

Receptor Editing in Self-reactive Bone Marrow B Cells

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Summary

A central paradigm of immunology is clonal selection: lymphocytes displaying clonally distributed antigen receptors are generated and subsequently selected by antigen for growth or elimination. Here we show that in mice transgenic for anti-H-2K^b antibody genes, in which a homogeneous clone of developing B cells can be analyzed for the outcome of autoantigen encounter, surface immunoglobulin M⁺/idiotype⁺ immature B cells binding to self-antigens in the bone marrow are induced to alter the specificity of their antigen receptors. Transgenic bone marrow B cells encountering membrane-bound K^b or K^k proteins modify their receptors by expressing the V(D)J recombinase activator genes and assembling endogenously encoded immunoglobulin light chain variable genes. This (auto)antigen-directed change in the specificity of newly generated lymphocytes is termed receptor editing.

Each B lymphocyte bears a unique antigen receptor composed of a single Ig light chain and a single heavy chain, despite its potential to simultaneously produce two different heavy chains and six different light chains (two κ 's and four λ 's). This so-called "allelic exclusion" is thought to be important in conferring on the B cell receptor a high degree of antigen specificity, which is critical for the process of clonal selection (1, 2). In adult mice, B cell production and V gene assembly occur in the bone marrow (3). Rearrangement and expression of the Ig genes is a highly ordered process (4), initiated by DNA rearrangement at the heavy chain loci in pro-B cells, where D elements are specifically ligated to J elements. V_H-to-DJ rearrangement then ensues, completing heavy chain gene assembly and leading to functional μ heavy chain protein synthesis. The resulting pre-B cells then rearrange light chain genes, leading to their expression of surface IgM. Light chain rearrangement appears to occur first at the κ locus and, because the light chain loci have no D minigenes, involves only V to J rearrangement (5, 6). λ light chain V-to-J gene rearrangement also occurs at this stage of development, but cells rearranging and expressing λ light chains generally have previously undergone nonproductive rearrangements at the κ locus, such as RS recombination; thus, λ rearrangement may represent a salvage pathway (7, 8). In developing B cells, expression of membrane-bound μ heavy chains mediates allelic exclusion by feedback inhibition of heavy chain gene rearrangement (9–12). Light chain rearrangement is believed to be blocked by the expression of membrane IgM that

results from the assembly of heavy and light Ig chain proteins (11, 13–15). However, the recent finding that circular DNAs excised by nested κ gene rearrangements frequently contain rearranged VJs that are in frame and potentially functional (16) contradicts this notion and suggests that light chain gene rearrangement often continues in IgM⁺ cells.

Immature lymphocytes that have successfully recombined receptor genes display their antigen receptors on the cell surface where they are then selected by antigen for activation, inactivation, or elimination. Autoreactive lymphocytes are probably produced continuously in the primary lymphoid organs. How are such autoreactive lymphocytes controlled? To study negative selection of autoreactive B cells in the mouse, many model systems have been used. These have included the treatment of immature B cells from normal mice with tolerogens or antibodies specific for IgM (reviewed in reference 17) and the generation of transgenic mice bearing functional, rearranged Ig genes encoding autoantibodies (reviewed in reference 18). These studies have yielded a wealth of information supporting the idea that B cells can be tolerized to self-antigens and suggest that more than one mechanism may play a role in this tolerance. Experiments using transgenic mice have shown that one way that autoreactive B lymphocytes are selected against in vivo is "clonal deletion" (19–25). In the bone marrow, encounter with cell membrane-associated self-antigens, capable of crosslinking Ig receptors on B cells with high avidity, leads to their elimination from secondary lymphoid tissues. However, a large pool of self-reactive

sIgM^{low} B cells is present in the bone marrow, suggesting that immature B cells are developmentally blocked (19, 20, 24). In this paper we address the mechanism by which deletion occurs in B cells reactive to membrane-bound autoantigens. We test the hypothesis that receptor selection, rather than clonal selection, occurs in immature, bone marrow B cells, and present data in support of this hypothesis indicating that immature autoreactive B cells encountering self-antigen attempt, and often succeed, in altering their specificities through secondary Ig light chain gene rearrangement.

Materials and Methods

Mice and Bone Marrow Chimeras. 3–83 μ / δ transgenic mice were produced as described (23). This transgenic mouse line bears approximately four copies of a 42-kb heavy chain genomic construct containing the genomic VDJ₃₋₈₃-containing R1 fragment upstream of a genomic C μ -C δ construct and approximately three copies of an 18-kb genomic fragment containing the intact VJ₃₋₈₃-C κ light chain gene. The light and heavy chain transgenes cosegregate in breeding experiments and are probably integrated at the same locus (our unpublished results). 3–83 μ / δ transgenic mice were bred and maintained in the animal care facility at the National Jewish Center for Immunology and Respiratory Medicine. MTK^b mice (26) were backcrossed twice onto a BALB/c (H-2^d) background. 3–83 μ / δ transgenic mice were bred three generations onto B10.D2/oSnJ (H-2^d) and subsequently bred one generation with B10.D2/oSnJ (H-2^d), C57BL/10J (H-2^b), B10.A/SgSnJ (H-2K^d), MRL/MpJ-lpr (H-2^k), or MTK^b mice. The F₁ mice resulting from the final cross were analyzed. As the results with (Tg-B10.D2 \times B10.A)F₁ and (Tg-B10.D2 \times MRL/lpr)F₁ mice were indistinguishable, the results obtained with these mice were pooled in Tables 1 and 2.

Bone marrow chimeras were prepared as follows. Femurs were removed aseptically, ground in a mortar containing 10 ml ice-cold, sterile, HBSS (Gibco Laboratories, Grand Island, NY), and filtered through cotton to remove bone and debris. The cells were depleted of erythrocytes using Gey's solution, washed twice in HBSS, and incubated for 30 min on ice in HBSS containing a cocktail of anti-Thy-1 and anti-CD4 antibodies. Cells were then centrifuged, resuspended in rabbit complement (Cedarlane, Hornby, Canada), diluted to 10% in HBSS, and incubated at 37°C for 45 min. Cells were then centrifuged, resuspended in HBSS, refiltered through cotton, washed twice in HBSS, and counted. Viable cell concentration was adjusted to 5 \times 10⁷/ml in HBSS and 0.1 ml was injected intravenously per irradiated recipient. Aliquots of cells taken before and after antibody/complement treatment were analyzed for the presence of T cells using immunofluorescence to determine the efficiency of the treatment. T cell contamination was reduced to <0.5%. Recipients were 4-mo-old (B6.D2 \times DBA/2) (H-2^d) or (C57BL/6 \times DBA/2)F₁ (H-2^{b/d}) mice that received 950 rad whole-body radiation 1 h before transfer. Mice were analyzed 3–4 wk after transfer.

Antibodies and Immunofluorescence. Lymphoid cells were prepared as previously described (19) and stained with mAbs: anti-3-83 clone-type, 54.1/biotin (19); anti-B200/FITC, RA3-3A1 (27); anti-IgM/biotin, Ak2 (28); goat anti- λ /FITC (Fisher Scientific, Pittsburgh, PA) was used at a dilution of 1:1,000 (similar results were obtained with anti- λ /FITC from a different supplier [Southern Biotechnology Associates, Birmingham, AL]); anti-IgD^a, AMS 9.1/biotin (29); and anti- κ /PE (Becton Dickinson & Co., Mountain View, CA). Biotin-conjugated antibodies were revealed with

PE-streptavidin (Becton Dickinson & Co.). Purification and fluorochrome conjugation of the antibodies was as described (30). Flow cytometry analysis was done using a Profile machine (Coulter Electronics Inc., Hiialeah, FL), and the two-color data are presented using its density plot function. In two-color experiments the percentages of cells in each quadrant, rounded to the nearest 0.1%, are indicated in the upper right corner of each plot (see Fig. 4). Except for the cell sorting experiments, histogram analysis data for bone marrow cells were gated by side scatter to exclude the predominant myeloid cell population from analysis.

