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

By Julie Lang,* Michael Jackson,‡ Luc Teyton,‡ Anders Brunmark,‡ Kevin Kane,§ and David Nemazee*‖

*From the *Department of Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80220; ‡Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; §Department of Immunology, University of Alberta, Edmonton, Edmonton, Alberta, Canada T6G 2H7; and ‖Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206*

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 K^{bm3} and D^k, showed remarkably low, but detectable, affinity to 3-83. The 3-83 antibody bound K^b with $K_A \sim 2 \times 10^5\ M^{-1}$ and bound 10-fold more weakly to the K^{bm3} ($K_A \sim 2 \times 10^4\ M^{-1}$) and D^k antigens. Breeding 3-83 immunoglobulin transgenic mice with mice expressing these ultralow affinity K^{bm3} and D^k 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 λ light chains in the spleen. Reactive class I antigens were also able to mediate deletion of mature B cells when injected into the peritoneal cavity of 3-83 transgenic mice. Although the highest affinity ligand, K^k, 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), anergy (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 reeptor (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 (NJCIRM). 3-83 μ/δ mice were backcrossed a minimum of 10 times onto a B10.D2 back-

[1] *Abbreviations used in this paper:* BCR, B cell receptor; RU, resonance units.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/11/1685/13 $2.00
Volume 184 November 1996 1685–1697

ground and subsequently bred one generation with C57Bl/6 J-H-2^{bm3}/EgAoEg (H-2^{bm3}), C57Bl/6^{bm6} (H-2^{bm6}), C57Bl/6 Kh-H-2^{bm8}/KhEg (H-2^{bm8}), B6.C-H-2^{bm11}/KhEg (H-2^{bm11}), C3H-H-2^{o2}/SfSn (C3H.OH: K^dD^k), B10.M/Sn (H-2^f), B10.BR/Sg-SnJ (H-2^k), C57Bl/6 (H-2^b), and B10.D2/nSnJ (H-2^d) 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-$2^{bm1,3,4,8,9,10;11}$ 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 μ/δ transgenic, β2-microglobulin$^{-/-}$ mice on a H-2^d homozygous background. These resulting mice were analyzed as negative controls.

Cell Lines. Sp2/0-derived H-2^b and H-2^b 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-2^k) cell line was obtained from L. Wysocki (NJCIRM). The Sp2/0 (H-2^d) cell line (40) was purchased from American Type Tissue 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/FITC, RA3-3A1 (41); anti–IgM/biotin, Ak2, 33.24.12 (42); anti–H-$2K^{k,b,mutants,r,s,q}$, D^k/biotin, Y3 (43) and 3-83P (44); anti–mouse IgD^a, AMS 9.1/biotin (45), (Pharmingen, San Diego, CA); and anti–IgM^a/FITC, RS3 (46); and with the following polyclonal antibodies: rat anti–mouse IgM, LO-MM-9/FITC (47), (Zymed Laboratories, South San Francisco, CA); and goat anti–λ/FITC (Fisher 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 K^b and K^{bm3} were purified as described previously (49). Briefly, *Drosophila* S2/M3 cells were cotransfected with a soluble form of H-2K^b or K^{bm3} 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 K^b was purified on an anti-$K^{b,\,bm3}$ (Y3) affinity column and the His-tagged K^{bm3} was purified on a nickel column, both of which were followed by ion-exchange chromatography. Approximate yields were 0.5 mg H-2K/liter of supernatant. The K^b and K^{bm3} were concentrated, dialyzed against PBS, filter sterilized, and stored at 4°C.

F_{ab} Preparation. F_{ab} fragments of the 3-83 antibody were prepared using the ImmunoPure F_{ab} Preparation Kit (Pierce Chemical Co., Rockford, IL). F_{ab} preparations were verified on reducing and nonreducing SDS–polyacrylamide gels. Specifity of F_{ab} fragments was verified upon biotinylation of the F_{ab} fragments, followed by staining of H-2 K^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 K^b, and 20 μg/ml for all antibodies. The K^{bm3} 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: K^b (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), K^b (2821 RU), and 33.18.12 (42) (anti–mouse κ; 2458 RU); chip 4: $K^{bm3\text{-}vsv}$ (3246 RU), K^{bm3} (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 BIAcore™ or 1:1,000 Triton-X 100 surfactant) to the following concentration ranges representing two-fold dilutions: run 1 (chip 1): 3-83 (25–800 nM) over K^b; run 2 (chip 2): soluble K^b 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 F_{ab} (250–8,000 nM), Y3 IgG (62–200 nM), and Hopc-1 (25–800 nM) over K^b; run 4 (chip 2): soluble K^{bm3} (39–625 nM), $K^{bm3\text{-}vsv}$ (125–500 nM) over Y3 and Hopc-1; run 5 (chip 5): soluble K^{bm3} (32–4,140 nM) and $K^{bm3\text{-}vsv}$ (143–9,140 nM) over 3-83 and Hopc-1; run 6 (chip 4): 3-83 IgG (25nM) 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 $K^{bm3\pm vsv}$. 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 K^{bm3} 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 2 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 K^b, 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 K^b were performed on the BIACore 1000, which allows passage of analyte over a single flowcell (single protein bound to chip), while the K^{bm3} 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 10^4 M^{-1}, while the BIACore 1000 has limitations in the 10^5 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 BIA-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 (k_d) was determined initially by the AB = A + B regression model to fit slope of $\ln(R_0/R)$ vs. time plot. Because of steepness of the slope and the short length of dissociation period, this method proved inacurrate 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 k_d was then calculated as $0.693/t_{1/2}$ (s_{-1}) and averaged over several concen-

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_d. The data from this analysis showed large variations, with the k_a value decreasing with concentration. The second method, referred to as the k_s vs. concentration method, does not assume k_d values, and calculates k_a based on several concentrations of analyte. For each concentration of analyte, the program determines the slope (k_s) of dR/dt vs. R. The association constant was then calculated as the negative slope of k_s vs. concentration. The K_a value determined by the k_s vs. concentration method correlated well with the K_a 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 (51). 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_s vs. concentration method did not always generate a straight line, and in these cases, analysis for k_a was restricted to the regression analysis. Also in instances where analyte dissociated very slowly, analyses of both k_d and k_a 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_a over k_d.

