B Cells Are Exquisitely Sensitive to Central Tolerance and Receptor Editing Induced by Ultralow Affinity, Membrane-bound Antigen

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Summary

To assess the sensitivity of B cell tolerance with respect to receptor/autoantigen affinity, we identified low affinity ligands to the 3-83 (anti-major histocompatibility complex class I) antibody and tested the ability of these ligands to induce central and peripheral tolerance in 3-83 transgenic mice. Several class I protein alloforms, including Kbm3 and Dk, showed remarkably low, but detectable, affinity to 3-83. The 3-83 antibody bound Kb with $K_a$ ~2 × 10^5 M$^{-1}$ and bound 10-fold more weakly to the Kbm3 ($K_a$ ~2 × 10^4 M$^{-1}$) and Dk antigens. Breeding 3-83 immunoglobulin transgenic mice with mice expressing these ultralow affinity Kbm3 and Dk ligands resulted in virtually complete deletion of the autoreactive B cells from the peripheral lymphoid tissues. These low affinity antigens also induced receptor editing, as measured by elevated RAG mRNA levels in the bone marrow and excess levels of $id^-$ variant B cells bearing $\lambda$ light chains in the spleen. Reactive class I antigens were also able to induce deletion of mature B cells when injected into the peritoneal cavity of 3-83 transgenic mice. Although the highest affinity ligand, Kk, was consistently able to induce elimination of the 3-83 peritoneal B cells, the lower affinity ligands were only partially effective. These results demonstrate the remarkable sensitivity of the deletion and receptor-editing mechanisms in immature B cells, and may suggest a higher affinity threshold for deletion of peripheral, mature B cells.

Self/nonself discrimination is initiated by antigen receptor engagement, but, unlike other receptor/ligand systems, the antigen receptors do not have predefined ligands. It is therefore important to understand how antigen form, affinity, and avidity affect activation and subsequent biological consequences, including tolerance. In B cells, multiple tolerance mechanisms appear to work together to maintain control of autoreactivity (reviewed in 1–6). These mechanisms, including central deletion (7–11), receptor editing (12–16), energy (17–19), follicular exclusion (19, 20), peripheral deletion of resting B cells (21–26), and antigen- and Fas-mediated death of germinal center B cells (27–30), have been described. Importantly, the affinity and avidity of the antigen/B cell receptor (BCR)$^1$ interaction may determine extent of tolerance induction, as well as tolerance phenotype (1, 31–36).

Tolerance susceptibility and mechanisms are also affected by the developmental stage at which the autoreactive B cell encounters antigen. In adult mice, the initial antigen-specific negative selection step in B cell development occurs in the bone marrow through a process of developmental arrest (37) associated with ongoing light chain gene rearrangement that can alter BCR specificity, a process referred to as receptor editing (12–16). B cells encountering self-antigen later in development can be controlled by a number of other mechanisms, all of which accelerate B cell death (21–30).

To evaluate the relative significance of the various B cell tolerance mechanisms in controlling autoreactive B cells, it is important to determine the sensitivity of these mechanisms to BCR/antigen affinity. Here, we test the hypothesis that cell-surface antigens with very low affinity for BCR might fail to mediate central or peripheral tolerance.

Materials and Methods

Mice. The 3-83 μ/δ transgenic mice (21), which express IgM and IgD forms of the 3-83 antibody, were bred and maintained in the animal care facility at the National Jewish Center for Immunology and Respiratory Medicine (NJCRIM). 3-83 μ/δ mice were backcrossed a minimum of 10 times onto a B10.D2 back-
ground and subsequently bred one generation with C57Bl/6 J-H-2bm3/EgAoEg (H-2bm3), C57Bl/6bm6 (H-2bm6), C57Bl/6 Kb- H-2bm8/KHeG (H-2bm8), B6.C-H-2bm11/KHeG (H-2bm11), C3H- H-2b/KSn (C3H.H-2b/KSn), (CD1)F1, B10.M/Sn (H-2a), B10.BR/Sp- SnJ (H-2b), C57Bl/6 (H-2b), and B10.D2/nSnJ (H-2a) mice (all purchased from Jackson Laboratory, Bar Harbor, ME). The F1 mice were directly analyzed. Initial screening for low affinity ligands was performed on H-2bm1/h-h-h-h-h, mice, kindly provided by Dr. Roger Melvold (Northwestern University Medical School, Chicago, IL). β2-microglobulin-deficient mice were a generous gift from Beverly Koller (University of North Carolina, Chapel Hill, NC) (38). These mice were crossed with B10.D2 3-83 transgenics. The F1 mice were interbred to obtain 3-83 µg/ transgenic, β2-microglobulin-/- mice on a H-2b homozygous background. These resulting mice were analyzed as negative controls.

Cell Lines. Sp2/0-derived H-2b and H-2a mutant cell lines (bm3, bm5, bm8, and bm11) were a generous gift from Janko Nikolic-Zugic and M.J. Bevan (formerly at The Scripps Research Institute, La Jolla, CA) (39). The 4549 (H-2a) cell line was obtained from W. Ysosski (NJCTRIM). The Sp2/0 (H-2b) cell line (40) was purchased from American Type Tissue Type Collection (Rockville, MD).

