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T Cell-Independent Somatic Hypermutation in Murine B Cells with an Immature Phenotype

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Summary

Somatic hypermutation contributes to the generation of antibody diversity and is strongly associated with the maturation of antigen-specific immune responses. We asked whether somatic hypermutation also plays a role in the generation of the murine immunoglobulin repertoire during B cell development. To facilitate identification of somatic mutations, we examined mouse systems in which only antibodies expressing λ1, λ2, and λx light chains can be generated. Somatic mutations were found in cells, which, by surface markers, RAG expression, and rapid turnover, had the phenotype of immature B cells. In addition, expression of AID was detected in these cells. The mutations were limited to *V* regions and were localized in known hotspots. Mutation frequency was not diminished in the absence of T cells. Our results support the idea that somatic hypermutation can occur in murine immature B cells and may represent a mechanism for enlarging the *V* gene repertoire.

Introduction

In mice and humans the preimmune repertoire is achieved primarily by gene rearrangement mechanisms. Additional diversity in the repertoire can also be generated by other mechanisms, such as gene conversion, as in birds (Reynaud et al., 1987), rabbits (Becker and Knight, 1990), cattle (Parng et al., 1996), and swine (Butler et al., 1996).

Somatic hypermutation also serves to diversify B cell receptors. The process of somatic hypermutation has been shown to require activation-induced cytidine deaminase (AID) expression (Muramatsu et al., 2000). There is evidence that AID directly modifies the DNA of its target genes (Petersen-Mahrt et al., 2002). When expressed in fibroblast cells, AID can also mutate a target gene, indicating that AID is the only lymphocyte-specific component of the mutator complexes (Yoshikawa et al., 2002). In mice AID is most abundant in Peyer's patch and lymph node cells, and much less in spleen and bone marrow cells (Muramatsu et al., 1999).

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We asked whether somatic hypermutation contributes to the diversity of the repertoire of murine immunoglobulin genes and whether AID is expressed early during B cell development.

In order to address these questions, we studied somatic hypermutation in λ light chain genes from mice with a limited Ig repertoire. This strategy was based on the assumption that a limited primary Ig repertoire provides an experimental system in which normally rare somatic variants will be expanded because of strong selective pressure. Indeed, receptor editing in the early stages of B cell development, another mechanism of diversification of the repertoire, was first discovered in a similar experimental situation (Gay et al., 1993; Kleinfield et al., 1986; Tiegs et al., 1993).

Four models were chosen for analysis: $\kappa^{-/-}$ mice, QM mice, QM $C4^{-/-}$ mice, and QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mice. $\kappa^{-/-}$ mice have a λ-restricted repertoire (Chen et al., 1993; Zou et al., 1993). Since there are only three V_λ germ line genes ($V_\lambda 1$, $V_\lambda 2$, and $V_\lambda x$) (Blomberg et al., 1981; Dildrop et al., 1987), there is only limited light chain diversity. We also used the quasi-monoclonal (QM) mouse that has an even more restricted Ig *V* gene repertoire (Cascalho et al., 1996). QM mice are heterozygous at the heavy chain locus: on one allele, the J_H region is replaced by the rearranged V_HDJ_H of an anti-NIP hybridoma 17.2.25 (Boersch-Supan et al., 1985); the other allele is nonfunctional because of a targeted deletion of all J_H gene segments. Because QM mice are also homozygous for the J_κ deletion, they produce only λ light chains. Thus, in the QM mouse all newly derived B cells have the 17.2.25 VDJ heavy chain paired with a λ chain encoded by one of the three V_λ genes.

The complement C4 component and complement receptors CD21/CD35 contribute to negative selection of self-reactive B cells (Prodeus et al., 1998). Self-reactive immature B cells generated by rearrangement are known to mature in C4-deficient mice. One expectation is that in C4-deficient mice cells that are self-reactive will accumulate mutations after self-antigen stimulation. Thus, in $C4^{-/-}$ mice (Fischer et al., 1996) the frequency and range of mutations in B cells may be increased, making their study less labor intensive. We therefore studied QM mice that were bred to be C4 complement factor deficient ($C4^{-/-}$).

In general, in humans and mice, somatic hypermutation has been described as occurring in the context of germinal centers after antigen stimulation and in a manner that is dependent on T cell help (Kelsoe, 1995). Recent reports, however, suggest that somatic hypermutations can be T cell independent (Toellner et al., 2002; Monson, 2001; Weller et al., 2001). In addition, in sheep, rabbits, and cattle, somatic hypermutation in immature B cells appears to contribute to diversity of the preimmune repertoire (Lucier et al., 1998; Reynaud et al., 1995; Weinstein et al., 1994). Therefore, we also studied mice made T cell deficient by gene-targeted deletion of $CD3\epsilon$ (DeJarnette et al., 1998).

Overall, we asked whether mice can generate somatic mutations in bone marrow B cells with an immature

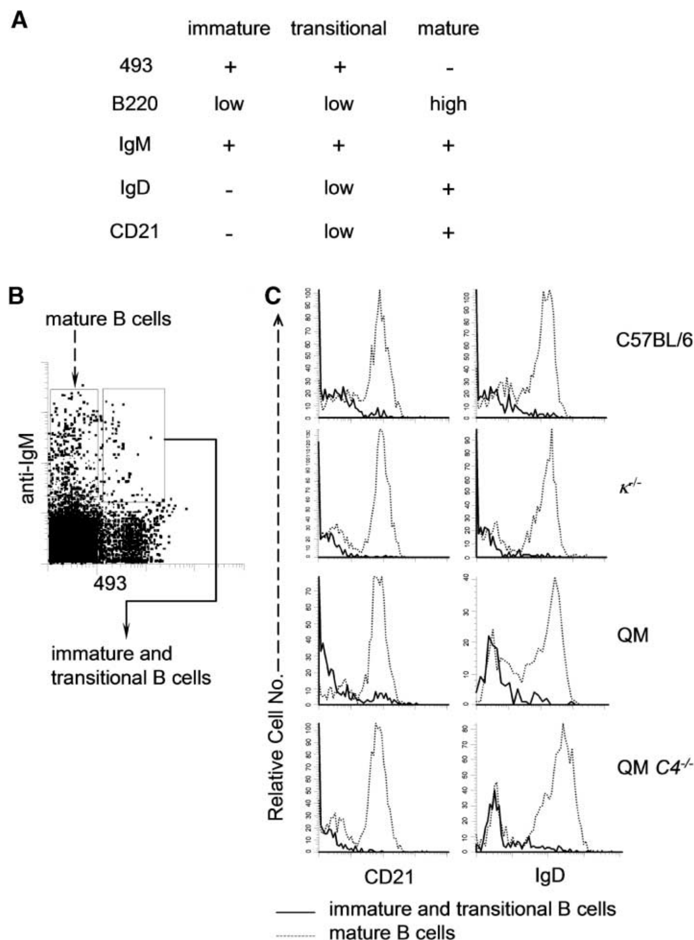


Figure 1. The 493^+IgM$^+$ Bone Marrow B Cells Have an Immature Surface Marker Phenotype

(A) Surface marker expression in immature, transitional, and mature B cells.

