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Ig Light Chain Receptor Editing in Anergic B Cells

Lina E. Tze, Elizabeth A. Baness, Keli L. Hippen, and Timothy W. Behrens

Receptor editing in the bone marrow (BM) serves to modify the Ag receptor specificity of immature self-reactive B cells, while anergy functionally silences self-reactive clones. Here, we demonstrate that anergic B cells in hen egg lysozyme Ig (HEL-Ig)/soluble HEL double transgenic mice show evidence of having undergone receptor editing in vivo, as demonstrated by the presence of elevated levels of endogenous \( \kappa \) light chain rearrangements in the BM and spleen. In an in vitro IL-7-driven BM culture system, HEL-Ig BM B cells grown in the presence of soluble HEL down-regulated surface IgM expression and also showed induction of new endogenous \( \kappa \) light chain rearrangements. Using a panel of soluble protein ligands with reduced affinity for the HEL-Ig receptor, the editing response was shown to correlate in a dose-dependent fashion with the strength of signaling through the B cell receptor. The finding that the level of B cell receptor cross-linking sufficient to induce anergy in B cells is also capable of engaging the machinery required for receptor editing suggests an intimate relationship between these two mechanisms in maintaining B cell tolerance. *The Journal of Immunology*, 2000, 165: 6796–6802.

Central tolerance in the B lineage is maintained by several distinct mechanisms. These include deletion, which purges from the repertoire B cells with highly self-reactive Ig receptors (1, 2), and induction of clonal anergy, which renders B cells nonresponsive to B cell receptor (BCR) \(^3\) cross-linking (3). In addition, it is now clear that central receptor editing, particularly at light chain loci, maintains tolerance by modifying the Ag receptor specificity of self-reactive cells through the initiation of new Ig light chain rearrangements (4, 5). The fate of individual autoreactive B cells depends on the strength of signaling through the BCR and the developmental stage at which BCR cross-linking by self-Ag occurs (6–9). For the most part, deletion, editing, and anergy have been considered independent mechanisms working to maintain B cell tolerance.

Our laboratory recently showed that transgenic (Tg) overexpression of the anti-apoptotic protein Bcl-x\(_L\) allowed self-reactive bone marrow (BM) B cells to escape central deletion in mice Tg for both a BCR recognizing hen egg lysozyme (HEL-Ig) and membrane-bound HEL (mHEL) (10). In the course of these studies, we noted that recombination-activating gene (Rag)-2 was strongly induced in BM B cells of HEL-Ig/mHEL animals in the absence of new \( \kappa \) light chain rearrangements. Since these mice showed nearly complete central deletion of B cells, this suggested an abortive attempt at receptor editing in response to extremely high levels of BCR cross-linking by membrane-bound self-Ag. We also found that bcl-x\(_L\) allowed HEL-Ig B cells to escape deletion in the mHEL environment. In the periphery, these “escaped” B cells were profoundly anergic in both in vitro and in vivo assays, and showed evidence of extensive receptor editing with up-regulated Rag-1 and Rag-2 mRNA expression and new endogenous \( \kappa \) rearrangements (10).

Based on these results, we were interested in further exploring the relationship between anergy induction and receptor editing. In anergic HEL-Ig/soluble HEL (sHEL) double Tg mice, the lysozyme self-Ag is present in the fluid phase and the level of Ig receptor cross-linking is significantly lower than in HEL-Ig/mHEL mice (6). The data presented here indicate that anergic B cells in HEL-Ig/sHEL mice indeed exhibit evidence of receptor editing, as determined by the detection of endogenous V-J-\( \kappa \) light chain rearrangements. In addition, new endogenous V-J-\( \kappa \) rearrangements could be induced in an IL-7-driven in vitro BM culture system following incubation of HEL-Ig B cell cultures with exogenous sHEL or several lysozyme variants with lower affinity for the HEL-Ig BCR. These data have interesting implications for our understanding of the nature of B cell tolerance induction in the BM.

Materials and Methods

**Mice**

MD4 HEL-Ig and ML5 sHEL Tg mice (3) were kindly provided by C. Goodnow (Australian National University, Canberra, Australia). Mice were genotyped by PCR of genomic DNA obtained from tail biopsy at the time of weaning. All animals were maintained in specific pathogen-free isolation at the University of Minnesota animal facility. Mice used for experiments were generally 4–8 wk of age.