Cell Sorting. Bone marrow cells were stained with 54.1 anti-idiotypic/biotin (or Ak2 anti-IgM/biotin) followed by PE-streptavidin in ice-cold PBS, 1% BSA, 0.1% sodium azide, washed extensively in the same medium, and sorted using a EPICS 752 cell sorter (Coulter Electronics Inc.) equipped with a Cicero/PDP11 computer system (Cytomation, Ft. Collins, CO). Sorted cells were collected in RPMI supplemented with 10% FCS and 0.1% sodium azide.

Detection of mRNA. RNA preparation was as described (31). For PCR detection of mRNA, cDNA was synthesized from total RNA using a kit (Superscript; Bethesda Research Laboratories, Gaithersburg, MD) according to the instructions of the manufacturer. In the cell sorting experiments, cDNA was synthesized in a 20- μ l reaction volume containing RNA from 10⁶ cell equivalents. 1 μ l of this reaction mixture was amplified with 18 cycles of PCR, run on 1.5% agarose gels, transferred to a Zetaprobe membrane (Bio-Rad Laboratories, Richmond, CA), and hybridized with probes recognizing gene transcripts of the GTP-binding protein G α s (32), and recombinase activator gene (RAG)¹-1 (33), RAG-2 (34), or terminal transferase (TdT) (35). PCR reactions were done in a final volume of 30 μ l containing 1 μ l of reverse transcription reaction, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 20 μ g/ml gelatin (0.3 mM each of dATP, dCTP, dGTP, and dTTP, 1 μ M of each oligonucleotide primer, and 1 U of Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). Except for the first cycle, which had a 2-min 94°C denaturation step, each cycle consisted of 30 s at 94°C, 30 s at 62°C, and 1.5 min at 72°C. A thermal cycler was used (Twinblock™ System; Ericomp, San Diego, CA). The appropriate cloned cDNAs were used as hybridization probes after labeling with α -[³²P]dCTP. PCR primers were: RAG-1, primers F3 and R6 (36); RAG-2, 101F and 102R (36); G α s primers were as in Russell et al. (23); TdT, P7 (766–786), 5'-GCTGTGTTAAATGATGAGCGA-3'; and P35 (1115–1134), 5'-GTGGCTTCTGGGCTGGTAAAT-3' (35). PCR product sizes were: RAG-1, 562 bp; RAG-2, 472 bp; G α s, 820 bp; TdT, 369 bp. Control experiments showed that the dose-response of the RNA PCR assay is linear, and mock cDNA synthesis controls verified that the amplified material was not the result of DNA contamination from genomic DNA or other sources. The ubiquitous G α s message served as an internal control for the efficiency of cDNA synthesis and PCR amplification. All of the primer pairs we used span introns and thus distinguish between genomic DNA and cDNA based on the predicted sizes of the amplified fragments.

Excision Product PCR Detection Assays. PCR reactions were done in a final volume of 30 μ l containing 1 μ l of purified DNA solution (at the concentration indicated) in PCR reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 20 μ g/ml gelatin 0.3 mM each of dATP, dCTP, dGTP, and dTTP, 1 μ M of each oligonucleotide primer, and 1 U of Taq polymerase [Perkin-Elmer Corp.]). V_HJ558-to-D excision products were detected using the following primers: P5 (conserved 3' of V_HJ558), 5'-TGAGGA-

¹ Abbreviations used in this paper: RAG, recombinase activator gene; TdT, terminal transferase.

GGAGGTAATAAATGGACA-3' (37); P71 (conserved 5' of D_{FL16.1}/D_{SP2.2}), 5'-TCAAAGCACAAATGCCTGGCTTGGG-3' (38). Each PCR cycle consisted of 30 s at 94°C, 30 s at 62°C, and 1.5 min at 72°C. A thermal cycler was used (Ericomp). 1 µg of input DNA was subjected to 27 amplification cycles under these conditions. The amplified product was 563 bp long. V_{λ1}-to-J_{λ1} excision products were detected using the following primers: P46 (3' of V_{λ1}), 5'-GCTGCATACATCACAGATGC-3'; P47 (5' of J_{λ1}), 5'-CAATGATTCATGTTGTGCC-3' (39). D_{Q52}-to-J_H excision products were detected using the following primers: P25 (3' of D_{Q52}), 5'-AAGACCTGTCCACAGTAACTCG-3'; P24 (5' of J_H), 5'-ATAGGAACAGAGGCCAGAACAGA-3' (40).

For PCR amplification of V_{λ1}-to-J_{λ1} and D_{Q52}-to-J_H excision products each cycle consisted of 60 s at 94°C, 2 min at 64°C, and 2 min at 72°C. 0.1 µg of input DNA was subjected to 27 amplification cycles under these conditions. The amplified products are 290 bp for V_{λ1}-to-J_{λ2} excision product and 182, 502, 883, and 1,451 bp for D_{Q52}-to-J_{H1,2,3,4}, respectively. PCR products were run on 1.5% agarose gels and transferred onto zeta probe membranes (Bio-Rad Laboratories) in 0.4 N NaOH. The appropriate cDNA or PCR products were used as hybridization probes after labeling with α-³²P]dCTP using the hexamer labeling method (41). Filters were hybridized with probes for 16 h at 68°C in 0.5 M NaPO₄ (pH 7.2), 1 mM EDTA, 7% SDS. Filters were washed to a final stringency of 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate [pH 7.4]), 0.1% SDS at 65°C, and then exposed to X-ray film. Linear dose-response of the excision product assays was verified by including different input amounts of normal mouse bone marrow DNA in the assays and measuring the amounts of PCR product de-

tected in Southern blotting by ³²P radioactivity quantitation using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Membrane Autoantigens Expressed on Peripheral Cells or on Cells throughout the Whole Body Delete Autoreactive B Cells. To analyze the mechanism of B cell tolerance we produced transgenic mice bearing functional, rearranged heavy and light chain genes encoding the H-2K^{k,b} antibody specificity 3-83 (19, 23). In this study we used the 3-83µ/δ transgenic line, which encodes 3-83 Ig of the IgM and IgD isotypes (23). 3-83µ/δ mice that were H-2^d (nondeleting mice) were virtually monoclonal with respect to B cells, with >95% bearing the 3-83 idotype (23). In contrast, when membrane K^k or K^b autoantigen was present on the whole body (centrally deleting mice) most B cells, including virtually all idiotypic B cells, were deleted from the spleen and lymph nodes (Table 1), but were present in the bone marrow (Table 2). Cell surface immunofluorescence staining with mAb to the pan B cell marker B220 confirmed that autoreactive B cells were deleted from the peripheral lymphoid organs and retained in the bone marrow (compare Tables 1 and 2). A similar profound deletion of autospecific B cells in the lymph nodes, and to a lesser extent in the spleen, was observed in 3-83µ/δ mice bred with MT-K^b transgenic mice (26), in which the

Table 1. Increased Number of λ Light Chain-bearing B Cells in Centrally Deleting, but Not in Peripherally Deleting, Transgenic Mice

