Light Chain Editing in κ-deficient Animals: A Potential Mechanism of B Cell Tolerance

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Summary

The genetic organization of the κ and λ light chain loci permits multiple, successive rearrangement attempts at each allele. Multiple rearrangements allow autoreactive B cells to escape clonal deletion by editing their surface receptors. Editing may also facilitate efficient B cell production by salvaging cells with nonproductive light chain (L chain) rearrangements. To study receptor editing of κ L chains, we have characterized B cells from mice hemizygous for the targeted inactivation of κ (JCkD/wt) which have an anti-DNA heavy chain transgene, 3H9. Hybridomas from JCkD/wt mice exhibited an increased frequency of rearrangements to downstream Jk segments (such as Jk5) compared with most surveys from normal mice, consistent with receptor editing by sequential κ locus rearrangements in JCkD/wt. We observed an even higher frequency of rearrangements to Jk5 in 3H9 JCkD/wt animals compared with nontransgenic JCkD/wt, consistent with editing of autoreactive κ in 3H9 JCkD/wt. We also recovered a large number of 3H9 JCkD/wt lines with Vk12/13–Jk5 rearrangements and could demonstrate by PCR and Southern analysis that up to three quarters of these lines underwent multiple κ rearrangements. To investigate editing at the λ locus, we used homozygous κ-deficient animals (JCkD/JCkD and 3H9 JCkD/JCkD). The frequencies of Vλ1 and Vλ2 rearrangements among splenic hybridomas in 3H9 JCkD/JCkD were reduced by 75% whereas VλX was increased 5–10-fold, compared with nontransgenic JCkD/JCkD animals. This indicates that Vλ1 and Vλ2 are negatively regulated in 3H9 JCkD/JCkD, consistent with earlier studies that showed that the 3H9 heavy chain, in combination with λ1 binds DNA. As successive λ rearrangements to VλX do not inactivate Vλ1, the consequence of λ editing in 3H9 JCkD/JCkD would be failed allelic exclusion at λ. However, analysis of 18 3H9 JCkD/JCkD hybridomas with Vλ1 and VλX DNA rearrangements revealed that most of these lines do not have productive λ1 rearrangements. In sum, both κ and λ loci undergo editing to recover from nonproductive rearrangement, but only κ locus editing appears to play a substantial role in rescuing autoreactive B cells from deletion.

It is striking that nearly all animals ranging from sharks to humans have two light (L) chain isotypes, referred to as κ and λ (1). It may be important to maintain two isotypes if each provides critical specificities to the germline repertoire. However, in certain species a given isotype, for instance λ in mice or κ in horses, makes up a negligible part of the expressed L chain pool (2). Moreover, one occasionally encounters a mutant animal or a human who lacks κ entirely (3–7). Yet these κ-deficient animals are able to survive.

Rather than providing essential, nonoverlapping specificities, two L chain isotypes may be necessary to guarantee L chain production. This is because the potential for generating a nonproductive L chain may be very high. Nonproductive L chains can result from aberrant joins incurred during rearrangement, the use of pseudo V or J segments, rearrangements that delete L chain exons and deleterious somatic mutations. If the frequency of aberrant rearrangement is high, indeed a high frequency may be essential to accomplish allelic exclusion (8), then rearrangements on both alleles of an isotype should often fail and the other isotype provides an option for successful L chain production.

The frequency of generating dysfunctional receptors may be even higher if one includes rearrangements that form autoreactive receptors. Although a B cell expressing an autoreactive receptor can be deleted or inactivated (9–11), B cells can sometimes escape these self-tolerance mechanisms by editing (12–14). This process involves secondary L chain rearrangements that substitute new V genes for autoreactive V genes. Multiple L chain isotypes extend the range of options for editing. For example, an autoreaction κ antibody may be modified by rearrangement and expression of a λ L chain (15). Such a fail-safe rearrangement mechanism extends to rear-

rangements within a L chain locus. The genetic organization of both κ and λ loci permits multiple rearrangement attempts and hence corrective recombinations of autoreactive rearrangements are possible. The κ locus in the mouse has four functional Jκ segments (16) and 150–180 functional Vκ segments (17). If the first κ rearrangement results in an aberrant or autoreactive L chain, it may be possible to correct that rearrangement by a secondary, leap-frogging type of rearrangement (18–20). The organization of the λ locus also permits multiple rearrangement attempts on each allele. Unlike the κ locus, the λ locus has two clusters each containing Vλ and JλCλ recombination elements (21, 22). If rearrangement in one cluster is nonproductive, the other clusters are still available for rearrangement.

Mice in which one κ L chain allele has been inactivated by gene targeting (JCkD/wild-type [wt])[1] can be used to assess these mechanisms for coping with dysfunctional receptors. Cells with an aberrantly rearranged κ gene (κ⁻) may survive by continued rearrangement of κ or λ L chain loci. If rescue of κ⁻ cells occurs by λ rearrangement, then JCkD/wt animals should have a higher frequency of λ positive B cells than wt/wt animals. Zou et al. (23) and Chen et al. (24) in fact observe that JCkD/wt have roughly twice as many λ positive B cells as wt/wt controls. This increase in λ positive B cell number is found not only in the periphery, but also in bone marrow of JCkD/wt animals, suggesting that increased production rather than selective outgrowth of λ positive B cells takes place (23, 24). Continued κ rearrangement in JCkD/wt will result in a skewing towards the more distal Jk segments because these animals can only rearrange on one κ allele.

JCkD/wt mice can also be used to assess the role L chain editing plays in correcting autoreactive rearrangements. In the JCkD/wt mouse, editing of Vκs that contribute to an autospecificity would further skew Vκ rearrangements to the distal Jκ segments. To test this prediction of κ editing, we compared Jκ usage in JCkD/wt mice with JCkD/wt animals that have a transgene coding for an autoreactive (anti-DNA) heavy (H) chain, 3H9 (11). We found an increased frequency of rearrangement to Jκ5 in 3H9 JCkD/wt compared with JCkD/wt, consistent with editing of autoreactive L chains. This approach can be extended to JCkD/JCkD, or λ-only mice. Here editing would lead to lymphocytes with multiple λ rearrangements. We used nontransgenic JCkD/JCkD animals to analyze the frequencies of the various λ subtypes, as a basis for comparison with 3H9 JCkD/JCkD subtype frequencies. Hybridomas from 3H9 JCkD/JCkD had more frequent rearrangements to λX and less frequent rearrangements to λ1 and λ2. However, L chain allelic exclusion was maintained in most lines analyzed from 3H9 JCkD/JCkD. This indicates that continued rearrangement of λ plays a limited role in salvaging B cells with autoreactive λ rearrangements.