Detection of mRNA. The levels of $G_{\alpha s}$, RAG-1, and RAG-2 mRNA expression in the bone marrow of F1 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-2^d) antigen-negative control cells, were washed twice in sterile PBS and resuspended at 5.0×10^7cells/ml. Using 26-gauge needles, 100 μl/mouse of hybridoma cells were injected into the peritoneums of age-matched, nondeleting (H-2^d background) 3-83 hemizygous transgenic mice. One mouse from each group received 100 μl 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^b and Natural Mutations in the K^b Molecule Further Lower the Relative Binding Affinity. The 3-83 antibody was raised in a BALB/c (H-2^d) mouse by immunization with C3H (H-2^k) spleen cells, and has specificity for K^k, but also binds very weakly to D^k and to other alloforms of H-2^k (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^b cells required far higher concentrations of antibody relative to that required for comparable binding to K^k cells (reference 44 and Fig. 1 *A*). Despite the relatively low affinity for K^b, 3-83 μ/δ Ig transgenic mice bred onto a H-2^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^b molecule, the so-called "bm" series (52), had further reduced affinity to the 3-83 antibody, relative to K^b. Spleen and lymph node cells of H-$2^{bm1,3,4,8,9,10,11}$ 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^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^k 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^{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^b mutations confirmed these results (Fig. 1, *C* and *D*). When reacted with 3-83 antibody, K^{bm3} was bound less well than K^b, whereas both were bound similarly by the Y3 antibody. The reactivity of 3-83 to the D^k molecule was also much weaker than its cross-reactivity to the K^b molecule. When the data are corrected for nonspecific binding, it is apparent that the very low affinity ligands K^{bm3} and D^k required >10-fold higher concentrations of 3-83 to achieve a level of binding comparable to that observed with K^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^b with an Association Constant in the 10^5 M^{-1} Range. Using the technique of surface plasmon resonance (54, 55), the equilibrium association constant (K_A) for the interaction between K^b and 3-83 antibody was measured (Table 1). Appropriate control antibodies were studied in parallel to assure the specificity of the interaction and the activity of the antibodies. 3-83 F_{ab} binding to immobilized K^b demonstrated a very fast off rate ($t_{1/2}$ ~7.4 s) largely responsible for the low K_A value of 1.7×10^5 M^{-1}. Soluble K^b binding to immobilized 3-83 was consistent with this result, yielding a K_A value of 3.3×10^5 M^{-1}. The bivalent, IgG form of 3-83 bound to immobilized K^b (K_A ~5.5×10^6 M^{-1}) with only 10-fold higher affinity than did F_{ab} 3-83, whereas bivalency improved the binding of the Y3 antibody by >500-fold (Table 1). Consistent with the binding studies (Fig. 1), the monovalent affinity of 3-83 for the putative very low affinity ligand K^{bm3} was ~10-fold lower: K_A ~2×10^4 M^{-1}, and the bivalent affinity was K_A ~5×10^5 M^{-1}. This binding appeared to be peptide independent, since K^{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*

Ligand	Analyte	Valency	Concentration	k_a ($M^{-1}s^{-1}$)	R^2	Half-life	SD	k_d (s^{-1})	SD	K_A(M^{-1})
						s				
K^b	Y3 IgG	Bi	62 nM	4.11E + 05	4.21*	>3,600		2.43E-04	4.77*	1.7E + 09
$K^{b\text{-}ova}$	3-83 IgG	Bi	800 nM	2.90E + 04	0.991	135.3	28.1	5.29E-03	1.09E-03	**5.5E + 06**
K^{bm3}	3-83 IgG	Bi	1,875 nM	1.42E + 04	0.999	26.5	1.5	2.63E-02	1.61E-03	**5.4E + 05**
$K^{bm3\text{-}vsv}$	3-83 IgG	Bi	1,875 nM	1.25E + 04	0.984	50.8	5.8	1.38E-02	1.73E-03	**9.1E + 05**
$K^{b\text{-}ova}$	Hopc IgG	Bi	800 nM							No binding
K^{bm3}	Hopc IgG	Bi	3,500 nM							No binding
$K^{bm3\text{-}vsv}$	Hopc IgG	Bi	3,500 nM							No binding
$K^{b\text{-}ova}$	3-83 Fab	Mono	8,000 nM	1.67E + 04	0.93	7.4	2	9.98E-02	2.93E-02	**1.7E + 05**
K^{bm3}	3-83 Fab	Mono	2,4450 nM	3.74E + 03	0.977	3.2	0.8	2.27E-01	6.05E-02	**1.6E + 04**
$K^{bm3\text{-}vsv}$	3-83 Fab	Mono	2,4450 nM	3.82E + 03	0.979	5.1	1	1,40E-01	2.89E-02	**2.7E + 04**
54.1	3-83Fab	Mono	2,000 nM	4.12E + 04	0.997	79.5	0	8.72E-03	0.00E + 00	4.7E + 06
54.1	3-83 IgG	Poly	25 nM	6.57E + 05	0.01*	NA		3.49E-04	0.07*	1.9E + 10
54.1	Y3 IgG	Poly	200 nM							No binding
33.18.12	3-83Fab	Mono		1.40E + 05	6.49*	NA		3.39E-06	2.77*	4.1E + 10
3-83	$K^{b\text{-}ova}$	Mono	1,000 nM	1.42E + 04	0.898	18.6	7.6	4.27E-02	1.83E-02	**3.3E + 05**
3-83	K^{bm3}	Mono	4,140 nM	3.44E + 03§	0.996	3.5	2.1	2.43E-01	1.47E-01	**1.4E + 04**
3-83	$K^{bm3\text{-}vsv}$	Mono	9,140 nM	3.44E + 03	0.996	13.1	1.2	5.31E-02	4.80E-03	**6.5E + 04**
Y3	$K^{b\text{-}ova}$	Mono	500 nM	7.41E + 04	0.966	30.4	3.1	2.30E-02	2.27E-03	3.2E + 06
Y3	K^{bm3}	Mono	625 nM	2.03E + 04	0.25‡	25.9	4.3	2.74E-02	4.71E-03	7.4E + 05
Y3	$K^{bm3\text{-}vsv}$	Mono	500 nM	3.52E + 04	0.22‡	16.8	2.2	4.19E-02	5.80E-03	8.4E + 05
Hopc	$K^{b\text{-}ova}$	Mono	1,000 nM							No binding
Hopc	K^{bm3}	Mono	4,140 nM							No binding
Hopc	$K^{bm3\text{-}vsv}$	Mono	9,140 nM							No binding

*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 χ^2 value for the regression analysis of the on and off rates.