Mouse type (n)	Lymph nodes				Spleens			
	B220	λ	κ	id	B220	λ	κ	id
	%				%			
Unmanipulated mice*								
H-2 ^d Tg (9) (nondeleting)	24.4 ± 5.7	0.08 ± 0.05	24.6 ± 5.8	24.4 ± 5.7	44.4 ± 6.5	0.3 ± 0.2	47.8 ± 6.5	45.8 ± 6.4
H-2 ^d Tg X MT-K ^b (7) (peripherally deleting)	0.9 ± 0.8	0.07 ± 0.07	0.6 ± 0.3	0	9.6 ± 4.3	0.5 ± 0.6	8.6 ± 4.9	7.3 ± 3.1
H-2 ^d Tg X H-2 ^b (7) (centrally deleting)	1.5 ± 0.8	0.6 ± 0.6	1.0 ± 0.5	0	6.4 ± 4.3	2.6 ± 2.2	3.1 ± 1.6	0.7 ± 1.1
H-2 ^d Tg X H-2 ^k (4) (centrally deleting)	3.7 ± 1.8	1.1 ± 0.4	2.4 ± 1.4	0	13.5 ± 6.7	4.9 ± 2.2	9.1 ± 4.8	0.2 ± 0.3
Non-Tg (7) (normal)	29.0 ± 5.9	1.0 ± 0.33	29.1 ± 6.7	0	51.5 ± 7.6	2.3 ± 0.8	53.4 ± 7.7	0
Bone marrow chimeras†								
H-2 ^d Tg→H-2 ^d (3) (nondeleting)	58.9 ± 10.8	0.13 ± 0.05	53.1 ± 15.4	50.7 ± 16.4	52.1 ± 16.7	0.2 ± 0.1	51.5 ± 19.7	48.4 ± 16.4
H-2 Tg→H-2 ^{b/d} (4) (centrally deleting)	4.4 ± 1.5	1.0 ± 0.20	3.4 ± 1.2	0.1 ± 0.2	11.6 ± 4.0	2.9 ± 1.4	9.0 ± 1.2	0.3 ± 0.3
Non-Tg→H-2 ^{b/d} (3) (normal)	51.9 ± 1.6	3.5 ± 0.5	52.3 ± 5.2	0	57.7 ± 8.9	4.1 ± 0.8	57.4 ± 4.2	0
Non-Tg→H-2 ^d (3)	67.7 ± 3.4	4.5 ± 0.6	64.4 ± 0.7	0	67.7 ± 3.4	4.4 ± 0.4	73.2 ± 7.7	0

* Mean ± SD from four to six experiments. Mice were 2 mo. old.

† Mean from three experiments. Mice were analyzed 21–26 d after transfer in three independent experiments.

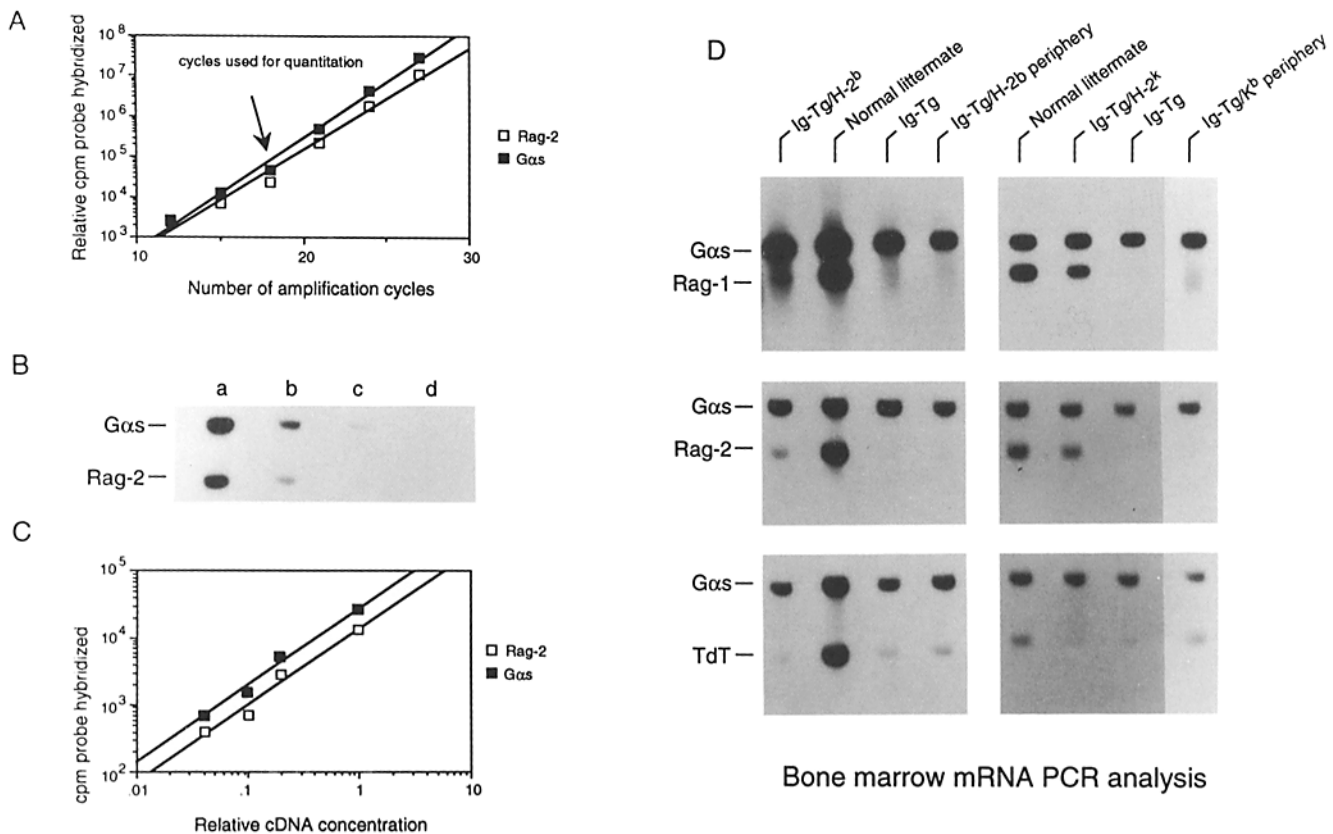


Figure 1. Elevated expression of RAG-1 and RAG-2, but not TdT, in the bone marrow of centrally deleting mice detected using a PCR assay. (A) cDNA synthesized from the bone marrow RNA of an 8-wk-old B10.D2 nontransgenic littermate mouse was subjected to various numbers of PCR amplification cycles as described in Materials and Methods using pairs of oligonucleotide primers specific for RAG-2 and the ubiquitous GTP-binding protein, G α s. Synthesized fragments were detected by Southern blotting, and the amount of probe hybridized was quantitated. Based on these results 18 cycles of amplification using the conditions described in Materials and Methods were chosen for the subsequent quantitation experiments. (B and C) Various amounts of cDNA synthesized from the bone marrow RNA were subjected to 18 cycles of the PCR with primers specific for RAG-2 and G α s. (B) Autoradiogram of Southern blot used to detect specific amplification of RAG-2 and G α s. (C) Measurement of radioactivity in the visualized bands of B. Dilution of input cDNA: lane a, undiluted; lane b, 1:5; lane c, 1:10; lane d, 1:25. The RAG-2 cpm/G α s cpm ratios were very similar at all cDNA dilutions tested: 0.52, 0.53, 0.47, 0.56 for a–d, respectively. (D) Comparison of recombinase message levels in bone marrow RNA of individual transgenic and nontransgenic mice. PCR detection of RAG-1, RAG-2, and TdT mRNA in bone marrow cells was performed as described in Materials and Methods. Shown are autoradiograms of Southern blots used to detect PCR products. (Left) Samples were from 5-wk-old mice; (right) samples were from 8-wk-old mice.