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[1] *Abbreviations used in this paper:* ds, double-stranded; ss, single-stranded; wt, wild-type.

Materials and Methods

Hybridomas. B cell hybridomas were prepared from spleens of transgenic 6–12-wk-old mice with the following genotypes: JCkD/wt (one animal); Vh3H9 JCkD/wt (one animal); JCkD/JCkD (two animals); and 3H9 JCkD/JCkD (five animals). The Vh3H9 (41 μ) line is described elsewhere (11). κ-deficient mice were obtained from GenPharm International (Mountain View, CA; reference 24). The κ locus in these animals was inactivated by deletion of the Jk and Ck elements in AB-1 ES cells (24). 3H9 H chain transgenic animals were backcrossed onto BALB/c and mated to JCkD homozygotes or hemizygotes. 3H9 JCkD/wt offspring were mated to JCkD/JCkD animals to produce 3H9 JCkD/JCkD animals. To prepare the hybridomas, splenocytes were stimulated with 20 μg/ml *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO) for 2–3 d before fusion with sp2/0-Ag14 (25). Hybridomas were selected using Azaserine-hypoxanthine (Sigma Chemical Co.). Supernatants were tested for antibody production and L chain isotypes, where applicable (see ELISA section).

Genomic DNA Preparation. Genomic DNA for PCR and Southern analysis was prepared as described by Ramirez-Solis et al. (26) with some modifications. Briefly, hybridoma cells were grown to high density in 24-well plates, washed twice with PBS, and incubated overnight at 55°C in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% sarkosyl, and 1.0 mg/ml proteinase K [Boehringer Mannheim Corp., Indianapolis, IN]). The next day 200 μl of ice-cold ethanol/salt mix (15 μl 5M NaCl/ml of 100% EtOH) were added to each well and incubated for 20 min at room temperature. Precipitated nucleic acid was washed three times with ice-cold 70% ethanol, air dried, and dissolved in Tris-EDTA pH 8.0.

κ PCR Assays. 100 ng of genomic DNA were used in each 50 μl reaction with 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 0.25 mM of each dNTP, 0.25 μM forward primer (Vs or Vk12/13-specific), and 0.50 μM reverse primer (Jk5, Jk2, or Jk4). DNA samples in PCR buffer and oligonucleotide primers were heated separately to 85°C for 10 min before mixing and running the amplification program: 4 min at 94°C (primary denaturation), followed by 40 cycles of 94°C 30 s, 65°C 30 s, and 72°C 90 s, followed by a final extension step at 72°C for 10 min. Samples were run on 3.0% NuSieve GTG low melting temperature agarose gels (FMC BioProducts, Rockland, ME). The Vs PCR primer is described in Schlissel et al. (27) and amplifies 80–90% of Vκ genes. The Vk12/13-specific primer sequence is 5'-CGAGCAAGTGAGAATATTTACAGTAATTTAGC-3'. The specificity of the Vk12/13 primer was tested using DNA samples from hybridomas with sequenced L chains. The Jk5 and Jk2 reverse primers are described by Ramsden et al. (28). The Jk4 primer is 5'-TTCCAACTTTGTCCCCGAGCCGAACGTG-3'. The size of the PCR product corresponds to the Jk segment participating in the rearrangement event (see Table 1).

λ PCR Assays. λ Rearrangements were amplified from genomic DNA using the following primer combinations: Vλ1/2 + Jλ1 for λ1, Vλ1/2 + Jλ2/3 for λ3 and λ2, Vλ2 + Cλ2 for λ2, and VλX + Cλ2 for λX. λ1 and λ2/3 PCR reaction conditions and primers are described in Zou et al. (23). λX- and λ2-specific primer sequences are given in Nadel et al. (29). λX- and λ2-specific PCR reactions consisted of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 0.25 mM each dNTP, 0.5 μM of each primer, and 100 ng genomic DNA. Amplifications were carried out as follows: 4 min at 94°C (primary denaturation) followed by 40 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 90 s, and a final extension step at 72°C for 5 min.

Southern Blotting. Genomic DNA was purified from hybridomas as described above and digested overnight with BamHI for κ analysis or with EcoRI for analysis of H chain rearrangements. Digested DNA was run on 0.8% agarose gels in Tris/Acetate/EDTA buffer and transferred to Zeta Probe nylon membranes (Bio-Rad Laboratories, Hercules, CA) in 0.4 N NaOH (30). κ blots were probed with 1.6R1, a 1.6 kb genomic EcoRI fragment upstream of Jk1. H chain blots were probed with pJ11, a 2.0-kb EcoRI–BamHI fragment spanning Jh3, Jh4, and the H chain enhancer region (31).

ELISA. Immulon II 96-well ELISA plates (Dynatech Laboratories, Chantilly, VA) were coated overnight with 5 μg/ml goat anti–mouse IgM antibodies (Fisher BioTechnology, Pittsburgh, PA) in 0.1 M carbonate buffer (pH 9.5). Plates were blocked for 1–2 h with 10% FCS in PBS or 1% BSA in PBS. Serial dilutions of hybridoma supernatants were added to the plates and incubated for 2 h at 37°C. Subsequently, anti–mouse κ alkaline phosphatase coupled antibodies (Fisher Scientific, Pittsburgh, PA) were added. In λ B cell assays, biotinylated anti-λ antibodies (Fisher Scientific, Pittsburgh, PA) or a monoclonal biotinylated anti-λ1 antibody (PharMingen, San Diego, CA) was used. In assays with biotinylated antibodies, plates were incubated with streptavidin-alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1,000 in PBS with 1% BSA. In the final step after extensive washing, plates were developed with the *p*-nitrophenyl phosphate substrate (Sigma Chemical Co.) for alkaline phosphatase at 1.0 mg/ml in 0.1 M carbonate buffer (pH 9.5), and 10 mM MgCl₂. Optical density at 405 nm was measured in an automated plate reader (Bio-Rad Laboratories).

DNA Binding Assays. Binding to single-stranded DNA (ssDNA) was measured in a solid phase ELISA as described previously (32). Double-stranded DNA (dsDNA) binding was determined in a solution phase ELISA with biotinylated dsDNA as described by Radic et al. (14). The same solution phase assay with denatured biotinylated DNA (ssDNA) was used to confirm ssDNA binding observed in some of the samples that typed positive in the solid phase assay for ssDNA binding.

