Receptor Editing Occurs Frequently during Normal B Cell Development

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Summary

Allelic exclusion is established in development through a feedback mechanism in which the assembled immunoglobulin (Ig) suppresses further V(D)J rearrangement. But Ig expression sometimes fails to prevent further rearrangement. In autoantibody transgenic mice, reactivity of immature B cells with autoantigen can induce receptor editing, in which allelic exclusion is transiently prevented or reversed through nested light chain gene rearrangement, often resulting in altered B cell receptor specificity. To determine the extent of receptor editing in a normal, non-Ig transgenic immune system, we took advantage of the fact that λ light chain genes usually rearrange after κ genes. This allowed us to analyze κ loci in IgM λ+ cells to determine how frequently in-frame κ genes fail to suppress λ gene rearrangements. To do this, we analyzed recombinant VκJκ genes inactivated by subsequent recombining sequence (RS) rearrangement. RS rearrangements delete portions of the κ locus by a V(D)J recombinase-dependent mechanism, suggesting that they play a role in receptor editing. We show that RS recombination is frequently induced by, and inactivates, functionally rearranged κ loci, as nearly half (47%) of the RS-inactivated VκJκ joins were in-frame. These findings suggest that receptor editing occurs at a surprisingly high frequency in normal B cells.

Key words: receptor editing • recombining sequence recombination • immune tolerance • B lymphocytes • V(D)J rearrangements

The fact that virtually all B cells express a single H and L chain prompted many studies to elucidate the underlying mechanism. One process that clearly contributes to allelic exclusion is the imprecision of V(D)J rearrangement that generates a maximum of one in-frame rearrangement per three attempts (1), but more active feedback processes are also involved. Classic studies showing the ability of a H chain transgene (2, 3) or an L chain transgene (4, and for review see reference 5) to mediate feedback suppression of H and L chain rearrangements, respectively, established important paradigms that have been widely accepted. But in the case of L chain allelic exclusion, this paradigm was weakened by an increasing number of “exceptions”, in which ongoing L chain rearrangement occurred despite expression of functional κ chain (6–9). Studies with autoantibody transgenic (Tg)1 mice suggested that many of the exceptions to the feedback regulation model of L chain allelic exclusion could be explained by postulating self-tolerance-induced receptor editing (10–15). In addition, recent in vitro studies (16–18) and analyses of autoantibody Ig knock-in mice (19, 20) have shown that L chain gene receptor editing can be an important mechanism of B cell tolerance. Despite these findings, it is unclear how frequently receptor editing is used for tolerance induction in normal, non-Ig Tg autoreactive B cells, in part because the extent of autoreactivity in the preselected B cell repertoire is unknown.

The organization of the κ locus, with arrangement of Vκ genes in both sense and antisense transcriptional orientations, the absence of D region gene segments, and the presence of several Jκ gene segments facilitates sequential, nested Vκ-to-Jκ rearrangement attempts (for review see reference 21). In developing B cells, these secondary rearrangements can both rescue receptor expression in cells that fail to assemble in-frame L chains (1, 22) and rescue autoreactive B cells from tolerance elimination by replacing rearranged κ genes with new ones that alter specificity (for review see reference 23). Another way that the organization of the κ locus promotes receptor editing is suggested by the existence of the conserved element known as recombining sequence (RS) in the mouse (or the homologous “κ deleting element” in humans, reference 24). RS is located ~25 kb...
Materials and Methods

Mice: Mice homozygous for the targeted deletion of the Jκ-Cκ locus (Jκx-D/Jκx-D); a gift from D. Huszar, GenPharm International, San Jose, CA; reference 33) were maintained under specific pathogen-fee conditions in the animal care facility at National Jewish Medical and Research Center. Jκx-D/Jκx-D mice were bred with B10.D2Sn/J mice to generate B10.D2Sn/J-Jκx-D/+; mice (Jκx-D/+), which were used at 6-8 wk of age.

