Editing Anti-DNA B Cells by V\(\lambda x\)

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Abstract

Receptor editing is performed by replacement of Vk genes that contribute to autoreactivity. In addition, the Ck locus can be deleted by Vk rearrangement to intronic or 3' of Ck RS sequences (also referred to as k deletion elements). B cells that delete the Ck can then express \(\lambda\) light chains. However, the \(\lambda\) locus, either of man or mouse, does not allow V gene replacement. Nor does it appear to be deleted. Therefore, editing of autoreactive \(\lambda\) B cells may require alternative pathways. We have found that in anti-DNA heavy chain transgenic mice (tgs) \(V_{H9}/56R\), B cells that express anti-DNA receptors comprised of \(\lambda\)1 in association with an anti-DNA heavy chain often coexpress a \(k\) chain that prevents DNA binding. We speculate that such iso-typically 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 VA family, \(V_\lambda x\). The \(\lambda\)x 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 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 (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 \(V_\lambda\) 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 \(V_L\)s have no influence on binding but some \(V_L\)s (editors) can modify or inhibit the \(V_H\–DNA\) interaction (11). Healthy \(H9\) H chain tgs express the \(H9\) 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 \(3H9/Vk8\) 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 (\(k\) and \(\lambda\)) 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

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Abbreviations used in this paper: IEP, isoelectric point; MBP, myelin basic protein; tg, transgenic mouse; tgs, transgenic mice.
extremely biased toward V\textalpha x-J\textalpha 2. 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 \lambda genes are thought to rearrange after \kappa 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\textalpha 3H9/56R H chain tgs on BALB/c or CB17 background have been described (17). The tgs were crossed to the immunoglobulin \kappa-deficient (Jkdel) mice (18). All mice used in the experiments were between 6–8 wk old.

Genotyping of the tgs. The PCR for typing the V\textalpha 3H9/56R tgs has been described (17). For typing the Jkdel mice, two separate PCR assays were performed with the following primer pairs: to detect the targeted inactivation of the \kappa gene by Jk\kappa deletion (18), the forward primer 5’-AAGAGCTTGCGGCGAATGG-3’ corresponding to the inserted neo gene and the reverse primer 5’-AAACCTACCATGGCCAGAGA-3’ corresponding to the remaining 3’ region of the \kappa locus were used. The wild-type \kappa locus (\kappa^+) was detected by the forward primer 5’-CTGTAATCACATTGAGTGATGG-3’ corresponding to Jk3 and the reverse primer 5’-CCGACCAGCAGCTGATCACA-3’ corresponding to Jk4. Both PCRs were performed with a final concentration of 250 \mu M of each dNTP and 2 mM MgCl\textsubscript{2} at 60°C annealing temperature.

Flow Cytometry. The following antibodies were used for flow cytometry: FITC-conjugated polyclonal goat anti-mouse \kappa (Fisher Scientific), biotin-conjugated anti-\lambda (R26-46), PE-conjugated anti-CD25 (3C7), APC-conjugated anti-B220 (RA3-6B2), FITC-conjugated anti-IgD\textsuperscript{\beta} (AMS9.1), biotin or PE-conjugated anti-IgD\textsuperscript{\gamma} (BD Biosciences), FITC-conjugated anti-IgD (11-26), and biotin-conjugated goat anti-IgM (Southern Biotechnology Associates, Inc.). Stained cells were analyzed using a FACSscan™ flow cytometer and CELLQuest™ software (Becton Dickinson).

Cell Sorting. Splenic B cells from V\textalpha 3H9/56R 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\textsuperscript{+}/CD19\textsuperscript{+} and the CD25\textsuperscript{−}/CD19\textsuperscript{+} 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 V\textalpha x rearrangement was performed as previously described (19). The PCR products were cloned into pGEM\textendash T Easy vector (Promega). Approximately seven to eight clones of each PCR were randomly picked and sequenced with the T7 primer. Ck 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’-GTTGTCATGATGGATTGGGT-3’ and the reverse primer 5’-GATGTCGCGCCACAATCCTCAGTTTC-3’ (21).