(B) An example of expression of 493 and IgM on bone marrow cells. Gates used to identify 493^+IgM$^+$ (immature and transitional) B cells and 493^-IgM$^+$ (mature) B cells are shown.

(C) Analysis of CD21 and IgD on gated 493^+ IgM$^+$ cells (solid line) and on gated 493^-IgM$^+$ cells (dotted line). Representative data from one of three mice of each genotype are shown.

phenotype and whether such mutations are T dependent. We found T-independent AID expression and somatic mutations in immature B cells in all of the four models examined.

Results

Most 493^+ Bone Marrow B Cells from $\kappa^{-/-}$, QM, and QM *C4*$^{-/-}$ Mice Have an Immature B Cell Phenotype

Because we wanted to look for somatic mutation in developing B cells, we needed a strategy to isolate pre-B, immature, and mature B cells from the bone marrow. Newly emergent immature B cells are 493^+B220^{low} HSA^{high}IgM$^+$IgD$^-$CD21$^-$ (Hardy and Hayakawa, 2001; Loder et al., 1999). These immature B cells develop into 493^+B220^{low}IgM$^+$IgD^{low}CD21^{int} transitional B cells, which further develop into 493^-B220^{hi}IgM$^+$IgD^{hi}CD21$^+$ mature B cells (Figure 1A). We identified bone marrow 493^+ B cells in $\kappa^{-/-}$, QM, and QM *C4*$^{-/-}$ mice (Figure 1B), and characterized them further by staining for IgD, B220, HSA, and CD21. The 493^+ B cells from these mice were B220^{low}HSA^{high} (data not shown; see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/2/133/DC1) as expected (Rolink et al., 1998). Most of the 493^+ B cells were IgD$^-$, CD21$^-$. Analysis of bone marrow cells from three individual mice per group indicated that only a small fraction was CD21^{low} (17 $\pm$ 6%), or IgD^{low} (8 $\pm$ 5%) (Figure 1C). We conclude that $\sim$80% of the 493^+ bone marrow B cells from our mutant mice have an immature surface phenotype and the remaining $\sim$20% have transitional B cell markers (for convenience, we refer to them together as immature B cells in the rest of the text), whereas 493^- bone marrow B cells from our mutant mice have mature B cell markers.

It is generally agreed that in wild-type mice pro-, pre-, and immature B cells have a rapid turnover. This contrasts with the longer half life of mature B cells and the observation that memory B cells are essentially resting (Allman et al., 2001; Forster et al., 1989; Rolink et al., 1998). To determine whether the 493^+ bone marrow B cell populations in our mutant mice are also rapidly turning over, we labeled bone marrow cells from $\kappa^{-/-}$, QM, and QM *C4*$^{-/-}$ in vivo with BrdU and followed BrdU labeling over time. We monitored the level of BrdU labeling in pre-, immature, and mature B cells by surface staining with 493 and anti-IgM (or anti-λ), and intracellular BrdU staining. Estimated initial rates of BrdU labeling in QM *C4*$^{-/-}$ mice were: 22% per hour for 493^+ large pro-B, 2% per hour for 493^+ small pre-B, 1.7% per hour for 493^+ immature B cells, and 0.7% per hour for 493^- mature B cells (Figure 2A). These rates are in agreement with the published data (Opstelten and Osmond, 1983). The large pro-B, small pre-B, and immature B cell popu-

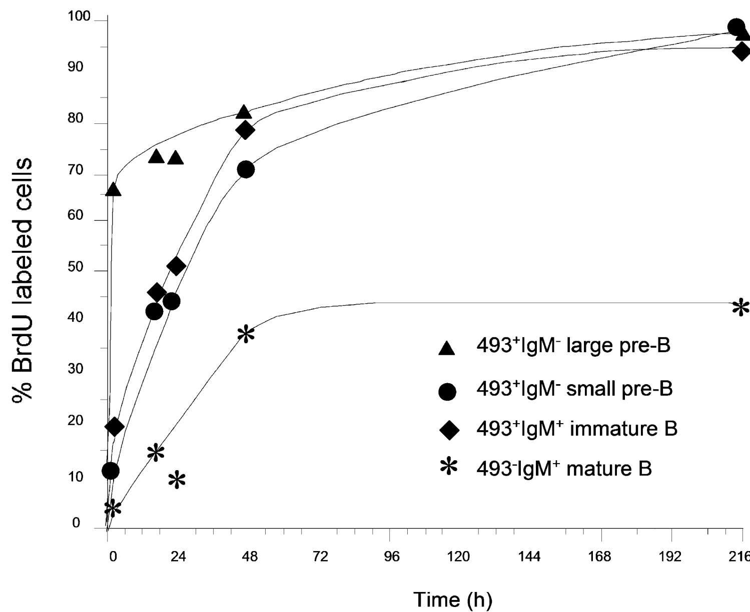


Figure 2. In Mutant Mice Only Pre-B and Immature B Cells Rapidly Incorporate BrdU

Ten-week-old QM $C4^{-/-}$ mice were injected with BrdU at 0 hr and then fed continuously with drinking water containing BrdU. At the indicated time points during BrdU feeding, bone marrow cells were harvested for analysis by FACS. The bone marrow cells were stained with 493 and anti-IgM, permeabilized, and stained with anti-BrdU antibodies. The percent of $BrdU^+$ cells in the indicated B lineage populations at different time points is shown. Results from identical time points represent a single mouse. Similar results were obtained with $\kappa^{-/-}$ and QM mice (data not shown).