‡The K_s 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 χ^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 $K^{bm3\text{-}vsv}$ 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 decribed in Matherials 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 irrevelant 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 Autoreactive 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 μ/δ transgenic mice were bred with mice that expressed these weakly reactive class I haplotyes (Fig. 2). Transgenic mice bred on the non–3-83–reactive H-2^d or H-2^f 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 $K^{k,b,bm3,6,8,11}$ 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 F1 transgenic mice that expressed ultralow affinity ligands, we used two inde-

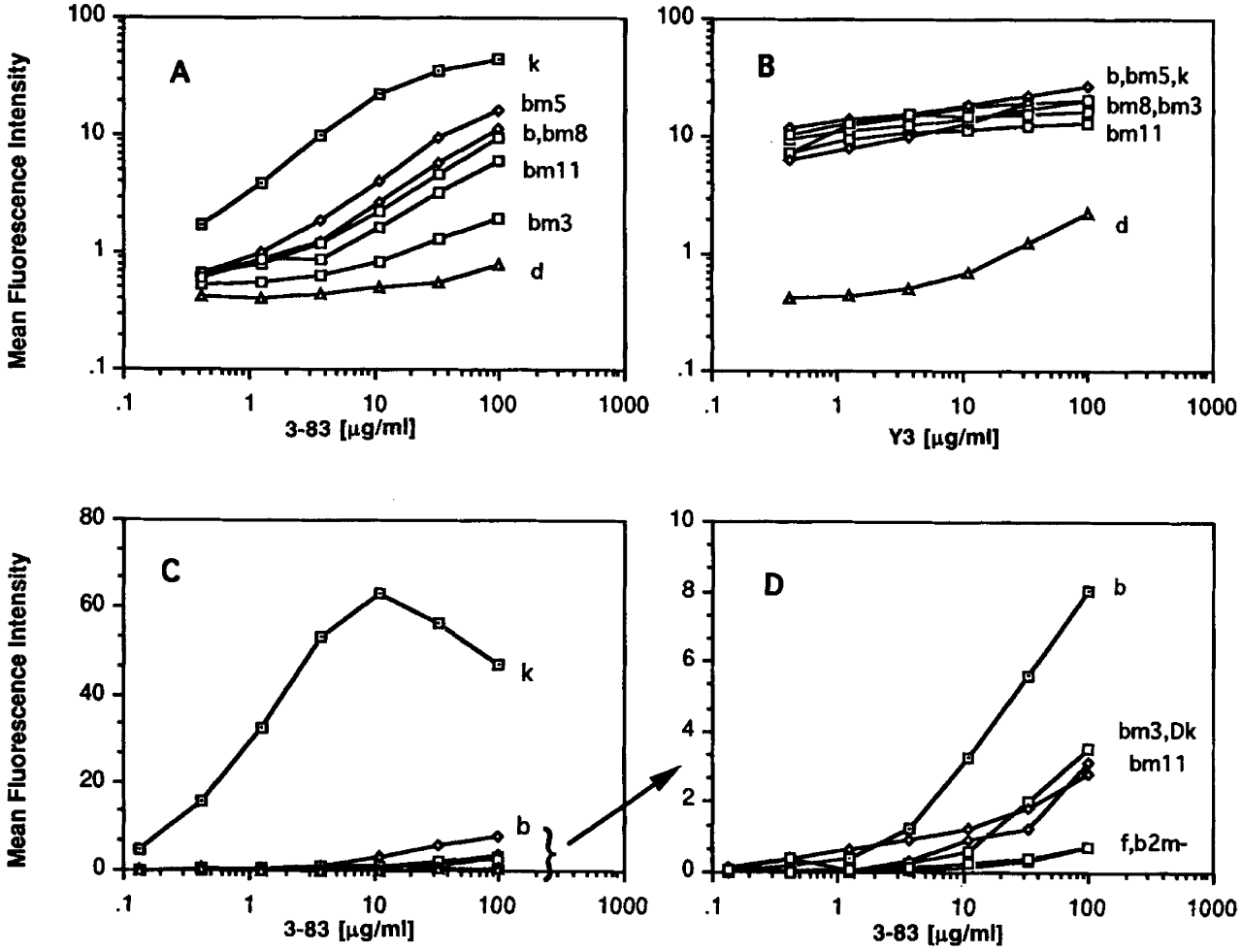

Figure 1. Relative binding of 3-83 antibody to various class I molecules in a flow cytometry assay. Binding of (*A*) 3-83 and (*B*) Y3 anti–class I antibodies to H-2 disparate, Sp2/0-derived hybridomas, or (*C*) binding of 3-83 antibody to spleen cells from MHC disparate mice. (*D*) Expands the lower range of the ordinate of *C* to differentiate poorly bound class I antigens. The data shown are representative of four independent experiments.

pendent 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^a (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 endogeneous heavy chain antibodies are of the b allotype and the 3-83 transgenic light chain is κ. In transgenic mice of the $H\text{-}2^f$ or $H\text{-}2^d$ (nondeleting) background, the vast majority of B cells expressed the IgD^a 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 κ 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 IgD^a, λ double-positive cells was not observed in 3-83 mice expressing a K^b 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), $H\text{-}2^d$ 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 $H\text{-}2^d$ backgrounds (Fig. 4), again suggesting that these

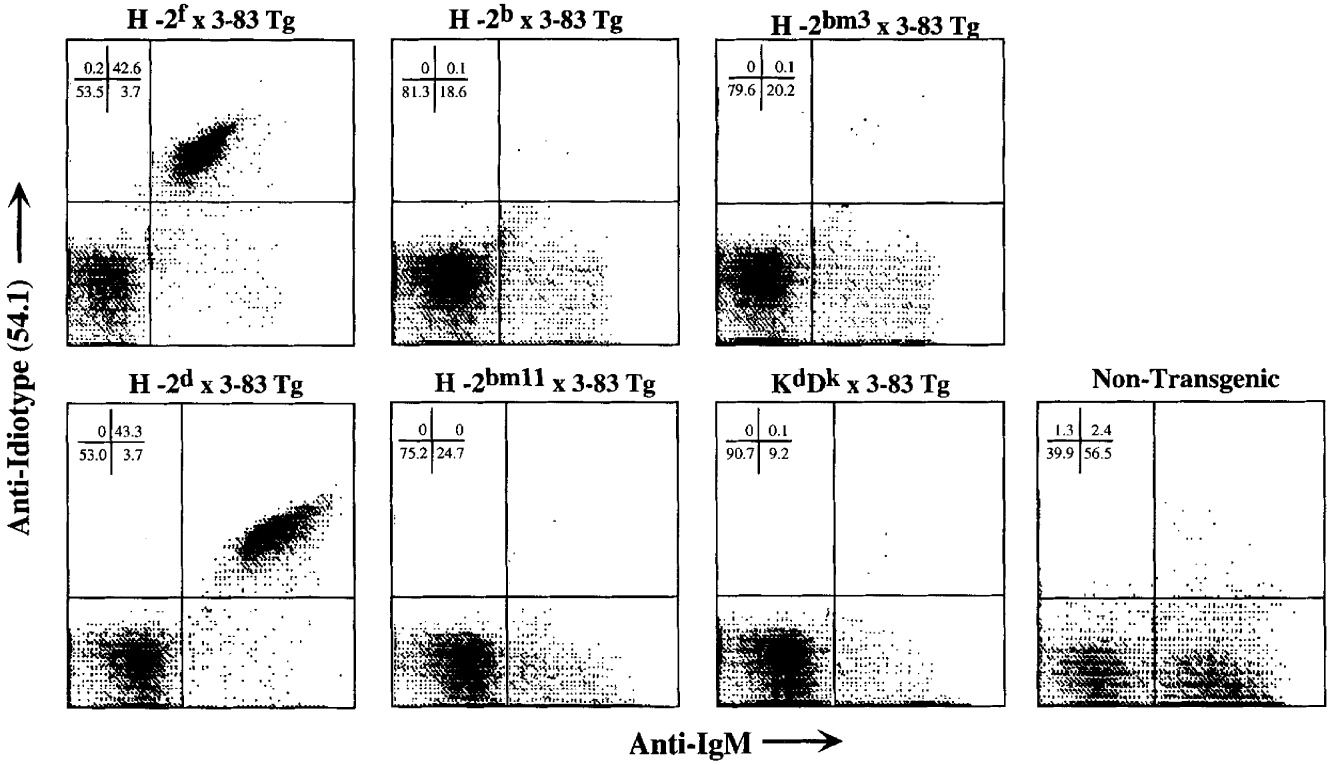

Figure 2. Deletion of idiotype-positive B cells from spleens of 3-83 μ/δ transgenic mice expressing low affinity self-antigens. 3-83 μ/δ transgenic mice from H-2^d × H-2^x breedings (x = haplotype including k, b, b mutations, d, and f) were killed at 2–4 mo of age. Spleen cells were stained with biotinylated anticlonotype 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.