Cell Sorting and Genic DNA Isolation. Splenic cells from Jκx-D/+ mice were isolated and stained with goat anti-mouse IgM+ λ+ B cells and determined their nucleotide sequences in order to ascertain the extent to which R S inactivates functional κ genes in a normal, non-Ig Tg immune system. The results indicate that in normal IgM+ B cells R S-mediated receptor editing is induced by and frequently inactivates functionally rearranged κ genes, probably because of immune tolerance.

Receptor Editing in Normal B Cells

This study, we have isolated such Vκxκ joins from a large number of individual IgM+ λ+ B cells and determined their nucleotide sequences in order to ascertain the extent to which R S inactivates functional κ genes in a normal, non-Ig Tg immune system. The results indicate that in normal IgM+ B cells R S-mediated receptor editing is induced by and frequently inactivates functionally rearranged κ genes, probably because of immune tolerance.

One approach to estimate the extent of receptor editing in normal B cells is to analyze V(D)J recombinational rearrangements that are the predicted residues of editing. In mouse B cells, which contain both κ and λ L chain loci, λ gene rearrangement almost always occurs after κ rearrangement (for review see references 29, 30). Thus, if an appropriate κ gene is not assembled, rearrangement at the λ locus often follows. In λ+ B cells, R S rearrangements usually have deleted the Cκ loci (27, 28, 31) either by recombining to Vκκ through the well characterized heptamer–nonamer recombination signal sequences (Fig. 1 B), or to heptamer sites in the Jκ-Cκ intron (Fig. 1 C) (27, 28, 32). Besides destroying the function of the κ locus, this latter mode of R S recombination has two important effects: first, unlike nested Vκκ recombinations, it eliminates the Cκ-associated δ-acting enhancer elements that are critical for Vκκ expression and rearrangement (33–36), and second, it retains any Vκκ join that was previously adjacent to Cκ. This physiological knockout of regulatory sequences required for κ gene rearrangement thus “freezes” the locus, allowing an analysis of the Vκκ gene that was assembled adjacent to the Cκ exon just before R S and λ gene rearrangement.

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V_{k}\kappa\text{-RS rearrangement from hybridoma 15E11 was also cloned by PCR using a leader intron oligo (5' TGGATCCAGTCTTCTACTGCCATTCTG-3')} and an oligo which spanned the XbaI site downstream of J_{k}k (5' ACAGATCTGTCTAGAGACATTTGG-3'). The resulting product was digested with EcoRI and subcloned into a shuttle vector containing V_{k}2IC leader and promoter elements. An XbaI fragment that contained the promoter elements, leader, and the 15E11 V_{k}\kappa\text{ rearrangement was isolated and cloned into the XbaI site in pSV2-neo-C (42). The 2H11 and 15E11 H and L chain constructs were then cotransfected into SP2/0 myeloma cells and selected by H_{1233} and 15E11 H and L chain transfectoma clones were assayed by the PCR strategy outlined in Fig. 1 for expression of IgM}_{k}. This cell sorting strategy should exclude from the template pool cells that are \kappa\text{-}, H chain isotype switched, surface (s)Ig{\kappa}-, or cells of a sIg{k}+, germinal center phenotype. In a second series of experiments, IgM \lambda secreting hybridomas were isolated and their \kappa loci analyzed in detail. To simplify these analyses, all B cells analyzed were heterozygous for a targeted deletion of the J_{k}/-C \kappa (J_{k}/-*) locus, which is responsible for elimination of the known \kappa loci and RS allele could rearrange (33). The potential \kappa gene and RS element rearrangements are depicted in Fig. 1.