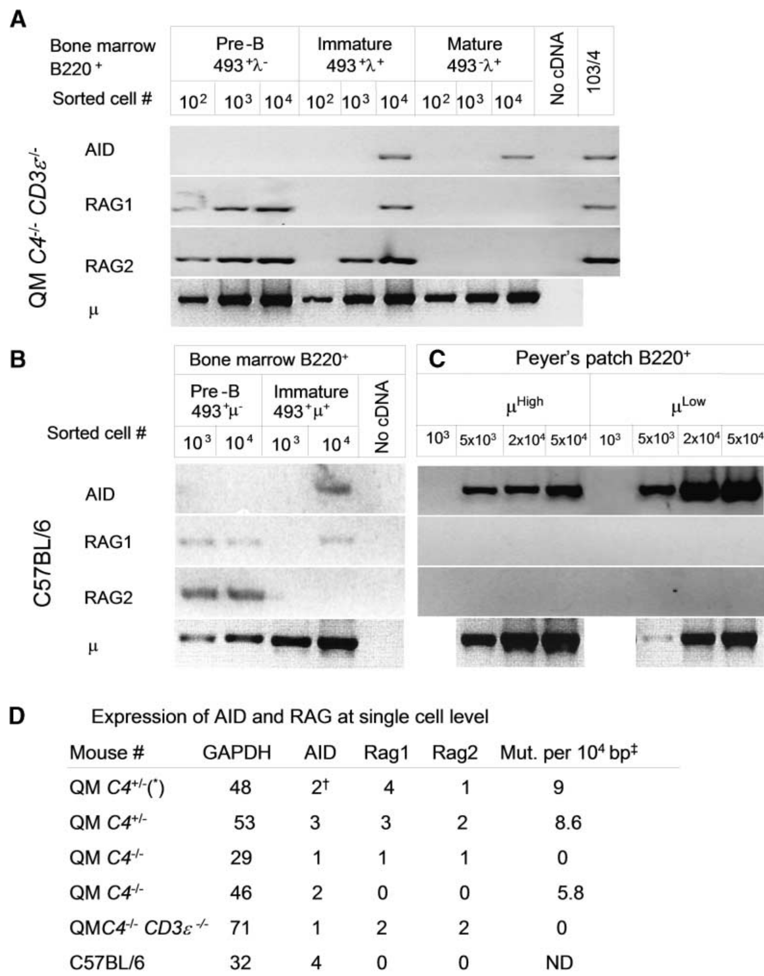
lations were 70% labeled before incorporation tapered off. All three populations eventually plateau at more than 90% labeled cells, which indicates that these cells are all rapidly turning over. In contrast, the maximum labeling of mature B cells was 40%, indicating a low turnover rate. Similar results were obtained when anti-λ was used instead of anti-IgM (data not shown). Thus, as in wild-type mice, bone marrow 493^+ B cells in QM $C4^{-/-}$ mice have a rapid turnover rate. Moreover, the similarity of BrdU labeling kinetics of the 493^+ small pre-B cells and the 493^+ B cells suggests that the latter were the direct descendants of the 493^+ small pre-B cells (Figure 2A). This is consistent with the notion that the 493^+ B cells from mutant mice are newly derived immature B cells. Similar results were obtained with bone marrow cells from $\kappa^{-/-}$ and QM mice (data not shown). Thus, we conclude that the 493^+ and 493^- B cells from $\kappa^{-/-}$, QM, and QM $C4^{-/-}$ mice are immature and mature B cells, respectively, as in wild-type mice.

493^+ Immature B Cells Express *RAG* Genes

Immature and pre-B cells express RAG, while mature B cells do not (Grawunder et al., 1995). Thus immature B cells can be identified by their expression of *RAG* genes. In order to test for RAG1 and RAG2 expression, total RNA was extracted from sorted bone marrow B lineage populations and cDNA was synthesized by RT reactions using oligo-dT primer. The cDNA was then PCR amplified using specific primers for *RAG1*, *RAG2*, and *Igμ* genes. The intensity of the μ bands was taken as a measure of the number of cells sampled. *RAG1* and *2* gene expression were easily detected by RT-PCR in 10^2 pre-B cells ($493^+\lambda^-$) of a QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mouse (Figure 3A). In the immature B cells ($493^+\lambda^+$) RAG1 was detected in a 10^4 cell sample, while RAG2 can be detected in a 10^3 cell sample. Cell samples from QM, QM $C4^{-/-}$ mice produced similar results (data not shown). In a C57BL/6 mouse, RAG1/2 expression in pro-/pre-B could be detected in 10^3 and 10^4 cell samples (10^2 was not done) (Figure 3B). In the immature B cells, RAG1 expression can be seen in the 10^4 cell sample. RAG2 was not detected. Another source of mature B cells, Peyer's patch B cells, was also tested for *RAG* gene expression. No RAG was detected in samples of 5×10^4 $B220^+\mu^{low}$ and $B220^+\mu^{high}$ cells from Peyer's patches of C57BL/6 mice (Figure 3C). In conclusion, the data show that RAG1/2 are expressed at high levels in pro-/pre-B cells, less in immature B cells, and are not detected in mature B cells. These data indicated that sorted $493^+B220^+\lambda^+$ B cells from QM, QM $C4^{-/-}$, and QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mice are indeed immature B cells and are similar to those found in normal, wild-type mice.

AID Is Expressed in Sorted 493^+B220^+ μ^+ or λ^+ Immature B Cells

As AID has been shown to be absolutely required for somatic hypermutation in B cells (Muramatsu et al., 2000), we asked whether immature B cells express AID. For this purpose we PCR amplified AID using the cDNA obtained from the mRNA of the sorted populations shown in Figure 3. As expected, AID expression was abundant in B cells isolated from Peyer's patches of the C57BL/6 mouse (Figure 3C). AID expression was detected in the samples of 10^4 immature B cells from the bone marrows of QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ and C57BL/6 mice (Figure 3). AID expression was also detected in the 10^4 cell sample of mature B cells from the QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mouse (Figure 3A). However, no AID expression was found in any samples of pro-/pre-B cells from the QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ or C57BL/6 mice (Figure 3). These data indicate that AID is expressed in immature phenotype B lymphocytes from QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ and C57BL/6 mice, as well as in mature phenotype cells from QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mice. We also conclude that AID expression in the bone marrow B cells is T cell independent. Similar data regarding AID and Rag expression was obtained from a QM $C4^{-/-}$ mouse (data not shown).



Mouse #	GAPDH	AID	Rag1	Rag2	Mut. per 10^4 bp‡
QM $C4^{+/-}$(*)	48	2†	4	1	9
QM $C4^{+/-}$	53	3	3	2	8.6
QM $C4^{-/-}$	29	1	1	1	0
QM $C4^{-/-}$	46	2	0	0	5.8
QM$C4^{-/-}$ $CD3\varepsilon^{-/-}$	71	1	2	2	0
C57BL/6	32	4	0	0	ND

Figure 3. RT-PCR Detection of RAG1, RAG2, and AID in Sorted Bone Marrow Cells

(A–C) From (A) a QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mouse, (B) sorted cells of bone marrow, and (C) Peyer's patches of a C57BL/6 mouse. The no cDNA lane is a negative control, and 103/4 (an Abelson murine leukemia virus transformed bone marrow pre-B cell line) was used as a positive control. RT-PCR for μ was done to confirm the presence of RNA in the samples.
(D) Summary of results of RAG and AID expression at the single-cell level. Total number of single cells analyzed is represented by the total number of wells positive for GAPDH. #, single immature B cells from various QM mice and mature B cells from C57BL/6 Peyer's patch. *, one cell expressed AID and Rag1 simultaneously. ‡, mutation frequency was obtained from genomic PCR of bulk samples. †, numbers represent number of positive cells obtained with RT-PCR. ND, not done.

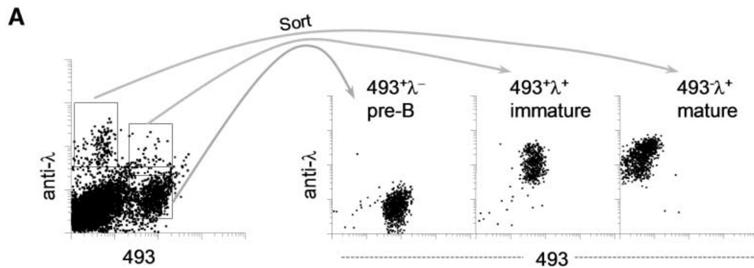
In order to determine whether the individual cells express both AID and RAG, single-cell RT-PCR for these genes was performed. Mature single B cells from C57BL/6 Peyer's patch were used as controls, and as expected, they express AID but no RAG (Figure 3D). We found that only a small fraction of immature B cells express AID (Figure 3D). In addition, *AID* and *RAG* genes were not coexpressed in most immature B cell analyzed, but we found that one of nine AID-positive immature B cells also expressed Rag1 (Figure 3D). These results might indicate that AID expression is initiated at a stage in B cell maturation at which RAG expression is ending.