Results and Discussion

Recovery from Nonproductive Rearrangement at the κ Locus. Vk gene replacement, as opposed to rearrangement on the other κ allele, leads to a bias favoring downstream Jk segments. This bias should be more pronounced in JCkD/wt than in wt/wt for two reasons. In wt/wt mice a nonproductive κ rearrangement can be corrected by rearrangement on the other (germline) allele. In the case of a nonproductive Jk1 rearrangement that is corrected by a Jk1 rearrangement on the other allele, the relative frequency of Jk1 to downstream Jk rearrangements will increase. The JCkD/wt mouse, however, can only correct by rearrangement to a downstream Jk segment. The second reason that the Jk bias should be more pronounced in JCkD/wt has to do with the method of analyzing Jk usage. If usage is assayed by typing the primary rearrangement regardless of whether it is functional (κ⁺) or nonfunctional (κ⁻) (see 33, 34), then the relative frequency of Jk1 to downstream Jk segments will be much higher in wt/wt than in JCkD/wt. This is because many (~50%) nonproductive Jk1 segments will be deleted as a result of editing. Although such editing can also occur in wt/wt, nonproductive rearrangements to Jk1 will be retained in examples of correction on the other allele.

In our survey we typed only productive κ rearrangements (Table 1) because we used κ expressing hybridomas from JCkD/wt. In Table 2 we compared the frequencies of Jk usage in JCkD/wt to those previously reported for wt/wt mice (33, 34). JCkD/wt has a decreased frequency of Jk1 rearrangements and an increased frequency of Jk5 rearrangements. If Jk usage were solely determined by a single κ rearrangement in each B cell, then the frequencies should have been the same. The differences observed are consistent with an increased frequency of secondary κ rearrangement in JCkD/wt.

The patterns of Jk usage in JCkD/wt and wt/wt of Kalled et al. (17) are nearly identical. The data reported by Kalled et al. were obtained from two cDNA libraries and represent a large collection of productive Vk–Jk rearrangements (17). This is in contrast to the DNA survey by Nishi et al. (34) which includes nonproductive as well as productive κ rearrangements. As discussed above, nonproductive rearrangements may be biasing the DNA frequency estimates in favor of Jk1 rearrangement. However, this explanation fails to account for the high frequency of Jk1 rearrangements observed in splenic B cell RNA by Wood et al. (33). Since it is known that some nonproductive alleles are transcribed (35), the survey of Wood et al. may also include nonproductive rearrangements. In contrast, our survey only includes productive rearrangements as also appears to be the case of the Kalled survey (17).

To obtain direct evidence of corrective recombination we identified primary rearrangements in lines that had undergone secondary rearrangement by inversion. Again the JCkD/wt animal provides an important tool for this experiment since any such product must have arisen from the cor-

Table 1. *Jk Rearrangement Frequencies in JCkD/wt and 3H9 JCkD/wt hybridoma DNA*

series	Jk1	Jk2*	Jk4	Jk5	Total
JCkD/wt	22	17	9	19	67
3H9 JCkD/wt	25	4	2	32	63

DNA from 130 κ expressing JCkD/wt or 3H9 JCkD/wt hybridomas was typed using the Schlissel degenerate Vk forward primer (Vs) and the Wu/Paige Jk5 reverse primer. The size of the amplified band on ethidium stained gels indicates which Jk segment was involved in the rearrangement (see Materials and Methods). Amplifications using the Vs and Jk5 primers yield a 1,590-bp fragment if the rearrangement is to Jk1, while the product is 1,230 bp for Jk2, 600 bp for Jk4, and 270 bp for Jk5 rearrangement. Rearrangements to Jk1 were confirmed by amplification with the Vs forward and Jk2 reverse primers (a rearrangement to Jk1 gives a band of 540 bp, a Jk2 rearrangement is 190 bp). Note that the Vs +Jk5 primer combination by definition types only productive rearrangements in JCkD/wt hybridomas (see text). 84% of all lines typed for rearrangements to Jk1, 4, or 5. The remaining 16% (listed under Jk2* in the table) harbor rearrangements to Jk2 or do not amplify with Vs. This is because the fusion partner, sp2/0, has a Jk2 rearrangement which amplifies with Vs. Thus the lines listed under Jk2* represent a maximum estimate of the number of Jk2 positive lines. As expected, we found no rearrangements to the Jk3 pseudogene segment (16).

Table 2. *Jk Frequencies Among Splenic B Cells or Splenic B Cell Hybridomas*

Reference	Stimulation	Sample	Jk1	Jk2	Jk4	Jk5
				%		
Wood et al. (33)	in vitro LPS/dextran sulfate	polyA RNA	~40	~40	~7.5	~12.5
Wood et al. (33)	in vitro LPS	total RNA	~40	~40	~10	~10
Nishi et al. (34)	in vivo LPS	DNA	42	33	8	17
Kalled et al. (17)	in vitro LPS	polyA RNA	33	29	13	24
This paper	in vitro LPS JCkD/wt	DNA	33	25	13	28

Jk rearrangement frequencies are compared in four different studies. Data from each study were reported as or converted to percentages of all Jk rearrangements surveyed. For example, in this study 22 out of 67 typed rearrangements in JCkD/wt were to Jk1 (Table 1). This corresponds to 33% of all Vk-Jk rearrangements typed. No rearrangements to Jk3 were observed in any of the studies, thus rearrangements to Jk1, 2, 4, and 5 sum to 100%. All previous surveys used B cells from mice with two functional κ alleles. Jk frequencies vary depending on the method of stimulation and nucleic acid sample characterized. Reasons for this are discussed in the text.

rected allele. By contrast, surveys of products in wt/wt cannot distinguish the chromosomal location. Our strategy was to analyze JCkD/wt lines with Jk5 rearrangements for additional rearrangements by PCR. Out of 18 JCkD/wt hybrids with Jk5 rearrangements, none had a Vk-Jk4 rearrangement. However, two out of the same 18 Vk-Jk5 lines contained primary rearrangements to Jk1 (data not shown). The fraction of Vk-Jk5 JCkD/wt lines that underwent multiple κ rearrangements is likely to be at least twice as great as 2/18 because primary rearrangements to Jk2 could not be identified (the fusion partner, sp2/0, harbors a Vk-Jk2 rearrangement). Also, our analysis only detects primary rearrangements in lines that have retained these rearrangements in their chromosomal DNA. Assuming that deletion occurs as frequently as inversion among secondary rearrangements, then 1/4 to 1/3 of JCkD/wt lines rearranged Jk5 by secondary recombination.