**PCR and RT-PCR**

Genomic DNA and mRNA were isolated from fresh spleen and BM cells or IL-7 culture-derived cells as described previously (11). Endogenous V-J-\( \kappa \) rearrangements were detected using a semiquantitative PCR assay, essentially as previously described (10, 11). Control experiments determined that the number of cycles used for each PCR (28 for V-J-\( \kappa \) and 23 for CD14 loading control) were within the linear range of amplification. The V-J-\( \kappa \) PCR was performed with an upstream V\(_k\) degenerate primer: 5'-GGCTGCAG/C(G/T)TGAGTGCCAGTG/G(T/A)(T)-3' and a primer that annealed just downstream of J-\( \kappa \): 5'-GCAACAGCATAGA-CACGGGAAGAA-3' (12). Amplification conditions were as follows: genomic DNA (from 4 × 10\(^4\) cells) in 1× PCR buffer (Boehringer Mannheim, Indianapolis, IN), 2.5 mM MgCl\(_2\), 200 µM dNTPs, 200 ng V\(_k\) degenerate primer, 100 ng J-\( \kappa \) primer and 1.25 U Taq polymerase (Boehringer Mannheim), with cycling conditions: 97°C for 45 s, 70°C for 1 min, and 72°C for 2.5 min for 5 cycles, followed by 94°C for 45 s, 70°C for 1 min, and 72°C for 2.5 min for another 23 cycles, and final extension at 72°C for 6 min. PCR amplification of C\(_\kappa\) was as described elsewhere (13).

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3. Abbreviations used in this paper: BCR, B cell receptor; HEL, hen egg lysozyme; mHEL, membrane-bound HEL; sHEL, soluble HEL; BM, bone marrow; Rag, recombination-activating gene; DEL, duck egg lysozyme; MFI, mean fluorescence intensity.
CD14 control PCR amplifications were performed using the forward primer 5'-GCTCAAACTTTCAGAATCTACCGAC-3' in combination with a reverse primer, 5'-AGTCAATGCTGGAGGCGGAAATC-3' (14). Amplification conditions were similar to that of the V-J1 PCR except that the reactions contained 100 ng of each primer, products were amplified with AmpliTaq Gold polymerase (Perkin-Elmer, Norwalk, CT), the denaturing temperature was 66°C, and 23 total cycles were used. RT-PCR was performed using oligo(dT)-primed RT of total RNA, followed by PCR with Rag-2 and β-actin primers as described previously (10). PCR products were separated on 1% agarose gels, transferred to nitrocellulose, and visualized by autoradiography. Densitometry was performed using a Bio-Rad GS-700 Imaging Densitometer and Bio-Rad Molecular Analyst software (version 2.1; Bio-Rad, Richmond, CA). In some instances blots were also directly analyzed using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (version 4.1), with comparable results.

**Flow cytometry**

Splenocytes and in vitro cultured cells were stained in FACS buffer (1× PBS, 2.5% FCS, 0.1% sodium azide) with FITC-, PE- or Cy-Chrome-conjugated mAbs to B220 (RA3-6B2), IgM, IgG1, CD43, CD21, CD23, and isotype controls (all Abs from PharMingen, San Diego, CA). Three-color flow cytometry was performed as described (10) using a FACScalibur (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest (Becton Dickinson) or Flowjo (Treestar, San Carlos, CA) software.