Somatic Mutation in V_λ Genes in B Cells from $\kappa^{-/-}$, QM, and QM $C4^{-/-}$ Mice

Using cells isolated from the bone marrow of 11-week- and 6-month-old mice, we looked for mutations in the cDNA of rearranged V_λ genes. In the sorted $493^+\lambda^-$, pre-B cells (Figure 4A), V_λ RNA transcripts were detected by RT-PCR (data not shown). This result suggests that light chain rearrangement had just been completed in some of the sorted pre-B cells. This conclusion was supported by the finding that in the pre-B cell samples, λ light chain cDNA sequence analysis showed that the frequency of reading frame shifts at VJ junctions was 58 ± 15%, which was significantly higher than the 20 ± 11% found in immature and mature B cell samples ($p < 10^{-7}$, one-tailed, unequal variance t test). The frequency of mutations in λ cDNA clones from pre-B samples from all the mice analyzed was 2.5 ± 3.0 per 10^4 bp ($n = 233$, n being the number of clones sequenced; data not shown). This represents a slight increase in the frequency of mutations when compared to the mutation frequency of C_λ regions (0.6 ± 1.4 per 10^4 bp) in these clones ($p < 0.024$, one-tailed, paired t test). However, when compared to the frequency of mutations in all ($n = 1589$) sequenced C_λ regions (1.5 ± 1.5 per 10^4 bp), it is not significantly different ($p < 0.065$, two-tailed, unequal variance t test). The frequency of mutations in the C_λ is quite similar to the calculated error rate of the pfu DNA polymerase, which, under our experimental conditions was 1.2 ± 1.2 per 10^4 bp for the housekeeping gene, *S14* ($n = 54$) (Figure 4B and data not shown).

In contrast to the findings in pre-B cells, mutations were found in the V_λ genes of immature 493^+ B cells of two of the six $\kappa^{-/-}$ and two of two QM mice. These occurred at similar frequencies (Figure 4B) and ranges (Figure 5). The average mutation frequency was 10×10^{-4} per bp, roughly ten times higher than our error rate. The 493^- mature B cells from these mice had variable

A



B

By RT-PCR		Frequency of mutations per 10^4 bp			
Mouse type number of mice analyzed		V region		C region	
	#[a]	immature	mature	immature	mature
$\kappa^{-/-}$ 6 mice in 4 experiments	1 (2.5 mo)	11	1	2	1
	2 (6 mo)	7	7	0	0
QM 2 mice	1 (2.5 mo)	11	13	3	1
	2 (7 mo)	10	15	3	3
QM *C4*$^{-/-}$ 3 mice	1 (2.5 mo)	17	12	0	0
	2 (2.9 mo)	8	35	1	0
	3 (6 mo)	13	42	1	3
QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$ 2 mice	1 (4.5 mo)	11	1	2	1
	2 (6.5 mo)	8	8	1	5
By genomic PCR QM *C4*$^{+/-}$ 2 mice	1 (6 mo)	9			
	2 (2.5 mo)	8.6			
QM *C4*$^{-/-}$ 4 mice	1 (5.5 mo)	5.8			
	2 (5.5 mo)	5			

[a]: name of individual mouse having somatic mutations. Their age (months) is in parenthesis. Mice having no mutations were not further analyzed.

Figure 4. V_λ Genes from the BM-Derived B Cells Contain Mutations

(A) An example of cell sorting of three cell subsets as shown in the boxed populations $493^+\lambda^-$ pre-B cells, $493^+\lambda^+$ (immature) B cells, and $493^-\lambda^+$ (mature) B cells. The BM cells from $\kappa^{-/-}$, QM, QM *C4*$^{-/-}$, and QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$ were stained with 493 and anti-λ antibodies. The purity of the sorted cells was routinely around 94% to 99%.

(B) Mutation frequency in the V_λ and C_λ regions of the immature and mature B cells. Rearranged λ genes were amplified either by RT-PCR or genomic PCR as indicated and cloned. The average mutation frequency for the *C* regions is 1.5 ± 1.5 per 10^4 bp.

mutation frequencies, ranging from background to 42×10^{-4} per bp (Figures 4B and 5). Although there were similar mutation frequencies in the immature and mature B cells (Figures 4B and 5), the underlying mutations in these two groups were different. For example, they differed in the frequency of mutations of G/C base pairs (see below), implying different mutation and/or selection processes in mature and immature B cells. These data clearly indicate that the 493^+ (immature) B cells had somatic mutations. The variability in occurrence of mutations in individual mice and cell populations could be due to the degree of heavy chain diversity in these different groups of mice or reflect the different genetic backgrounds; however, the issue needs to be further investigated.

The mutation frequencies and range of mutations in 493^+ B cells from QM *C4*$^{-/-}$, QM, and $\kappa^{-/-}$ mice were similar (Figures 4B and 5). Mature 493^- B cells of two of the three QM *C4*$^{-/-}$ mice had about a 4-fold greater mutation frequency than their immature counterparts ($p < 0.03$, two-tailed, unequal variance t test). These mutations were associated with a higher percentage of mutant clones and more mutations per clone (Figure 5). Thus, loss of C4 has little influence on somatic mutation in 493^+ B cells but is associated with an increase in mutations in mature B cells. These results provide evidence that the mutated 493^+ B cells were neither contaminating mature 493^- B cells nor were they likely to be their descendants.

As a small number of contaminating plasma cells, producing large amounts of Ig mRNA, could distort RT-PCR results, genomic-PCR of rearranged λ genes was done. In two QM *C4*$^{+/-}$ mice and two of four QM *C4*$^{-/-}$ mice, the mutation frequencies found in genomic DNA samples from immature B cells ($B220^+493^+\lambda^+$) were comparable to those obtained with RT-PCR (Figure 4). In addition, AID was found to be expressed in some of these cells (Figure 3D). Taken together, the data from