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-K^b transgene, which restricts K^b antigen expression to extra bone marrow tissues and demonstrated little endogenous light chain rearrangement (Fig. 3 *B*). Taken together with the changes in levels of spleen IgD^a/λ double-positive cells, these data

Table 2. *Deletion of Idiotype-positive B Cells from Peripheral Lymph Organs of (3-83 × H-2^x) F_1 Transgenic Mice*

3-83 transgenic mice H-2 haplotype (F_1)	Percent of 54.1$^+$ cells			Percent of B220$^+$ cells		
	Lymph node	Spleen	Bone marrow	Lymph node	Spleen	Bone marrow
d	16.6 ± 1.2* (18)‡	39.3 ± 1.3 (19)	27.0 ± 1.8 (11)	13.4 ± 1.2 (17)	36.1 ± 1.5 (18)	26.7 ± 2.0 (8)
k	0.2 ± 0.2 (3)	0.3 ± 0.2 (3)	0.1 ± 0.1 (2)§	5.4 ± 3.4 (2)‖	10.0 ± 3.9 (2)‖	26.5 ± 7.5 (2)
b	0.1 ± 0.1 (8)	1.1 ± 0.5 (10)	2.7 ± 2.3 (8)§	2.2 ± 0.8 (7)‖	7.9 ± 0.8 (9)‖	36.9 ± 2.0 (5)
bm3	0.1 ± 0 (7)	0.4 ± 0.2 (7)	2.1 ± 1.9 (4)§	2.4 ± 0.5 (6)‖	8.6 ± 1.7 (6)‖	35.9 ± 5.6 (3)
bm6	0 ± 0 (4)	1.2 ± 0.3 (4)	ND	1.9 ± 0.3 (4)‖	17.3 ± 2.8 (3)‖	31.7 (1)
bm8	0.2 ± 0.1 (6)	1.4 ± 0.6 (6)	2.8 ± 2.7 (2)§	2.8 ± 0.4 (6)‖	14.6 ± 2.0 (6)‖	ND
bm11	0 ± 0 (4)	0.9 ± 0.9 (4)	3.1 ± 0.9 (3)§	3.9 ± 0.5 (4)‖	13.9 ± 3.0 (4)‖	35.4 ± 0.7 (2)
K^dD^k	0 ± 0 (6)	0.5 ± 0.2 (5)	2.4 ± 2.1 (2)§	0.9 ± 0.1 (6)‖	7.4 ± 0.6 (6)‖	41.0 (1)
Nontransgenic mice	0.3 ± 0.1 (15)	0.7 ± 0.2 (15)	0.3 ± 0.1 (9)	23.1 ± 2.0 (15)	40.8 ± 2.8 (15)	52.8 ± 4.9 (6)

*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.

