Editing Anti-DNA B Cells by Vλx

Yijin Li,[1] Yoram Louzoun,[2] and Martin Weigert[1]

[1]*Department of Molecular Biology, Princeton University, Princeton, NJ 08544*
[2]*Department of Mathematics, Bar Ilan University, Ramat Gan 52900, Israel*

Abstract

Receptor editing is performed by replacement of Vκ genes that contribute to autoreactivity. In addition, the Cκ locus can be deleted by Vκ rearrangement to intronic or 3′ of Cκ RS sequences (also referred to as κ deletion elements). B cells that delete the Cκ can then express λ light chains. However, the λ locus, either of man or mouse, does not allow V gene replacement. Nor does it appear to be deleted. Therefore, editing of autoreactive λ B cells may require alternative pathways. We have found that in anti-DNA heavy chain transgenic mice (tgs) V_H3H9/56R, B cells that express anti-DNA receptors comprised of λ1 in association with an anti-DNA heavy chain often coexpress a κ chain that prevents DNA binding. We speculate that such isotypically included cells may have low anti-DNA receptor densities, a feature that may lead to self-tolerance. Here we describe a mechanism of preventing DNA binding by expression of a rarely used member of the Vλ family, Vλx. The λx B cells of the tgs also express CD25 and may represent B cells that have exhausted light chain editing possibilities.

Key words: autoimmunity • lupus • CD25 • light chain • myelin basic protein

Introduction

Self-reactive B cells are rendered tolerant by several mechanisms including inactivation, receptor editing, and deletion. In addition, interaction with self-antigen may impede exit from the bone marrow, in which case the B cells may die in situ. Which mechanism(s) is used depends on the state of self-antigen (i.e., soluble vs. membrane-bound) and on the avidity for a self-antigen (1–6). Anti-DNA transgenic mice (tgs) are important because the transgene-encoded antibody is directed against a constitutive self-antigen, DNA, the major target of autoantibodies in SLE (7). The 3H9 anti-DNA model is especially relevant because the genes coding for 3H9 anti-DNA were derived from a diseased MRL/lpr mouse and the 3H9 antibody is typical of the pathogenic anti-DNAs in SLE (8). Understanding how this autoantibody is controlled in healthy mice is important for understanding why self-tolerance fails in SLE.

The 3H9 anti-DNA has unique and useful properties. Although most antibodies bind antigen through a combination of V_H and V_L residues (9), DNA binding by 3H9 is mediated mainly by arginine residues in V_H (10). Hence, the H chain tgs by themselves are informative because they can be studied in the context of the endogenous L chain repertoire. The H chain–only tgs show that the majority of V_Ls have no influence on binding but some V_Ls (editors) can modify or inhibit the V_H–DNA interaction (11). Healthy 3H9 H chain tgs express the 3H9 H chain, but only in combination with V_L editors (5, 6).

The range and efficiency of editing is influenced by the antigen-binding characteristics of the antibody: anti-ssDNA (represented by the 3H9/Vκ8 combination) are inactivated (4), whereas anti-dsDNA and anti-phosphatidylserine are edited (5, 6, 12). Anti-DNAs, such as 3H9, are useful in this regard because the stringency of DNA binding can be modified simply by adding or subtracting arginine residues (10). Thereby we can extend our study to transgenes with a range of affinities for DNA. Studies on anti-DNAs tgs derived from such modified H chains show that the tolerance is mainly achieved by receptor editing. Although editing is an efficient mechanism, it can result in allelic and isotypic (κ and λ) inclusion. We have observed increased inclusion in V_H3H9 and V_H3H9/56R tgs (5, 13). Typically, allelically and isotypically included B cells coexpress an L chain that permits DNA binding and an L chain editor.

Another effect of receptor editing is to bias the repertoire of V genes toward those that rearrange late or rarely in the sequence of rearrangement. We have discovered a population of B cells in the V_H3H9/56R transgenic mouse (tg) that is

Address correspondence to Yijin Li, Gwen Knapp Center for Lupus and Immunology Research, The University of Chicago, 924 East 57th St., Rm. 413A, Chicago, IL 60637. Phone: (773) 702-2719; Fax: (773) 702-1576; email: yijinli@uchicago.edu

Abbreviations used in this paper: IEP, isoelectric point; MBP, myelin basic protein; tg, transgenic mouse; tgs, transgenic mice.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/02/337/10 $8.00
Volume 199, Number 3, February 2, 2004 337–346
http://www.jem.org/cgi/doi/10.1084/jem.20031712

extremely biased toward Vλx-Jλ2. This L chain is an editor as it has a low isoelectric point (IEP) and, when combined with 3H9/56R, the combination does not bind DNA (14). Because λ genes are thought to rearrange after κ genes (15, 16), this population is another example of the influence of editing on V gene repertoire. This population is unusual because it bears the activation marker, CD25, and we discuss the possible origin of this population.

Materials and Methods

Mice. Site-directed V_H3H9/56R H chain tgs on BALB/c or CB17 background have been described (17). The tgs were crossed to the immunoglobulin κ-deficient (Jκdel) mice (18). All mice used in the experiments were between 6–8 wk old.

Genotyping of the tgs. The PCR for typing the V_H3H9/56R tgs has been described (17). For typing the Jκdel mice, two separate PCR assays were performed with the following primer pairs: to detect the targeted inactivation of the κ gene by JκCκ deletion (18), the forward primer 5′-AAGAGCTTGGCGGCGAATGG-3′ corresponding to the inserted neo gene and the reverse primer 5′-CAAACCTACCATGGCCAGAGA-3′ corresponding to the remaining 3′ region of the κ locus were used. The wild-type κ locus (κ^+) was detected by the forward primer 5′-CTGTAAAT-CACATTCAGTGATGG-3′ corresponding to Jκ3 and the reverse primer 5′-CCGAGCCGAACGTGAATCACA-3′ corresponding to Jκ4. Both PCRs were performed with a final concentration of 250 μM of each dNTP and 2 mM $MgCl_2$ at 60°C annealing temperature.

Flow Cytometry. The following antibodies were used for flow cytometry: FITC-conjugated polyclonal goat anti–mouse κ (Fisher Scientific), biotin-conjugated anti-λ (R26-46), PE-conjugated anti-CD25 (3C7), APC-conjugated anti-B220 (RA3-6B2), FITC-conjugated anti-IgD^a (AMS9.1), biotin or PE-conjugated anti-IgD^b (217-170; BD Biosciences), FITC-conjugated anti-IgD (11-26), and biotin-conjugated goat anti–IgM (Southern Biotechnology Associates, Inc.). Stained cells were analyzed using a FACScan™ flow cytometer and CELLQuest™ software (Becton Dickinson).

Cell Sorting. Splenic B cells from V_H3H9/56R tgs were stained with FITC-conjugated anti-CD19 (1D3) and PE-conjugated anti-CD25 (3C7; BD Biosciences). Dead cells were excluded by propidium iodide staining. The $CD25^+/CD19^+$ and the $CD25^-/CD19^+$ fractions were sorted using a FACS Vantage™ flow cytometer (Becton Dickinson).

PCR. Total RNA was prepared from sorted B cells with RNeasy Mini Kit (QIAGEN). RT was performed using First-Strand cDNA Synthesis Kit (Amersham Biosciences). In addition, genomic DNA from sorted cells was purified using the previously described protocol (19). PCR-detecting Vλx rearrangement was performed as previously described (19). The PCR products were cloned into pGEM®-T easy vector (Promega). Approximately seven to eight clones of each PCR were randomly picked and sequenced with the T7 primer. Cκ deletion was detected using Vs as a forward primer and RS-101 as a reverse primer as previously described (20). The control actin PCR was performed using the forward primer 5′-GGTGTCATGGTAGGTATGGGT-3′ and the reverse primer 5′-GATGTCGCGCACAATCTCACGT-TCAG-3′ (21).