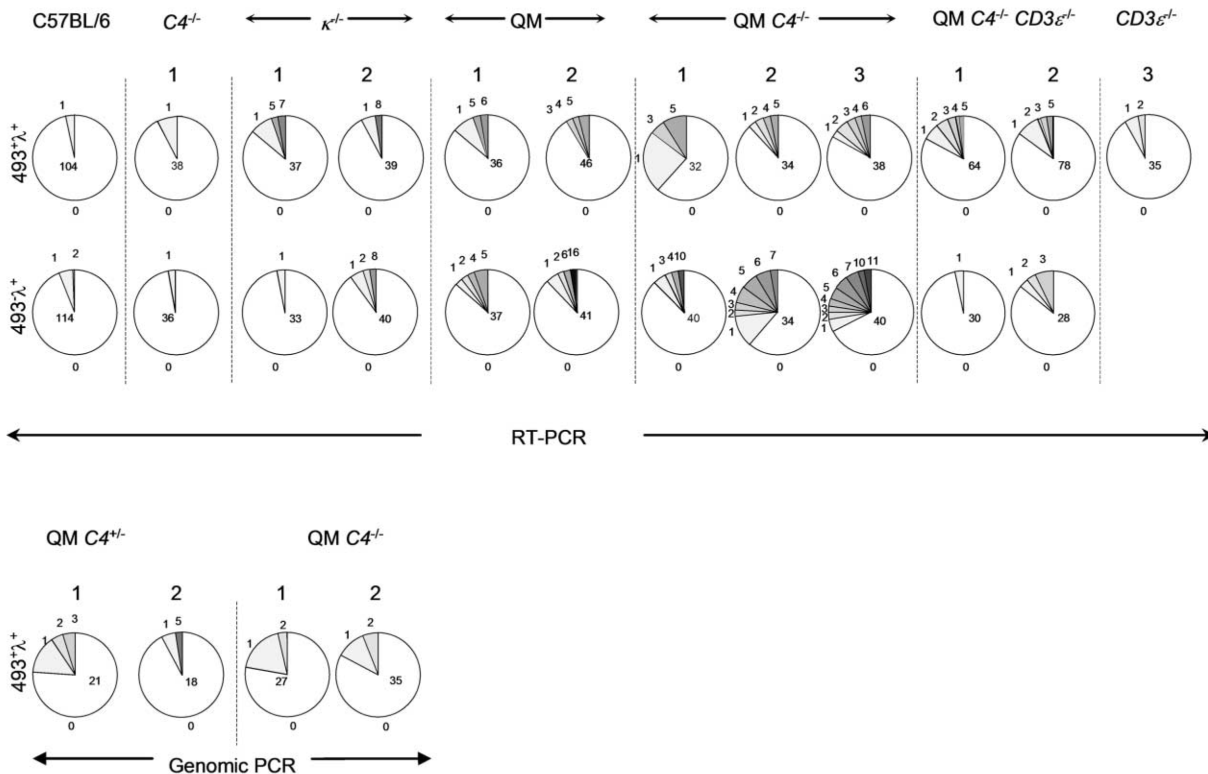


Figure 5. Mutation in the V_λ Clones from the $493^+\lambda^+$ (Immature) and $493^-\lambda^+$ (Mature) B Cells

The mouse source is shown above. Each pie chart depicts the proportion of sequences with mutations. Numbers outside of each pie are the number of mutations. The size of each wedge coded with varying shades of gray is proportional to the percentage of clones carrying that number of mutations. Inside each pie is the number of clones sequenced.

both cDNA and genomic-DNA clones suggest that immature B cells from these mutant mice have somatic hypermutation.

T Cell-Independent Mutation in Immature B Cells

Somatic hypermutation occurs in newly derived B cells in sheep ileal Peyer's patches in a T-independent manner (Reynaud et al., 1995). The observation of somatic hypermutation in murine 493^+ immature B cells raises the question whether T cells are required for this process. To determine this, we looked for mutations in QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$ mice.

Somatic mutation has been associated with DNA double-strand breaks (Bross et al., 2000; Sale and Neuberger, 1998), and is regulated by the cell cycle (Papavasiliou and Schatz, 2000). We therefore focused our analysis on the large, dividing bone marrow 493^+ (data not shown) and 493^- B cells (therefore, the results from these mice can not be directly compared with those of other mice). We sorted the large $493^+\lambda^+$ and $493^-\lambda^+$ B cells from a QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$ mouse in two separate experiments. Mouse one was 17 weeks old; mouse two was 28 weeks old. In the immature B cells of both of these mice, a significant number of mutations were found in the V_λ compared to the C_λ regions in the same clones ($p < 0.002$, one-tailed, paired t test) (Figures 4B and 5). Mutations were also found in the mature B cells from one of the two mice (mouse two) (Figures 4B and 5). The frequency of mutation (Figure 4B) in the constant region of λ light chain gene of mature B cell population from mouse two seems higher (5×10^{-4} per bp) than the average mutation frequency (1.6×10^{-4} per bp). This result could be due to random variation or to somatic mutation in the λ constant region as previously reported (Motoyama et al., 1991). These data indicate that T cells are not required for somatic mutation in the V region early in B cell development.

Mutations Were Not Detected in Bone Marrow B Cells in Mice with an Intact Ig Repertoire

In order to determine whether mutations can be found in mice with an intact V gene repertoire, we chose C57BL/6, *C4*$^{-/-}$, and *CD3ε*$^{-/-}$ mice for analysis (Figure 5 and data not shown). Mutations were not found in the sorted pre-, immature, or mature B cells of C57BL/6 mice (250 clones in three experiments, six age-matched mice). In addition, no mutations were found in either an eleven-week-old *C4*$^{-/-}$ mouse (103 clones) or in two *CD3ε*$^{-/-}$ mice (4.5 months old, 35 clones). These results indicate either that, when κ chains are expressed, mutation of λ genes does not occur, or, more likely, that in the presence of κ-expressing B cells λ-mutated cells are not clonally expanded to the point that allows their detection by the methods employed.

Nature of the Mutations

Analysis of the pattern of nucleotide targeting showed that 58 ± 4% of the mutations in immature and 54 ±

Table 1. Pattern and Frequency of Mutations in Vλ Genes of Immature B Cells

A. Significant Increase of C to T Mutations in Immature B Cells Compared to Those in Mature B Cells

Cell Type	Original Nucleotide (Germline)	Final Nucleotide (Mutated)[a] T	C	G	A	Total[c]
Immature B cells	T		6[b]	1	3	10
	C	23[d]		4	2	29
	G	1	6		22	29
	A	8	8	16		32
Mature B cells	T		6	1	2	9
	C	16		4	3	23
	G	3	6		22	29
	A	12	10	16		37

B. Lower Mutation Frequency of V_λ Genes from Immature B Cells Compared to the Published V_λ Mutations from Putative Memory B Cells

Segment[g]	Nucleotides	Mutations[e] 493$^+$ (n = 61)	493$^-$ (n = 51)	Mutations/bp in $V_\lambda J_\lambda$[f] 493$^+$	493$^-$	Peyer's Patch[h]	QM PB[i]
L	57	5	4	1.4	1.4		
FR1	69	21	16	5	4.5	5	24
CDR1	42	38	60	15	28	38	15
FR2	45	22	25	8	11	14	19
CDR2	21	18	32	14	30	51	60
FR3	96	25	38	4	8	14	22
CDR3	21	21	28	16	26	44	
J	39	10	9	4	4.5	7	
V/J	384	160	202	6.8	10	20	23
R/S[j]				1.9	2.6	3	3.8

[a] The total number of observed mutations in V_λ1 and 2 from $\kappa^{-/-}$, QM, QM *C4*$^{-/-}$, and QM *C4*$^{-/-}$ *CDε*$^{-/-}$ was 155 in immature and 199 in mature B cells. Numbers represent the percentage of nucleotide changes in V_λ genes. Deletions were excluded. Data in this table were from RT-PCR.
[b] Percentage of nucleotide change is corrected for base composition of V_λ1 according to the method of Gojobori et al. (1982). Correction based on V_λ 2 gives almost identical results.
[c] Total by row.
[d] By χ^2 test $p < 0.015$ compared to C to T mutations in mature B cells.
[e] Number of mutations observed within each segment is indicated. N, number of mutant clones analyzed. Data in this table were from RT-PCR.
[f] Mutations/bp is number of mutations/segment length/mutated clone number × 1000.
[g] L, leader; FR, framework; CDR, complementary determining region; J, joining region.
[h] From B220$^+$ PNA^{hi} Peyer's patch B cells (Gonzalez-Fernandez et al., 1994).
[i] Adapted from B220$^-$, idiotype-positive B cells from peripheral blood (PB) (Cascalho et al., 1998).
[j] Replacement versus silent mutations.