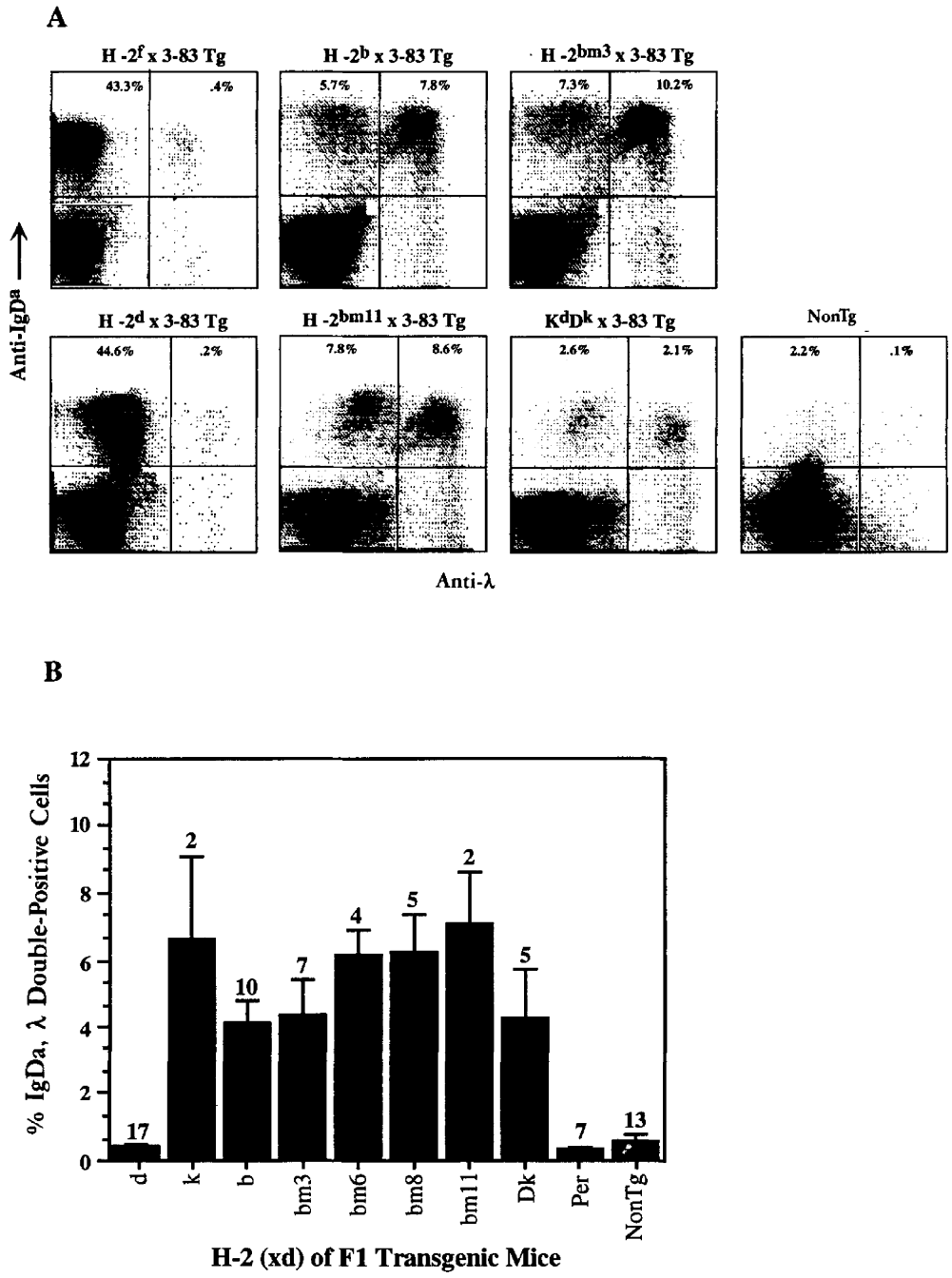

Figure 3. Increased percentage of B cells coexpressing IgD^a (transgene heavy chain) and λ light chain (endogeneous) in spleens of autoantigen-bearing transgenic mice. (*A*) Spleen cells from 3-83 transgenic mice bred with non-antigen bearing mice ($H\text{-}2^{d,f}$) or antigen-expressing mice ($K^{b,bm3,bm11}$ or D^k) were stained with FITC anti-λ and biotinylated anti-IgD^a antibodies, followed by PE-streptavidin. On these genetic backgrounds, only the transgenic heavy chain is of the 'a' allotype. A nontransgenic control is included to show specificity of the 'a' allotype reagent for transgene. 50,000 cells from each sample were collected in a gate defined by forward and side scatter to include the predominant lymphocyte population. Each row represents an individual experiment and is representative of a minimum of three experiments for each H-2 haplotype. (*B*) The combined data are shown as the mean ± SEM (error bars) percent of IgD^a, λ double-positive cells in transgenic mice of the indicated H-2 haplotype. The number above each bar indicates the number of mice per group. *Per*, peripherally deleting, MT-K^b/3-83 μ/δ double-transgenic mice; *Non Tg*, nontransgenic littermates.

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 K^k 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 ($H\text{-}2^d$)–derived cell lines coexpressing the following class I molecules: K^k, K^b, K^{bm11}, and K^{bm3}, 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 ($H\text{-}2^d$ 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 K^k-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).

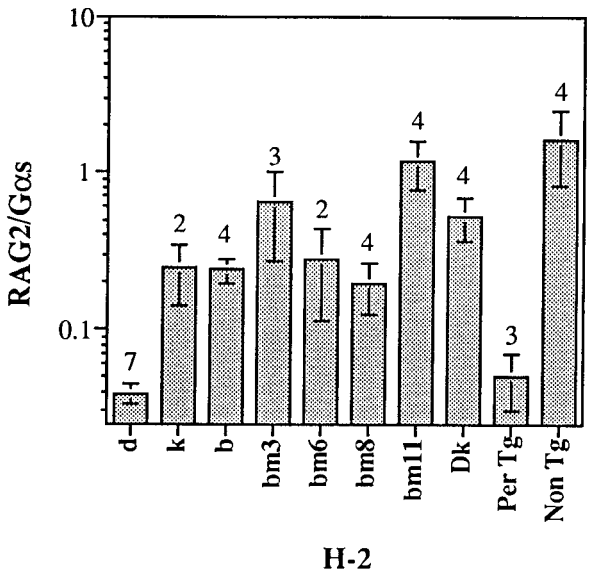

Figure 4. Elevated expression of RAG-2 in the presence of bone marrow autoantigen, including low affinity ligands. Reverse transcription PCR analysis of bone marrow RNA of (3-83μδ Tg/H-2^x) F_1 mice, where x includes H-2 haplotypes both reactive and nonreactive to the 3-83 antibody. cDNA was amplified with 17–22 cycles of PCR using oligos specific for RAG-2 and, as a normalization control, the ubiquitously expressed GTP-binding protein, Gαs. Southern blot analysis revealed specific fragments that were quantitated using a Phospho-γ-imager. The data represent the mean ± SEM RAG-2/Gαs values for each haplotype. Numbers of mice per group are shown above each bar. 3-83μδ/MT-K^b peripherally deleting mice (*Per Tg*), in which K^b antigen driven by the metallothionein promoter is expressed in the periphery, but not in the bone marrow, are included as negative controls for the nonspecific effects of peripheral B cell deficiency (13, 21). Results with nontransgenic mice (*Non Tg*) define normal levels of bone marrow RAG-2 expression.

The remaining id-positive B cells usually showed downregulation 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 K^k ligand, we tested the sensitivity of this tolerance mechanism to the antibody/antigen–binding affinity. Intraperitoneal injection of the low affinity (K^b) and ultralow affinity ($K^{bm3.11}$) ligand–expressing cell lines resulted in only twofold reduction of the 3-83–bearing B-cells (Fig. 5 and Table 3). Compared to K^k-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 K^k molecule and the lower affinity $K^{b,bm3,bm11}$ ligands, whereas even these weakly reactive molecules were capable of inducing complete deletion of immature, autoreactive B cells, as was clear from analysis of F_1 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 monovalent interaction between K^b and 3-83 to be in the range of $1–3 \times 10^5\ M^{-1}$. K^{bm3} and D^k were bound by 3-83 more weakly than K^b, 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 $4 \times 10^4\ M^{-1}$ 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 K^{bm3} ($1–6 \times 10^4\ M^{-1}$). 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 tolerance revealed clear differences between high and low affinity ligands in the efficiency of peripheral deletion.