Single Cell PCR. Spleen B cells were enriched by purification with anti-B220 magnetic beads (Miltenyi Biotec). The cells were then stained with anti-CD19 FITC and anti-CD25 PE and sorted into 96-well plates. cDNA was synthesized according to the previously described protocol (22) and was used in two types of PCR-detecting Vλx-Jλ2 and V_H3H9/56R transcripts. Each type of PCR has two rounds of reactions (40 μl reactions with 10 pmol of each primer, 1.56 mM $MgCl_2$, and 0.2 μl AmpliTag Gold). 50 cycles of PCR were performed at 94°C for 20 s, 55°C for 40 s, and 72°C for 1 min. The following primers were used for Vλx-Jλ2: first round ACCTTGAGTAGTCAGCACAG and CTAGGACAGTGACCTTGGTT; second round GAGCTTA-AGAAAGATGGAAGCCA and GTTCCACCGCCGAAAAC-ATA. The following primers were used for V_H3H9/56R: first round CAGGTTCAACTGCAGCAGTC and GGGATCCTG-GGAAGGACTGACTCTC (Cμ; reference 23); second round AGTAGCTCCTGGATGAACTGG and CATAACATAGGA-ATATTTACTCCTCGC.

Generation of Rabbit Anti–Vλx Antibody. A peptide corresponding to a sequence spanning the FWR2 and CDR2 region of Vλx (N′-CPPKYVMELKKDGSHSTGD-C′), which has no homology to Vλ1 or Vλ2, was synthesized. The amino-terminal cysteine was added for conjugation. The peptide was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (Pierce Chemical Co.). 0.5 mg immunogen in PBS was mixed with Freund's adjuvant (GIBCO BRL) and injected into a rabbit. The animal was boosted every 28 d with 0.2 mg immunogen mixed with Freund's adjuvant (GIBCO BRL). Blood was collected 14 d after each injection. IgG was precipitated from the serum using Na_2SO_4 at a final concentration of 0.18 g/ml of serum. The precipitates were dissolved in 17.5 mM sodium phosphate buffer. Anti-Vλx antibody was purified using an affinity column of the synthetic peptide conjugated to SulfoLink Coupling Gel (Pierce Chemical Co.).

Magnetic Sorting and Western Blot. $CD25^+$ B cells were enriched magnetically after staining with PE-conjugated anti-CD25 (3C7) followed by attachment to anti-PE magnetic beads (Miltenyi Biotec). The $CD25^-$ B cells from the same mice (flow through of anti-CD25 purification) and B cells from nontransgenic BALB/c mice were purified with anti-B220 magnetic beads (Miltenyi Biotec). Cells were lysed on ice for 30 min in buffer containing 1% Triton X-100, 50 mM Tris, pH 7.6, 150 mM NaCl, and 1 mM AEBSF (Sigma-Aldrich). The lysate was sonicated and centrifuged for 20 min at 12,000 *g*. Supernatant was mixed with SDS sample buffer and boiled for 5 min. Proteins were resolved on a 10% SDS PAGE under reducing conditions and transferred to PVDF membrane (Bio-Rad Laboratories) at 200 mV for 2 h. After blocking with 5% nonfat milk in PBS, the blot was probed for 1 h at room temperature with rabbit anti–Vλx. The membrane was washed three times for 5 min with PBS containing 0.05% Tween 20, incubated with horseradish peroxidase–conjugated goat anti–rabbit IgG (Bio-Rad Laboratories), and developed using SuperSignal West Pico Kit (Pierce Chemical Co.).

Computation of κ and λ Expression Frequency. The frequency of κ and λ L chain expression was computed using a Monte Carlo computer simulation. The simulation design and mathematical details have been described (24) and are at http://www.cs.biu.ac.il/~louzouy/simulations/kl.htm. We describe hereby the details relevant to the current analysis. The simulation contains one κ locus (with each allele rearranging separately) and two λ loci (λ1 and λ2/λx). Rearrangement is assumed to occur simultaneously and independently on each allele. Rearrangement is modeled as a stochastic process. Rearrangement was determined by the probability of rearrangement initiation (0.1 per cycle) and by the length of the rearrangement process. The low initiation probability reduces the number of cells presenting two different functional κ alleles and is

consistent with Schlissel's (25) estimate. κ allelic exclusion is thus practically guarantied by the low initiation probability. κ/λ isotypic exclusion is guarantied by the longer rearrangement process in λ (50 cycles) than in κ (5 cycles).

At the κ locus rearrangement is between any Vκ and a Jκ. Jκ is chosen according to a quasi-sequential model (unpublished data). In the λ1 locus rearrangement is between Vλ1 and Jλ1 (i.e., we ignore the possible rearrangement to λ3). At the λ2/λx locus, we assumed that half the rearrangements are Vλ2 to Jλ2 and half are Vλx to Jλ2 (26).

Further rearrangements are required if the resulting L chain is either out of frame or anti-self. We assumed that 33% of all rearrangements are in frame and that in the non-tg self-antigen binding is rare (and thus in this simulation neglected). In the V_H3H9/56R tg, we assumed that all Vκs except for Vκ21D, Vκ20, and Vκ38c (editors) combined with any Jκ gene and all Vλs except for Vλx are anti-self. Note that rearrangement on all alleles was assumed to be simultaneous, thus multiple functional L chains can be produced. If the L chain does not bind self-antigen, a nonself L chain double expresser can be produced. If one of the L chains binds self-antigen (in the context of the given H chain) and the other one does not, we assumed that the cell would mature only if the majority of L chains are from the nonself type (24). An example of a partially self-reactive double expresser cell would be the combination of a Vκ editor combined with a Vλ1 rearrangement.

If rearrangement is either anti-self or nonfunctional on all L chain loci, rearrangement continues until either all κs and λs are rearranged or the cell dies. Cell death is introduced using a crash factor added to the Langman and Cohn concept (27) to explain the observed κ to λ usage ratio (15–20:1) in non-tg (see Discussion). Note that λ rearrangement cannot be deleted. Thus, an anti-self λ rearrangement must lead either to cell death or to partially self-reactive double expresser.

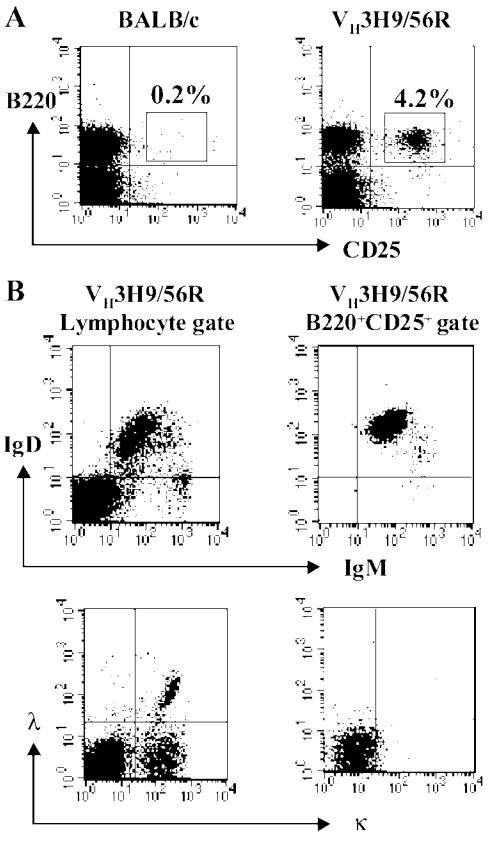

Figure 1. CD25+/IgM+/IgD+ spleen B cells of V_H3H9/56R mice. (A) Spleen cells from V_H3H9/56R BALB/c mice and its nontransgenic littermate were stained with anti-B220 and CD25. Percentages of CD25+/B220+ cells in a lymphoid gate are indicated. (B) Spleen cells from V_H3H9/56R BALB/c mice were stained with κ, λ, IgM, and IgD. Results of IgM/IgD and κ/λ staining are shown for the whole lymphocyte gate (left) and the B220+/ CD25+ gate (right).