16% in mature B cells were in G/C base pairs (Table 1A and Supplemental Table S1 at http://www.immunity.com/cgi/content/full/20/2/133/DC1). 23 ± 9% of mutations in immature B cells were C to T transitions compared to an average of 16 ± 8% in the mature B cells ($p < 0.015$, χ^2 test). The average of C to T changes in mouse Peyer's patches was reported to be 17 ± 4% (Bertocci et al., 2002; Betz et al., 1994; Frey et al., 1998; Gonzalez-Fernandez et al., 1994; Gonzalez-Fernandez and Milstein, 1993; Yelamos et al., 1995) ($p < 0.022$, compared to C to T from immature B cells, χ^2 test). We conclude that C to T changes occur more frequently in immature than mature B cells.

Many studies have shown that the location of mutations in *V* genes is higher in so-called hotspots with the motifs RGYW (R = A or G, Y = C or T, W = A or T) and TAA (Betz et al., 1993; Rogozin and Kolchanov, 1992). We therefore analyzed the somatic mutations we found in bone marrow-derived B cells to determine if they were also localized to hotspots. We calculated the percentage of mutations that falls within these hotspots. In the clones examined, 46% of immature (160 total in VJ, five deletions included) and 55% of the mature mutations (202 total, three deletions included) were in these hotspots or their inversions (WRCY and TTA) (Figure 6). Thirty-four of the 73 hotspot mutations from the 493$^+$ B cells were in RGYW, 32 in WRCY, 2 in TTA, 1 in TAA, 2 in overlap of TAA and RGYW, and 2 in an overlap of RGYW and WRCY. Fifty four of the 108 hotspot mutations from the 493$^-$ B cells were in RGYW and TAA, 50 in WRCY and TTA, and 4 in an overlap. The mutations we found in the immature B cells thus show features previously described as common in somatic hypermutation of Ig genes: they occur at hotspots and in both strands of DNA (Dorner et al., 1998; Milstein et al., 1998).

Several positions are favored mutation targets (Oprea et al., 2001; Shapiro et al., 1999, 2002). In the immature B cells, mutations in nucleotide positions 77 (A$\underline{C}$T, Thr27) (where the mutated nucleotide position is underlined followed by the amino acid three-letter code and its location), 91 ($\underline{A}$CT, Thr29), 101 (T$\underline{A}$T, Tyr32), 170 (G$\underline{C}$T, Ala55), and 284 (A$\underline{G}$C, Ser93) were the favored targets for mutation (Figure 5). The A$\underline{G}$C and G$\underline{C}$T previously described as the two most targeted triplets for mutation

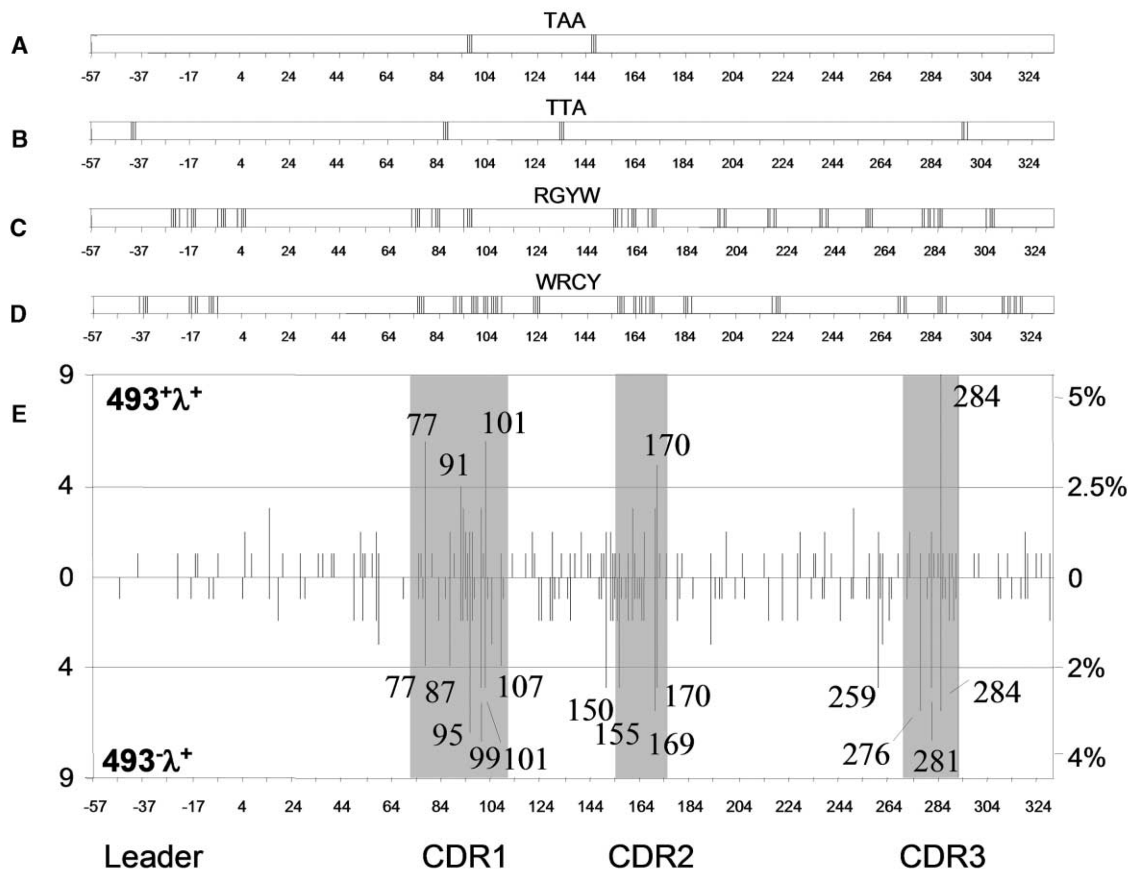


Figure 6. Point Mutations in the $V_\lambda J_\lambda$ Gene Segment of the BM B Cells Are Preferentially Located in the Hotspots and Their Inversions

(A–D) Hotspot TAA, its inversion TTA, hotspot RGYW, and its inversion WRCY in the $V_\lambda J_\lambda$ ($V_{\lambda x}$ is not included).