Editing of Autoreactive κ Rearrangements. The primary κ rearrangements of Jk1 in Vk-Jk5 positive lines from JCkD/wt could be nonproductive or productive (18–20). If the rearrangements to Jk1 were productive, perhaps they encoded autoantibodies (12–14). To assess the role of editing of autoreactive κ rearrangements, we studied 3H9 JCkD/wt. If editing revises both nonproductive and autoreactive κ rearrangements, then the recombination bias favoring downstream Jk segments should be even greater in the 3H9 JCkD/wt.

Studies in MRL/lpr lpr animals and in vitro transfection experiments have shown that the 3H9 H chain can form anti-dsDNA antibodies with a large variety of L chains, both of the κ and λ isotype (36, 37). Known anti-dsDNA combinations such as 3H9-Vk4 and 3H9-Vλ1 are not found in the periphery of normal 3H9 H chain transgenic animals (13, 14). Indeed, no hybridoma from 3H9 H chain transgenic animals was found to express anti-dsDNA antibodies (14). Instead, 3H9 H chain transgenic animals used a reduced number of different L chains and exhibited a marked skewing

towards distal Jk rearrangement (14). One particular L chain group, Vk12/13, was found in 17 out of 42 clonally unrelated hybridomas from 3H9 H chain transgenic animals (14).

First we characterized the specificity and Vk gene usage of hybridomas from 3H9 JCkD/wt animals. None out of eleven lines tested had detectable anti-dsDNA activity (data not shown). Thus, as in 3H9 H chain transgenic animals with normal κ loci, 3H9 JCkD/wt animals appear to negatively regulate anti-dsDNA B cells. Furthermore, the 3H9 transgene was detectable in 16/16 hybridomas assayed by PCR (data not shown). This suggests that the absence of anti-dsDNA activity in these hybridomas is not due to inactivation (by deletion) of the H chain transgene (38). Instead, the absence of dsDNA binding activity is probably due to L chain usage in 3H9 JCkD/wt.
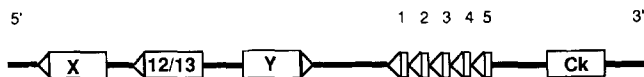
If 3H9 JCkD/wt animals avoid generating anti-dsDNA B cells by choice of L chains, this should be reflected in the expressed L chain repertoire. To test this idea, 3H9 JCkD/wt hybridomas were surveyed for rearrangements of Vk12/13, a frequently recovered L chain in 3H9 H Tg animals (14). Remarkably, 20 out of 24 hybrids from 3H9 JCkD/wt were PCR positive for Vk12/13 rearrangement. These results demonstrate that selection in 3H9 JCkD/wt favors lines with productive Vk12/13 rearrangements, since rearrangement to Vk12/13 in JCkD/wt or in wt/wt is relatively rare (none out of 14 lines from JCkD/wt had a Vk12/13 rearrangement).

κ editing of anti-DNA antibodies should result in skewing towards distal Jk segments in 3H9 JCkD/wt. To test this prediction, we typed κ rearrangements for Jk usage in κ expressing hybridomas from 3H9 JCkD/wt (Table 1). Over half of the 3H9 JCkD/wt hybrids have Jk5 rearrangements, significantly higher than the frequency of Jk5 rearrangements in JCkD/wt (by Fisher's exact test, in a 2 × 2 table of Jk5 and non-Jk5 rearrangements from 3H9 JCkD/wt and JCkD/wt panels, $p < 0.015$). Unexpectedly, the frequency of
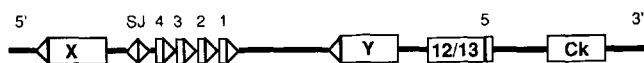
Jk1 rearrangements was similar in 3H9 JCkD/wt and JCkD/wt whereas the frequencies of Jk2 and Jk4 rearrangements were reduced in 3H9 JCkD/wt. These Jk frequencies suggest that editing follows a restricted pathway: the secondary rearrangements to Jk5 may arise mainly from primary rearrangements to Jk2 or Jk4. To search for traces of such primary rearrangements, 51 Jk5 positive lines were typed with Vs + Jk2 primers and 17 of these lines were also typed with Vs + Jk4 primers. Only one line out of 51 had retained a primary rearrangement to Jk1 and none had evidence of a primary rearrangement to Jk4 (data not shown). This finding suggests that either the secondary rearrangement to generate Vk12/13–Jk5 nearly always occurs by deletion rather than by inversion (see Fig. 1) or that L chain editing is not a common event and that recombinations to Jk5 in 3H9 JCkD/wt are primary events.

To test the first alternative we asked whether Vk12/13-Jk5 rearrangements were generated by inversion or deletion (Table 3). Southern analysis revealed that most Vk12/13–Jk5
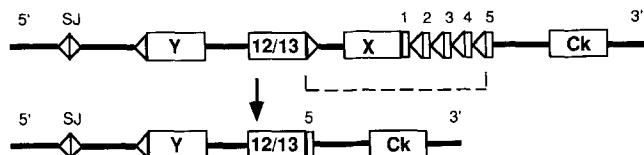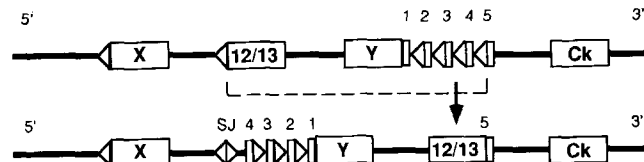

Figure 1. Rearrangement pathways to generate Vk12/13–Jk5 in 3H9 JCkD/wt hybridomas. The starting point for all murine κ locus rearrangements is the germline κ locus, shown in the top of the figure. Shown from 5' to 3' are: VkX, a hypothetical Vk segment situated upstream of Vk12/13, in the inversional orientation; Vk12/13 (there are at least three different Vk12/13 sequences, but, for simplicity, only one box depicts them in this figure); the hypothetical Vk segment VkY, in the deletional orientation; five joining (Jk) segments and Ck. Exons are denoted with *boxes*, introns with *heavy black lines*, and recombination signal sequences with *open triangles*. SJ, signal joint. Elements are not drawn to scale.