Single Cell PCR. Spleen B cells were enriched by purification with anti-B220 magnetic beads (Miltenyi Biotech). 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 V\textalpha x-J\textalpha 2 and V\textalpha 3H9/56R transcripts. Each type of PCR has two rounds of reactions (40 pmol of each primer, 1.56 mM MgCl\textsubscript{2}, and 0.2 \mu M AmpliTaq 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 V\textalpha x-J\textalpha 2: first round ACCCTTGAGTAGCTCAGCAG and CTAGGACATGACCTTGTGTT; second round GAGCTTAAGAAGATGGAAGGCA and GTTCGCCAGCGGAAACAT. The following primers were used for V\textalpha 3H9/56R: first round CAGGTTCACCTGCACAGTC and GGGATCTCG- GGAAGAAGCTGACCTC (C\kappa; reference 23); second round AGTAGCTCTGGATGAACTGG and CATACATAGGATTATTTACTCCTGCG.

Generation of Rabbit Anti–V\textalpha x Antibody. A peptide corresponding to a sequence spanning the FRW2 and CDR2 region of V\textalpha x (N\textsuperscript{\textendash}CQPKYVMEKKDDHSTG–C), which has no homology to V\kappa1 or V\kappa2, 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\textsubscript{3}SO\textsubscript{4} at a final concentration of 0.18 g/ml of serum. The precipitates were dissolved in 17.5 mM sodium phosphate buffer. Anti-V\textalpha x 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\textsuperscript{+} B cells were enriched magnetically after staining with PE-conjugated anti-CD25 (3C7) followed by attachment to anti-PE magnetic beads (Miltenyi Biotech). The CD25\textsuperscript{−} 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 Biotech). 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 ABEFS (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 m\textsuperscript{2}V for 2 h. After blocking with 5% nonfat milk in PBS, the blot was probed for 1 h at room temperature with rabbit anti–V\textalpha x. 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 \kappa and \lambda Expression Frequency. The frequency of \kappa and \lambda 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 \kappa locus (with each allele rearranging separately) and two \lambda loci (\lambda1 and \lambda2/\lambdax). 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 \kappa alleles and is
consistent with Schlissel's (25) estimate.κ allelic exclusion is thus practically guaranteed by the low initiation probability.κ/λ isotypic exclusion is guaranteed by the longer rearrangement process in λ (50 cycles) than in κ (5 cycles).

At the λ locus rearrangement is between any Vλ and a Jκ. Jκ is chosen according to a quasi-sequential model (unpublished data). In the λ1 locus rearrangement is between Vλ1 and JA1 (i.e., we ignore the possible rearrangement to λ3). At the λ2/λx locus, we assumed that half the rearrangements are to JA2 and half are to JA2 (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 VH3H9/56R 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 does not bind self-antigen, a nonself L chain double expresser cell would be the combination of a Vκ edit combined with a VκA1 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.

Results

VH3H9/56R 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 VH3H9/56R 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 VH3H9/56R, but these B cells are not detectable in either the nontransgenic littermates of this tg (Fig. 1A) 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. 1B). However, the frequency of these CD25+ B cells is higher in VH3H9/56R 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 Vκx. Although this Vκ is located in the Jκ2-CAκ locus and is usually rearranged to Jκ2, its V region is only 33% identical to the VκA1 (Identity to VA1 and mouse Vκs is also <35% but homology to human VA5-6 and VA5-1 is high, at 75 and 70%, respectively.). If anti-λ antibodies are directed to the V region, then Vκx/CAκ may not be recognized by these reagents. Indeed, molecular analysis of L chain rearrangement (see below) shows that Vκx is rearranged in the CD25+ population.

Vκx is barely detectable in normal mouse serum but reaches significant levels in κ-deficient mice (26, 30). Vκx 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 Jκ/del/Jκdel mice have no L chain according to either the anti-κ or anti-λ antibodies discussed above. We presume that they express Vκx. 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/AX B cells per se express the activation marker. Other activation markers such as CD69 or elevated Fas are not found on

Figure 1. CD25+/IgM+/IgD+ spleen B cells of VH3H9/56R mice. (A) Spleen cells from VH3H9/56R 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 VH3H9/56R 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).
these cells. Nor do they express CD11c or CD103, markers that are expressed by hairy cells, the CD25+/CD4− clonally expanded mature activated B cells (31 and unpublished data).