(E) Distribution of point mutations in $V_\lambda J_\lambda$ in the $493^+\lambda^+$ (immature) and $493^-\lambda^+$ (mature) B cells. Mutant clones from $\kappa^{-/-}$, QM, QM $C4^{-/-}$, and QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ made from RT-PCR are included in this analysis. The immature B cells analyzed resulted in 61 mutant/404 total clones and the mature B cells in 51 mutant/323 total clones. The x axis indicates nucleotide position; y axis indicates number of mutations. The axis on the right represents the percent of mutations located at a particular nucleotide position among total mutations. The dominant individual hotspots are indicated with the position of the nucleotide.

(Oprea et al., 2001; Shapiro et al., 1999) were mutated nine and five times, respectively. The ACT and TAT were mutated six times each in the immature B cells. The ACT was mutated four times. All except the TAT are in known hotspots. The A in the TAT (Tyr 32) is flanked by a hotspot on each side. Mutations occurred in more hotspots and TAT triplets in the mature B cells. The combined mutations at positions 77, 101, 170, and 284 account for 16% of the total mutations in immature B cells compared to 10% of the total mutations in mature B cells. Thus, these data suggest that the immature B cells have more focused targeting of mutations in a few hotspots compared to the mature B cells.

There is an association of an enhanced replacement (R)/silent (S) mutation ratio (R/S ratio) with increased affinity maturation of B cell receptors (Berek and Milstein, 1987; Weigert et al., 1970). Our data show that, in the immature B cells, the R/S in the three CDRs was similar to that in the mature B cells: 3.3 and 2.9 for the immature and mature B cells, respectively (data not shown). These values are only slightly higher than the expected ratio of 2.7, assuming unselected random mutations. The overall R/S ratios for the 493^+ immature and 493^- mature B cells were 1.9 and 2.6, respectively, which is lower than the reported R/S ratios of 3 and 3.8 in Peyer's patch B cells (Gonzalez-Fernandez et al., 1994) and in circulating B lymphocytes of peripheral blood of QM mice (Cascalho et al., 1998), respectively (Table 1B). Furthermore, mutations per 1000 bp in $V_\lambda J_\lambda$ were lower in the 493^+ than in 493^- B cells. In addition, the reported frequency of mutations in the $\lambda 1$ genes of putative memory B cells from Peyer's patches in normal mice (Gonzalez-Fernandez et al., 1994) or peripheral blood of QM mice (Cascalho et al., 1998) (Table 1B) were much higher than we observed in the bone marrow B cells. Thus, it is unlikely that mutations found in 493^+ B cells were from circulating memory type B cells found previously in QM mice. We conclude that mutation occurs in B cells with immature phenotype in the bone marrow. Unlike mutations in germinal center B cells the mutations in immature B cells are C biased and are more focused to a few hotspots.

Discussion

To date, somatic hypermutation in mice was found to be associated with the postantigenic stages of the immune response and limited to mature, memory, and plasma

cells. Thus, our finding of mutations in the bone marrow-derived 493$^+$ B cells raises a question regarding the identity of this population in the $\kappa^{-/-}$, QM, and QM *C4*$^{-/-}$ mouse models. We addressed this in three ways: by the analysis of surface markers, by BrdU labeling, and by molecular analysis. The majority of 493$^+$ cells from the bone marrow of these mice have immature surface markers and the rest transitional markers. Furthermore, they are rapidly turning over (Figures 1 and 2), a feature characteristic of immature B cells in normal mice. The 2% renewal rate of the 493$^+$ cells is similar to rates seen in several mouse strains (Allman et al., 2001; Opstelten and Osmond, 1983). In addition, these 493$^+$ B cells express *RAG* genes (Figure 3), a hallmark molecular marker of pre-B and immature B cells (Grawunder et al., 1995; Monroe et al., 1999).

Bone marrow is a rich source of memory and plasma cells (Manz et al., 1997; Slifka et al., 1998; Smith et al., 1997), which, by contamination, could account for the somatic mutations in sorted "493$^+$" B cells. These memory and plasma cells have several common features: their B cell receptors are frequently class switched and they have a high frequency of somatic mutations in their V genes (Smith et al., 1997). However, more than 98% of the 493$^+\lambda^+$ were IgM$^+$ (data not shown). Comparisons of mutations in V_λ genes of immature 493$^+$ B cells with putative memory circulating B cells of QM mice, or with putative memory cells from Peyer's patches (Gonzalez-Fernandez et al., 1994) indicate that they differed significantly in mutation frequency and in pattern of nucleotide bias (Table 1B). Thus, contaminating memory and plasma cells are unlikely sources of the mutations seen in the 493$^+$ B cell populations. That the mutations found in λ light chain genes in sorted 493$^+$ B cells are not due to contaminating mature 493$^-$ B cells is supported by the observation that, in the QM *C4*$^{-/-}$ mice, an increase in the frequency of mutations in the mature 493$^-$ B cells is not reflected by an increase in the mutation frequency in the 493$^+$ cells.

C4 participates in generating B cell tolerance and enhances B cell signaling by antigen through linking of the CD19/CD21 coreceptor with the BCR (Fearon and Carroll, 2000). It has been proposed that C4 transports soluble self-antigens to CD21/CD35 within the bone marrow and secondary lymphoid tissues and thereby plays a critical role in the induction of anergy (Prodeus et al., 1998). Thus, one possible explanation for the increased frequency of mutation in mature B cells of QM *C4*$^{-/-}$ mice is that self-reactive B cells are chronically activated by self-antigens, leading to an accumulation of mutations. Alternatively, as previously suggested (Chen et al., 2000), because C4 complexed with antigen crosslinks the BCR with the CD21/CD19 coreceptor, an absence of C4 may lead to the selection of B cells producing higher-affinity antibodies. This would allow maintenance of a full number of B cells despite a reduction in signaling via the coreceptor CD21/CD19 due to *C4* deficiency.

Another important element for the process of somatic hypermutation is AID. AID expression has been detected in Peyer's patch, splenic, and bone marrow cells by RT-PCR (Muramatsu et al., 1999). We extend these findings by showing AID expression in the bone marrow immature and mature B cells of wild-type and mutant mice (Figure 3). In addition, we find expression of AID in cells from QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$ mice, providing evidence of T-independent activation of AID in vivo. These results extend a previous report that T-independent activation of a B cell line can induce AID expression in vitro (Faili et al., 2002).

Weller et al. have proposed a hypothesis of two pathways of somatic hypermutation, one that is dependent on and the other independent of CD40 signals (Weller et al., 2001). In addition, somatic hypermutation studies of Ig genes in *Msh2*$^{-/-}$ mice led to the proposal of a two-stage model of mutations, in which hotspot-focused, G/C-biased mutations are followed by Msh2-dependent mutations that are biased toward A/T (Rada et al., 1998). Our data showing high C to T nucleotide changes in immature B cells suggests the possibility that somatic hypermutation operates in two different fashions. During B cell development mutation is AID dependent and T cell independent but in germinal centers it is T dependent and requires AID plus Msh2 and/or other factors.