Results

V_H3H9/56R tgs Have a Population of CD25+ Spleen B Cells That Express the λx L Chain. In our studies on site-directed anti-DNA H chain tgs, we found that the V_H3H9/56R BALB/c tg has an unusual set of spleen B cells that express the activation marker CD25. CD25 is the IL-2 receptor α chain and is reported to be expressed on both pre–B cells and activated B cells (28, 29). The CD25+ B cells represent 13.2% (SD 2.2, $n = 7$) of the B220+ B cell population of V_H3H9/56R, but these B cells are not detectable in either the nontransgenic littermates of this tg (Fig. 1 A) or tgs with anti-DNA H chain transgenes such as 3H9 and 3H9/56R/76R (unpublished data).

Like normal mature follicular B cells, these CD25+ B cells are IgM+ and IgD+. Yet they attracted our attention because no L chain expression could be detected by the commercially available anti-κ or anti-λ antibodies (Fig. 1 B). However, the frequency of these CD25+ B cells is higher in V_H3H9/56R κ-deficient mice, suggesting that these B cells do express λ chain. The CD25+ population size is inversely proportional to the number of κ alleles. Deletion of one κ allele increases the percentage to 18.1 (SD 3.6, $n = 4$) and 55.0% (SD 18.1, $n = 4$) when both κs are deleted (Fig. 2). The most likely λ chain is the Vλx. Although this Vλ is located in the Jλ2-Cλ2 locus and is usually rearranged to Jλ2, its V region is only 33% identical to the Vλ2 (Identity to Vλ1 and mouse Vκs is also <35% but homology to human Vλ5-6 and Vλ5-1 is high, at 75 and 70%, respectively.). If anti-λ antibodies are directed to the V region, then Vλx/Cλ2 may not be recognized by these reagents. Indeed, molecular analysis of L chain rearrangement (see below) shows that Vλx is rearranged in the CD25+ population.

Vλx is barely detectable in normal mouse serum but reaches significant levels in κ-deficient mice (26, 30). Vλx has also been found in the hybridoma panel generated from the tgs that express the 3H9/56R H chain (14). As shown in Fig. 2, we find that ~21% of B220+ B cells of nontransgenic Jκdel/Jκdel mice have no L chain according to either the anti-κ or anti-λ antibodies discussed above. We presume that they express Vλx. In the nontransgenic κ-deficient mouse, the κ−/λ− (putative λx population) is not CD25+, whereas most or all of the comparable population is CD25+ in the tg (Fig. 2). Hence, 3H9/56R/λx B cells per se express the activation marker. Other activation markers such as CD69 or elevated Fas are not found on

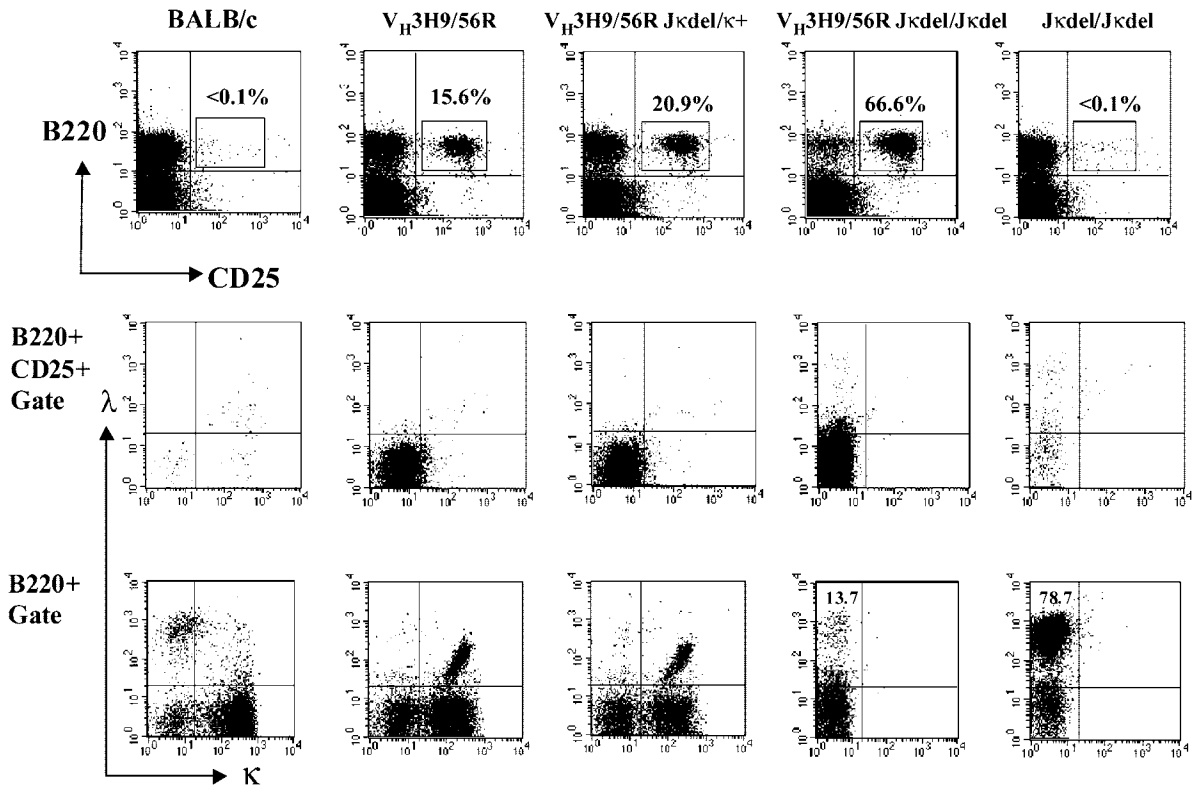

Figure 2. Deletion of κ L chain loci increases the frequency of $CD25^+$ B cells that express Vλx. Spleen cells from V_H3H9/56R and κ L chain–deficient mice (Jκdel/κ+, Jκ/Cκ deletion on one κ allele; Jκdel/Jκdel, deletion on both alleles) with or without the 3H9/56R transgene were stained with anti-B220, CD25, κ, λ, IgM, and IgD. The percentages of the $CD25^+/B220^+$ cells in the $B220^+$ B cells are indicated in the top. On the middle and bottom panels are the κ/λ staining of cells in the $CD25^+/B220^+$ and the $B220^+$ gates, respectively.

these cells. Nor do they express CD11c or CD103, markers that are expressed by hairy cells, the $CD25^+$ clonally expanded mature activated B cells (31 and unpublished data).