Table 3. *Southern Analysis of Vk12/13 Clones from 3H9 JCkD/wt*

Clone	Jk	1.6R1 band	Rearrangement path
		kb	
80-11	1	10	A
80-13	1	10	A
80-35	1	10	A
80-41	1	10	A
80-5	1	10.5	A
80-7	1	14.5	A
80-48	1	14.5	A
80-53	1	14.5	A
80-18	5	11.5	A
80-4	5	16	A
80-58	5	16	A
80-60	5	16	A
80-63	5	16	A
80-51	5	8	B
80-2	5	10	B
80-57	5	13.5	B
80-61	5	13.5	B
80-25	5	15	B
80-33	5	15	B
80-37	5	15	B
80-38	5	15	B
80-6	5	none	C
80-49	5	none	C
80-59	5	none	C
80-67	5	none	C

All of the hybridomas shown in the table have productive rearrangements involving Vk12/13, as determined by a Vk12/13-specific PCR assay (see Materials and Methods). Shown in the top half of the table are 3H9 JCkD/wt lines with Vk12/13-Jk1 rearrangements. These rearrangements, by definition, are primary. In the bottom portion are lines with Vk12/13-Jk5 rearrangements. L chain Southern blots were probed with 1.6R1, a 1.6-kb EcoRI-fragment derived from genomic BALB/c DNA. The 3' border of the 1.6R1 probe is ~1.2 kb upstream of the Jk1 exon. In addition to the reciprocal product band (shown in the second column of the table), all lines had a germline κ locus and at 12.7 kb due to the fusion partner and a targeted κ locus band at 8.7 kb. All lines listed in this table have also been analyzed for H chain rearrangements, using the H chain J region probe pJ11 (31). All of the lines listed in this table have distinct H chain banding patterns, indicating that they are clonally unrelated (see text). The probable κ rearrangement pathway is listed in the right most column. These editing pathways are diagrammed in Fig. 1 and discussed in the text. In the case of the Vk12/13-Jk1 lines the rearrangement pathway is primary inversion (pathway A). At least three different types of rearrangement pathways are postulated for the Vk12/13-Jk5 lines: primary inversion (path A) and two pathways (B and C) that require multiple κ rearrangements.

lines in 3H9 JCkD/wt (13/17) had retained a reciprocal product as expected by inversion. Furthermore, several of the reciprocal products were the same size (13.5 kb [2 lines], 15 kb [4 lines], and 16 kb [4 lines]). This implies a common rearrangement pathway or clonal relatedness. Vk12/13–Jk5 lines 80-33, 80-37, and 80-38 for example all share a 15-kb band which hybridizes to 1.6R1. But these same lines have very distinct banding patterns when analyzed for endogenous H chain rearrangements, indicating that they are clonally unrelated (data not shown). Similarly, lines 80-4, 80-58, 80-60, and 80-63, all of which share a 16-kb 1.6R1 band, each have different H chain bands (data not shown). Therefore, these lines share a rearrangement pathway.

To further characterize the rearrangement pathways followed by Vk12/13–Jk5 lines (Fig. 1) we carried out a Southern analysis on a panel of Vk12/13–Jk1 lines. All Vk12/13–Jk1 lines retained a 1.6R1 hybridizing band (Table 3). There were three species of bands of ~10, 10.5, and 14.5 kb, suggesting that there are at least three Vk12/13 genes. Three Vk12/13 genes have been described (39–41). Each of these three genes might produce one of the three different sized bands on the Vk12/13–Jk1 Southern blot.

If this is the case, a model of primary inversional recombination to generate Vk12/13–Jk5 in 3H9 JCkD/wt would predict only three species of bands on the Vk12/13–Jk5 blot, each ~1.5 kb larger than the corresponding Vk12/13–Jk1 band (due to the presence of Jk1–Jk4 sequences in the Vk12/13–Jk5 inverted segment). For example, Jk5 lines 80-4, 80-58, 80-60, and 80-63, (all of which share a 16-kb 1.6R1 fragment) may have rearranged by inversion to the same Vk12/13 segment as Jk1 lines 80-7, 80-48, and 80-53 (which have a 14.5-kb 1.6R1 fragment in common). Similarly, Jk5 line 80-18 (11.5-kb 1.6R1 fragment) may have rearranged by primary inversion to the same Vk as the four Jk1 lines sharing a 10-kb 1.6R1 band. However, Jk1 line 80-5 has a 10.5-kb 1.6R1 band, while none of the Jk5 lines has the predicted 12 kb band. Furthermore, there are other Vk12/13–Jk5 lines with 1.6R1 bands at 8, 10, 13.5, and 15 kb that do not fit any of the sizes predicted by the three observed species of Vk12/13–Jk1 1.6R1 bands. The only way in which an inversional model of Vk12/13–Jk5 rearrangement can account for these additional 1.6R1 bands is if they represent rearrangements to Vk segments that were not found among the three observed species of Vk12/13–Jk1 rearrangements. Alternatively, these lines underwent a rearrangement pathway (pathway B in Fig. 1) which involves secondary κ rearrangements.

In pathway B (Fig. 1), the first κ rearrangement is inversional and involves a Vk segment (VκX) situated upstream of Vk12/13. This inversional recombination places the Vk12/13 genes in a deletional orientation. When one of the Vk12/13 genes subsequently rearranges to Jk5, the primary Vk–Jk rearrangement is deleted. Nevertheless, the 1.6R1 hybridizing fragment is retained in Vk12/13–Jk5 lines because the initial inversion places it upstream of the Vk12/13 genes. This pathway can explain 8 of the 17 characterized Vk12/13–Jk5 rearrangements (see Table 3).

Not all Vk12/13–Jk5 rearrangements can be accounted for by rearrangement pathways A and B (Fig. 1). For example, line 80-67 has two Vk–Jk rearrangements, one to Jk1 and the other to Jk5. This line lacks a reciprocal product, suggesting that it arose by pathway C (Fig. 1): First, rearrangement by deletion, eliminating the 1.6R1 band, and then rearrangement of Vk12/13 by inversion to Jk5, thereby retaining the primary rearrangement. There are three other Vk12/13–Jk5 lines that lack a 1.6R1 band but also lack evidence of a prior Vk–Jk rearrangement. In these lines it is possible that the prior rearrangement was to Jk2, which the PCR assay cannot type or perhaps these lines underwent primary rearrangement by deletion to a Vk12/13 segment that was not identified on the Vk12/13–Jk1 Southern blot.

These data indicate that up to 75% (12/17, Table 3) of Vk12/13–Jk5 rearrangements in 3H9 JCkD/wt hybridomas arise secondarily. This is not simply because Vk12/13 rearrangements are generated more easily by secondary than by primary rearrangement because a large fraction of Jk1 rearrangements in 3H9 JCkD/wt hybridomas also involve Vk12/13. We believe instead that the high frequency of secondary recombinations is due to editing of autoreactive primary rearrangements. The frequency of secondary rearrangements among Jk5 lines from 3H9 JCkD/wt is two to three times greater than our estimates for JCkD/wt, suggesting that approximately two thirds of Vk12/13–Jk5 lines in 3H9 JCkD/wt began with an autoreactive receptor.