**Results**

**Evidence for endogenous light chain rearrangements in anergic B cells in vivo**

To determine whether anergic B cells exhibit evidence for light chain editing in vivo, we prepared single-cell suspensions of spleen and BM from young (4- to 6-wk old) HEL-Ig and anergic HEL-Ig/sHEL mice, and isolated genomic DNA and total RNA. Since the HEL-Ig light chain is the product of the V-J κ rearrangement (16), we used degenerate Vκ framework primers along with 1 μCi of [3H]thymidine before harvest and scintillation counting. Cells from first-stage cultures were also harvested and washed, then recultured in complete media in the absence or presence of 10 ng/ml IL-4 (R&D Systems), 200 ng/ml HEL, or 10 μg/ml F(ab')2 goat anti-mouse IgM Abs (Jackson ImmunoResearch, West Grove, PA). [3H]Thymidine incorporation was measured for the last 16 h of a 2-day culture. Duck egg lysozyme was custom produced by Rockland Immunochemical (Gilbertsville, PA) and was >98% pure by SDS-PAGE. J558 cells engineered to secrete site-directed HEL mutants were kindly provided by F. Battista and M. Neuberger (London, U.K.) (15). Cells were grown in complete RPMI 1640 media, and recombinant proteins were purified from culture supernatants by ion exchange chromatography on CM-52 cellulose as described elsewhere (15). Protein purities were ~50% for HEL sincere and 25% for HELΔ456 by SDS-PAGE. Total protein was quantitated using the bicinchoninic acid protein assay (Pierce, Rockford, IL).
RT-PCR analysis demonstrated mice compared with HEL-Ig mice as determined by densitometry. BM but not the spleen of HEL-Ig/sHEL mice (Fig. 2), suggesting of V-J examined, there was an overall 11-fold increase in the abundance mice, with some enrichment in the spleen. In four pairs of animals both spleen and BM cells of anergic HEL-Ig/sHEL (Ig/sHEL) cell line; positive control (lane 6), C57BL/6 BM. Representative of three experiments.

FIGURE 2. Rag-2 is expressed in the BM of HEL-Ig/sHEL anergic mice. Total RNA was obtained from spleen (Spl) and BM of HEL-Ig/sHEL anergic mice. RT-PCR was used to amplify Rag-2 and β-actin cDNAs, and the PCR products were analyzed as described in the legend to Fig. 1. H2O control (lane 3); genomic DNA control (lane 4); negative control (lane 5), WEHI 231 mouse B cell line; positive control (lane 6), C57BL/6 BM. Representative of three experiments.

with a primer downstream of the Jκ1 gene segment in PCR of genomic DNA to detect endogenous V-Jκ1 joints (10, 12). Fig. 1, A and B, illustrates the control experiments that were performed to validate this semiquantitative assay.

As shown in Fig. 1C, spleen and BM cells of single Tg HEL-Ig (Ig) mice showed almost no detectable endogenous V-Jκ1 rearrangements, consistent with tight allelic exclusion by the Tg light chain. In contrast, V-Jκ1 rearrangements were easily detected in both spleen and BM cells of anergic HEL-Ig/sHEL (Ig/sHEL) mice, with some enrichment in the spleen. In four pairs of animals examined, there was an overall 11-fold increase in the abundance of V-Jκ1 rearrangements in the spleen of HEL-Ig/sHEL anergic mice compared with HEL-Ig mice as determined by densitometry. RT-PCR analysis demonstrated Rag-2 mRNA expression in the BM but not the spleen of HEL-Ig/sHEL mice (Fig. 2), suggesting that the endogenous κ rearrangements occurred centrally at the time when IgM receptors were first expressed on immature B cells. Rag-2 mRNA levels in the BM of HEL-Ig/sHEL mice were ~2-fold higher than in HEL-Ig BM (data not shown).

The total number of B220+ cells in the BM of HEL-Ig and HEL-Ig/sHEL mice was comparable (3.3 ± 0.2 × 10^6 vs 3.3 ± 0.9 × 10^6, respectively; n = 3), while there were approximately twice as many B220+ cells in HEL-Ig spleens compared with HEL-Ig/sHEL spleens (2.6 ± 0.6 × 10^7 vs 1.1 ± 0.3 × 10^7, respectively; n = 6). IgMα-positive, non-HEL-binding B cells (i.e., nontransgenic B cells) comprised <2% of the total cells found in either HEL-Ig or HEL-Ig/sHEL mice. Thus, the increases in V-Jκ1 rearrangements and Rag-2 expression observed in HEL-Ig/sHEL anergic mice were not due to the presence of an expanded population of endogenous nontransgenic B cells.