Antibodies and Immunofluorescence. Lymphoid cells were prepared as described previously (7), and were stained with the following mAbs: anti-3-83 clonotype, 54.1/biotin (7), anti-B220/ELITE (Coulter Electronics Inc., Hialeah, FL). Purification and polyclonal antibodies: rat anti-mouse IgM, LO-MM-9/FITC (47), (Zymed Laboratories, South San Francisco, CA); and goat anti-κ/FITC, RA3-3A1 (41); anti-IgM/biotin, Ak2, 33.24.12 (42); mouse IgD ~, AMS 9.1/biotin (45), (Pharmingen, San Diego, CA); and anti-μ/biotin, Y3 (43) and 3-83P (44); anti-λ/biotin, AM56 (46) (Pharmingen, San Diego, CA); and anti-μ/biotin, Y11.2.6.14 (47), (Zymed Laboratories, South San Francisco, CA); and goat anti-κ/FITC, (Fish Scientific, Pittsburgh, PA). Biotin-conjugated antibodies were revealed with PE-streptavidin (Becton Dickinson & Co, Mountain View, CA). Samples were analyzed on a FACScan® (Becton Dickinson), FACS® Profile, or Profile ELITE (Coulter Electronics Inc., Hialeah, FL). Purification and fluorochrome conjugation of the antibodies were performed as described previously (48). Except for the cell-staining binding assays, histogram analysis data were gated by side scatter to include the predominant lymphocyte population.

Class I Molecules. Soluble Kb and Kb3 were purified as described previously (7). Briefly, Drosophila S2/M3 cells were cotransfected with a soluble form of H-2Kb or Kb3 cDNA and a full-length cDNA encoding murine β2m. Stable transfectants grown in serum-free medium were induced by cupric sulfate (0.7 mM). Culture supernatants were collected by centrifugation and concentrated. Soluble Kb was purified on an anti-Kb, bm3 (Y3) affinity column and the His-tagged Kb3 was purified on a nickel column, both of which were followed by ion-exchange chromatography. Approximate yields were 0.5 mg H-2Kb/liter of supernatant. The Kb and Kb3 were concentrated, dialyzed against PBS, filter sterilized, and stored at 4°C.

Fab Preparation. Fab fragments of the 3-83 antibody were prepared using the ImmunoPure Fab Preparation Kit (Pierce Chemical Co., Rockford, IL). Fab preparations were verified on reducing and nonreducing SDS–polyacylamide gels. Specificity of Fab fragments was verified upon biotinylation of the Fab fragments, followed by staining of H-2 K cells and flow cytometry analysis as described above.

Affinity Measurements. Kinetic measurements were made with surface plasmon resonance apparatus (BIAcore; Pharmacia, Piscataway, NJ). Proteins to be coupled to the sensor chip were concentrated using Centricon-10 microfuge tubes (W.R. Grace & Co., Beverly, MA), washed twice, and resuspended in 10 mM Na-acetate, pH 5.0, to a concentration of ~50 µg/ml for soluble Kb, and 20 µg/ml for all antibodies. The Kb3 molecules were resuspended in 10 mM morpholino propane sulfonic acid buffer, pH 6.0, to a concentration of 20 µg/ml. Ligands were covalently bound to the sensor chips using the BIAcore® Amine Coupling Kit (Pharmacia Biosensor), according to the manufacturer's instructions. The amount of protein bound to the CM5 sensor chips was as follows: chip 1: Kp (3421 resonance units [RU]); chip 2: Y3 (6854 RU), 3-83 (7236 RU), and Hopc-1 (50) (purchased from American Type Culture Collection; 6123 RU); chip 3: 54.1 (3671 RU), Kp (2821 RU), and 33.18.12 (42) (anti-mouse κ; 2458 RU); chip 4: K33.18.12 (3246 RU), Kb3 (3864 RU), and 54.1 (2188 RU); chip 5: 3-83 (6667 RU) and Hopc-1 (5364 RU). Analyte solutions were diluted in running buffer (PBS, 1 mM EDTA, 0.05% P20 BIAcoreTM or 1:100 Triton-X 100 surfactant) to the following concentration ranges representing two-fold dilutions: run 1 (chip 1): 3-83 (25–800 nM) over Kp; run 2 (chip 2): soluble Kb over Y3 (62–500 nM), 3-83 (62–1,000 nM), and Hopc-1 (62–1,000 nM); run 3 (chip 3): 3-83 IgG (25–800 nM), 3-83 Fab (250–8,000 nM), Y3 IgG (62–200 nM), and Hopc-1 (25–800 nM) over Kp; run 4 (chip 4): soluble Kb3 (39–625 nM), Kb3 (125–500 nM) over Y3 and Hopc-1; run 5 (chip 5): soluble Kp (32–4,140 nM) and K33.18.12 (143–9,140 nM) over 3-83 and Hopc-1; run 6 (chip 4): 3-83 IgG (25–800 nM) over 54.1; and run 7 (chip 4): 3-83 IgG (59–1,875 nM), 3-83 Fab (96–24,450 nM), and Hopc-1 (109–3500 nM) over K33.18.12. Since initial experiments showed a probability of aggregated antibodies, the antibody preps were spun at 100,000 g for 45 min immediately before dilution and passage over the Kb3 bound sensor chips. Each analyte preparation was injected over the covalently bound ligand on the BIASensor chip at a flow rate of 15–25 µl/min for 5–60 min. The analyte was allowed to dissociate with buffer for adequate time to remove analyte from the chip (5–60 min). For binding of IgG Y3 antibody to Kp, 3-83 IgG to 54.1, and 3-83 Fab to 33.18.12, the dissociation rate was too slow to completely remove all analyte. For this reason, kinetic data was limited to the first injection only. The experiments with soluble Kp were performed on the BIAcore 1000, which allows passage of analyte over a single flowcell (single protein bound to chip), while the Kb3 experiments used the BIAcore 2000, which allows flow of analyte over all four flowcells on a single chip in series. The BIAcore 2000 has the added advantage of lower signal/noise ratio and higher sensitivity, measuring affinities as low as 10 M–1, while the BIAcore 1000 has limitations in the 10 M–1 range.