The finding of AID expression in early B cell development clearly indicates that somatic hypermutation could play an important role in enlarging the Ig repertoire and/or rescuing self-reactive B cells. In some respects, mutation in the immature B cells of mice is reminiscent of mutations found in sheep (Reynaud et al., 1995). In sheep, somatic hypermutation occurs in newly derived B cells without T cell help. Similarly, in the adult mice with a restricted Ig repertoire that we describe, mutation was limited to some animals, and in these, only about 20% of the immature B cells were mutated.

No mutations were found in bone marrow-derived mature or immature λ light chain genes of C57BL/6, *C4*$^{-/-}$, or *CD3ε*$^{-/-}$ mice, yet mutations were found in λ light chain-restricted mice: $\kappa^{-/-}$, QM, QM *C4*$^{-/-}$, and QM *C4*$^{-/-}$ *CD3ε*$^{-/-}$. We did find, however, expression of AID in immature B cells from wild-type mice (Figure 3B). The extreme pressure on B cells to diversify due to the limited repertoire of V_λ genes might cause an expansion of many clones of mutated B cells in the $\kappa^{-/-}$, QM, and QM-derived mice, while in normal mice expansion might occur only at a much lower frequency. Thus, although these mutations were only detected in these genetically manipulated mice, that they occur at all indicates that this type of mutation mechanism is generally available.

However, the mechanisms generating somatic hypermutation in developing B lymphocytes may not be operative in normal mice. Like the secondary rearrangements of V_H and V_L genes found in cells expressing self-reactive Ig transgenes (Gay et al., 1993; Pelanda et al., 1997; Tiegs et al., 1993), or in self-reactive B cells of autoimmune mice (Klonowski et al., 1999), somatic mutation at the immature B cell stage might also occur in response to self-antigen binding. As with secondary rearrangements, this would be a mechanism that would serve to alter the specificity of otherwise self-reactive cells. Such a mechanism would provide an explanation for results obtained when the anti-ssDNA Ig *3H9*/*Vκ8* transgenes were bred onto the autoimmune MRL/*lpr* background (Brard et al., 1999). In this case, the targeted $V_\kappa 8$ light chain gene had G/C-biased mutations, one of which rendered the gene nonfunctional, allowing subsequent rearrangement of endogenous $V_\kappa 23$ light chain genes. The targeted *3H9 VH* gene also had G/C-biased mutations. Some of these mutations could have taken

place during B cell development without the need to invoke reactivation of *RAG* genes at the mature B cell stage. It will be very important to investigate whether the higher rate of somatic mutations found in systemic lupus erythematosus patients (Dorner et al., 2001) is in part due to an increased rate of somatic mutation in developing B cells.

Experimental Procedures

Mice

C57BL/6 and C57BL/6-*Igk-C*^{tm1Cgn} were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our facility. The QM mice were a gift from Drs. M. Cascalho and M. Wabl (University of California, San Francisco). $C4^{-/-}$ mice (Fischer et al., 1996) were crossed with QM mice to produce QM $C4^{-/-}$ animals. $CD3\epsilon^{-/-}$ mice were kindly provided by Dr. P.E. Love (NICHD, NIH, Bethesda, MD). We crossed the $CD3\epsilon^{-/-}$ mice with QM $C4^{-/-}$ mice to produce QM $C4^{-/-}$ $CD3\epsilon^{-/-}$ mice. Oligonucleotide primers and conditions for PCR used for mouse genotyping are described in the Supplemental Data at http://www.immunity.com/cgi/content/full/20/2/133/DC1.

5-Bromo-2′-Deoxyuridine Labeling of Cells

0.2 ml solution containing 0.8 mg of 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St Louis, MO) in PBS was injected i.p. at the start of the experiment. The animals were fed BrdU (0.8 mg/ml) containing drinking water protected from light and changed daily for 9 days.

Flow Cytometry and Cell Sorting

In order to isolate immature B cells, we used mAb 493 (493) (Rolink et al., 1998). Dr. A.G. Rolink (Basel Institute for Immunology, Switzerland) graciously provided this reagent. The following anti-mouse antibodies were used: 493 labeled with Alexa 488 (Molecular Probes, Eugene, OR); biotinylated anti-λ, anti-IgM (μ chain specific) (both from Southern Biotechnology, Birmingham, AL), and anti-IgD (11–26); PE anti-BrdU (3D4) (all from BD Pharmingen, San Diego, CA); anti-CD21 (from Dr. J.F. Kearney, University of Alabama at Birmingham); APC anti-B220 (RA3.6B2, Caltag, Burlingame, CA). Biotinylated antibodies were revealed with streptavidin-PE (Pharmingen) or streptavidin-CyChrome (Pharmingen). Bone marrow cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA). Dead cells were excluded based on ability to include propidium iodide (PI) and forward scatter gating.

Intracellular BrdU was stained as described (Geiselhart et al., 2001). In brief, bone marrow cells were first stained with 493 and anti-IgM or anti-λ, followed by streptavidin-CyChrome. Then, the steps to stain BrdU were followed. Dead cells were excluded by forward scatter gating. The initial rate was estimated as: (% of BrdU$^+$ cells at time 2 − % of BrdU$^+$ cells at time 1)/(time 2 − time 1) in (% per hr).

For λ RT-PCR, bone marrow cells were stained with 493 and anti-λ, and the cells in the lymphocyte gate were sorted into 493$^+$λ$^-$ (pre-B), 493$^+$λ$^+$ (immature), and 493$^-$λ$^+$ (mature) B cells on a MoFlow instrument (Cytomation, Fort Collins, CO).

To detect AID, Rag1, and Rag2 expression, bulk cells of different numbers were sorted into 30 to 50 μl Trizol (Invitrogen, Carlsbad, CA). Single cell was sorted into 10 μl RT buffer containing 0.06 μl of 10 mg/ml yeast tRNA. For λ genomic PCR, 5000 cells were sorted into 30 μl DNAzol (Invitrogen).

Reverse Transcription PCR

Up to 1 μg of RNA was used to synthesize the first strand of the cDNA in a 20 μl reaction with oligo-dT and superscript II (GIBCO BRL). Three microliters of the first strand cDNA was amplified in a 20 μl reaction using pfu DNA polymerase (Stratagene, La Jolla, CA). *S14* gene, which encodes a ribosomal protein, was used to monitor PCR error rates.

To detect expression of *AID*, *Rag1*, *Rag2*, *GAPDH*, and μ, cDNA was prepared using oligo-dT and ThermoScript RT-PCR system (Invitrogen). For single-cell samples, 1 μl of 5% NP40 was added in the RT reaction. Separate PCR was carried out for each gene with 1 μl cDNA in 15 μl reaction (2 mM $MgCl_2$, 0.2 μM primers, and 0.5 U Platinum Taq DNA polymerase) (Invitrogen). Oligonucleotide primers used for RT-PCR amplification are described in the Supplemental Data.

Genomic PCR of λ Genes

PCR using purified genomic DNA and pfu DNA polymerase (Stratagene) was done at 95°C, 1 min 45 s; 35 cycles at 95°C, 20 s; 60°C, 30 s; and 72°C, 40 s, followed by a 30 min final extension at 72°C with primers 0.2 μM UVLN, 0.1 μM JL1R, and 0.1 μM JL23R.

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