In vitro HEL-Ig BM B cell cultures

We next turned to an IL-7-driven in vitro BM culture system (17–20) to examine the response of immature HEL-Ig B cells to incubation with soluble self-Ag in vitro. Unfractionated BM cells from HEL-Ig mice were cultured for 5 days with recombinant IL-7 to expand the population of precursor B cells. At the end of this first-stage culture period, >80% of lymphoid-gated cells in the culture were B220+, indicating a selective outgrowth of B cells. Fig. 3 compares the cell surface phenotypes of splenocytes from HEL-Ig and HEL-Ig/sHEL mice (upper two rows) with those of HEL-Ig BM B cells generated in 5-day IL-7 cultures in the absence or presence of 200 ng/ml soluble HEL (lower two rows). Strikingly, HEL-Ig BM B cells cultured with HEL showed reduced surface IgMα expression similar to that observed on HEL-Ig/sHEL splenocytes (the “a” allotype is specific for the Tg IgM receptor). In this system, the levels of IgDα were significantly lower on the cultured cells than the splenic cells of either Ig or Ig/sHEL mice, consistent with an immature phenotype (IgMα+, IgDα[low–neg]) of the majority of cultured B cells. Additional evidence for an immature phenotype of the cultured cells included the persistence of the pro-B cell marker CD43 and low surface levels of the maturation markers CD23 and CD21 (Fig. 3). The levels of IgMα and IgDα expression correlated with HEL binding as determined by a flowcytometric HEL sandwich assay (3) (data not shown).

We noted approximately a 40% reduction in the number of viable cells recovered at day 4 in IL-7 cultures of HEL-Ig BM when incubated in the presence of HEL compared with cultures lacking the HEL Ag (Fig. 4A). Significant reductions in DNA synthesis were also observed at the end of the first-stage culture in the presence of self-Ag (Fig. 4B). These data were consistent with a modest Ag-mediated growth inhibition rather than Ag-induced death, since there were no consistent differences in the number of apoptotic B220+ cells as determined by 7-amino-actinomycin D staining, either in the presence or absence of self-Ag (data not shown).

FIGURE 3. Comparison of surface markers on cultured HEL-Ig BM B cells and splenic B cells from HEL-Ig and HEL-Ig/sHEL mice. Splenocytes from HEL-Ig (Ig) and HEL-Ig/sHEL (Ig/sHEL) mice were stained with Cy-Chrome-conjugated anti-B220 mAbs to identify B cells, along with FITC or PE-labeled mAbs recognizing IgMα, IgDα, CD43, CD23, or CD21, and analyzed by flow cytometry. In parallel, the cells from 5-day IL-7-driven HEL-Ig (Ig) BM cultures, grown in the presence or absence of soluble HEL (200 ng/ml), were similarly stained and analyzed. Results show lymphoid cells gated electronically by forward and side scatter. The dotted lines provide a reference to compare levels of staining between the various samples.

FIGURE 3. Comparison of surface markers on cultured HEL-Ig BM B cells and splenic B cells from HEL-Ig and HEL-Ig/sHEL mice. Splenocytes from HEL-Ig (Ig) and HEL-Ig/sHEL (Ig/sHEL) mice were stained with Cy-Chrome-conjugated anti-B220 mAbs to identify B cells, along with FITC or PE-labeled mAbs recognizing IgMα, IgDα, CD43, CD23, or CD21, and analyzed by flow cytometry. In parallel, the cells from 5-day IL-7-driven HEL-Ig (Ig) BM cultures, grown in the presence or absence of soluble HEL (200 ng/ml), were similarly stained and analyzed. Results show lymphoid cells gated electronically by forward and side scatter. The dotted lines provide a reference to compare levels of staining between the various samples.
Cells were next harvested at the end of the first-stage culture, washed, and recultured in the presence or absence of IL-4, soluble HEL, polyclonal anti-IgM Abs, or combinations of these reagents for another 2 days. In these second-stage cultures, cells incubated with HEL during the first stage showed reduced proliferation following stimulation compared with cells not exposed to Ag in the first-stage culture (Fig. 4C). These differences were less striking than the defects observed in anergic HEL-Ig/sHEL splenic B cells analyzed in parallel (Fig. 4C), likely due to residual IL-7 cytokine effects in the BM cells. Together, these results indicated that exposure of HEL-Ig BM cells to HEL in an IL-7-driven BM culture system leads to lowered levels of surface IgM associated with modestly reduced proliferative responses following subsequent BCR cross-linking.