**Molecular Analysis of V\textsubscript{x}x.** To confirm that conventional L chains are replaced by V\textsubscript{x}x, we sorted the CD25+ cells from the spleen of V\textsubscript{H}3H9/56R tg and performed a V\textsubscript{x}x RT-PCR. We used a J\textsubscript{x}2 primer and a V\textsubscript{x}x primer corresponding to a sequence spanning the FW2 and CDR2 region, which has no homology to V\textsubscript{x}1, V\textsubscript{x}2, or V\textsubscript{x}0. 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 V\textsubscript{x}x 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 V\textsubscript{x}x, which has no homology to V\textsubscript{x}1 or V\textsubscript{x}2. Western blot using cell lysate of V\textsubscript{x}x, V\textsubscript{x}1, and V\textsubscript{x}0 hybridomas demonstrates that the antibody recognizes a single band with the molecular weight of 27 kD from the lysates of V\textsubscript{x}x hybridomas and does not cross-talk with V\textsubscript{x}1 or V\textsubscript{x}0 L chains (Fig. 4 A). The observed molecular weight of V\textsubscript{x}x 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 V\textsubscript{x}x. The CD25−/B220+ cells sorted from the same mice show a much weaker band and B220+ cells from non-
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\_4 (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\_x tgs were crossed to CB17 mice (Ig\_p) 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\_4 is replaced and that the Ig\_p C region is retained (33). To address this, we tested the expression of the 3H9/56R H chain together with V\_x by single cell PCR. 22 of 48 (45.8%) CD25+ cells are positive for the V\_x-J\_2 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 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 V\_k1 or V\_k2, is an editor of 3H9/56R.

V\_x Can Rearrange to Different J\_x. V\_x1 and V\_x2 can and do rearrange to different J\_x sequences (Fig. 7 A, J\_x1, J\_x2, or J\_x3; reference 26). Alternate J\_x usage of V\_x has not been described. Whether rearrangement to other J\_x occur in the tgs was tested by sequence analysis. RT-PCR was performed using cDNA from sorted CD25+ cells together with the upstream V\_x primer and the downstream J\_x1 or J\_x2,3 primers. PCR products of both V\_x-J\_x1 and V\_x-J\_x2,3 were cloned and sequenced at random. Eight sequences of V\_x-J\_x2 rearrangements were in frame and have the same junction but only two of the seven V\_x-J\_x1 rearrangements were in frame (no V\_x-J\_x3 rearrangements were found). These V\_x-J\_x1 junctions have interesting features including possible N addition and large deletions (Fig. 7 B). V\_x-J\_x sequences usually do not exhibit this sort of diversity but SCID mice do have deletions at J\_x 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\_H\_3H9/56R tgs and non-tgs. The results presented in Table I show the expected repertoire, if rearrangement had been accounted for in our analysis. The apparent order of rearrangement is shown to be: V\_H\_3H9/56R 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 (κ\^del/κ\^+), and no κ alleles (κ\^del/κ\^del). The first row represents the total κ populations, and the second row represents the λ (A1 and A2, 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...
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 VA1, VA2, or Vλx. In the V_{H}3H9/56R tg, both VA1 and VA2 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 VA1 (19, 35). In the absence of selection, we expect the frequency of Vλx to be 50% that of VA1 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 VA(1+2)/Vλx to be 3:1 (Table I). A completely

![Figure 5.](image)

Figure 5. The CD25^+ B cells from V_{H}3H9/56R express the transgene allele (Ig^α). Spleen cells from BALB/c (Ig^α), CB17 (Ig^α), and CB17 mice carrying the 3H9/56R transgene (Ig^α) were stained with antibodies to B220, CD25, IgD^a, and IgD^b. The top panel shows the frequency of CD25^+ B220^+ from the V_{H}3H9/56R 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_{H}3H9/56R CB17, and CD25^+ B220^+ cells of the V_{H}3H9/56R CB17.

![Figure 6.](image)

Figure 6. The CD25^+ B cells in V_{H}3H9/56R 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_{H}3H9/56 tgs. PCRs that detect Ck deletion (top, Vs-RS101) and Vλx-Jκ2 rearrangement (middle), and the control actin PCR (bottom) were performed using serial dilutions of DNA from each sample.
A different picture emerges in the $V_{H9}/56R$ tg. In this mouse $V_{H}$ is an editor, whereas $V_{H1}$ and $V_{H2}$ are not. Therefore, the $L$ chain should be (unless allelically included) $V_{H1}$. Moreover, the frequency of $V_{H1}$ is greater than any $V_{H0}$ editor (2 vs. 0.66%). Because there are only three $V_{H0}$ editors, we expect roughly the same amount of $V_{H0}$ and $V_{H1}$ $L$ chain expressers in the $V_{H9}/56R$ tg, as is observed in our simulation.