Table 2. Increased Abundance of Bone Marrow RAG mRNA and B Cells in Centrally Deleting, but Not in Peripherally Deleting, Transgenic Mice

Mouse type	RAG-2 mRNA* (n)	% B220 ⁺ -cells† (n)	% Ig ⁺ -cells‡ (n)
H-2 ^d Tg (non deleting)	1.0 ± 0.38 (5)	11.7 ± 1.9 (5)	9.7 ± 1.4 (5)
H-2 ^d Tg × MT-K ^b (peripherally deleting)	1.2 ± 0.38 (4)	13.3 ± 5.9 (4)	10.6 ± 4.7 (4)
H-2 ^d Tg × H-2 ^b (centrally deleting)	3.4 ± 0.75 (4)	19.1 ± 4.9 (3)	16.0 ± 5.7 (3)
H-2 ^d Tg × H-2 ^k (centrally deleting)	8.3 ± 2.1 (2)	15.2 ± 0.9 (3)	11.7 ± 0.7 [§] (3)
Non-Tg (normal)	11.6 ± 6.5 (6)	23.8 ± 6.9 (3)	6.4 ± 2.0 (3)

* PCR detection of RAG-2 mRNA was performed as described in Materials and Methods using detection of G α s mRNA as internal control. Relative abundance was calculated as: (cpm RAG-2 probe hybridized/cpm G α s probe hybridized)_{experimental} / (cpm RAG-2 probe hybridized/cpm G α s probe hybridized)_{mean of nondeleting samples}. Data shown are expressed as mean relative abundance. All mice were 2 mo old.

† Percent of total viable bone marrow cells.

§ As the sIg⁺ cells in this group had a very low level of staining, this value is only approximate.

K^b antigen is only detectably expressed on the surfaces of hepatocytes, exocrine pancreatic cells, and kidney tubules (peripherally deleting mice) (Table 1) (see also reference 23). As shown below, comparison of the peripherally and centrally deleting mice provided insights into the mechanism of deletion.

Elevated RAG Expression in Bone Marrow IgM⁺ Cells of Centrally Deleting Mice. To examine the possibility that secondary Ig gene rearrangements are induced upon encounter of B cells with tolerogen, we measured RAG-1 and RAG-2 (33, 34) expression in bone marrow cells using a quantitative PCR assay (Fig. 1). Nondeleting, H-2^d 3-83 μ / δ mice have greatly reduced levels of both RAG-1 and RAG-2 relative to nontransgenic littermates, presumably because of the feedback regulation that maintains allelic exclusion (15) (Fig. 1 D, lanes *Ig-Tg*, and Table 2). Peripherally deleting mice showed similar low levels of RAG-1 and RAG-2 (Fig. 1 D, lanes *Ig-Tg/H-2K^b periphery*, and Table 2). In striking contrast, and consistent with the possibility that secondary Ig gene rear-

rangements are induced in B cells by interaction with autoantigen, centrally deleting mice had greatly increased RAG-1 and RAG-2 levels that approached the levels in nontransgenic mice (Fig. 1 D, lanes *Ig-Tg/H-2^b* and *Ig-Tg/H-2^k*, and Table 2).

To determine which cells in the bone marrow of centrally deleting mice were responsible for the elevated RAG expression, we separated idiotype-positive and idiotype-negative cells and measured their RAG-1 and RAG-2 levels. For these experiments we chose to analyze centrally deleting mice bearing the antigen K^b, which is a low affinity ligand for the 3-83 antibody, because the level of idiotype detected on the surface of bone marrow B220⁺ cells is higher in these mice than in H-2^k centrally deleting mice and thus allows a cleaner cell separation. Fig. 2 A shows that the idiotype-positive cells were highly enriched for RAG-2 expression and accounted for most of the bone marrow RAG-2 message, as demonstrated by the depletion of RAG-2 in the idiotype-negative cells. The lack of RAG expression in the idiotype-

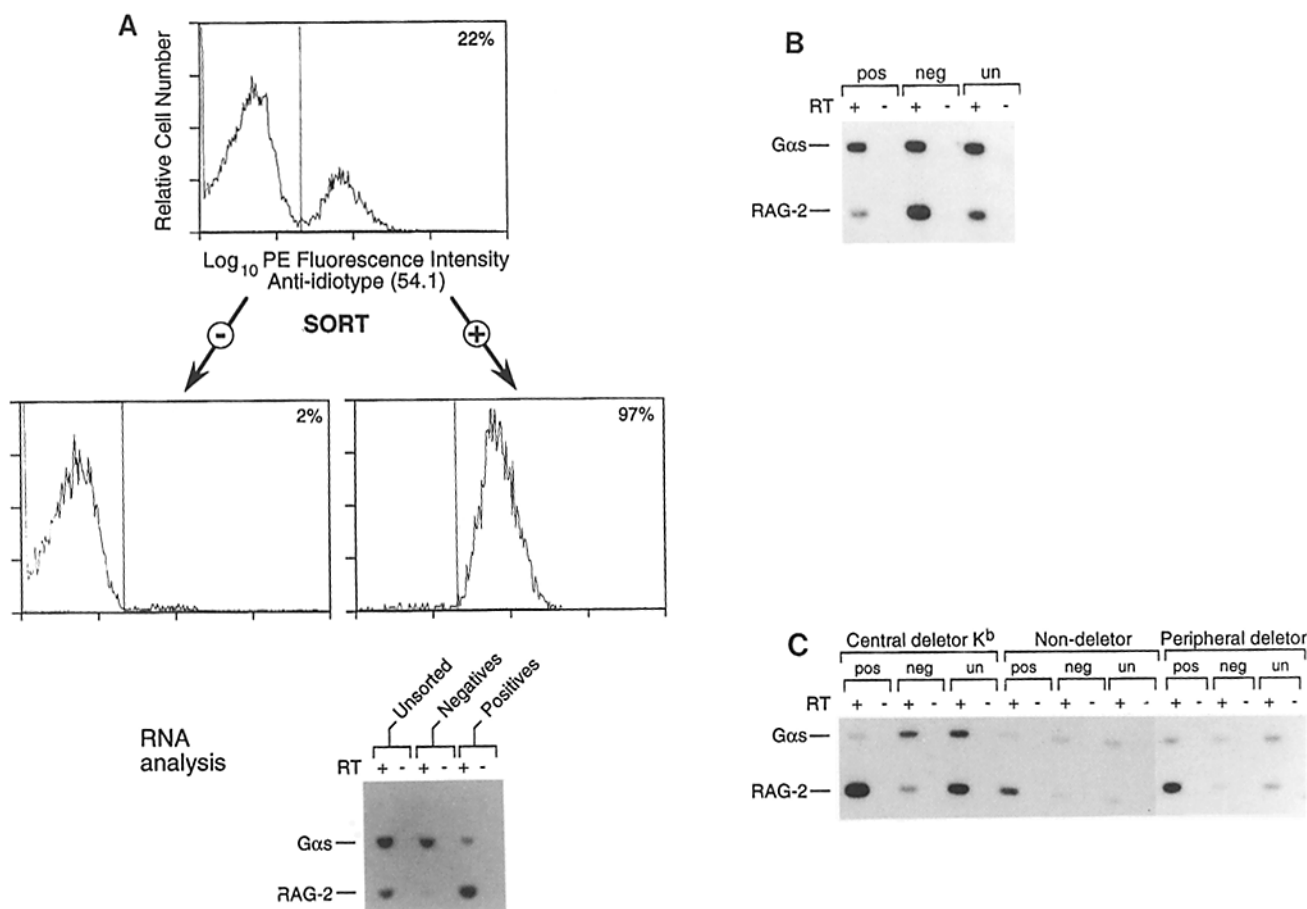


Figure 2. Most RAG-2 mRNA from H-2^b centrally deleting bone marrow is contained in sIgM⁺ cells. (A) 3-83 idiotype-positive cells were stained with the anticonotype antibody 54.1/biotin, followed by PE-avidin, and sorted as described in Materials and Methods. RNA was isolated from sorted and nonsorted populations and PCR detection was carried out as in Fig. 1. (Bottom) Autoradiogram of Southern blot used to detect PCR products. (RT, +/-) Addition or omission of reverse transcription during the cDNA synthesis procedure before PCR amplification. (B) A similar analysis as in A for bone marrow cells from a normal, nontransgenic mouse sorted on the basis of sIgM expression. (C) RAG-2 expression analysis of sorted nondeleting, peripherally deleting, and centrally deleting mouse bone marrow cells. In this experiment Gαs probe of lower specific activity was used to better reveal the RAG-2 signal.