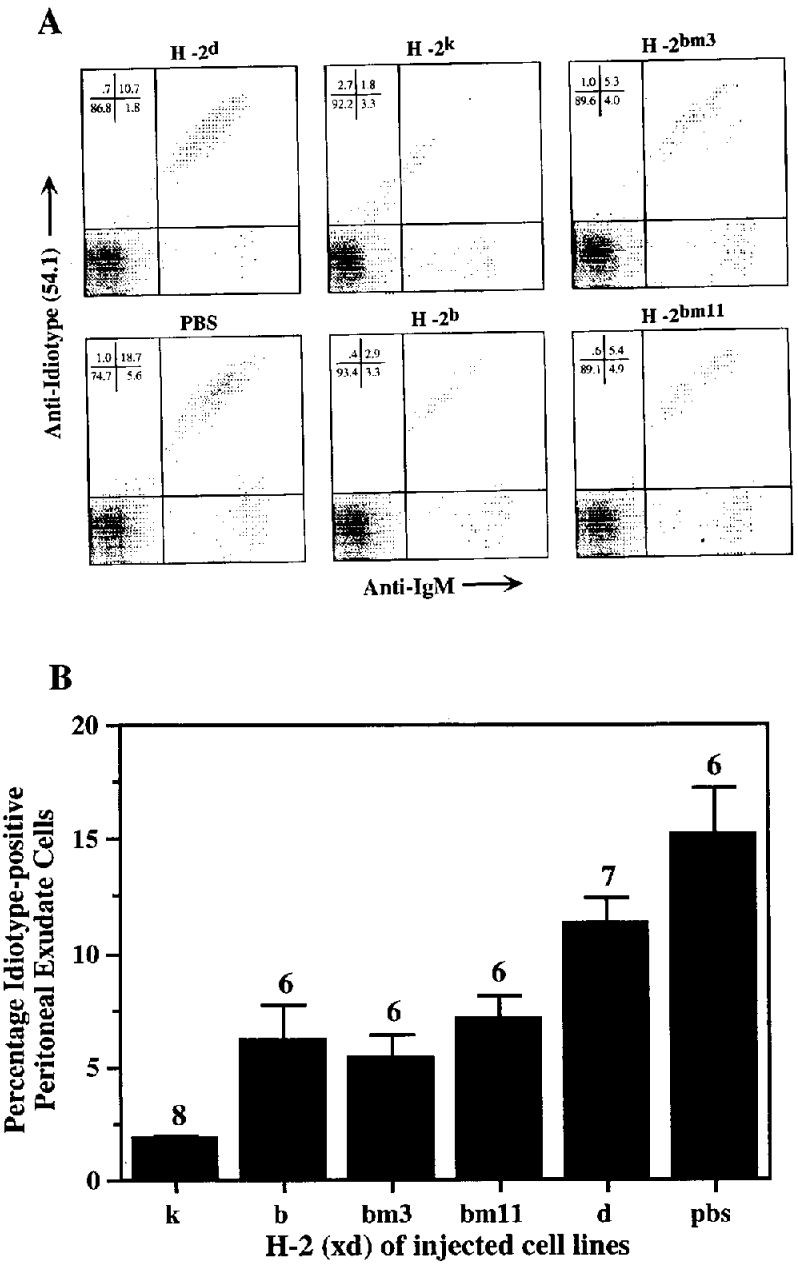

Figure 5. Reduction of idiotype-positive cells in the peritoneal cavity of transgenic mice upon antigenic challenge. Nondeleting (H-2^d) 3-83 transgenic mice were injected intraperitoneally with 5×10^6 MHC-disparate Sp2/0 spleen hybridomas. Sp2/0 (H-2^d) cells were injected to control for antigen nonspecific effects and one mouse per group received 100 μl of PBS alone. (*A*) Approximately 16 h after injection, nucleated peritoneal cells were isolated and stained with biotinylated anticlonotype 54.1 and FITC-labeled rat anti-mouse IgM antibodies. Biotin-conjugated 54.1 was revealed with PE-streptavidin. Fluorescence analysis was gated by forward and side scatter to exclude the large myeloid and injected tumor cells. The density plot data are from one experiment and represent six such experiments. (*B*) The compiled data as the percentage of idiotype-positive cells (quadrant 2) in the peritoneal cavity after injections of cell lines. The letter on the *x* axis indicates the H-2 haplotype of injected cells. The data are mean ± SEM for each group. The number of mice in each group is indicated above each bar.

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_{ab} 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 affinites 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 K^{bm3} ligands by 3-83 actually represents expression of a lower density of higher affinity ligands rather than a comparable density of low af-

Table 3. *Degree of Reduction in the Number of Idiotype-positive B Cells from the Peritoneum of Antigenically Challenged Transgenic Mice Correlates with Affinity of 3-83 Antibody for Antigen*

H-2 (X × d) of injected cells	Peritoneal exudate cells				Spleen cells		
	%54.1$^+$	%IgM^{+*}	%B220^{+*}	%B220$^+$, IgM$^-$	%54.1$^+$	%IgM$^+$	%B220$^+$
d	11.2 ± 1.2‡ (7)§	23.2 ± 6.7 (6)	15.4 ± 2.5 (6)	2.2 ± 0.5 (4)	48.6 ± 4.7 (5)	53.1 ± 2.9 (4)	49.1 ± 3.7 (4)
k	1.8 ± 0.2 (8)	7.8 ± 1.1 (8)	8.4 ± 1.0 (8)	2.2 ± 0.3 (5)	48.3 ± 6.3 (5)	52.3 ± 5.9 (4)	49.9 ± 6.0 (4)
b	6.1 ± 1.6 (6)	14.2 ± 5.2 (5)	11.7 ± 3.1 (5)	2.6 ± 0.6 (4)	41.5 ± 2.4 (5)	50.2 ± 3.2 (4)	48.5 ± 3.2 (4)
bm3	5.4 ± 1.0 (6)	14.6 ± 2.6 (6)	10.7 ± 1.9 (6)	2.0 ± 0.5 (5)	44.9 ± 4.1 (4)	54.3 ± 0.5 (3)	52.8 ± 2.0 (3)
bm11	7.0 ± 1.1 (6)	16.4 ± 4.3 (6)	11.4 ± 2.4 (6)	1.5 ± 0.3 (5)	34.8 ± 7.9 (3)	48.1 ± 2.7 (3)	48.4 ± 2.4 (3)
pbs	15.2 ± 2.1 (6)	26.0 ± 3.8 (5)	18.3 ± 2.4 (5)	1.3 ± 0.4 (3)	38.3 ± 3.3 (5)	42.1 ± 0.5 (3)	41.2 ± 2.0 (3)

*The peritoneal cells consist of a variable population of IgM$^+$, B220$^{-/lo}$ cells, which are not gated as B220$^+$ cells, and account for the discrepancy between the percentage of IgM$^+$ and B220$^+$ cells.
‡Data are mean ± SEM.
§Numbers in parentheses represent number of mice examined.

finity ligands. We do not favor this notion, however, because it is inconsistent with the observation that binding to putative low affinity ligands fails to plateau at high 3-83 concentrations (Fig. 1 *D*) and the similar binding affinities of 3-83 to peptide-bearing and peptide-free K^{bm3}. Nonetheless, if the weak binding to certain class I molecules such as D^k does represent a low density of high affinity antigens, the interpretation of our data would be that tolerance can be induced by higher affinity membrane antigens present at very low epitope density, as opposed to the interpretation that we favor, namely that tolerance is induced by very low affinity self-antigens with moderate to high cell surface abundance. We have attempted to address the issue of antigen density by breeding β2-microglobulin–deficient mice with 3-83 transgenic mice bearing H-2^{bm3}. The F_1 mice expressed one half the normal class I molecules because of heterozygosity of the β2-microglobulin locus deficiency, and only one half of the H-2^k molecules were antigenic (K^{bm3}), since the mice were heterozygous at the MHC locus (bm3/d). With one quarter the normal number of class I molecules, the resulting transgenic mice were still capable of fully deleting the autoreactive 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 tolerance.