Data sensograms (showing relative RU changes caused by injection, buffer changes, and binding of ligand) were analyzed to calculate association and dissociation rate constants using the BIAcore Evaluation 2.0 program and time points relevant to the association and dissociation phases, excluding the first few seconds of each phase from analysis to avoid buffer and mass transport effects. The dissociation rate constant (kd) was determined initially by the AB = A + B regression model to fit slope of ln(R0/R) vs. time plot. Because of steepness of the slope and the short length of dissociation period, this method proved inaccurate for instances of very fast off rates. Therefore, the half-life of bound protein was determined by subtracting the initial time of dissociation phase from the time at which half of the bound protein was dissociated. The amount of protein bound was calculated as the maximal RU of sensogram over experimental chip minus maximal RU of same analyte over a blank or irrelevant protein chip. The kd was then calculated as 0.693/t_{1/2} (s) and averaged over several concent-

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trations. For each experiment, the association rate constant \( (k_a) \) was calculated by both the \( A + B = AB \) type 1 and 4 options of the BIAEvaluation program. The first model is a regression analysis for first-order kinetic reactions and assumes a known \( k_p \). The data from this analysis showed large variations, with the \( k_i \) value decreasing with concentration. The second method, referred to as the \( k_i \) vs. concentration method, does not assume \( k_p \) values, and calculates \( k_i \) based on several concentrations of analyte. For each concentration of analyte, the program determines the slope \( (k_i) \) of \( \frac{d[R]}{dt} \) vs. \( R \). The association constant was then calculated as the negative slope of \( l_q \) vs. concentration. The \( K_a \) value determined by the \( k_i \) vs. concentration method corresponded well with the \( K_i \) calculated by the regression analysis for the highest analyte concentration. BIACore kinetic analysis is considered most accurate in cases where the least-free ligand is available for rebinding. This occurs at high concentrations of analyte. Because of the very low affinity binding, the data were difficult to interpret in some experiments. The \( k_i \) vs. concentration method did not always generate a straight line, and in these cases, analysis for \( k_i \) was restricted to the regression analysis. Also in instances where analyte dissociated very slowly, analyses of both \( k_i \) and \( k_p \) were restricted to the regression models for the first concentration of analyte tested. The equilibrium association constant \( (K_a) \) was calculated as the dividend of \( k_i \) over \( k_p \).

Detection of mRNA. The levels of \( G_{\alpha}, \) RAG-1, and RAG-2 mRNA expression in the bone marrow of PI mice were detected using reverse transcription-PCR and Southern analysis, and the quantitation of radioactive signals was performed as described previously (13).

Intraperitoneal Injection Assays. Sp2/0 hybridomas derived from bm mutant spleen cells (39), in addition to Sp2/0 (H-2b) antigen-negative control cells, were washed twice in sterile PBS and resuspended at \( 5.0 \times 10^7 \) cells/ml. Using 26-gauge needles, \( 100 \mu l/mouse \) of hybridoma cells were injected into the peritoneums of age-matched, nondeleting (H-2b background) 3-83 hemizygous transgenic mice. One mouse from each group received 100 \( \mu l \) of PBS alone. Approximately 16 h later, the mice were killed and the peritoneal cells were removed by flushing with 10 ml of either HBSS or PBS. In some experiments, the lymph nodes, spleen, and/or bone marrow were also analyzed. The peritoneal cells were centrifuged, treated with either Gey’s solution or buffered ammonium chloride to remove red blood cells, washed twice in staining buffer, counted, and stained. Samples were analyzed on either the Profile or ELITE flow cytometers (Coulter Electronics). To exclude the larger, injected tumor cells, detection of idiotype-positive B cells was limited to the lymphocyte population, as defined by forward and side scatter. Detection of class I molecules by Y3 antibodies was analyzed for both the small lymphocytic and larger, tumor-containing myeloid populations.