negative cells was not the result of selective death of pre-B cells, because in normal mouse bone marrow cells that were sorted similarly using anti-IgM antibodies most, but not all, of the RAG-2 mRNA was present in the sIgM⁻ population (Fig. 2 B). The idiotype-positive cells of the nondeleting and peripherally deleting transgenic mice were also enriched in RAG-2 mRNA, but this message was present at a lower level than in the idiotype-positive cells isolated from centrally deleting mouse bone marrow (Fig. 2 C). These results reflect the relative amounts of RAG-2 message seen in the total mouse bone marrow RNA preparations (Table 2). The significance of the residual RAG expression in the sIgM⁺ cells of the nondeleting and peripherally deleting mice is unclear, as these cells demonstrate little functional Ig gene rearrangement (see below). Because binding by the antiidiotypic antibody is de-

pendent on coexpression of the transgenic light and heavy chains, these experiments demonstrate that bone marrow B cells bearing intact surface (s)IgM can express RAG-1 and RAG-2, and that the levels of RAG mRNAs are highest in cells that have bound (auto)antigen in the bone marrow. Interestingly, centrally deleting mice bearing the high affinity ligand K^k expressed more RAG-1 and RAG-2 in their bone marrow, but had no more bone marrow B cells than centrally deleting mice expressing the low affinity ligand K^b (Table 1). This suggests that the affinity of the antigen-receptor interaction affects the probability that a bone marrow IgM⁺ cell will express recombinase.

Elevated Light Chain Gene Recombination in Centrally Deleting Bone Marrow Cells. To test whether or not the elevated RAG expression in centrally deleting bone marrow cells reflected

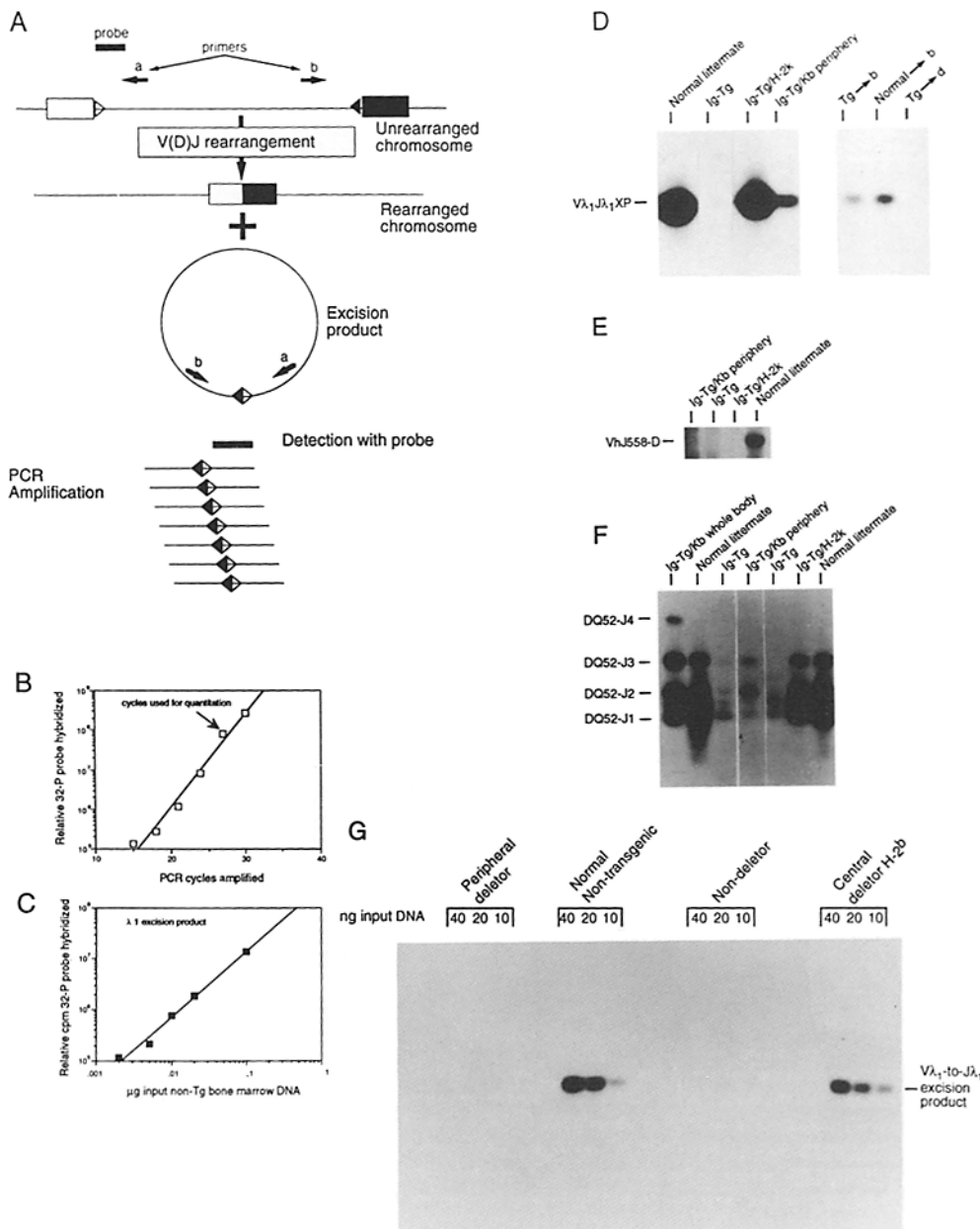


Figure 3. PCR detection of Ig gene rearrangement excision products. (A) General strategy for the PCR detection of Ig gene rearrangement excision products. Filled and open rectangles represent Ig coding segments. Open and filled triangles represent rearrangement signal sequences with 12- and 23-bp spacers, respectively. Primers a and b are oligonucleotides that initiate DNA synthesis on opposite DNA strands in the indicated directions. (B) Amplification of V_{λ1}-to-J_{λ1} excision product from purified normal, nontransgenic mouse bone marrow DNA. Effect of altering the number of PCR cycles on the amount of amplified product. Based on these results 27 cycles of amplifications using the conditions described in Materials and Methods was chosen for the subsequent quantitation experiments. (C) Dose-response of input normal bone marrow DNA amount on V_{λ1}-to-J_{λ1} excision product signal detected after 27 cycles of amplification. Similar linear dose-response curves were seen in experiments detecting the other types of excision product (not shown). (D-G) Detection of Ig gene rearrangement excision products in bone marrow DNA. Bone marrow DNA from 8-wk-old mice was analyzed for excision products of: (D and G) V_{λ1}-to-J_{λ1} joining (D, comparison of a number of individual 2-mo-old mice; G, comparison of one mouse of each group using different starting amounts of bone marrow DNA); (E) V_hJ558-to-D joining; (F) DQ52-to-J_H joining. PCR products were visualized by Southern blotting followed by autoradiography.