Rearrangement in the λ Locus. The organization of the murine λ locus precludes secondary, leap-frogging rearrangements. The λ locus is organized into two independent clusters (see Fig. 2 and references 21, 42–44). The upstream cluster contains Vλ2, VλX, JC2, and JC4 (JC4 is nonfunctional). Because of the orientation, Vλ2 rearrangement to JC2 will delete VλX, which is in between Vλ2 and JC2. VλX rearrangement excludes Vλ2 rearrangement by occupying JC2, the only functional J segment in the same cluster. The downstream cluster contains only one Vλ segment, Vλ1. This cluster can only undergo a single rearrangement event, Vλ1 to either JC1 or JC3. Rearrangements between λ clusters (from Vλ2 or VλX to JC1 or JC3) appear to occur infrequently (45, 46).

The inability of λ to undergo secondary, leap-frogging rearrangements limits λ editing. λ Editing, if it occurs at all, will result in multiple λ rearrangements. Therefore genotypic allelic exclusion would not be maintained in B cells with edited autoreactive λ L chains. λ Editing may be occurring at the level of H/L pairing, and depend upon the ability of the nonautoreactive L chain to displace the autoreactive L chain. Such phenotypic allelic exclusion has been observed for κ by Gay et al. (13).

To learn more about the nature of λ editing, we chose to study B cell lines from κ-deficient JCkD/JCkD and 3H9 JCkD/JCkD animals. Nontransgenic JCkD/JCkD animals provide a baseline measurement of λ subtype frequencies. These λ subtype frequencies in JCkD/JCkD allow us to ask whether rearrangements to the rarer λ subtypes (λ2, λ3, and λX) occur directly or indirectly by editing of nonproductive rear-
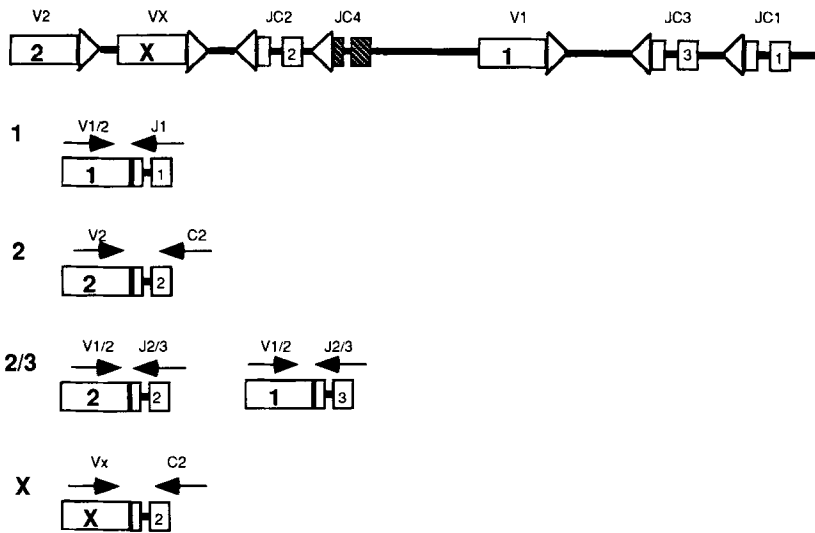
Figure 2. λ locus PCR assays. Shown are the germline λ locus and the various λ PCR primer combinations. From 5' to 3' the germline λ locus consists of Vλ2, VλX, JC2, JC4 (a pseudogene), Vλ1, JC3, and JC1. Exons are shown in *boxes*, introns with *heavy black lines*, and recombination sequences with *open triangles*. All murine Vλ segments are in the deletional orientation. Elements are not drawn to scale. Four λ PCR reactions were performed on genomic DNA from each JCkD/JCkD and 3H9 JCkD/JCkD hybridoma. As shown in the figure, they are: 1, 2, 2/3, and X. These PCR reactions do not distinguish productive from nonproductive λ rearrangements. Also note that lines with a λ2 rearrangement cannot be distinguished from lines with rearrangements of both λ2 and λ3. This is because λ2 rearrangements are amplified in the 2/3 PCR reaction. For more details concerning the λ PCR please see the Materials and Methods section and references (23) and (29).

rangements. By comparing λ subtypes and lines with multiple λ rearrangements in JCkD/JCkD with 3H9 JCkD/JCkD, we can characterize λ editing of anti-DNA antibodies.

λ *Subtype Frequencies in JCkD/JCkD.* It is known that murine λ subtypes (λ1, λ2, λ3, and λX) are not equally abundant. Surveys of λ proteins show a 5–10-fold excess of λ1 over λ2 in normal mouse sera (47). Reilly et al. (48) reported that the ratios of different λ subtypes in resting splenocytes were λ1 (Vλ1JC1)/λ2(Vλ2JC2)/λ3 (Vλ1JC3) = 1.0:0.7:0.3. The more recently discovered λX (VλXJC2, references 49, 50) occurs as frequently as λ2 among hybridomas from mice immunized with rabbit anti-λ2 antibodies (51). Preferential usage of the λ1 subtype is even more pronounced in specific immune responses (52–55). The skewing of λ subtype levels in normal mouse serum could be due to disparate frequencies of λ1, λ2, λX, an λ3 B cells, with λ1 B cells being more common than λ2 and λX B cells and so forth (56, also see discussion in 53). Alternatively, the frequencies of different λ B cells might be similar, but λ1 B cells produce more antibody than λ2 or λX B cells which, in turn express more antibody than λ3 B cells (57).

λ Subtypes are not equally represented in our panel of hybridomas from JCkD/JCkD. Of 102 hybridomas with single λ rearrangements, 66 have λ1 rearrangements, 31 have λ2 rearrangements, and 5 have λX rearrangements (Table 4). The remaining 23 lines have more than one kind of λ subtype rearrangement (Table 4). 15 of these have rearrangements to both λ1 and λ2. As in previous studies on κ wt/wt, λ1 JCkD/JCkD B cells occur more frequently than other B cells. However, in contrast to one report of λ subtype frequencies in κ wt/wt (51), cells with λX and λ3 rearrangements are uncommon in JCkD/JCkD.