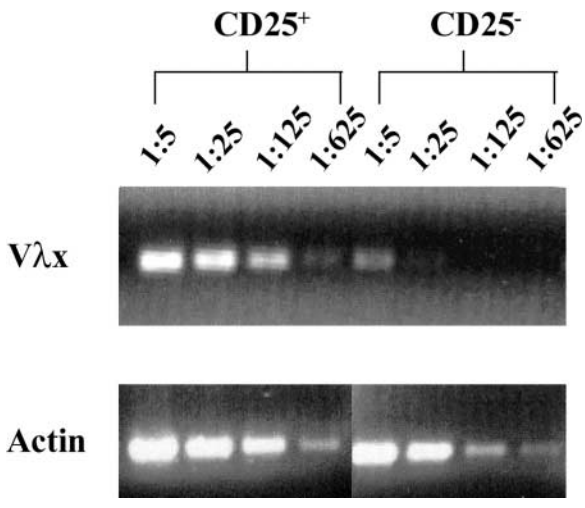

Figure 3. $CD25^+$ B cells express the Vλx-Jλ2 mRNA. $CD25^+/CD19^+$ and the $CD25^-/CD19^+$ B cells were isolated from the spleens of V_H3H9/56R mice using anti-CD19 and CD25 antibodies and a FACS Vantage™ flow cytometer. cDNAs were synthesized from total RNA extracted from both fractions. Vλx-Jλ2 PRC was performed on serial dilutes of cDNA from each sample. The bottom panel shows the actin PCR.

Molecular Analysis of Vλx. To confirm that conventional L chains are replaced by Vλx, we sorted the $CD25^+$ cells from the spleen of V_H3H9/56R tg and performed a Vλx RT-PCR. We used a Jλ2 primer and a Vλx primer corresponding to a sequence spanning the FW2 and CDR2 region, which has no homology to Vλ1, Vλ2, or Vκ. A stronger signal is detected in the $CD25^+$ fraction than the $CD25^-$ B cells sorted from the same mice (Fig. 3). PCR using genomic DNA from sorted cells shows similar results (see Fig. 6, middle).

To detect the expression of Vλx at protein level, a polyclonal antibody was generated by immunizing a rabbit with a peptide corresponding to a sequence spanning the FWR2 and CDR2 region of Vλx, which has no homology to Vλ1 or Vλ2. Western blot using cell lysate of λx, λ1, and κ hybridomas demonstrates that the antibody recognizes a single band with the molecular weight of 27 kD from the lysates of λx hybridomas and does not cross talk with λ1 or κ L chains (Fig. 4 A). The observed molecular weight of λx is about the same as that which has been reported by another group using their antibody (32). We then did the Western blot using cell lysates from purified $CD25^+$ B cells, $CD25^-/B220^+$ B cells from the tg, and $B220^+$ B cells from non-tg BALB/c mice. The $CD25^+$ cells shows a strong band of λx. The $CD25^-/B220^+$ cells sorted from the same mice show a much weaker band and $B220^+$ cells from non-

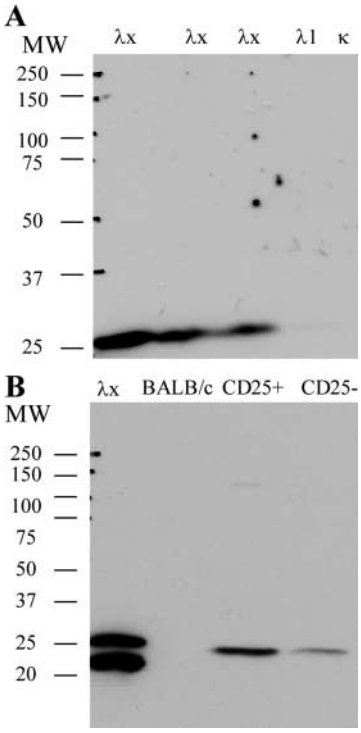

Figure 4. Western blot analysis of λx. (A) Verifying the specificity of anti-Vλx antibody. Cell lysates of three different λx hybridomas (lanes 1–3; chosen because they are positive for Vλx-Jλ2 by PCR but negative in both κ and λ ELISA), a λ1 (lane 4), and a κ hybridoma (lane 5) were used for the analysis. (B) Detection of λx protein expression in $CD25^+$ cells. Cell lysates of purified $CD25^+$ and $CD25^-/B220^+$ B cells from the tg and $B220^+$ B cells from non-tg BALB/c mice were used for the Western blot. The positive control (lane 1) is the supernatant collected from a λx hybridoma. The lower 23-kD band is most likely a degradation fragment of λx.

transgenic BALB/c mice show no signal (Fig. 4 B). We were not able to use the antibody to perform flow cytometry as it only recognizes the denatured λx protein.

The $CD25^+$ B Cells Express the Transgene Allele. The $CD25^+/λx$ population appears to depend on the presence of the 3H9/56R transgene. As discussed above, λx B cells in non-tgs are $CD25^-$. However, hybridoma panels from this tg show that 17% of the hybridomas have undergone replacement of the transgene V_H (17). Therefore, it was important to confirm that the $CD25^+$ B cells coexpress λx and the 3H9/56R H chain. We tested transgene expression by allotype analysis. The Ig^a tgs were crossed to CB17 mice (Ig^b) and the B cells from the allotype heterozygous mice were stained with allotype-specific antibodies. Nearly all of the $CD25^+$ B cells express the transgene allotype (Fig. 5). This, however, does not rule out the possibility that the transgene V_H is replaced and that the Ig^a C region is retained (33). To address this, we tested the expression of the 3H9/56R H chain together with Vλx by single cell PCR. 22 of 48 (45.8%) $CD25^+$ cells are positive for the Vλx-Jλ2 PCR, whereas only 4 of 48 (8.3%) $CD25^-$ cells sorted from the same mice are positive. 12 of the 22 $CD25^+/λx$ cells (54.5%) are positive for the PCR detecting the 3H9/56R H chain. We do not know if this is the actual percentage of transgene$^+$ cells because the efficiency of the assay has not been determined. However, this result shows that at least 50% of the cells in this population retain the transgene.

The $CD25^+$ B Cells Show Extensive Rearrangement at the κ L Chain Loci. The apparent order of L chain rearrangement is κ and then λ. Consequently, κ B cells usually have not rearranged λ loci, and λ B cells have rearranged and/or deleted κ alleles (15, 16). Therefore, we would expect the $CD25^+/λx$ population to have extensive κ rearrangement. To test this prediction, we performed a PCR on genomic DNA from sorted $CD25^+$ B cells using primers that detect Cκ deletion. A stronger signal was detected in the $CD25^+$ fraction than in the $CD25^-$ fraction (Fig. 6). This implies that these λx B cells failed to rearrange a functional κ or rearrange a κ with the ability to prevent, i.e., edit, the DNA-binding capacity of the 3H9/56R H chain. It should be noted that λx, but not Vλ1 or Vλ2, is an editor of 3H9/56R.

Vλx Can Rearrange to Different Jλs. Vλ1 and Vλ2 can and do rearrange to different Jλs (Fig. 7 A, Jλ1, Jλ2, or Jλ3; reference 26). Alternate Jλ usage of Vλx has not been described. Whether rearrangement to other Jλs occur in the tgs was tested by sequence analysis. RT-PCR was performed using cDNA from sorted $CD25^+$ cells together with the upstream Vλx primer and the downstream Jλ1 or Jλ2,3 primers. PCR products of both Vλx-Jλ1 and Vλx-Jλ2,3 were cloned and sequenced at random. Eight sequences of Vλx-Jλ2 rearrangements were in frame and have the same junction but only two of the seven Vλx-Jλ1 rearrangement were in frame (no Vλx-Jλ3 rearrangements were found). These Vλx-Jλ1 junctions have interesting features including possible N addition and large deletions (Fig. 7 B). Vλ-Jλ sequences usually do not exhibit this sort of diversity but SCID mice do have deletions at λ junctions (34). Thus, these B cells may have atypical TdT and DNA-PK expression.

Computation of L Chain Isotype Expression Frequency. The computer simulation of κ and λ rearrangement was used to compare the expected and observed repertoire of V_H3H9/56R tgs and non-tgs. The results presented in Table I show the expected repertoire, if rearrangement had been responsible for all differences between the non-tgs and V_H3H9/56R tgs. The differences between the simulation and the observed results probably represents elements that are not strictly due to the mechanism of rearrangement, such as positive and negative selection by antigen (other than negative selection by DNA, which is already taken into account in our analysis) or differential longevity in the various compartments of the immune system.