Rows four to seven in Table I represent the frequency of included cells. The observed $V_{H0}/V_{H1}$ inclusion frequency is $2\%$ as in the study by Casellas et al. (36). The level of $\kappa/\lambda$ inclusion is lower in the $V_{H9}/56R$ tg because the probability of producing a functional nonself $\kappa$ is lower than non-$tg$. $\kappa/\lambda$ inclusion is also very rare in non-$tg$ and its level in the $V_{H9}/56R$ $tg$ is lower or of the same magnitude as the non-$tg$. Rearrangement by itself cannot explain the high level of $\kappa/\lambda$ inclusion observed in the $V_{H9}/56R$ $tg$. The $L$ chains that are coexpressed include $V_{H1}$, an $L$ chain that permits DNA binding by the $V_{H9}/56R$, and a $V_{H0}$ editor ($V_{H20}$, $V_{H21D}$, and $V_{H38c}$). We think this population is resistant to activation because the coexpression of an anti-DNA receptor and a

This inclusion is expected because the cell cannot remove the anti-self $V_{H1}$ by editing. The only way for this cell to survive might be by diluting the $V_{H1}$ allele with $V_{H0}$. Thus, the $V_{H1}/V_{H0}$ inclusion frequency is high and it is rising as the total frequency of $V_{H0}$ 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 $V_{k}$ genes (3 out of 93 functional $V_{k}$ genes) than $3H9$. Therefore, the probability of rearranging a $V_{k}$ 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 $\lambda 1$, an $L$ chain that permits DNA binding by the $3H9/56R$, and a $\kappa$ editor ($V_{k20}$, $V_{k21D}$, and $V_{k38c}$). We think this population is resistant to activation because the coexpression of an anti-DNA receptor and a
The Expected Frequency of Different L Chain Isotypes

| κ Genotype | V_{3H9/56R} | Nontransgenic |
|------------|-------------|---------------|
|            | −/−  | −/+  | +/+  | −/−  | −/+  | +/+  |
| 1. κ       | 0.00  | 25.83 (NA) | 41.47 (NA) | 0.00  | 79.09 (NA) | 91.72 (87.0) |
| 2. λ       | 0.00 (NA) | 0.00 (NA) | 0.00 (NA) | 74.49 (89.0) | 15.40 (NA) | 6.10 (3.9) |
| 3. λx      | 93.49 (55.0) | 68.21 (18.1) | 53.03 (13.2) | 24.55 (12.0) | 5.11 (NA) | 1.96 (0.8) |
| 4. κ/κ     | 0.00  | 0.00  | 0.63 (NA) | 0.00  | 0.00  | 1.38 (1.5) |
| 5. κ/λ     | 0.00  | 0.06 (14.0) | 0.10 (23.8) | 0.00  | 0.14 (NA) | 0.10 (2.1) |
| 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 κ1 and λ1, 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 (Jkdel/Jkdel, first and fourth columns), a single κ allele (Jkdel/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_{3H9/56R} 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_{3H9/56R} tg, we assumed all L chains lead to DNA binding except for the editors Vx21D, Vx20, Vx38c, and Vxα. All the data obtained by simulation are listed and the observed values are shown in parentheses.

1Reference 36.
2Reference 38.
3Reference 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 Vαx-Jα2 L chain. The Vαx is one of the three functional V genes of mice, Vα1, Vα2, and Vαx. Vα1 and Vα2 allow DNA binding by 3H9/56R and related V{13}, but Vαx prohibits DNA binding of 3H9/56R. H chain. Thus, these edited 3H9/56R/Vαx B cells are not unexpected. Vαx is only distantly related to the other Vαs and among the differences from Vα1 and Vα2 is the IEP of its CDR. Vαx has a very low IEP (in the range of κ editors), whereas the other Vαs have IEPs in the upper range of L chain IEP scale. Furthermore, Vα1 in combination with 3H9/56R has been shown to bind DNA (17).

Vαx 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 Vαx-Jα2. 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 Vαx (and the aforementioned κ editor/α1) B cell populations, are prominent in the V_{3H9/56R} 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, CA1 or CA2, 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 κ/λ = 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 κ/λ = 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 VH3H9/56R 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 VH3H9/56R 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 VH3H9/56R (both non-tg and VH3H9/56R = 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 VH3H9/56R. As expected, the simulation does yield a much higher frequency of λx in VH3H9/56R 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 VAX is the only λ editor. This means that the λ1 and λ2 populations are deleted and the λx population remains (κs do not have kdel elements, as far as we know, hence this rearrangement is permanent). In addition, the simulation yields a VAX + VAX ratio of ~3 in non-tg. Here we make the reasonable assumption that λ1 and λ2 loci rearrange at the same rate and that VAX and VAX rearrange at the same rate. Because VAX and VAX 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 VH3H9/56R/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 affin-

ity 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.

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