A analysis of IgM \lambda Cells Reveals Frequent Receptor Editing. Genomic DNA from sorted IgM \lambda cells was used as template for a PCR using a panspecific V_{k} FWR 3 oligonucleotide primer, which recognizes ~80% of V_{k} genes (37), together with an R S-specific primer to amplify V_{k}\kappa-intron-R S rearrangements (Fig. 1 C, primers A and B). V_{k}\kappa-intron-R S rearrangements containing V_{k} genes rearranged to each of the four functional J_{k} genes were detected by PCR amplification and Southern blotting (data not shown), but V_{k}\kappa5-intron-R S rearrangements were most abundant, in part because their smaller size promoted preferential amplification. Amplified V_{k}\kappa5-intron-R S rearrangements were gel-purified and cloned, and a total of 52 clones were sequenced across both the V_{k}\kappa and the J_{k}-intron-R S joins (Fig. 2). These two different recombination join, present on each PCR product analyzed, provided markers for uniqueness. PCR products that were identical to one another, or that differed by just one nucleotide, were assumed to represent repeated isolates derived from the same initial template (i.e., derived from a single B cell clone). This represents an underestimate because the single base changes could have reflected real differences and because it was possible that some of the apparent repeats were independent events that happened to have identity in the portions of the genes studied, but not in upstream portions of the V genes. In this sample, at least 37 of the 52 clones represented independent events. Analysis of the V_{k}\kappa join sequences allowed an assessment of the potential prior functionality of the V_{k}\kappa5 joins just upstream of intron-R S rearrangements. Surprisingly, 15 of the 37 clones (41%) contained V_{k}\kappa5 joins that were in-frame (Fig. 2), and if the apparent repeats were not excluded 23 out of 52 (44%) were in-frame.

To verify the analysis of the PCR-amplified V_{k}\kappa-intron-R S rearrangements and to increase the sample size, an independent sampling of V_{k}\kappa-intron-R S rearrangements was derived from J_{k}C_{k}/+ splenocytes in the form of B

![Diagram.png]

Figure 1. RS rearrangements inactive and preserve V_{k}\kappa joins. A rearranged, potentially functional \kappa locus (A) can be silenced by two types of RS recombination: V_{k}\kappa-R S (B) or V_{k}\kappa-intron-R S (C). Type C retains the prior V_{k}\kappa join, and the RS recombination event eliminates the known ds acting elements that are critical for efficient rearrangement and expression, thus freeing the locus from further V_{k}\kappa recombination. Also shown are the intronic recombination sequence 1 (RS 1) (32), the intronic (IE) and 3' kappa (3'E) enhancers (35, 36), and the recombination sequence (RS) (27, 28) element. Probes IVS (1) and RS 0.8 (27, 28) are indicated by filled boxes.
cell hybridomas. A total of 133 IgM-κ expressing hybrids were obtained from five separate fusions and their κ locus rearrangements were analyzed. Genomic Southern blot and PCR analysis revealed that at least 74% of the λ+ hybrids (99 out of 133) had inactivated the wild-type κ locus by RS rearrangements (Table 1), a value in accord with previous estimates (31, 34). Two hybridomas apparently had undergone inversional Vκ-RS rearrangements, as they had unique restriction fragments that retained the Cκ locus as revealed by the intron (IVS) probe (data not shown), but scored positive in a Vκ-RS PCR (Fig. 1B). Approximately 25% (26 out of 99) of the hybridomas with RS rearrangements had Jκ-intron-RS joins (Table 1), as detected with primers B and C (Fig. 1C). Genomic Southern blot analysis of 18 out of 20 hybrids scoring PCR positive for Jκ-intron-RS rearrangements demonstrated that the RS rearrangements colonized with EcoR I restriction fragments hybridizing with the IVS probe (data not shown), thus independently confirming the Vκ-Jκ-intron-RS rearrangement phenotype. Vκ-Jκ-intron-RS rearrangements from individual hybridomas were PCR amplified and directly sequenced, rather than cloned, a procedure that diminishes potential Taq polymerase-generated mutations. Like the Vκ-Jκ-intron-RS PCR clones, most of the Vκ-Jκ-intron-RS loci from hybridomas used Jκ5, although four hybridomas had rearrangements to upstream Jκ5, including one to Jκ2 and three to Jκ4, suggesting that developing B cells do not frequently undergo RS rearrangement until all of the Jκs are rearranged. Sequence analysis over both the Vκ-Jκ and intron-RS joins clearly showed that each cell line had a unique sequence at the Vκ-Jκ join and that, remarkably, 12 of 20 (60%) of the Vκ-Jκ joins were in-frame (Fig. 3A).