We further analyzed the effect of deleting κ alleles. We modeled the repertoire with both κ allele ($κ^+/κ^+$), a single κ allele (κdel/$κ^+$), and no κ alleles (κdel/κdel). The first row represents the total κ populations, and the second row represents the λ (λ1 and λ2, not including λx) population. The regular non-tg has a 15:1 κ/λ ratio as is indeed observed. This ratio is the result of the introduction of the crash factor model introduced by Coleclough et al. (16). A high crash factor increases the probability that cells should

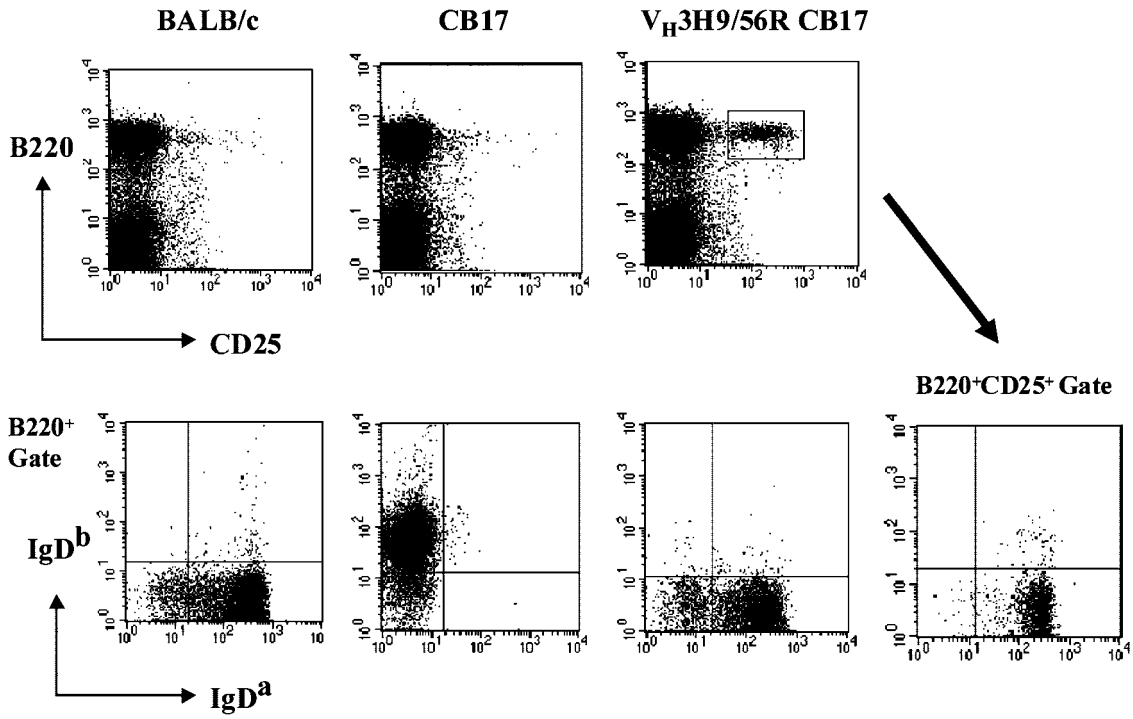

Figure 5. The CD25$^+$ B cells from V_H3H9/56R express the transgene allele (Ig^a). Spleen cells from BALB/c (Ig^a), CB17 (Ig^b), and CB17 mice carrying the 3H9/56R transgene (Ig^a) were stained with antibodies to B220, CD25, IgD^a, and IgD^b. The top panel shows the frequency of CD25$^+$ B220$^+$ from the V_H3H9/56R CB17. BALB/c or CB17 mice have background levels of these cells. The bottom panel (left to right) shows the IgD^a/IgD^b staining of the B220$^+$ cells from BALB/c, CB17, V_H3H9/56R CB17, and CD25$^+$ B220$^+$ cells of the V_H3H9/56R CB17.

die before they manage to rearrange the λ allele, and thus decreases the λ usage frequency. The κ/λ ratio is lower in the mice with a single κ allele than in mice with both κ alleles. This decrease is explained by the reduced probability of the obtainment of a functional κ (~50% with one allele compared with 75% with two alleles. We assume that each rearrangement has a 33% chance of being in frame and a 43 out of 140 chance of being a pseudo gene.). We computed the average number of rearrangement in the presence of the crash factor and the J order. In the absence of κ alleles, all L chains are obviously Vλ1, Vλ2, or Vλx. In the V_H3H9/56R tg, both Vλ1 and Vλ2 bind DNA in conjunction with the H chain transgene. Thus, if negative selection is 100% efficient, no pure λ B cell can mature and the κ/λ ratio is infinity. Note that not all cells will carry a κ L chain because the Vλx is an editor and will therefore not be negatively selected.

The third row represents the frequency of Vλx. The Vλx frequency in non-tg is lower than that of Vλ1 (19, 35). In the absence of selection, we expect the frequency of Vλx to be 50% that of Vλ1 because there is only one rearrangement possibility in the λ1 locus, and there are two such possibilities in the λ2/λx locus. Thus, we expect the ratio of Vλ(1+2)/Vλx to be 3:1 (Table I). A completely

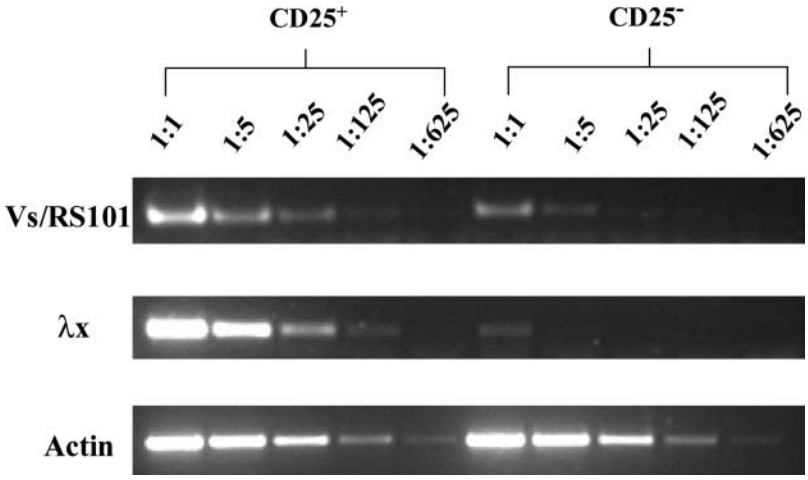

Figure 6. The CD25$^+$ B cells in V_H3H9/56R tgs have gone through extensive rearrangement at the κ L chain loci. Genomic DNA was prepared from CD25$^+$ CD19$^+$ and CD25$^-$ CD19$^+$ B cells sorted from the spleen of V_H3H9/56 tgs. PCRs that detect Cκ deletion (top, Vs-RS101) and Vλx-Jλ2 rearrangement (middle), and the control actin PCR (bottom) were performed using serial dilutions of DNA from each sample.