Results

3-83 Antibody Cross-reacts Weakly to K\textsuperscript{b} and Natural Mutations in the K\textsuperscript{b} Molecule Further Lower the Relative Binding Affinity. The 3-83 antibody was raised in a BALB/c (H-2\textsuperscript{b}) mouse by immunization with C3H (H-2\textsuperscript{b}) spleen cells, and has specificity for K\textsuperscript{b}, but also binds very weakly to D\textsuperscript{b} and to other allotypes of H-2\textsuperscript{b} (44). Relative rankings of 3-83 affinities to various class I molecules were determined by flow cytometry (Fig. 1). Cross-reactive binding of 3-83 to K\textsuperscript{b} cells required far higher concentrations of antibody relative to that required for comparable binding to K\textsuperscript{a} cells (reference 44 and Fig. 1 A). Despite the relatively low affinity for K\textsuperscript{b}, 3-83 \( \mu g \) Ig transgenic mice bred onto a H-2\textsuperscript{b} background completely deleted the autoreactive B cells from secondary lymphoid organs (13, 21, 34), indicating that this weak binding is physiologically relevant.

Natural mutants of the K\textsuperscript{b} molecule, the so-called “bm” series (52), had further reduced affinity to the 3-83 antibody, relative to K\textsuperscript{b}. Spleen and lymph node cells of H-2bm\textsuperscript{1,3,4,8,9,10,11} strains were analyzed directly for relative binding to 3-83 in complement-mediated cytotoxicity and flow cytometry assays. All target cells tested, except bm1, had lower binding to 3-83 than to the natural K\textsuperscript{b} molecule (not shown). Hybridomas derived from bm mutant spleen cells (39) were stained with 3-83 (Fig. 1 A) or the mAb Y3 (Fig. 1 B), which recognizes the same epitope on H-2K\textsuperscript{a} as that seen by 3-83 (53). All antigen-bearing cells showed similar levels of class I expression, as detected by Y3 binding. Mutations in K\textsuperscript{bm3} reduced 3-83 binding (Fig. 1 A), but not Y3 binding (Fig. 1 B). Staining of lymphocytes from mice homozygous at the H-2 locus for the K\textsuperscript{b} mutations confirmed these results (Fig. 1, C and D). When reacted with 3-83 antibody, K\textsuperscript{bm3} was bound less well than K\textsuperscript{b}, whereas both were bound similarly by the Y3 antibody. The reactivity of 3-83 to the D\textsuperscript{b} molecule was also much weaker than its cross-reactivity to the K\textsuperscript{b} molecule. When the data are corrected for nonspecific binding, it is apparent that the very low affinity ligands K\textsuperscript{bm3} and D\textsuperscript{b} required \( >10 \)-fold higher concentrations of 3-83 to achieve a level of binding comparable to that observed with K\textsuperscript{b} cells (Fig. 1, A and D). Thus, flow cytometry was useful in identifying and ranking the relative binding strength of ligands that were very weakly reactive to the 3-83 antibody.

The 3-83 Antibody Binds the Intermediate Affinity Ligand K\textsuperscript{b} with an Association Constant in the \( 10^7 \text{ M}^{-1} \) Range. Using the technique of surface plasmon resonance (54, 55), the equilibrium association constant \( (K_a) \) for the interaction between K\textsuperscript{b} and 3-83 antibody was measured (Table I). Appropriate control antibodies were studied in parallel to assure the specificity of the interaction and the activity of the antibodies. 3-83 F\textsubscript{ab} binding to immobilized K\textsuperscript{b} demonstrated a very fast off rate \( (t_{1/2} \sim 7.4 \text{ s}) \) largely responsible for the low \( K_a \) value of \( 1.7 \times 10^5 \text{ M}^{-1} \). Soluble K\textsuperscript{b} binding to immobilized 3-83 was consistent with this result, yielding a \( K_a \) value of \( 3.3 \times 10^5 \text{ M}^{-1} \). The bivalent, IgG form of 3-83 bound to immobilized K\textsuperscript{b} \( (K_a \sim 5.5 \times 10^6 \text{ M}^{-1}) \) with only \( 10 \)-fold higher affinity than did F\textsubscript{ab} 3-83, whereas bivalency improved the binding of the Y3 antibody by \( >500 \)-fold (Table I). Consistent with the binding studies (Fig. 1), the monovalent affinity of 3-83 for the putative very low affinity ligand K\textsuperscript{bm3} was \( \sim 10 \)-fold lower: \( K_a \sim 2 \times 10^5 \text{ M}^{-1} \), and the bivalent affinity was \( K_a \sim 5 \times 10^5 \text{ M}^{-1} \). This binding appeared to be peptide independent, since K\textsuperscript{bm3} molecules were bound with similar affinities whether or not the groove-binding peptide was present. We refer to these weak interactions as ultralow affinities.
**Table 1.** Measurement of Equilibrium Association Constants for Anti-class I Antibodies Using Real-time Kinetic Analysis with BIAcore