increased V(D)J recombination, we measured the levels of Ig gene rearrangement excision products in bone marrow DNA using a PCR assay (Fig. 3). This assay takes advantage of the fact that during recombination the heptamer-nonamer signal sequences flanking the two joined Ig gene coding elements are themselves ligated, and the resulting structure can be detected and amplified using PCR with appropriately designed oligonucleotide primers (shown schematically in Fig. 3 A). The strength of this approach, at least for V_H-to-D, DQ52-to-J_H, and V λ -to-J λ rearrangements, is that these excision products, unlike the corresponding coding products that are retained by the chromosome, are always deleted from the chromosome and so their quantity should not be affected by the possible subsequent growth of the cells. In addition, because each cell has a limited number of loci that can rearrange, the amount of excision product detected is likely to reflect the fraction of cells undergoing gene rearrangement. These experiments showed that the level of λ_1 light chain excision products in bone marrow DNA preparations were extremely high in centrally deleting but not peripherally deleting or nondeleting mice (Fig. 3, D and G). Thus, autoantigen expression in the bone marrow is correlated with increased Ig gene recombination activity. Similar results were obtained with λ_2 -specific assays (not shown). By contrast, V_H-to-D excision products were not elevated in bone marrow cells of centrally deleting mice, indicating that light chain, but not heavy chain, rearrangement is induced in the centrally deleting mice (Fig. 3 E).

Interestingly, increased levels of D_{Q52}-to-J_H excision products were detected in the bone marrow cells of centrally deleting mice, relative to the levels seen in the bone marrow cells of nondeleting and peripherally deleting control mice (Fig. 3 F). The appearance in centrally deleting B cells of D_H-to-J_H joining, but not V_H-to-D joining, is similar to the IgH rearrangement patterns seen in T cell lines (42) and probably reflects a less restrictive control of the accessibility of the elements needed for D_H-to-J_H joining compared with the control of the accessibility of elements needed for V_H-to-D rearrangements.

Bone marrow DNA preparations also were analyzed for V κ -to-J κ rearrangements by the excision product assay. As for the λ loci tested, the bone marrow cells of centrally deleting mice had greatly elevated levels of detectable excision products, relative to the bone marrow cells of peripherally deleting or nondeleting transgenic mice (data not shown). These results support the idea that κ light chain genes are also induced to rearrange in centrally deleting mice. However, these data are more difficult to interpret for κ than for the other loci tested, because in κ , in contrast to the other Ig loci, many V_s rearrange by inversion. In the case of inversion the signal joints that our assay recognizes are retained on the chromosome upstream of the C κ , and their quantity is therefore potentially subject to subsequent cellular selection.

Elevated λ Light Chain Expression in Centrally Deleting Mice. In deleting transgenic mice small numbers of B cells that lack the autoreactive specificity escape deletion and appear in the peripheral lymphoid organs. A large fraction

($\sim 1/3$) of the B cells found in centrally deleting mouse lymph nodes expressed λ light chains (Table 1). These λ chains must have been the result of rearrangement and expression of endogenous λ loci because the transgenic light chain is κ . Lymph node cells of centrally deleting mice had ~ 10 -fold more λ^+ cells than lymph node cells of peripherally deleting and of nondeleting mice, both in terms of the absolute numbers of λ^+ cells and in terms of the percentage of total lymph node cells. This high frequency of λ^+ cells in the centrally deleting mice was not a simple result of selective outgrowth of idiotype-negative cells, because peripherally deleting mice, which had even more profoundly depressed lymph node B cell levels, had as few λ -bearing lymph node cells as the nondeleting 3-83 μ / δ mice. Although the peripherally deleting mice had nearly twofold more λ -bearing cells in the spleen than the nondeleting mice (0.5 vs. 0.3%), this increase was relatively slight compared with the 8–16-fold increase seen in the spleens of centrally deleting mice (Table 1). We note that the slight increase in the percentages of B220⁺ cells in the lymph nodes of centrally deleting mice, relative to that percentage in peripherally deleting mice, can be largely accounted for by the increase in the fraction of λ -bearing cells. These data document the increased rearrangement of endogenous light chain genes in the B cells of centrally deleting mice at the level of light chain protein expression.

To more directly examine the potential of bone marrow B cell precursors developing in deleting or nondeleting environments to give rise to λ -bearing cells, we constructed radiation chimeras. Aliquots of bone marrow cells from nondeleting, H-2^d 3-83 μ / δ mice were used to reconstitute lethally irradiated H-2^d or H-2^{d/b} recipients. 3–4 wk after transfer the mice were analyzed. Again, centrally deleting mice demonstrated a greatly increased frequency of λ -bearing cells, while the nondeleting transgenic chimeras demonstrated excellent light chain isotype exclusion (Table 1). These cells were of donor origin because double staining of peripheral B cells with anti-I-A^b and anti-B220 antibodies revealed that >95% of the B cells in all samples were donor derived and virtually all detectable bone marrow B cells in the deleting chimeras had low levels of 3-83 idiotype (not shown). Since the transgenic donor bone marrow was H-2^d, this experiment also shows that for the induction of λ^+ B cell production in these chimeras the B cell receptor had to emerge onto the cell surface and receive an antigenic signal from another cell. This result is important because pre-B cells and B cells express class I MHC molecules and might have conceivably required autoantigen expression internally, for example in the endoplasmic reticulum, to induce λ production. It also suggests that any antigen that is present in the bone marrow, and is capable of crosslinking B cell receptors with sufficient avidity, can induce recombinase expression and secondary light chain rearrangement in slgM⁺ cells.

In Centrally Deleting Mice λ Chain-bearing B Cells Arise from Self-reactive B Cells. To determine whether or not λ^+ B cells from centrally deleting mice arose from autoreactive precursors, we analyzed bone marrow cells by two-color immunofluorescence with antiidiotype and anti- λ antibodies (Fig.

BONE MARROW

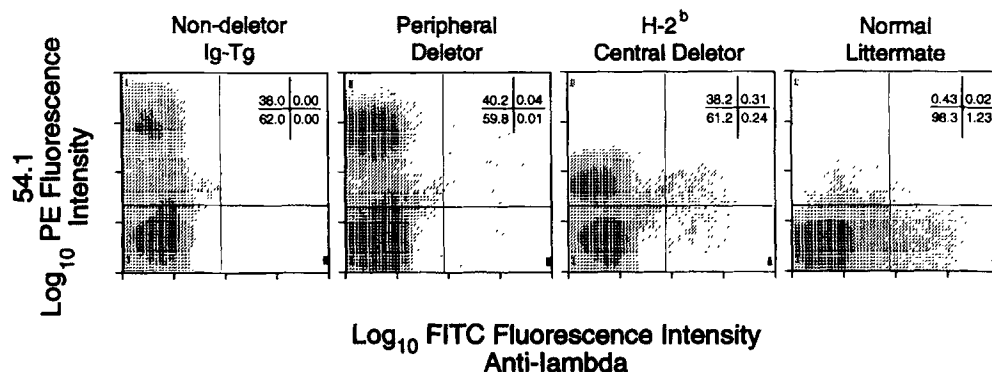


Figure 4. Detection of idiotype⁺/λ⁺ B cells in the bone marrow of a centrally deleting mouse. Idiotype was detected with the 3-83 clonotype-specific rat mAb 54.1, which recognizes an epitope created by the pairing of 3-83 heavy and light chains. λ-bearing cells were visualized with a goat anti-mouse λ/FITC conjugate. The binding of biotin conjugated 54.1 was revealed with PE-streptavidin. For each sample 100,000 events were analyzed. Data were electronically gated based on 90° light scatter to exclude from analysis the predominant myeloid cell population.