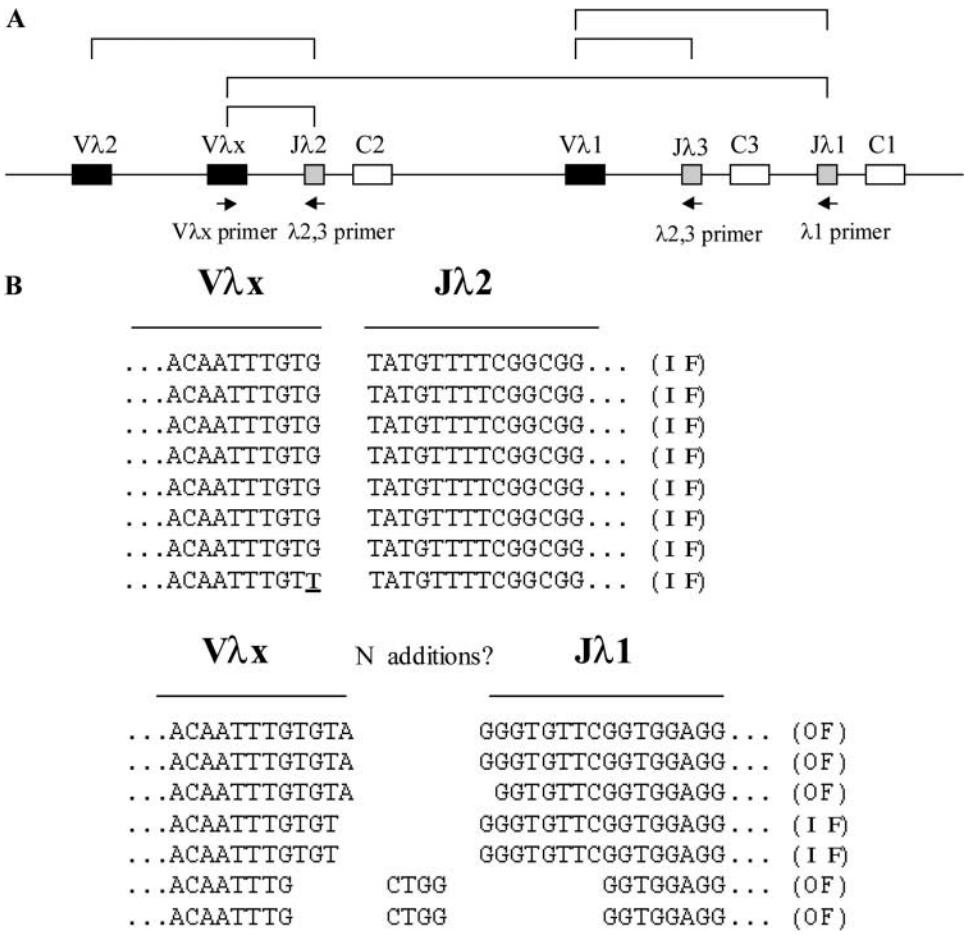

Figure 7. Sequences of the Vλx-Jλ2 and Vλx-Jλ1 junctions. (A) Mouse λ locus and the primers used in the experiments. (B) Eight Vλx-Jλ2 and seven Vλx-Jλ1 rearrangements were sequenced from randomly chosen PCR products cloned into pGEM®-T easy vector. IF, in frame rearrangement; OF, out-of-frame rearrangement. In the last two sequences of Vλx-Jλ1 rearrangement (bottom), the four nucleotides CTGG following Vλx have the same sequence as the beginning of germ line Jλ1 gene. After this sequence several nucleotides of Jλ1 are missing. Either a deletion occurred within the rearranged Jλ1 or these four nucleotides are N additions and not the beginning of Jλ1. The latter case would mean that there is a 10 nucleotide deletion at the beginning of the Jλ1.

different picture emerges in the V_H3H9/56R tg. In this mouse Vλx is an editor, whereas Vλ1 and Vλ2 are not. Therefore, the L chain should be (unless allelically included) Vλx. Moreover, the frequency of Vλx is greater than any Vκ editor (2 vs. 0.66%). Because there are only three κ editors, we expect roughly the same amount of κ and Vλx L chain expressers in the V_H3H9/56R tg, as is observed in our simulation.

Rows four to seven in Table I represent the frequency of included cells. The observed κ/κ inclusion frequency is ~2% as in the study by Casellas et al. (36). The level of κ inclusion is lower in the V_H3H9/56R tg because the probability of producing a functional nonself κ is lower than non-tg. κ/λ inclusion is also very rare in non-tg and its level in the V_H3H9/56R tg is lower or of the same magnitude as the non-tg. Rearrangement by itself cannot explain the high level of κ/λ inclusion observed in the V_H3H9/56R tg. The low inclusion frequency is due to the fact that most of the time the κ allele is rearranged to extinction before the λ allele starts rearrangement. The probability of inclusion is further decreased in the V_H3H9/56R tg because the Vλ1 is self-reactive, and the only way for a κ/λ included cell to mature is if the Vκ is an editor, itself a rare event. A much higher level of inclusion is expected in the V_H3H9/56R tg between Vλ1 and Vλx (Table I, row 7). This inclusion is expected because the cell cannot remove the anti-self Vλ1 by editing. The only way for this cell to survive might be by diluting the Vλ1 allele with Vλx. Thus, the Vλ1/Vλx inclusion frequency is high and it is rising as the total frequency of Vλx increases. Note that we never observed in our simulation the theoretically possible triple inclusion (Table I, row 8).

Discussion

The extent of rearrangement caused by editing depends on the number of editor L chains encoded in the germ line. 3H9/56R, a mutant of 3H9 H chain with a high affinity for DNA, has more stringent requirements for editing than 3H9 in that it appears to be edited by fewer Vκs (3 out of 93 functional Vκ genes) than 3H9. Therefore, the probability of rearranging a Vκ editor for 3H9/56R is low and alternative ways of achieving self-tolerance such as V_H replacement are used (17). In addition, B cells that retain the 3H9/56R transgene have a large population of B cells that express two L chains (13). The L chains that are coexpressed include λ1, an L chain that permits DNA binding by the 3H9/56R, and a κ editor (Vκ20, Vκ21D, and Vκ38c). We think this population is resistant to activation because the coexpression of an anti-DNA receptor and a

Table I. *The Expected Frequency of Different L Chain Isotypes*

	V_H3H9/56R			Nontransgenic		
κ Genotype	−/−	−/+	+/+	−/−	−/+	+/+
1. κ	0.00	25.83 (NA)	41.47 (NA)	0.00	79.09 (NA)	91.72 (87.0[a])
2. λ	0.00 (NA)	0.00 (NA)	0.00 (NA)	74.49 (89.0[b])	15.40 (NA)	6.10 (3.9[b])
3. λx	93.49 (55.0)	68.21 (18.1)	53.03 (13.2)	24.55 (12.0[b])	5.11 (NA)	1.96 (0.8[b])
4. κ/κ	0.00	0.00	0.63 (NA)	0.00	0.00	1.38 (1.5[a])
5. κ/λ	0.00	0.06 (14.0)	0.10 (23.8[c])	0.00	0.14 (NA)	0.10 (2.1[c])
6. κ/λx	0.00 (NA)	0.97 (NA)	1.50 (NA)	0.00 (NA)	0.04 (NA)	0.03 (NA)
7. λ/λx	6.51 (NA)	4.94 (NA)	3.90 (NA)	0.97 (NA)	0.22 (NA)	0.08 (NA)
8. κ/λ/λx	0.00 (NA)	0.00 (NA)	0.00 (NA)	0.00 (NA)	0.00 (NA)	0.00 (NA)

A cell is defined as κ, λ, or λx if it expresses only κ, λ1 or λ2, or λx L chains. A cell expressing two different L chains is defined as an "included cell" and is designated κ and λ, κ and λx, and λ and λx. Note that we define a cell expressing λx on both alleles or λ on both alleles as an allelically excluded cell. The simulation was performed using a crash factor to obtain the observed κ/λ ratio in non-tg. We have performed the simulation with no κ alleles (Jκdel/Jκdel, first and fourth columns), a single κ allele (Jκdel/κ+, second and fifth column), and both κ alleles (κ+/κ+, third and sixth columns). The only difference between the non-tg and the V_H3H9/56R tg in the simulation is the probability that an L chain will sustain DNA binding. In the non-tg no DNA binding was assumed. In the V_H3H9/56R tg, we assumed all L chains lead to DNA binding except for the editors Vκ21D, Vκ20, Vκ38c, and Vλx. All the data obtained by simulation are listed and the observed values are shown in parentheses.
[a]Reference 36.
[b]Reference 38.
[c]Reference 13.
NA, data not available.

non–anti-DNA receptor dilutes either receptor to the point that cross-linkage by self-antigen is not efficient. In addition, the partial self-reactivity of the B cell may confer special properties to this population, among them the homing to the marginal zone (13).