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>Valency</th>
<th>Concentration</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$R^2$</th>
<th>Half-life</th>
<th>SD $k_d$ (s$^{-1}$)</th>
<th>SD</th>
<th>$K_a$(M$^{-1}$)</th>
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<td>$K^b$</td>
<td>Y3 IgG</td>
<td>Bi</td>
<td>62 nM</td>
<td>4.11E + 05</td>
<td>4.21*</td>
<td>&gt;3,600</td>
<td>2.43E-04</td>
<td>4.77*</td>
<td>1.7E + 09</td>
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<td>3-83 IgG</td>
<td>Bi</td>
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<td>2.90E + 04</td>
<td>0.991</td>
<td>135.3</td>
<td>5.29E-03</td>
<td>1.09E-03</td>
<td>5.5E + 06</td>
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<td>1,875 nM</td>
<td>1.42E + 04</td>
<td>0.999</td>
<td>26.5</td>
<td>2.63E-02</td>
<td>1.61E-03</td>
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<td>0.996</td>
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*The dissociation rate was very slow disabling regeneration of the chip. Kinetic analysis is limited to one concentration of analyte. The statistical analysis is the $\chi^2$ value for the regression analysis of the on and off rates.

The $K_a$ vs. concentration plot did not generate a linear slope, therefore the $A + B = AB$ regression model was used to calculate an on rate. The statistical analysis is the $\chi^2$ value for the regression analysis of the highest concentration measured.

Association rate analysis for this experiment was uninterpretable, therefore the on rate for the $K^{bm3-yv}$ experiment was assumed to be comparable. As described in Materials and Methods, the BIAcore was used to measure association constants for divalent and monovalent binding of Y3 and 3-83 antibodies to soluble $K^b$ and $K^{bm3}$ with and without peptide. The ligand refers to the protein bound to the sensor chip and the analyte the protein passed over the chip. The method for association constant calculation is described in Materials and Methods. The $R^2$ and SD columns represent statistical analysis for $k_a$ and $k_d$ calculations, respectively. The concentration column shows the highest concentration of analyte evaluated. Several rungs of two fold dilutions from this concentration were also evaluated. The irrelevant IgG2a Hopc-1 antibody was used to control for specificity.

Binding of the 3-83 antibody to its anticlonotype 54.1 antibody was included as a positive control. Blank cells of the sensor chip were also used as negative controls.

Immature B Cells Bearing Ultralow Affinity Autoimmune Receptors for Membrane-bound Self-antigens Are “Deleted” from Peripheral Lymph Organs. To examine the effect of the ultralow affinity ligands on B cell tolerance, 3-83 1*/8 transgenic mice were bred with mice that expressed these weakly reactive class I haplotypes (Fig. 2). Transgenic mice bred on the non-3-83-reactive H-2a or H-2f backgrounds had a nearly monoclonal population of 3-83 idiotype-positive B cells in the peripheral lymph organs, as expected, whereas idiotype-positive B cells were not found in the lymph nodes or spleens of $F_1$ transgenic mice heterozygous for $K^{b,b,bm3,6,8,11}$ or $D^k$ alleles, regardless of the affinity for the 3-83 antibody (Fig. 2 and Table 2). Virtually no B220-positive, IgM-negative cells were present (Table 2), arguing against the possibility that the autoreactive B cells had downregulated surface Ig receptor expression. These data demonstrate that even ultralow affinity, membrane-bound self-antigens are capable of “deleting” autoreactive B cells from peripheral lymph organs.

Ultralow Affinity Ligands to Autoreactive Ig Receptors Are Capable of Mediating Receptor Editing in Immature B Cells. To determine the mechanism by which 3-83-bearing B cells were absent from the spleens of $F_1$ transgenic mice that expressed ultralow affinity ligands, we used two inde-
Figure 1. Relative binding of 3-83 antibody to various class I molecules in a flow cytometry assay. Binding of (A) 3-83 and (B) Y3 anti-class I antibodies to H-2 disparate, Sp2/0-derived hybridomas, or (C) binding of 3-83 antibody to spleen cells from MHC disparate mice. (D) Expands the lower range of the ordinate of C to differentiate poorly bound class I antigens. The data shown are representative of four independent experiments.

Independent assays based on previous work (13) to measure the extent of receptor editing. Lymphocytes from 3-83 transgene-positive, H-2 heterozygous mice were double stained with antibodies specific for the IgD transgene-encoded heavy chain and λ light chain. This assay identifies transgene-bearing B cells that have undergone endogenous light chain gene rearrangements because the endogenous heavy chain antibodies are of the b allotype and the 3-83 transgenic light chain is k. In transgenic mice of the H-2d or H-2d (nondeleting) background, the vast majority of B cells expressed the IgDk allotype (Fig. 3 A and summarized in Fig. 3 B), indicating expression of the transgenic heavy chain. These cells were not λ-positive, and anticlonotype staining indicated that the transgenic heavy chain paired with the 3-83 λ light chain. But in the presence of autoantigen-expressing bone marrow, regardless of the antigen affinities examined, the percentage of cells double-positive for the transgenic heavy chain and λ light chain was significantly increased (Fig. 3). Because the total spleen cell numbers among H-2 disparate mice were comparable (data not shown), the increased percentage of idiotype-negative B cells in the presence of antigen represented an increase in the absolute number of cells bearing these altered receptors. This increase in IgDk, λ double-positive cells was not observed in 3-83 mice expressing a Kb transgene only in the periphery (liver, kidney; 21; Fig. 3 B column marked Per and reference 13), demonstrating that the abundant λ-positive B cells found in the centrally deleting mice were antigen induced and not merely the outgrowth of preexisting variants.