4). A small, but significant population of double-positive cells could be clearly identified. Roughly half of the λ-positive cells expressed idiotype (id). Double-positive id⁺/λ⁺ cells do not appear in the peripheral lymphoid organs of centrally deleting mice; thus, in the bone marrows of centrally deleting mice, the predicted id⁺/λ⁺ intermediate in the receptor editing process could be detected. In addition, in the periphery of centrally deleting mice essentially all λ⁺ B cells coexpress the transgenic heavy chain, as shown by two-color immunofluorescence analysis with antibodies specific for λ chains and for IgD heavy chains (δ chains) of the transgenic allotype (Fig. 5). Because virtually all B cells in the centrally deleting mice bear Ig heavy chains of the transgenic allotype, these cells must retain and express the heavy chain transgene. These results indicate that the peripheral λ-expressing B cells

in centrally deleting mice coexpress transgenic heavy chain genes and they support the V_H-to-D excision product data, suggesting that receptor editing involves assembly of light chain, but not heavy chain, V genes (Fig. 3).

Discussion

Although Ig receptor generation and antigenic selection in B cells have been generally thought to be independent processes, we find that in immature, IgM⁺ bone marrow B lymphocytes these processes are intimately linked. Bone marrow B cells expressing receptors that are crosslinked by autoantigen express high levels of recombinase gene mRNAs and continue to rearrange Ig light chains. Evidence for rearrangement activity in bone marrow cells of centrally deleting mice came from experiments demonstrating: (a) elevated levels of RAG-1 and RAG-2 mRNA in bone marrow cells of centrally deleting mice; (b) elevated expression of these recombinase genes in sIgM⁺ bone marrow cells of centrally deleting mice; (c) elevated levels of light chain rearrangement excision products in bone marrow cells of centrally deleting mice; and (d) expression of endogenously encoded light chain protein in cells expressing transgenic Ig in bone marrow cells of centrally deleting mice. These results suggest that autoreactive bone marrow B cells are often rescued from elimination by an induced alteration of the structure and specificity of their antigen receptors. While our results in a sense reinforce the concept of clonal selection, as cells with autoreactive specificities do indeed disappear in our system, the receptor editing idea represents an important conceptual departure from the prevailing dogma of clonal selection, which holds that selection against self-reactive clones requires their functional or physical elimination. In essence, the distinction between clonal selection and receptor editing is that in receptor editing the fates of the autoreactive B cell and its receptor are uncoupled.

The Case against Feedback Regulation. The elevated expression of RAG-1 and RAG-2 accompanied by increased λ light chain gene rearrangement in the bone marrow of centrally

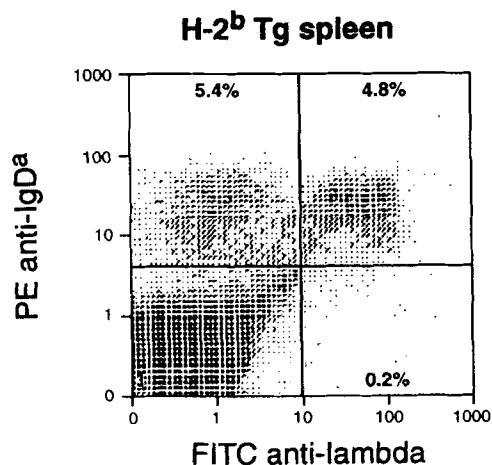


Figure 5. Peripheral λ light chain-bearing B cells in a centrally deleting mouse coexpress the transgenic heavy chain. Spleen cells from an 8-wk-old 3-83μ/δ transgenic mouse bred to the C57BL/10 background (Ig heavy chain allotype b, H-2K^b) were stained with anti-λ/FITC and anti-IgD^a/biotin. On this genetic background the anti-IgD^a antibody only reacts with transgene-encoded IgD. The binding of the biotin conjugate was revealed with PE-streptavidin.

deleting mice raises the question of feedback in hematopoiesis. It could be argued that a paucity of B cells in the periphery or in the bone marrow induces elevated RAG expression in bone marrow cells of centrally deleting mice as a consequence of an overall increase in B cell production. We think this is unlikely for several reasons: (a) the peripherally deleting mice, which are highly B cell deficient, fail to demonstrate RAG upregulation (Fig. 1); (b) the cells that are enriched for RAG expression in bone marrow of centrally deleting mice are not pre-B cells, as would be predicted by a feedback model, but sIgM⁺ cells (Fig. 2); (c) expression of terminal transferase, a pro-B stage-specific gene, is not elevated in bone marrow cells of centrally deleting mice (Fig. 2); (d) rearrangement in bone marrow B cells of centrally deleting mice occurs at the light chain loci, but not detectably at the heavy chain loci (Fig. 3); (e) in mixed bone marrow chimeras, in which an excess of normal, nontransgenic B cells of all developmental stages develops alongside a minority population of transgenic B cells, the autoreactive transgenic B cells continue to demonstrate elevated λ light chain rearrangement (data not shown); (f) a simple feedback model cannot explain the different λ/κ ratios in peripherally deleting and centrally deleting mice; and (g) studies on anti-IgM-suppressed mice, SCID mice, and SCID mice bearing active Ig transgenes indicate that depletion of mature and immature B cells, late-pre-B cells, or late pro-B cells does not increase the rate of formation, or steady-state levels, of their precursors in the bone marrow (3, 43, 44).

The Receptor Editing Hypothesis. Based on these results we have formulated the following model for the role of Ig gene rearrangement in B cell tolerance (Fig. 6). Cells at the pre-B to B cell transitional stage of development in the bone marrow are undergoing κ rearrangement. The appearance of IgM on the cell surface normally turns off further recombination. We propose that if the newly emerging or recently emerged receptors are autoreactive, and their surface IgM is crosslinked by membrane-bound autoantigen, the following sequence of events takes place. (a) Recombinase levels in the autoreactive B cells are high, either because expression fails to be shut down or because the recombinase machinery is newly expressed. (b) Because recombinase is now expressed in a cell with recombinase-accessible light chain V genes, but not recombinase-accessible heavy chain V genes, secondary rearrangement occurs in the κ locus. (c) If light chain rearrangement is successful, the previously active κ VJ is rendered inactive by nested rearrangement and deletion, or by inversion, which places the VJ far away from C κ . Rearrangement continues until a new, functional VJ is generated, or until the C κ loci are inactivated by using up the available J κ 's or by RS recombination. The functional light chain must fail to result in an autoreactive receptor or the editing process will be resumed, starting at step a. It is also possible at this stage that a new light chain that pairs preferentially with the cell's μ chain can in some cases extinguish the B cell's anti-self-specificity without eliminating the active, autospecific VJ on the other chromosome. This has been clearly shown by Gay et al. (45) in their anti-DNA transgenic mouse system. Their