As described here, the 3H9/56R tg has a population of B cells that express the 3H9/56R transgene and the Vλx-Jλ2 L chain. The Vλx is one of the three functional V genes of mice, Vλ1, Vλ2, and Vλx. Vλ1 and Vλ2 allow DNA binding by 3H9/56R and related V_Hs, but Vλx prohibits DNA binding of 3H9/56R H chain. Thus, these edited 3H9/56R/λx B cells are not unexpected. Vλx is only distantly related to the other Vλs and among the differences from Vλ1 and Vλ2 is the IEP of its CDR. Vλx has a very low IEP (in the range of κ editors), whereas the other Vλs have IEPs in the upper range of L chain IEP scale. Furthermore, Vλ1 in combination with 3H9/56R has been shown to bind DNA (17).

Vλx has been a neglected V-region for two reasons. One is its extremely low level of expression in normal mice. In fact, λx is only detectable in κ-deficient mice. A second reason is that conventional anti-λ reagents do not react or react weakly with Vλx-Jλ2. As illustrated here, the B cells from 3H9/56R include a population that typed κ^-/λ^- with these anti-λ reagents. This population was puzzling until molecular analysis of members of this population showed expression of λx L chain.

The Vλx (and the aforementioned κ editor/λ1) B cell populations, are prominent in the V_H3H9/56R tg but are undetectable in non-tgs or the lower affinity tgs. How might 3H9/56R produce or influence the size of these populations?

κ/λ isotypic exclusion, and for that matter allelic exclusion, are thought to emerge from a series of stochastic events and rearrangement product alternatives that reduce the probability of two functional alleles or isotypes. For example, the probability of productive rearrangement at κ is low. Furthermore, a rearrangement STOP signal is required to stabilize a B cell with a functional receptor. The combination of these factors accounts for the low frequency of allelic inclusion at the κ locus (36). Isotype exclusion can be explained by assuming that the probability of rearrangement of κ is greater than λ. The relative probabilities of κ versus λ could be determined by differences in initiation of expression, transcript abundance, quality of rearrangement sequences, etc. λ allelic and isotype exclusion, Cλ1 or Cλ2, might be determined in a similar fashion.

The number of κ and λ B cells must also be influenced by these mechanisms, but reconciling the predictions of purely probabilistic models with the observed frequencies of L chain isotypes and monospecific B cells is problematic. Mouse antibodies are mainly κ (the ratio $\kappa/\lambda = 20$), but, as pointed out by Langman and Cohn (27), the probabilistic model described above would predict a much lower ratio (~2). Langman and Cohn (27) derived this value by assuming that κ^-/κ^- B cells are the major source of the λ population (minus those that fail to rearrange λ productively) and that the frequency of κ-deficient B cells is high. The ratio $\kappa/\lambda = 2$, is a pre-receptor editing calculation and hence overestimates the frequency of failed κ loci. But ad-

justing the κ-deficient population to account for κ rescue by editing still yields a ratio (~4) far lower than observed. Coleclough et al. (16) solved this paradox by introducing the crash factor, which in effect limits rearrangement opportunities (a reasonable parameter given the finite lifespan of a B cell). This limit reduces the number of κ^-/κ^- B cells that can be produced and in turn reduces the number of B cells that make the transition to λ and, hence, increases the κ/λ ratio.

We wish to explain the λ contribution to the B cells of V_H3H9/56R tg in the context of the Coleclough et al. (16) modification of the Langman and Cohn (27) model. We explained the κ/λ population of V_H3H9/56R by postulating a significant overlap between κ and λ rearrangement. We thought this overlap was the result of extended periods of κ rearrangement in this tg (24) but simulations that include this factor preclude this explanation (Table I). Our explanation for the λx population was because of a shift toward late-stage rearrangement driven by receptor editing. This explains in part the existence of the λx B cells but not their overall frequency (Table I).

The simulation gives surprising (to us) results with regard to the frequencies of κ/λ doubles and λx. The frequency of κ/λ doubles does not change in the V_H3H9/56R (both non-tg and V_H3H9/56R = 0.1%). In the simulation, we assumed that there are three κ editors and one λx editor. The reason for a low κ/λ frequency in non-tg is the high probability of deleting the κ locus before λ even has the opportunity of rearranging. This probability is greatly increased in V_H3H9/56R. As expected, the simulation does yield a much higher frequency of λx in V_H3H9/56R than in non-tg. We account for this frequency in two ways. Most κs are not editors leading to rapid deletion of the κ locus and Vλx is the only λ editor. This means that the λ1 and λ2 populations are deleted and the λx population remains (λs do not have κdel elements, as far as we know, hence this rearrangement is permanent). In addition, the simulation yields a Vλ1+Vλ2: Vλx ratio of ~3 in non-tg. Here we make the reasonable assumption that λ1 and λ2 loci rearrange at the same rate and that Vλ2 and Vλx rearrange at the same rate. Because Vλ2 and Vλx rearrangements are mutually exclusive, the above ratio should be 3. However, there is disagreement on the actual ratio. A ratio close to 3 has been observed (35), but we find the ratio is 20 (19).

There are several ways to explain the difference between simulations and the data. The most interesting explanation is antigen selection. The maintenance of B cells is thought to require survival signals, one of which is thought to be antigen. To explain the consistent appearance of B cells with edited receptors like V_H3H9/56R/λx or partially edited receptors like κ/λ double expressers, these receptors must bind ubiquitous antigen(s). Such antigens are probably self and the edited populations found in the anti-DNA tgs might owe their existence to self-reactivity. The anti-DNA coreceptor in the 3H9/56R κ/λ B cell may promote its survival and homing to the marginal zone, a process thought to be driven by self-reactivity. The effective affinity of these receptors with respect to selection may in this case favor positive selection and clonal expansion, thereby explaining the high frequency of this population.

The odd association of 3H9/56R/λx B cells with the activation marker CD25 implies that the 3H9/56R/λx B cells are antigen activated and that these B cells may have reacted with antigen for extended periods before they rearranged the λx editor. Although the pre-λx DNA reactivity in the presence of endogenous DNA might explain the appearance of an activation marker, it does not explain the maintenance of this population after expression of the anti-DNA editor, λx. However, λx-associated antibodies, indeed λx alone, are themselves anti-self. Both have been shown to bind myelin basic protein (MBP; 32). In addition, a human mAb (a Waldenstrom's macroglobulinemia protein) that shares the same idiotype of mouse Vλx also binds MBP (37). The lower than expected numbers of λx B cells observed here and in κ-deficient mice (reference 19 and Fig. 2) might be explained by deletion of self-reactive λx B cells. Hence, editing might be a two-edged sword by which expression of acidic editors that have evolved to prevent DNA binding may also create reactivity to basic self-proteins such as MBP. The association between anti-DNAs and their editors may in turn edit the editors, but revision of the B cell receptor, for example, by H chain replacement or even H chain deletion might expose the autoreactivity of the editor.