We considered four artifacts that could, in theory, contribute to the low frequency of λ3 expressing lines in the JCkD/JCkD and 3H9JCkD/JCkD hybridoma panels. First, the λ3 PCR is not as sensitive as the other λ PCR assays. Perhaps all of the lines that failed to type for any λ rearrangement in fact had λ3 rearrangements. Even if this were the case, the number of PCR negative lines (<5% of all lines tested) would not be large enough to explain the difference between our λ3 frequency and previously reported λ3 frequencies. Second, the λ3 PCR reaction, which uses Vλ1/2

Table 4. λ *Rearrangements in JCkD/JCkD and 3H9 JCkD/JCkD*

	Single rearrangements			Multiple rearrangements						
	1	2	X	1+2	1+3	X+1	X+2	X+3	X12/X13	Total
JCkD/JCkD	66	31	5	15	3	0	2	1	1/1	125
3H9 JCkD/JCkD	14	7	40	2	0	18	6	7	6/5	105

Shown are numbers of hybridomas that type for rearrangements to λ1 (Vλ1JC1), λ2 (Vλ2JC2), λX (VλXJC2), λ3 (Vλ1JC3), and various combinations of these rearrangements. For example, the column labeled 1+2 is for cell lines which harbor DNA rearrangements for λ1 and λ2. PCR reactions summarized in the table were performed on genomic DNA samples from 228 hybridomas and did not discriminate between productive and nonproductive rearrangements. Note that any hybridoma with a λ2 rearrangement will also type positive for λ3 rearrangement (See Fig. 2 and Materials and Methods for a description of the primers used). These assays are also not sensitive to copy number. Thus, they cannot distinguish a cell line with a λ1 rearrangement on one allele from a line with λ1 rearrangements on both alleles.

and JΛ2/3 primers (see Fig. 2), is only capable of amplifying λ2 rearrangements. This is unlikely because λ3 was observed among lines that typed negative in the λ2-specific PCR assay (Vλ2 and Cλ2 primers), such as lines with λ 1+3, X+3, and 1+X+3 rearrangements. Third, the λ1 PCR falsely amplifies λ3 as well as λ1 rearrangements, also seems unlikely because the number of λ 1+3 doubles is small compared with the number of λ1 singles. Fourth, we note that hybridomas were derived from JCkD/JCkD splenocytes that were stimulated with LPS in vitro before fusion (see Materials and Methods). This method of polyclonal B cell activation does not appear to favor the expression or outgrowth of B cells producing particular λ subtypes (57). These considerations support the notion that the frequencies of λ subtypes observed in JCkD/JCkD approximate the true distribution of λ subtype frequencies in peripheral B cells from normal mice: λ1>λ2>λX~λ3.

Unequal frequencies of different λ subtypes could arise by biased recombination and/or by selection. Biased recombination could lead to sequential λ rearrangements, with λ1 rearrangement always preceding rearrangements to λ2, λX, or λ3, in much the same way as κ rearrangement is thought to precede λ rearrangement (58). This regulated model of λ rearrangement implies a functional connectivity between the different λ subtypes. For example, λ1 rearrangement, according to this model, may be required for rearrangements of other λ subtypes to occur. This could, in part explain the rarity of λ3 B cells, since a rearrangement to λ1 ablates λ3 rearrangement on the same allele. The prediction of such an ordered model of λ rearrangement is that a line with λ2, λ3, or λX rearrangements should also have a nonproductive λ1 rearrangement(s). Our data do not support this prediction because a largely majority of λ2 and λX lines from JCkD/JCkD have not rearranged λ1 (Table 4).

Nevertheless, there could still be a mild preference for λ1 rearrangement. Zou et al. (23) found about twice as many λ1⁻ rearrangements as λ3⁻ rearrangements and approximately twice as many λ3⁻ rearrangements as λ2⁻ rearrangements. As the authors point out, these data suggest that λ1/λ3 rearrangements occur more often than λ2 rearrangements.

JCkD/JCkD Lines with Multiple λ Rearrangements. A prediction of λ editing is B cells with multiple λ rearrangements. Consistent with λ editing, 18% of JCkD/JCkD lines have more than one λ subtype rearrangement. (Our results differ from those of Nadel et al. [59], who surveyed 67 λ hybridomas by Southern analysis and found only 2 which scored positive for more than one λ rearrangement.)

λ Editing could be part of an ordered rearrangement program for λ subtypes, or it could occur in a probabilistic fashion, rescuing some fraction of lines with a nonproductive rearrangement to any λ subtype. If rearrangement is promptly terminated after the formation of a productive λ, then any nonproductive λ rearrangement in a λ⁺ B cell must precede the productive rearrangement. Thus if λ rearrangement is ordered, with λ1 preceding λ2, most lines with λ1 and λ2 rearrangements should have nonproductive λ1 rearrangements. When we analyzed 15 JCkD/JCkD λ 1+2 doubles for λ1

expression, we found that 11 had detectable λ1 reactivity (data not shown). Most JCkD/JCkD λ 1+2 doubles appear to have productive λ1 rearrangements. Reverse transcriptase PCR analysis indicates that the λ2 L chain is not transcribed in two out of four λ1+λ2 JCkD/JCkD lines (Luning Prak, E., D. Ni, and M. Weigert, unpublished observations). The

Table 5. *3H9 JCkD/JCkD Hybridomas with λ1 Rearrangements*

Clone	λ PCR	λ1	ssDNA	3H9 transgene
59-8	X+1	−	−	+
59-10	X+1	−	−	+
59-15	X+1	−	−	+
59-30	X+1	+	+	+
59-31	X+1	+/−	+	+
59-32	X+1	+/−	−	+
59-36	X+1	+	−	+
59-41	X+1	−	−	+
3H9-7	X+1	−	−	+
3H9-9	X+1	−	−	+
83-9	X+1	−	−	+
66-2	X+1	ND	−	+
66-6	X+1	−	ND	+
66-8	X+1	−	ND	+
66-18	X+1	−	ND	+
66-26	X+1	−	ND	+
66-33	X+1	−	ND	+
66-34	X+1	+	+/−	+
66-4	1	+	+	+
66-11	1	+	+	+
66-24	1	−	+	+
66-28	1	+	+	+
66-35	1	+	+	+
66-37	1	+	+	+
66-46	1	+	−	+
83-6	1	+	−	−
3H9-8	1	+	−	−
3H9-3	1	+	−	−
3H9-4	1	+	+	−
83-10	1	−	−	−
59-35	1	−	ND	−
59-43	1	+	ND	−

3H9 JCkD/JCkD λ expressing hybridomas with λ1 or with λ1 and λX DNA rearrangements (typed by PCR, see Materials and Methods) were assayed for λ1 expression, ssDNA binding, and 3H9 transgene DNA. λ1 binding was determined by an ELISA using an anti-λ1–specific mouse monoclonal antibody (PharMingen; see Materials and Methods). ssDNA binding was measured in a solid phase ELISA. Positives did not display significant binding to protamine-only control plates. The 3H9 transgene was assayed by PCR (described in reference 11). ND, not done.

simplest explanation is that a nonproductive λ2 rearrangement preceded a productive λ1 rearrangement in most of the λ1 + λ2 double rearrangers from JCkD/JCkD. This excludes an ordered model of λ subtype rearrangement in which editing is restricted to cells with nonproductive λ1 rearrangements.