Evidence for receptor editing was also apparent in assays that measured expression levels in the bone marrow of the V(D)J recombinase genes RAG-1 (56) and RAG-2 (57). As observed previously (13), H-2d 3-83 μ/δ transgenic mice expressed a low level of RAG-2, consistent with the observation that endogenous Ig rearrangements are suppressed by expression of the transgenic receptor (Fig. 4). Breeding of the 3-83 μ/δ transgenic mice onto class I haplotypes that were even weakly reactive with the 3-83 antibody resulted in significant upregulation of the RAG-2 gene expression, relative to that observed on the nonreactive H-2d backgrounds (Fig. 4), again suggesting that these
low affinity ligands can induce receptor editing. Similar results were obtained in assays of RAG-1 levels (data not shown). In contrast, little or no increase in RAG-2 expression was observed in mice bearing the MT-Kb transgene, which restricts Kb antigen expression to extra bone marrow tissues and demonstrated little endogenous light chain rearrangement (Fig. 3B). Taken together with the changes in levels of spleen IgD+/μ double-positive cells, these data

Table 2. Deletion of Idiotype-positive B Cells from Peripheral Lymph Organs of (3-83 × H-2b) F1 Transgenic Mice

<table>
<thead>
<tr>
<th>3-83 transgenic mice H-2 haplotype (F1)</th>
<th>Percent of 54.1+ cells</th>
<th>Percent of B220+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lymph node</td>
<td>Spleen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>16.6 ± 1.2* (18)*</td>
<td>39.3 ± 1.3 (19)</td>
</tr>
<tr>
<td>k</td>
<td>0.2 ± 0.2 (3)</td>
<td>0.3 ± 0.2 (3)</td>
</tr>
<tr>
<td>b</td>
<td>0.1 ± 0.1 (8)</td>
<td>1.1 ± 0.5 (10)</td>
</tr>
<tr>
<td>bm3</td>
<td>0.1 ± 0 (7)</td>
<td>0.4 ± 0.2 (7)</td>
</tr>
<tr>
<td>bm6</td>
<td>0 ± 0 (4)</td>
<td>1.2 ± 0.3 (4)</td>
</tr>
<tr>
<td>bm8</td>
<td>0.2 ± 0.1 (6)</td>
<td>1.4 ± 0.6 (6)</td>
</tr>
<tr>
<td>bm11</td>
<td>0 ± 0 (4)</td>
<td>0.9 ± 0.9 (4)</td>
</tr>
<tr>
<td>KdDk</td>
<td>0 ± 0 (6)</td>
<td>0.5 ± 0.2 (5)</td>
</tr>
<tr>
<td>Nontransgenic mice</td>
<td>0.3 ± 0.1 (15)</td>
<td>0.7 ± 0.2 (15)</td>
</tr>
</tbody>
</table>

*Data are mean ± SEM of three to six experiments.
*Numbers in parentheses indicate number of mice examined.
*The lower percentage of id-positive cells is primarily the result of receptor downmodulation, which prevented detection at the concentration of 54.1 used.
*These cells coexpressed IgM receptors and are presumably the result of receptor editing.

Figure 2. Deletion of idiotype-positive B cells from spleens of 3-83 μ/β transgenic mice expressing low affinity self-antigens. 3-83 μ/β transgenic mice from H-2d × H-2b breedings (x = haplotype including k, b, b mutations, d, and f) were killed at 2–4 mo of age. Spleen cells were stained with biotinylated anticonalotype 54.1 antibody and FITC rat anti-mouse IgM antibody. 54.1 binding was revealed with PE-streptavidin. Each row represents an independent experiment and is representative of a minimum of three experiments for each H-2 haplotype.
demonstrate the ability of even ultralow affinity ligands to fully induce central tolerance and receptor editing in autoreactive B cells.

In Acute Challenge with Antigen, Ultralow Affinity Ligands Are Only Moderately Effective at Deleting Mature B Cells as Compared to the "High" Affinity Kk Ligand. To test the effect of the receptor/antigen interaction on tolerization of mature B cells, we took advantage of a system originally described by Murakami et al. in which it was shown that ligands of surface Ig could induce rapid apoptosis of peritoneal B cells (9, 22, 58). We injected mice intraperitoneally with Sp2/0 (H-2b)-derived cell lines coexpressing the following class I molecules: Kk, Kp, Kbm11, and Kbm3, which bind 3-83 with decreasing affinities. Approximately 16 h later, the peritoneal cavity cells were isolated and deletion of the id-positive B cells was scored. The Sp2/0 (H-2b control)-injected mice generally showed a modest, non–antigen-specific reduction in transgenic B cells compared to PBS-injected controls (Fig. 5, A and B). In contrast, injection of the high affinity Kk-expressing tumors led to a consistent 5–10-fold decrease in the percentage of id-positive B cells over eight experiments (Fig. 5 and Table 3).
gens was less consistent over several experiments, and lines resulted in only twofold reduction of the 3-83-bearing B-cells (Fig. 5 and Table 3). Compared to Kk-injected cells, the amount of deletion with the lower affinity anti-injection of high affinity Kk ligand, we tested the sensitivity of peripheral deletion mice (Non Tg) define normal levels of bone marrow RAG-2 expression.