We thank Dongyao Ni for technical support, Cristina J. DeCoste for doing the sorting, Rimanatas Kirvelevicius for rabbit immunization, Dr. Eric Meffre, Dr. Hedda Wardemann (members of Dr. Michel Nussenzweig's Lab), and Klara Velinzon (The Rockefeller University) for helping us with the single cell PCR experiment, Dr. Jan Erikson (The Wistar Institute) for providing one of the λx hybridomas, and Dr. Eline Luning Prak (University of Pennsylvania) for critical reading of the manuscript.

This work is supported by National Institutes of Health grant GM20964 and the Lupus Research Foundation. The work of Y. Louzoun was partly covered by a fellowship from the Yeshaya Horowitz foundation, a grant from the Israel Science foundation, as well as by a Burroughs Wellcome fellowship from the Program in Mathematics and Molecular Biology.

Submitted: 3 October 2003
Accepted: 5 December 2003

References

1. Goodnow, C.C., J. Crosbie, H. Jorgensen, R.A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature*. 342:385–391.
2. Nemazee, D., and K. Burki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class-I antibody genes. *Nature*. 337:562–566.
3. Tiegs, S.L., D.M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med*. 177:1009–1020.
4. Erikson, J., M.Z. Radic, S.A. Camper, R.R. Hardy, C. Carmack, and M. Weigert. 1991. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature*.

349:331–334.
5. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 177:999–1008.
6. Radic, M.Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med.* 177:1165–1173.
7. Tan, E.M. 1988. Antinuclear antibodies: diagnostic markers and clues to the basis of systemic autoimmunity. *Pediatr. Infect. Dis. J.* 7:S3–S9.
8. Shlomchik, M.J., A.H. Aucoin, D.S. Pisetsky, and M.G. Weigert. 1987. Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. *Proc. Natl. Acad. Sci. USA.* 84:9150–9154.
9. Kabat, E., T. Wu, H. Perry, K. Gottesman, and C. Foeller. 1991. Sequences of Proteins of Immunological Interest. US Government Printing Office, Bethesda, MD.
10. Radic, M.Z., J.J. Mackle, J. Erikson, C. Mol, W.F. Anderson, and M.G. Weigert. 1993. Residues that mediate DNA binding of autoimmune antibodies. *J. Immunol.* 150:4966–4977.
11. Ibrahim, S.M., M. Weigert, C. Basu, J. Erikson, and M.Z. Radic. 1995. Light chain contribution to specificity in anti-DNA antibodies. *J. Immunol.* 155:3223–3233.
12. Li, H., Y. Jiang, H. Cao, M. Radic, E.L. Prak, and M. Weigert. 2003. Regulation of anti-phosphatidylserine antibodies. *Immunity.* 18:185–192.
13. Li, Y., H. Li, and M. Weigert. 2002. Autoreactive B cells in the marginal zone that express dual receptors. *J. Exp. Med.* 195:181–188.
14. Chen, C., M.Z. Radic, J. Erikson, S.A. Camper, S. Litwin, R.R. Hardy, and M.G. Weigert. 1994. Deletion and editing of B cells that express antibodies to DNA. *J. Immunol.* 152: 1970–1982.
15. Hieter, P.A., S.J. Korsmeyer, T.A. Waldmann, and P. Leder. 1981. Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. *Nature.* 290:368–372.
16. Coleclough, C.C., R.P. Perry, K. Karjalainen, and M.G. Weigert. 1981. Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression. *Nature.* 290:372–378.
17. Li, H., Y. Jiang, E.L. Prak, M. Radic, and M. Weigert. 2001. Editors and editing of anti-DNA receptors. *Immunity.* 15: 947–957.
18. Chen, J., M. Trounstine, C. Kurahara, F. Young, C.C. Kuo, Y. Xu, J.F. Loring, F.W. Alt, and D. Huszar. 1993. B cell development in mice that lack one or both immunoglobulin kappa light chain genes. *EMBO J.* 12:821–830.
19. Prak, E.L., M. Trounstine, D. Huszar, and M. Weigert. 1994. Light chain editing in kappa-deficient animals: a potential mechanism of B cell tolerance. *J. Exp. Med.* 180:1805–1815.
20. Retter, M.W., and D. Nemazee. 1998. Receptor editing occurs frequently during normal B cell development. *J. Exp. Med.* 188:1231–1238.
21. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre–pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213–1225.
22. Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science.* 301:1374–1377.
23. Young, D., and J.F. Kearney. 1995. Sequence analysis and antigen binding characteristics of Ig SCID Ig+ mice. *Int. Immunol.* 7:807–819.
24. Louzoun, Y., T. Friedman, E. Luning Prak, S. Litwin, and M. Weigert. 2002. Analysis of B cell receptor production and rearrangement. Part I. Light chain rearrangement. *Semin. Immunol.* 14:169–190.
25. Schlissel, M. 2002. Allelic exclusion of immunoglobulin gene rearrangement and expression: why and how? *Semin. Immunol.* 14:207–212.
26. Sanchez, P., B. Nadel, and P.A. Cazenave. 1991. V lambda-J lambda rearrangements are restricted within a V-J-C recombination unit in the mouse. *Eur. J. Immunol.* 21:907–911.
27. Langman, R., and M. Cohn. 2002. Haplotype exclusion: the solution to a problem in natural selection. *Semin. Immunol.* 14:153–162.
28. Rolink, A., U. Grawunder, T.H. Winkler, H. Karasuyama, and F. Melchers. 1994. IL-2 receptor alpha chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* 6:1257–1264.
29. Ortega, G., R.J. Robb, E.M. Shevach, and T.R. Malek. 1984. The murine IL 2 receptor. I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. *J. Immunol.* 133:1970–1975.
30. Dildrop, R., A. Gause, W. Muller, and K. Rajewsky. 1987. A new V gene expressed in lambda-2 light chains of the mouse. *Eur. J. Immunol.* 17:731–734.
31. Goodman, G.R., K.J. Bethel, and A. Saven. 2003. Hairy cell leukemia: an update. *Curr. Opin. Hematol.* 10:258–266.
32. Galin, F.S., C.C. Maier, S.R. Zhou, J.N. Whitaker, and J.E. Blalock. 1996. Murine V lambda x and V lambda x-containing antibodies bind human myelin basic protein. *J. Clin. Invest.* 97:486–492.
33. Chen, C., Z. Nagy, E.L. Prak, and M. Weigert. 1995. Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing. *Immunity.* 3:747–755.
34. Ruetsch, N.R., G.C. Bosma, and M.J. Bosma. 2000. Unexpected rearrangement and expression of the immunoglobulin lambda1 locus in scid mice. *J. Exp. Med.* 191:1933–1943.
35. Nadel, B., P.A. Cazenave, and P. Sanchez. 1990. Murine lambda gene rearrangements: the stochastic model prevails over the ordered model. *EMBO J.* 9:435–440.
36. Casellas, R., T.A. Shih, M. Kleinewietfeld, J. Rakonjac, D. Nemazee, K. Rajewsky, and M.C. Nussenzweig. 2001. Contribution of receptor editing to the antibody repertoire. *Science.* 291:1541–1544.
37. Noerager, B.D., T. Inuzuka, J. Kira, J.E. Blalock, J.N. Whitaker, and F.S. Galin. 2001. An IgM anti-MBP Ab in a case of Waldenstrom's macroglobulinemia with polyneuropathy expressing an idiotype reactive with an MBP epitope immunodominant in MS and EAE. *J. Neuroimmunol.* 113:163–169.
38. Sanchez, P., D. Rueff-Juy, P. Boudinot, S. Hachemi-Rachedi, and P.A. Cazenave. 1996. The lambda B cell repertoire of kappa-deficient mice. *Int. Rev. Immunol.* 13:357–368.