Instead, it is possible to account for these results under a simple selection model and strict allelic exclusion. In this model, rearrangements of λ1 and λ2 are equally likely but half of the rearrangements to λ2 are selected against. Thus, before selection there would be equal numbers of λ1 and λ2 positive lines, but after selection there would be twice as many λ1 lines as λ2 lines. Among the λ 1+2 doubles, before selection, half would have functional rearrangements to λ1 and the other half to λ2. After selection, two thirds of the λ 1+2 doubles would have functional rearrangements to λ1.

λ *Rearrangements in 3H9 JCkD/JCkD.* To understand how B cells cope with an autoreactive λ antibody, we studied homozygous κ-deficient animals with the 3H9 H chain transgene (3H9 JCkD/JCkD). Previous studies showed that the antibody formed by the 3H9 H chain and the λ1 L chain binds dsDNA (37). Since anti-dsDNA antibodies are deleted in normal animals (13), it seemed reasonable to suppose a similar fate for 3H9-Vλ1. The 3H9 JCkD/JCkD animal provides an in vivo assay of the fate of 3H9-Vλ1, and a similar test for the other λ L chains (λ2, λX, and λ3).

That selective pressures can dramatically alter the λ subtype frequencies is illustrated by comparing the λ rearrangements of 3H9 JCkD/JCkD with those of JCkD/JCkD (Table 4). In 3H9 JCkD/JCkD, hybridomas with rearrangements to λX are more frequent whereas lines with rearrangements to λ1 or λ2 are less common. (The presence of λ1 and λ2 expressing B cells in 3H9 JCkD/JCkD is explained in large part by deletion of the 3H9 transgene and expression of endogenous H chains, as discussed below). Although there are fewer lines with only λ1 or λ2 rearrangements in 3H9 JCkD/JCkD, the ratio of λ1-only lines/λ2-only lines is nearly 2:1 in both strains (14 λ1:7λ2 in 3H9 JCkD/JCkD, 66λ1:31λ2 in JCkD/JCkD). Based on the altered frequency distribution in 3H9 JCkD/JCkD, it appears that λ1 and λ2 producing lines are disfavored, whereas λX lines are favored.

3H9 JCkD/JCkD Hybridomas with Multiple λ Rearrangements. In addition to the increased frequency of λX lines, lines with multiple rearrangements are more common in 3H9 JCkD/JCkD than in JCkD/JCkD. In particular, lines with multiple λ rearrangements including λX were more frequent in 3H9 JCkD/JCkD. Because the PCR assays do not distinguish productive from nonproductive λ rearrangements, we wondered whether these double or triple rearrangers were double or triple producers or, if they maintained allelic exclusion. Making this distinction has implications for the mechanism of tolerance. If allelic exclusion is not maintained in these cells, then one pathway of escape into the periphery for autoreactive λ B cells is to continue rearranging λ light chains until the autoreactive L chains (such as λ1 in 3H9 JCkD/JCkD) are either diluted or out-competed by nonautoreactive L chains (in this case λX). If, on the other hand, allelic exclusion remains intact, skewing towards productive

rearrangements of a particular subtype (in this case λX) would be achieved mainly by selective pressures.

To distinguish these alternatives we surveyed lines with λ1 and λX DNA rearrangements for λ1 antibody expression (Table 5). At least 12 out of 18 λ X+1 lines fail to bind to an anti-λ1 monoclonal antibody, suggesting that a majority of λ 1+X lines lack productive rearrangements to λ1. The absence of serologically detectable λ1 activity correlates with a lack of ssDNA binding. Furthermore, none of the λ X+1 lines has deleted the 3H9 H chain transgene. 2 out of 18 λ X+1 lines are positive for λ1 by serology and bind ssDNA whereas two other lines, 59-32 and 59-36, are positive for λ1 by serology but lack measurable ssDNA binding activity. The λ1 RNA sequence of 59-36 is identical to the wild-type λ1 sequence (Luning Prak, E., et al., manuscript in preparation). The absence of ssDNA binding, despite an unmutated λ1 sequence suggests that another L chain (λX) or an endogenous H chain is altering the antigen binding characteristics of this line. Sequencing of λX RNA from line 59-36 revealed an inframe VλXJλ2 junction, indicating that this line is a λ 1+X double producer (Luning Prak, E., et al., unpublished data).

Nevertheless, the large majority of λ 1+X hybridomas in 3H9 JCkD/JCkD do not express λ1 and have no measurable ssDNA binding activity. Despite the lack of serologically detectable λ1 activity, these lines could still have productively rearranged λ1 L chains that fail to be exported to the cell surface. DNA sequence analysis of three such lines (59-10, 3H9-9, and 3H9-7) reveals frame-shift mutations generated by base insertions and/or deletions at the VλJλ junction (Ni, D., E. Luning Prak, and M. Weigert, unpublished data). These results suggest that most λ 1+X doubles in 3H9 JCkD/JCkD in fact have nonproductive λ1 rearrangements.

These results differ from those for λ1 lines (Table 5). The presence of λ1 lines in 3H9 JCkD/JCkD was unexpected because the λ1 3H9 antibody binds dsDNA and was not recovered in hybridoma panels from normal background 3H9 H chain transgenics (37, 14). Further analysis revealed that several λ1 3H9 JCkD/JCkD hybridomas had deleted the H chain transgene DNA. This type of H chain editing has been observed in other anti-DNA transgenics (38) and accounts for roughly half of the λ1 3H9 JCkD/JCkD hybridomas. Other λ1 hybridomas may have escaped deletion by downregulation of the 3H9 H chain RNA and/or dilution of the transgene product by coexpression of endogenous H chains.

Nonproductive λ1 L chains among λ 1+X doubles and a paucity of λ1-only lines suggest that negative selection rather than λ editing of autoreactive B cells is the major B cell tolerance mechanism in mice that can only express λ. Selection also strongly influences the types of κ L chains that can be recovered from 3H9 JCkD/wt animals. However, the high frequency of secondary κ rearrangements observed in 3H9 JCkD/wt, is not predicted by selection. Instead, secondary rearrangement is an essential requirement for, and probable consequence of, κ editing in these animals. When successful, editing of autoreactive or nonproductively rearranged κ chains allows cells to avoid clonal elimination.

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