B cells expressing heavy chain-\(\lambda\) pairs entering the mature repertoire.

Our model will permit the study of signals and environmental factors involved in the generation and maintenance of germline mature B cells. Positive selecting stages are likely to be identified in B cell generation and maintenance, comparable to T cell development in which positive selection is well established. The process favoring certain B cell receptor heavy-light chain pairs would be analogous to the thymic positive selection at the double-positive stage, where pairs of TCR \(\alpha\)- and \(\beta\)-chains are selected together to

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**FIGURE 7.** Development of \(\lambda\) B cells in the spleen of several \(\mu\) transgenic mice (VH81X, M167, MD2, M54). A. Spleen cells stained with anti-IgM, anti-\(\lambda\), and anti-B220 Abs as well as B220\(^+\) cells within the lymphoid gate are displayed. All four transgenic mice (TG) are IgH6a, while littermates (LM) are IgH6b. Representative profiles of three independent experiments are shown. B. Quantitative representation of \(\lambda\) B cells as percentage of B220\(^+\) cells in the four \(\mu\) transgenic mice. Three to four mice analyzed on the same day are included in each group.
form a mature functional TCR repertoire, while the peripheral long term maintenance of B cell clones may depend on continuous low level interactions of the receptor with Ag in a self environment similar to that required by mature T cells (74).

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