**In vitro receptor editing in response to BCR cross-linking by HEL self-Ag**

We next tested whether HEL-Ig B cells in the BM culture system underwent endogenous light chain rearrangements in response to BCR ligation by the soluble self-Ag HEL. In first-stage IL-7 cultures, HEL-Ig B cells showed low background levels of endogenous V-Jκ1 rearrangements in the absence of Ag (Fig. 5, lanes 4 and 6), similar to background levels described previously in another Ig Tg system (12). Incubation of first-stage cultures with HEL (1 μg/ml) induced modest levels of new V-Jκ1 rearrangements (lanes 5 and 7). However, in second-stage cultures, following a wash to remove IL-7 and reculturing in the presence of HEL, there was a consistent and strong induction of endogenous V-Jκ1 rearrangements by HEL compared with cells cultured in the absence of HEL (compare lanes 8 and 12 with lanes 9 and 13, respectively). Background levels of Rag-2 mRNA were quite high in this in vitro system (see also Ref. 21), and the strong induction of new V-Jκ rearrangements in second-stage cultures was associated with only about a 2-fold induction of Rag-2 mRNA (data not shown).

**Receptor editing in response to low-affinity HEL ligands**

The data shown in Figs. 1C and 5 were consistent with the hypothesis that the level of receptor cross-linking sufficient to induce...
anergy in HEL-Ig B cells was also sufficient to induce editing. Thus, we next investigated the influence of lower affinity ligands for the HEL receptor on editing in the BM culture system. Two of the low-affinity ligands tested were site-directed mutants of HEL (15). The first HEL mutant, R21D101 (HEL안드), contains two alanine substitutions in the region of lysozyme that binds to the HyHEL10 receptor of HEL-Ig B cells. HEL안드 has an overall 12-fold reduced affinity compared with HEL, as determined by BiaCore measurements (15). The second HEL mutant, R21D101G102N103 (HEL안드전), has four alanine substitutions and a 100-fold lower affinity for the HEL-Ig receptor than HEL (15). We also tested duck egg lysozyme (DEL) with an ~3500-fold reduced affinity (22).

We first tested the influence of the various Ags to modulate HEL-specific surface IgM* levels in first-stage BM cultures. As shown in Fig. 6A, the ability of each of the ligands to down-regulate IgM* levels correlated with their affinity for the anti-HEL receptor, with HEL showing the strongest down-regulation of IgM levels and DEL the least. These results were consistent with previous studies in the HEL system, which have shown that the level of IgM* down-regulation is proportional to the strength of BCR signaling (23).

Next, we explored the ability of the various lysozyme molecules to induce editing. HEL-Ig BM cells were incubated with IL-7 in either media alone, or with HEL, HEL안드, HEL안드전, or DEL at 100, 500, or 2500 ng/ml for 5 days. At the end of the first-stage culture, cells were harvested for determination of V-J1 rearrangements (Fig. 6B). Significantly, the levels of V-J1 rearrangements observed were generally proportional to the affinity and concentration of the various Ags tested. At the higher concentrations, the various soluble ligands induced stronger levels of endogenous rearrangements, and even DEL, a low-affinity ligand for the HyHEL10 receptor, could induce weak editing.

In second-stage cultures, the induction of editing showed less of a dose response, particularly with the higher affinity ligands HEL and HEL안드 (Fig. 6C, lanes 2–7). It appeared that cells in the second-stage cultures following withdrawal of IL-7 were somewhat “primed” to edit, compared with first-stage cultures. This was clearly observed following Ag challenge (e.g., compare lanes 2–4 of Fig. 6, B and C), and was also seen in control cultures in the absence of Ag (lane 1 of Fig. 6, B and C). A similar brisk induction of editing after IL-7 withdrawal was also noted in in vitro 3-83 Tg B cell cultures following BCR crossing (12, 20).