**Table 1.** κ Locus Rearrangement Status of IgM λ H ybridomas

| Hybridoma | κ Locus genotype | Productive/Nonproductive |
|-----------|------------------|-------------------------|
| Hλαβγδεδ | Rk-RS | Vκ-Jκ-RS |
| n          | Fusion 1 | Fusion 2 | Fusion 3 | Fusion 4 | Fusion 5 |
| 1234      | 37 | 18 | 9/9* | 1          | 2/2 |
| 1234      | 44 | 10 | 4/4 | 2          | 1/1 |
| 1234      | 12 | 1  | 5/4 | 4/0        |       |
| 1234      | 25 | 9  | 5/2 | 1/1        |       |
| 1234      | 15 | 2  | 3/1 | 0/1        |       |
| Totals    | 133 | 34 | 26/20 | 12/8 |

RS-S denotes hybridomas that lacked a detectable RS rearrangement. Vκ-RS and Vκ-Jκ-RS are defined in Fig. 1. A asterisk indicates total number of Vκ-Jκ-intron-RS loci that were isolated followed by the number that we were able to PCR amplify with the consensus FWR3 oligo.
direct PCR–derived clones, whereas 6 hybridomas expressed distinct V_\kappa genes that were members of families observed in the PCR clone sample and 1 hybridoma expressed a V_\kappa32 gene, a V_\kappa family not seen in the PCR clone samples (Figs. 2 and 3 and data not shown).

### Intron/RS Joins

The sequences of the J_\kappa-intron-RS joins in both the PCR clones (Fig. 2) and hybridomas (Fig. 3) were quite varied and were dominated by deletions at both sides of the joins, as up to nine nucleotides were missing from either the J_\kappa-intron or RS heptamer-flanking sequences. There did appear to be a bias for a particular join (e.g., clone 4, Fig. 2 A), which was observed to be associated with 13 independent V_\kappaJ_\kappa rearrangements. Two of the intron-RS joins contained P nucleotides and one contained N-region addition nucleotides, consistent with findings described previously (7, 43).

### Rebuilding IgM_\kappa Antibodies for Analysis of H/L Pairing and Antigen Specificity

To determine if the high frequency of in-frame V_\kappaJ_\kappa rearrangements silenced by intron-RS recombination was due to the inability of H chains to pair with their \kappa L chains, the V(D)J and V_\kappaJ_\kappa rearrangements from hybridomas 2H11 and 15E11 were cloned into C_\mu and C_\kappa expression vectors, respectively. These H and L chain constructs were cotransfected into SP2/0 myeloma cells to generate transfectedoma clones. Analysis of transfectedoma supernatants by IgM_\kappa sandwich ELISA revealed that the in-frame \kappa L chains were able to pair with their hybridoma H chains (Fig. 4), suggesting that ongoing R S rearrangement was not due to the inability of H/L chain pairing. The specificity of the \kappa L transfectedoma antibodies remains unknown, however. Attempts in flow cytometry assays to detect recombinant antibody binding to the surfaces of bone marrow cells were unsuccessful (data not shown).

### Discussion

In this report we examined the DNA sequences of V_\kappaJ_\kappa joins located upstream of intronic-RS rearrangements in normal, non-Ig Tg B cells to determine the extent to
which RS-mediated recombination silences functionally rearranged κ genes. Nearly half of all VkJκ joins inactivated by RS recombination were in-frame (27 out of 57). This high frequency is clearly incompatible with a strict feedback suppression model of L chain allelic exclusion, which predicts no in-frame VkJκ joins upstream of the RS rearrangements. More strikingly, this high frequency is also significantly higher than 33%, the percentage of in-frame joins expected from random VkJκ rearrangement, indicating that productive VkJκ rearrangements actively induce intron-RS rearrangements. The data also demonstrate a physiological role for the RS element in normal B cell development—the inactivation of functionally rearranged κ genes.