The remaining id-positive B cells usually showed down-regulation of IgM receptor expression, indicating that these cells had encountered antigen (Fig. 5 A). To exclude the possibility that the B cells completely lost their receptor expression and thus were not actually deleted, cells were double stained with anti-IgM and anti-B220 antibodies. As can be seen in Table 3, no increase in the percentage of IgM-negative, B220-positive cells accompanied the decrease in id-positive B cells. Based on the previous work, we presume the id-positive cells that disappeared had undergone antigen-induced apoptosis and had not merely left the peritoneal cavity (9, 22, 58). This deletion effect induced by the intraperitoneal injection of antigen was not seen systemically, since no change in the percentage (or number) of splenic transgenic B cells was observed (Table 3).

Having shown deletion of reactive, mature B cells upon injection of high affinity Kk ligand, we tested the sensitivity of this tolerance mechanism to the antibody/antigen-binding affinity. Intraperitoneal injection of the low affinity (Kk) and ultralow affinity (Kbm3,13) ligand—expressing cell lines resulted in only twofold reduction of the 3-83-bearing B-cells (Fig. 5 and Table 3). Compared to Kk-injected cells, the amount of deletion with the lower affinity antigens was less consistent over several experiments, and downregulation of the IgM receptor was rarely seen. Overall, a difference in the extent of deletion of mature B cells upon acute antigenic challenge was clearly noted between the high affinity Kk molecule and the lower affinity Kbm3,13 ligands, whereas even these weakly reactive molecules were capable of inducing complete deletion of immature, autoreactive B cells, as was clear from analysis of F1 crosses (Fig. 2 and Table 2).

Discussion

In concordance with earlier studies investigating the sensitivity of B cell tolerance with respect to BCR/soluble antigen affinity (31, 32, 36), we have verified tolerance induction in immature B cells upon interaction with very low affinity, membrane-bound self-antigens. We were able to follow the fate of the immature, self-reactive B cells and to show that deletion of this cell population was indeed the mechanism of censorship. Our data also indicate that extremely low affinity membrane ligands are sufficient to signal the immature B cell to alter receptor specificity ("receptor editing").

In this study, the affinity between MHC class I autoantigen and Ig receptor that was sufficient to stimulate receptor editing/deletion of the immature B cell was so low that we were unable to place a lower limit on its value. In two independent studies, we measured the affinity constant of the noncovalent interaction between Kk and 3-83 to be in the range of 1-3 × 10^-6 M^-1. Kbm3 and Dk were bound by 3-83 more weakly than Kk, requiring more than 10-fold more IgG to achieve similar binding. Because these ultralow affinity ligands were fully as effective as high affinity ligands in mediating central deletion, an affinity constant of 4 × 10^-7 M^-1 between BCR and membrane self-antigen represents a very conservative value sufficient to induce tolerance in the immature B cell population, this range of affinity was in fact observed in the case of Kbm3 (1-6 × 10^-6 M^-1). This affinity is lower than many reported for TCR/class I peptide agonist interactions (59), suggesting that central deletion of autoreactive B cells in the bone marrow can be as sensitive as central T cell tolerance and indeed is in the affinity range for positively selecting ligands (60).

An important implication of a low affinity threshold for self-tolerance is that it may have profound effects on the B cell repertoire and directly influence the fate of a large proportion of generated B cells. Theoretical models predict that self-reactivity should be common in the preselected lymphocyte repertoire and that negative selection may play a powerful role in the evolution of lymphocyte specificity (61).

While the multivalent nature of the cell/cell interactions between the B cell and antigen-bearing cells is important in permitting weak interactions to lead to biologically significant avidities, such multimerization does not make receptor/ligand affinity irrelevant. Analysis of peripheral B cell tolerance using the same panel of ligands that led to central deletion revealed clear differences between high and low affinity ligands in the efficiency of peripheral deletion.
An unexpected finding in the present study was that the IgG form of the 3-83 antibody had only a slightly higher overall avidity for immobilized ligand than the corresponding F\(_a\)b fragment. The comparative increase seen in the bivalent vs. monovalent binding of the Y3 antibody to the same class I ligand was more than 500-fold and was consistent with results from previous studies on affinity constants for anti-class I antibodies to their ligands (62), whereas the affinity increase resulting from bivalent binding in the 3-83/Kb interaction was unusually small. Since Y3 and 3-83 were passed over the same K\(^b\)-containing sensor chip, a density difference resulting in spatial limitations could not account for this difference, particularly because Y3 and 3-83 reportedly see the same epitope (53). Importantly, there was excellent agreement between measured affinities of the IgG antibodies and the relative affinities observed in the flow cytometry analysis of binding to Kb-expressing cells: ~1,000-fold higher concentration of 3-83 was required to achieve levels of binding equivalent to that observed with Y3 (Fig. 1). These findings lend credibility to the extreme sensitivity of B cell tolerance induction with respect to affinity, since the polyvalency appeared to play a limited role in this particular Ig transgenic system.