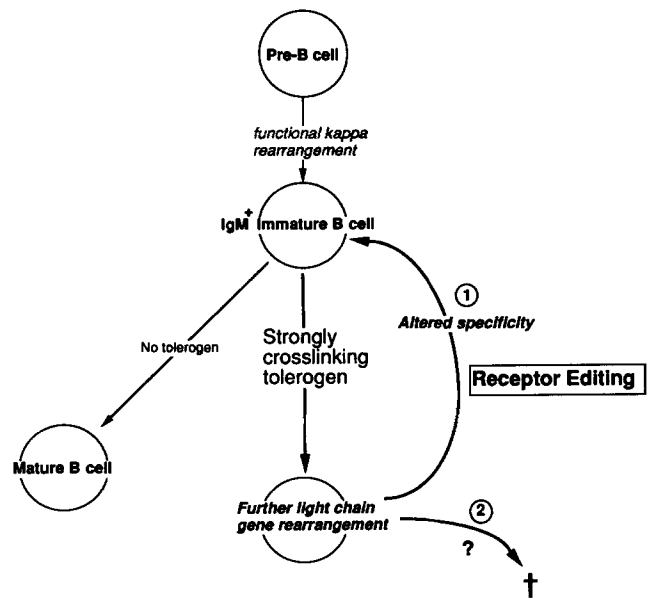


Figure 6. A model for receptor editing. Pre-B cells that have functionally rearranged light chain genes display surface IgM that can bind antigen. Absence of strong sIgM crosslinking by antigen allows the B cell to mature. (1) Strongly crosslinking antigen induces light chain rearrangement and altered antigenic specificity in immature B cells. (2) Cells that fail to alter their antiself specificity or that lack receptors may eventually die in situ.

work also underscores the importance of editing in eliminating potentially pathogenic specificities. (d) Those cells that have activated RS recombination, which deletes the C κ locus, now make λ loci available for rearrangement and expression. In the mouse, further editing of active λ light chain loci that encode autoantibodies is not generally possible, given the organization of the λ genes; however, in other species, such as humans, nested secondary V λ J λ rearrangements that can eliminate active V λ J λ 's may be possible.

The Importance of Secondary Ig Gene Rearrangements. Secondary Ig gene rearrangements are defined as rearrangements caused by the V(D)J recombinase system in loci that have already undergone V region gene assembly. In the case of nonfunctional rearrangements, such as out-of-frame coding joints, secondary (and higher order) rearrangements can allow multiple attempts at generating functional Ig genes. Mouse Ig gene organization has a number of interesting features that may be important in limiting, or facilitating, such secondary rearrangements. The heavy chain V, D, and J minigenes are in the same transcriptional orientation (46). Thus, VDJ recombination deletes the intervening DNA between the active V_H and D_H segments and that between the rearranged D_H and J_H segments. This results in the loss of all other D's from that allele of the Ig locus and eliminates the possibility of further rearrangements using conventional signal sequences. By contrast, the κ locus has V genes in different orientations relative to the J's, and thus can rearrange by deletion or inversion of the intervening DNA (47–49). Because in the κ locus the V's recombine directly to J's, and there are four functional J κ 's, nested rearrangement is possible. Furthermore,

as inversion leaves the entire repertoire of $V\kappa$'s intact, after inversional $V\kappa J\kappa$ rearrangement, the entire repertoire of $V\kappa$'s is available for secondary rearrangements. That such secondary rearrangements can occur in κ has been well documented (48–50). In addition, because $V\kappa$ rearrangements to $J\kappa 1$ and $J\kappa 2$ are most frequent (51), downstream J's are often available for secondary rearrangements in the κ loci. By contrast, λ loci each have a single J and, like the heavy chain V genes, rearrange by a deletional mechanism (52, 53). Thus, in the mouse, Ig gene organization appears to uniquely favor secondary rearrangements in κ .

Secondary Rearrangement of Ig Genes in B Cells Can Alter Specificity. How can the concept of receptor editing be reconciled with the observation of allelic exclusion? There are a number of well-documented reports of secondary Ig gene rearrangements in murine B cell tumor lines already expressing intact IgM on the cell surface (54–56). These rearrangements can create new, functional light or heavy chain genes, effectively changing the antigen specificity of the cells in the process. Two groups demonstrated a novel form of recombination at the IgH locus, called V gene replacement, in which coding sequences of the rearranged, functional V gene are replaced by upstream germline V gene segments (54, 57). This V_H -to- V_HDJ_H recombination appears to be mediated by cleavage and recombination in a highly conserved heptamer in the 3' portion of the rearranged V_H coding sequence. This heptamer is identical to, and in the same orientation as, that found 5' of D elements, suggesting that in V_H to V_HDJ_H replacement, the same enzymatic machinery that mediates the familiar V_H -to- DJ_H recombination is involved. It has not been determined whether or not this process occurs in normal cells. Nevertheless, we cannot exclude a role for V_H gene replacement in receptor editing. Evidence for a functional secondary rearrangement at the light chain locus has been provided by Levy et al. (55): independent idiotypic variants of the surface IgM⁺ tumor 38C13 were shown to have deleted and replaced the rearranged, functional $V_{J\kappa 2}$ by recombination between upstream $V\kappa$'s and downstream $J\kappa$'s. This type of nested rearrangement has been observed frequently in myelomas and hybridomas in situations where the rearrangement was inversional (48–50). In normal B cells, circular DNAs excised by nested κ gene rearrangements have been demonstrated. Interestingly, these circles frequently contain rearranged VJs, about one-third of which are in-frame and potentially functional (16). Finally, in the NFS-5 tumor, under conditions in which it expresses surface IgM (μ, κ), λ light chain rearrangement may occur. The λ protein product competes efficiently for binding to heavy chain with the func-

tional κ protein, extinguishing surface κ expression (56). In all the above examples, not only do the rearrangements create a new specificity, but they extinguish the original specificity of the B cells. Secondary rearrangements have been thought to occur in developing B cells that fail to make functional Ig receptors; the data presented in this paper suggest that autoreactive B cells can similarly be rescued and made nonautoreactive by secondary Ig gene rearrangement.

Our model may help to explain exceptions to the paradigm of feedback inhibition of Ig gene rearrangement. For example, Storb and her colleagues (58, 59) have observed that in κ chain transgenic mice, in which feedback inhibition of κ rearrangement is effective in most B cells, a subset of B cells appears to ignore the expressed transgene and continues to rearrange endogenous κ and λ light chains. We predict that in this subset of B cells the transgenic light chain paired with the cell's μ heavy chain confers autoreactive specificity that is sufficient to activate the receptor editing process.

Antigen Receptor Selection in the Primary Lymphoid Organs. Recent work on T lymphocyte selection suggests that thymic-positive selection or TCR crosslinking can terminate ongoing TCR α chain gene rearrangement in immature TCR⁺ T cells (60, 61), providing another example of a link between antigen receptor crosslinking in immature lymphocytes and V(D)J recombinase regulation. Our results demonstrating secondary light chain rearrangement in immature B cells encountering autoantigen in the bone marrow differ substantially from the results with T cells, however, because we find that receptor crosslinking in immature B cells turns recombinase on, rather than off. However, sIgM⁺ lymphomas generated in $E\mu$ -N- myc transgenic mice downregulate RAG expression upon sIg crosslinking (62). It is possible that B cells are uniquely adapted to use the receptor editing mechanism for the elimination of potentially harmful autoreactive B cells, whereas T cells make use of receptor editing to increase their chances of being positively selected. We assume that the receptor editing we detect in transgenic mice reflects a process occurring in normal cells as suggested by the presence of a subset of sIgM⁺/RAG⁺ cells in normal bone marrow (62) and the abundance of in-frame $V\kappa J\kappa$ joins in circular excised B cell DNA from normal mice (16). In both B and T lymphocytes receptor editing could relieve developmental "bottlenecks," in which large numbers of cells are selected against, by providing a means of rapidly generating cells with appropriate specificities at an early developmental stage through the sequential alteration and testing of their antigen receptors.

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