To understand why we conclude that the RS rearrangements were actively induced by functional κ L chains, consider the extreme hypothetical cases of mice in which all κ gene rearrangements result in either autoreactive B cell receptors or nonproductive κ chains (Table 2). If Vk-to-Jκ and RS rearrangements proceed randomly, albeit with different relative frequencies, then in either case VkJκ joins located upstream of intronic-RS rearrangements should be in-frame at a maximum frequency of one out of three. To significantly exceed this frequency, in-frame VkJκ joins must stimulate the relative rate of (intronic) RS rearrangement. This argument applies to our data because the observed frequency of in-frame joins, 47.4%, is significantly higher than one out of three (P < 0.04, single sample test of a proportion based on a normal approximation). Since it is exceedingly unlikely that the stimulus for increased in-frame rearrangements is mediated by anything other than κ protein, and because κ chains can probably only be perceived by the signaling machinery of B cells through their association with H chains, we conclude that functional κ chains actively stimulate the rate of RS rearrangement based on B cell receptor antigenic specificity. These data also predict that in mice in which the Cκ exon is inactivated, but surrounding os-acting elements are left intact, VkJκ rearrangement should be extensive, whereas RS rearrangement should be reduced. This is in fact the experimental observation (44).

The statistical argument also excludes the possibility that a high frequency of rearrangeable Vk pseudogenes, L chains that fail to pair with H chains, or a role for positive selection is responsible for our results. Furthermore, complete sequencing of the coding regions from all the in-frame VkJκ rearrangements derived from λ+ hybridomas revealed no stop codons or other obvious defects that would have precluded function (Fig. 3A). It is also unlikely that frequent aberrant H/L chain pairing is responsible for the high frequency of in-frame VkJκ joins in the VkJκ-intron-RS rearrangements, as demonstrated by the ability of H/κL chains from two hybridomas to pair (Fig. 4). Moreover, there are few examples of L chains that fail to pair with H chains and most experiments suggest that virtually all random H/L pairs can associate (40, 45–48). Finally, if a lack of positive selection of surface Ig was responsible for the high frequency of in-frame joins, this would predict that B cells should frequently express two κ chains, a result that has not been observed.

The receptor editing events documented in this study probably do not represent renewed V(D)J recombination in mature B cells, such as has recently been described in the germinal center (49–51), because the cells analyzed expressed high levels of IgM and λ chain and because they were isolated and, in the case of the hybridomas, stimulated in a manner that should not have induced V(D)J recombination. Another indication that receptor editing in mature B cells is unlikely to explain our results is that the fraction of λ+ cells in newly formed and mature splenic B cells is nearly identical, suggesting that in unmanipulated mice mature κ+ cells rarely give rise to λ+ B cells (44). Overall, it would appear from our data that the RS rearrangements that we studied were actually stimulated, rather than inhibited, by productive κ gene rearrangements, probably as the result of immune tolerance-mediated receptor editing in immature B cells. To definitively test the prediction that the κ chains of the cells that we have analyzed generate autoantibodies in association with the same cell’s heavy chain, it will be necessary to generate mice transgenic for these genes.

Table 2. Analysis of VkJκ Joins in VkJκ-intron-RS Sequences: Models and Predictions Compared with Experimental Data

| Models | Predicted fraction in-frame | Experimental data | Observed percentage in frame |
|--------|-----------------------------|-------------------|------------------------------|
| Perfect feedback regulation with functional κ chain preventing λ rearrangement | 0% | 27 out of 57 VkJκ-intron-RS loci | 47.4% (P < 0.04) |
| Poor feedback regulation | ≤33% | | |
| High frequency of Vk pseudogenes | ≤33% | | |
| High frequency of H/κ chain mispairing | ≤33% | | |
| Any combination of the above | ≤33% | | |
| Extreme model of editing with random RS rearrangements and all κs autoreactive | 33% | | |
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