The possible peptide dependency of anti–class I antibodies also presents limitations to our affinity measurements, since the recombinant soluble H-2^k 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 K^b 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 F_1 transgenic mice on any low affinity D^k, K^{bm3}, or K^{bm11} 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 miminum 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 K^k ligand. The intermediate affinity, K^b, and low affinity K^{bm3,11} ligands were only able to induce partial deletion, suggesting that low affinities ($<10^6$ M^{-1}) limit the potential to induce deletion. A difference in total H-2^k density could not account for this difference in deletion induction, since staining of the hybridomas showed equivalent surface expression. In contrast to the partial deletion seen upon intraperitoneal injection of K^b cells into 3-83 μ/δ mice, transgene-driven, tissue-specific expression of the intermediate affinity ligand K^b on the liver and kidney results in complete deletion of the 3-83–bearing B cells from the lymph node in MT-K^b/3-83 μ/δ double-transgenic mice (21). The inability of K^b-bearing cells to achieve the same level of deletion upon intraperitoneal injection could be caused by the more mature devel-

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 isoantibodies (65, 66). In view of these efficient tolerance mechanisms, one might ask 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.

The authors are grateful to Haruo Kozona for his assistance with the BIAcore; Roger Melvold for providing bm mutant mice, Janko Nikolic-Zugic and M.J. Bevan for providing cell lines, David Russell and Russell Yawger for technical assistance; Shirley Sobus and Bill Townsend for flow cytometry assistance; Leigh Landskroner for illustrations; and Amy Carbone and other members of our laboratory for critical review of the manuscript.

Julie Lang is a Howard Hughes Medical Institute Predoctoral Fellow. This work was supported by the Arthritis Foundation and the National Institutes of Health (RO1 GM 44809 and K04 AI01161).

Address correspondence to Dr. David Nemazee, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206. No reprints will be sent.

Received for publication 6 June 1996 and in revised form 24 July 1996.

References

1. Nossal, G.J.V. 1983. Cellular mechanisms of immunologic tolerance. *Annu. Rev. Immun.* 1:33–62.
2. Nemazee, D. 1995. B lymphocyte tolerance in the mouse. *In* Immunoglobulin Genes. 2nd ed. T. Honjo and F.W. Alt, editors. Academic Press, 365–378.
3. Radic, M.Z., and M. Weigert. 1994. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu. Rev. Immunol.* 12:487–520.
4. Fulcher, D.A., C.B. Koleda, and A. Basten. 1996. B cell activation and tolerance. *Int. Rev. Immunol.* In press.
5. Melchers, F., A. Rolink, U. Grawunder, T. Winkler, H. Karasuyama, P. Ghia, and J. Andersson. 1995. Positive and negative selection events during B lymphopoiesis. *Curr. Opin. Immunol.* 7:214–227.
6. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature (Lond.).* 381:751–758.
7. Nemazee, D.A., and K. Buerki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature (Lond.).* 337:562–566.
8. Hartley, S.B., J. Crosbie, R. Brink, A.B. Kantor, A. Basten, and C.C. Goodnow. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature (Lond.).* 353:765–769.
9. Nisitani, S., T. Tsubata, M. Murakami, M. Okamoto, and T. Honjo. 1993. The bcl-2 gene product inhibits clonal deletion of self-reactive B lymphocytes in the periphery but not in the bone marrow. *J. Exp. Med.* 178:1247–1254.
10. Spanopoulou, E., C.A.J. Roman, L. Corcoran, M. Schissel, D.P. Silver, U. Storb, D. Nemazee, M. Nussenzweig, S.A. Shinton, R.R. Hardy, and D. Baltimore. 1994. Expression of functional immunoglobulin transgenes allows ordered B-cell differentiation to progress in Rag-1 deficient mice. *Genes Dev.* 8:1030–1042.
11. Chen, C., Z. Nagy, M.Z. Radic, R.R. Hardy, D. Huszar, S.A. Camper, and M. Weigert. 1995. The site and stage of anti-DNA B-cell deletion. *Nature (Lond.).* 373:252–255.
12. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 177:999–1008.
13. Tiegs, S.L., D.M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177:1009–1020.
14. Radic, M.Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med.* 177:1165–1173.
15. Prak, E.L., M. Trounstine, D. Huszar, and M. Weigert. 1994. Light chain editing in kappa-deficient animals: a potential mechanism of B cell tolerance. *J. Exp. Med.* 180:1805–1815.
16. Prak, E.L., and M. Weigert. 1995. Light chain replacement: a new model for antibody gene rearrangement. *J. Exp. Med.* 182:541–548.
17. Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature (Lond.).* 334:676–682.
18. Erikson, J., M.Z. Radic, S.A. Camper, R.R. Hardy, C. Carmack, and M. Weigert. 1991. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature*