Finally, we analyzed cells from each of the first-stage culture conditions shown in Fig. 6B by flow cytometry to determine the relative level of V-J1 rearrangements and IgM* MFI in first-stage cultured cells incubated with the various lysozyme molecules. The data from three independent first-stage cultures (including the experiment shown in B) were analyzed by densitometry to determine the relative level of V-J1 rearrangements compared with control CD14 amplifications. An aliquot of each of the cultures was also analyzed by flow cytometry at day 5 for IgM* MFI (gated on B220* cells). Each data point represents the average of three independent determinations of IgM* MFI and V-J1ICD14 densitometric values for each of the 13 culture conditions shown in B. The control data point (media alone, lane 1) was arbitrarily assigned a V-J1ICD14 densitometric value of 1.0.
extent of IgM down-regulation. Plotting of the mean fluorescence intensity (MFI) of IgM levels for the various first-stage cultures against the densitometric determination of V-Jk1 rearrangements normalized to CD14 showed an interesting relationship between these two variables (Fig. 6D; each data point represents the average of three separate experiments). Cells receiving the lowest levels of BCR signaling (high IgM MFI) showed the lowest levels of editing (Fig. 6D, bottom right portion of the graph), whereas cells receiving the strongest levels of signaling (low IgM MFI) demonstrated the highest levels of editing (Fig. 6D, upper left portion of the graph). Thus, the magnitude of the editing response in this in vitro system was correlated with BCR signaling strength.

Discussion

The data presented here provide new insights into the relationship between anergy and receptor editing in maintaining central tolerance. The key findings of this report are as follows: 1) Anergic HEL-Ig/sHEL B cells show evidence for central Ig light chain receptor editing in vivo, as demonstrated by elevated Rag-2 gene expression in the BM along with new endogenous V-Jk1 rearrangements. 2) Some of the features typical of HEL-Ig/sHEL anergic B cells were reproduced in immature HEL-Ig/sHEL B cells incubated with soluble HEL in an IL-7-driven in vitro BM culture system, including down-regulation of surface IgM levels, reduced proliferation following BCR cross-linking, and induction of endogenous light chain rearrangements. 3) Lowered affinity ligands for the HEL receptor were also found to be capable of inducing endogenous κ editing responses in vitro, with the level of editing observed generally correlated with the strength of signaling through the BCR.

One conclusion from these data is that the level of BCR cross-linking sufficient to induce anergy, as classically defined in HEL-Ig/sHEL B cells, is also sufficient to induce new endogenous light chain rearrangements. In this system, the detection of V-Jk1 rearrangements in HEL-Ig Tg B cells really serves only as a marker for the receptor editing response. This is because in this Tg system “genuine” light chain receptor editing, defined as editing that leads to a change in the heavy and/or light chain composition of the BCR, does not occur. The HEL-Ig animals used in these experiments are conventional Tg mice, where the heavy and light chain Tg cassettes are present at multiple copies and are co-integrated in the genome, but not at the normal endogenous heavy and light chain loci (3). Because of this, induction of new rearrangements at the endogenous κ loci in HEL-Ig Tg B cells fails to remove or inactivate the autoreactive V-Jk gene segment, like that which occurs in normal B cells through nested V-Jk rearrangements (24) or RS-type recombinations (25). Thus, receptor editing in this system can be considered a form of “frustrated” editing. The coexistence of both anergy and editing in HEL-Ig/sHEL B cells indicates that these cells are attempting to edit away from “anergic” levels of self-reactivity. Thus, anergy is one potential outcome for B cells that fail to productively edit.

Another conclusion from this work is that the level of BCR cross-linking sufficient to induce editing occurs over a broad range of BCR signaling strengths. An advantage of the lysozyme Tg system is that the level of surface IgM expressed on HEL-Ig B cells provides a reliable readout for the level of BCR signaling strength experienced by the cells (23). Our data indicate that the level of IgM found on HEL-Ig/sHEL anergic splenic B cells was approximately equivalent to the IgM levels observed on in vitro cultured BM B cells grown in the presence of 200 ng/ml HEL (Fig. 3). We also found that editing was induced in the in vitro system after exposure to very low-affinity soluble ligands for the HEL receptor, most notably by DEL with ~3500-fold lower affinity than HEL. Editing in response to low-affinity ligands was previously reported in the 3-83 anti-class I MHC Tg system (26); however, because the class I molecules with low affinity for the 3-83 BCR were membrane bound and present at very high concentrations, it was difficult to estimate the true signaling strength experienced by the B cells. Taken together, these studies suggest that the BCR signaling threshold for B cell editing in immature cells may indeed be quite low. Furthermore, these findings suggest that developing B cells are likely to attempt to edit away from levels of self-reactivity that are far below the upper threshold for anergy induction.