Because of the peptide dependency of some anti-class I antibodies (63), it is difficult to formally exclude the possibility that the weak binding of D\(^k\) and Kbm3 ligands by 3-83 actually represents expression of a lower density of higher affinity ligands rather than a comparable density of low af-
Do weakly self-reactive B cells contribute to the emergence of low affinity autoantibodies? In contrast to the affinity studies in the HEL radiation chimera system (36), we do not see the emergence of 3-83-bearing cells in F1 transgenic mice on any low affinity Dk, Kbm3, or Kbm11 background in mice tested out to 18 mo of age (data not shown). Nevertheless, B cells with low affinity receptors may be more sensitive to a breakdown in tolerance. Although all of the ligands of 3-83 that we tested induced central deletion, it is likely that there exists an affinity threshold for membrane antigen at which the reactive B cell is no longer tolerized or the tolerance phenotype is altered, perhaps leading to an anergic state rather than to cell death. We have evidence for this change in tolerance phenotype to a membrane-bound antigen in cases of limiting antigen dose (our unpublished data), implying that the strength of the tolerance signal is dependent on the number or duration of B cell/antigen interactions. It will be important to determine if a low affinity ligand requires longer duration or more antigenic “hits” for a tolerance signal to satisfy a minimum threshold for tolerance.

Developing B cells are generally thought to be more sensitive to tolerance induction than mature B cells (1, 64), but this is by no means proven (32). Our data are consistent with, but do not prove, this notion, since deletion of the autoreactive mature B cells occurred consistently only in the presence of the high affinity Kk ligand. The intermediate affinity, Kk, and low affinity Kbm3,11 ligands were only sensitive to tolerance induction than mature B cells (1, 64), but this is by no means proven (32). Our data are consistent with, but do not prove, this notion, since deletion of the autoreactive mature B cells occurred consistently only in the presence of the high affinity Kk ligand. The intermediate affinity, Kk, and low affinity Kbm3,11 ligands were only sensitive to tolerization in B cells and inducing receptor editing (data not shown), suggesting that only a few thousand molecules per cell of a low affinity membrane self-antigen, when present in the bone marrow, can efficiently induce B cell tolerization.

The possible peptide dependency of anti-class I antibodies also presents limitations to our affinity measurements, since the recombinant soluble H-2k molecules contained only one or a limited number of peptides. However, the binding affinity measurements correlated well with binding of these antibodies to spleen or hybridoma cell-derived Kb molecules, which bear diverse peptides.

Do weakly self-reactive B cells contribute to the emergence of low affinity autoantibodies? In contrast to the affinity studies in the HEL radiation chimera system (36), we do not see the emergence of 3-83-bearing cells in F1 transgenic mice on any low affinity Dk, Kbm3, or Kbm11 background in mice tested out to 18 mo of age (data not shown). Nevertheless, B cells with low affinity receptors may be more sensitive to a breakdown in tolerance. Although all of the ligands of 3-83 that we tested induced central deletion, it is likely that there exists an affinity threshold for membrane antigen at which the reactive B cell is no longer tolerized or the tolerance phenotype is altered, perhaps leading to an anergic state rather than to cell death. We have evidence for this change in tolerance phenotype to a membrane-bound antigen in cases of limiting antigen dose (our unpublished data), implying that the strength of the tolerance signal is dependent on the number or duration of B cell/antigen interactions. It will be important to determine if a low affinity ligand requires longer duration or more antigenic “hits” for a tolerance signal to satisfy a minimum threshold for tolerance.
opmental state of the B cells that encounter autoantigen in this case, or because of a longer duration of exposure to antigen in the double-transgenic mice, compared to the short-term peritoneal injection assay. No increase in the deletion of transgenic B cells was observed 2 d after peritoneal injection of Kb-hybridomas, suggesting that a longer period of antigen exposure did not affect the phenotype, but further experiments are required to establish this point.

An important conclusion to be drawn from our study is that tolerance to membrane self-antigens can be extraordinarily efficient, and can eliminate precursors of antibody-forming cells that have very low affinity for self. This tolerance may be essential to the survival of the organism because low affinity IgM antibodies to cell-surface proteins are potentially toxic, as has been observed in tissue rejection by isoaotibodies (65, 66). In view of these efficient tolerance mechanisms, one might wonder how autoantibodies arise. One clue from the present study is the apparently higher affinity threshold for the deletion of mature B cells, at least in response to transiently expressed antigen. Perhaps failure in these mechanisms resulting from stricter requirements for tolerance induction in mature B cells contribute to the autoimmune process for peripheral antigens.

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