(Lond.). 349:331–334.
19. Cyster, J., and C.C. Goodnow. 1995. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity*. 3:691–701.
20. Cyster, J.G., S.B. Hartley, and C.C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature (Lond.)*. 371:389–395.
21. Russell, D.M., D. Zlatko, G. Morahan, J.F.A.P. Miller, K. Buerki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. *Nature (Lond.)*. 354:308–311.
22. Murakami, M., T. Tsubata, M. Okamoto, A. Shimizu, S. Kumagai, H. Imura, and T. Honjo. 1992. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature (Lond.)*. 357:77–80.
23. Parry, S.L., J. Hasbold, M. Holman, and G.B. Klaus. 1994. Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. *J. Immunol.* 152:2821–2829.
24. Fulcher, D.A., and A. Basten. 1994. Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J. Exp. Med.* 179:125–134.
25. Finkelman, F.D., J.M. Holmes, O.I. Dukhanina, and S.C. Morris. 1995. Cross-linking of membrane immunoglobulin D, in the abscence of T cell help, kills mature B cells in vivo. *J. Exp. Med.* 181:515–525.
26. Fulcher, D.A., A.B. Lyons, S.L. Korn, M.C. Cook, C. Koleda, C. Parish, B. Fazekas de St. Groth, and A. Basten. 1996. The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. *J. Exp. Med.* 183:2313–2328.
27. Linton, P.J., A. Rudie, and N.R. Klinman. 1991. Tolerance susceptibility of newly generating memory B cells. *J. Immunol.* 146:4099–4104.
28. Pulendran, B., G. Kannourakis, S. Nouri, K.G.C. Smith, and G.J.V. Nossal. 1995. Soluble antigen can cause enhanced apoptosis of germinal-centre B cells. *Nature (Lond.)*. 375:331–334.
29. Shokat, K.M., and C.C. Goodnow. 1995. Antigen-induced B-cell death and eliminiation during germnal centre immune responses. *Nature (Lond.)*. 375:334–338.
30. Han, S., B. Zheng, J. Dal-Porto, and G. Kelsoe. 1995. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182:1635–1644.
31. Teale, J.M., and N.R. Klinman. 1980. Tolerance as an active process. *Nature (Lond.)*. 288:385–387.
32. Riley, R.L., and N.R. Klinman. 1986. The affinity threshold for antigenic triggering differs for tolerance susceptible immature precursors vs mature primary B cells. *J. Immunol.* 136: 3147–3154.
33. Dintzis, H.M., and R.Z. Dintzis. 1990. Antigens as immunoregulators. *Immunol. Rev.* 115:243–251.
34. Nemazee, D., D. Russell, B. Arnold, G. Haemmerling, J. Allison, J.F.A.P. Miller, G. Morahan, and K. Buerki. 1991. Clonal deletion of autospecific B lymphocytes. *Immunol. Rev.* 122:117–132.
35. Bachmann, M.F., U.H. Rorher, T.M. Kundig, K. Burki, H. Hengartner, and R.M. Zinkernagel. 1993. The influence of antigen organization on B-cell responsiveness. *Science (Wash. DC)*. 262:1448–1451.
36. Hartley, S.B., and C.C. Goodnow. 1994. Censoring of self-reactive B cells with a range of receptor affinities in transgenic mice expressing heavy chains for a lysozyme-specific antibody. *Int. Immunol.* 6:1417–1425.
37. Hartley, S.B., M.P. Cooke, D.A. Fulcher, A.W. Harris, S. Cory, A. Basten, and C.C. Goodnow. 1993. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. *Cell*. 72:325–335.
38. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β2m MHC Class I protein, and $CD8^+$ T cells. *Science (Wash. DC)*. 248: 1227–1230.
39. Nikolic-Zugic, J., and F.R. Carbone. 1990. The effect of mutations in the MHC class I peptide binding groove on the cytotoxic T lymphocyte recognition of the K^b-restricted ovalbumin determinant. *Eur. J. Immunol.* 20:2431–2437.
40. Schulman, M., C.D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.)*. 276:269–270.
41. Coffman, R.L., and I.L. Weissman. 1981. B220: a B cell-specific member of the T200 glycoprotein family. *Nature (Lond.)*. 289:681–683.
42. Leptin, M., M.J. Potash, R. Grutzmann, C. Heusser, M. Shulman, G. Kohler, and F. Melchers. 1984. Monoclonal antibodies specific for murine IgM. I. Characterization of antigenic determinants on the four constant domains of the μ heavy chain. *Eur. J. Immunol.* 14:534–542.
43. Jones, B., and C.A. Janeway. 1981. Cooperative interaction of B lymphoctyes with antigen-specific helper T lymphocytes is MHC restricted. *Nature (Lond.)*. 292:547–549.
44. Ozato, K., N. Mayer, and D.H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533–540.
45. Stall, A.M., and M.R. Loken. 1984. Allotypic specificities of murine IgD and IgM recognized by monoclonal antibodies. *J. Immunol.* 132:787–795.
46. Schuppel, R., J. Wilke, and E. Weiler. 1987. Monoclonal anti-allotype antibody toward Balb/c IgM. Analysis of specificity and site of a V-C crossover in recombinant strain BALB-Igh-V^a/Igh-C^b. *Eur. J. Immunol.* 17:739–741.
47. Bristow, C.L., and P.M. Flood. 1993. T cell antigen receptor immune complexes demonstrating biologic and proteolytic activity. *Int. Immunol.* 5:79–88.
48. Hardy, R.R. 1986. Purification and characterization of monoclonal antibodies. *In* Handbook of Experimental Immunology. 4th ed. D.M. Weir, L.A. Herzenberg, C.C. Blackwell, and L.A. Herzenberg, editors. Blackwell Scientific Publications Ltd., London. 97.1–97.17.
49. Jackson, M.R., E.S. Song, Y. Yang, and P.A. Pederson. 1992. Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in *Drosophila melanogaster* cells. *Proc. Natl. Acad. Sci. USA*. 89: 12117–12121.
50. Weigert, M.G., I.M. Cesari, S.J. Yonkovich, and M. Cohn. 1970. Variability in the lambda light chain sequences of mouse antibody. *Nature (Lond.)*. 228:1045–1047.
51. van der Merwe, P.A., and A.N. Barclay. 1996. Analysis of cell-adhesion molecule interactions using surface plasmon resonance. *Curr. Opin. Immunol.* 8:257–261.
52. Bluestone, J.A., I.F.C. McKenzie, R.W. Melvold, K. Ozato, M.S. Sandrin, S.O. Sharrow, and D.H. Sachs. 1984. Serological analysis of H-2 mutations using monoclonal antibodies. *J. Immunogen.* 11:197–207.
53. Hammerling, G.J., E. Rusch, N. Tada, S. Kimura, and U. Hammerling. 1982. Localization of allodeterminants on $H\text{-}2K^b$

antigens determined with monoclonal antibodies and H-2 mutant mice. *Proc. Natl. Acad. Sci. USA.* 79:4737–4741.

54. Karlsson, R., A. Michaelsson, and L. Mattson. 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods.* 145:229–240.
55. Chaiken, I., S. Rose, and R. Karlsson. 1992. Analysis of macromolecular interactions using immobilized ligands. *Anal. Biochem.* 201:197–210.
56. Schatz, D.G., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell.* 59: 1035–1048.
57. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science (Wash. DC).* 248:1517–1523.
58. Tsubata, T., M. Murakami, and T. Honjo. 1994. Antigen-receptor cross-linking induces peritoneal B-cell apoptosis in normal but not autoimmunity-prone mice. *Curr. Biol.* 4:8–17.
59. Fremont, D.H., W.A. Rees, and H. Kozono. 1996. Biophysical studies of T-cell receptors and their ligands. *Curr. Opin. Immunol.* 8:93–100.
60. Alam, S.M., P.J. Travers, J.L. Wung, W. Nasholds, S. Redpath, S.C. Jameson, and N.R.J. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature (Lond.).* 381:616–620.
61. Nemazee, D. Antigen receptor "capacity" and the sensitivity of self tolerance. *Immunol. Today.* 17:25–29.
62. Ways, J.P., and P. Parham. 1983. The binding of monoclonal antibodies to cell-surface molecules. A quantitative analysis with immunoglobulin G against two alloantigenic determinants of the human transplantation antigen HLA-A2. *Biochem. J.* 216:423–432.
63. Sherman, L.A., S. Chattopadhyay, J.A. Biggs, R.F. Dick, and J.A. Bluestone. 1993. Alloantibodies can discriminate class I major histocompatibility complex molecules associated with various endogenous peptides. *Proc. Natl. Acad. Sci. USA.* 90: 6949–6951.
64. Monroe, J.G. 1996. Tolerance sensitivity of immature-stage B cells: can developmentally regulated B cell antigen receptor (BCR) signal transduction play a role? *J. Immunol.* 156:2657–2660.
65. Starzl, T.E., A. Tzakis, L. Makowka, B. Banner, A. Demetrius, G. Ramsey, R. Duquesnoy, and M. Griffin. 1987. The definition of ABO factors in transplantation: relation to other humoral antibody states. *Transplant. Proc.* 19:4492–4497.
66. Platt, J.L., and F.H. Bach. 1991. Mechanism of tissue injury in hyperacute xenograft rejection. *In* D.K.C. Cooper, E. Kemp, K. Reemtsma, and D.J.G. White, editors. *Xenotransplantation.* Springer Verlag, Berlin. p. 69.