Although receptor editing in the 3-83 anti-class I MHC Tg system was associated with increased expression of λ light chains in vivo (5), we have never observed elevated levels of λ (defined as >1% of B220− cells also positive for λ) in vivo, either on anergic HEL-Ig/sHEL B cells (data not shown) or previously on anergic HEL-Ig/hel-xj double Tg B cells escaping deletion in mHEL mice (10). Only when HEL-Ig B cells generated in the in vitro system were incubated with very high concentrations of HEL (e.g., 50 μg/ml) or received strong cross-linking from anti-IgM Abs have we observed significant λ staining (up to 3 or 4% of B220− cells, data not shown). Even under these extreme in vitro conditions, the cell populations did not show a detectable loss of HEL binding as determined by an HEL-specific sandwich assay (data not shown). The relative resistance of HEL-Ig Tg B cells to edit away from HEL specificity is probably explained by the fact that the HEL-Ig transgenes are conventional and randomly integrated in the genome and not subject to normal excision by deletion. In addition, it is possible that the highly somatically mutated anti-HEL heavy chain either does not pair well with endogenous κ or λ light chains, or that endogenous light chains do not compete well with the Tg κ chain for pairing with the Tg heavy chain.

Our finding of elevated levels of endogenous V-Jk1 rearrangements in anergic HEL-Ig/sHEL B cells is supported by gene-expression array analysis, which documented elevated levels of V-Jk1 transcripts in anergic double Tg B cells compared with naive B cells (27). These results, however, contrast with a recent paper by Kraus et al. (28), who found no evidence for κ light chain editing in anergic B cells compared with HEL-Ig B cells using a single-cell analysis. It is likely that at least two factors are contributing to this difference. The first is that Kraus et al. (28) performed their single-cell analysis on sorted BM cells, whereas we have consistently found higher levels of rearrangements in splenic B cells. Furthermore, the PCR strategy they used detected all of the potential V-Jk joints, including the Tg V-Jk2 rearrangement, using a degenerate Vκ primer and a primer downstream of Jk5. In their strategy, V-Jk1 was the longest of the PCR products to be amplified, and it had to compete with the Tg V-Jk2 rearrangement. Since V-Jk1 is the preferred Jk segment for de novo κ rearrangements (29, 30) and the data shown by Kraus et al. (28) did not identify a single V-Jk1 product, we conclude that their assay may have missed the Jk1 rearrangements in HEL-Ig/sHEL mice that we were able to detect using a V-Jk1-specific strategy in unfractionated BM and spleen. Definitive resolution of this issue would likely require a single-cell PCR analysis along with Southern blot detection of V-Jk1 joints.

Another question that arises from these data concerns the identity of the cell population that responds to BCR cross-linking with an editing response in vitro. Specifically, is this a broad response across the population of cells, or does this represent a selective response of an activated subpopulation? Our data at present are most consistent with the notion that the editing observed represents a broad response across the population. This is supported by the
proliferation data shown in Fig. 4, where incubation with Ag resulted in lower, not higher, levels of proliferation than cultures not containing Ag. In addition, in recent experiments we have labeled cells with CFSE at the end of first-stage cultures and then followed the fate of these cells in second-stage cultures. After 2 days, there were no significant differences detected in CFSE staining between second-stage B cells cultured in the absence or presence of HEL Ag. Thus, we find no evidence for the selective outgrowth of a subpopulation in response to incubation with HEL.

The data presented here are consistent with the hypothesis that the editing response is regulated primarily by BCR signaling strength. In one respect, this is not particularly surprising, given the critical role that BCR signaling has at virtually every stage in B cell development. Currently, there are a number of important and unresolved issues regarding central receptor editing. These include the nature of the signaling pathways downstream of the BCR that regulate editing, the molecular mechanism by which editing is confined to a specific developmental stage in BM B cell development, and the mechanism by which light chain allelic exclusion is maintained during editing. The in vitro system described here, where the degree of BCR signaling and receptor editing can be modulated using soluble ligands, may prove useful in addressing some of these issues.

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