

B Cell Tolerance in Mice Transgenic for Anti-CD8 Immunoglobulin μ Chain

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Summary

To analyze *in vivo* the induction of B cell tolerance against a T cell surface antigen, we generated transgenic mice expressing an anti-CD8.2 μ heavy chain gene. We show that self-specific B cells are efficiently tolerized if they express the membrane-bound form of the transgenic μ chain on their surface but that they can escape tolerization if they express only the secreted form. In the latter, we find an enhanced expression of anti-CD8.2 antibodies after polyclonal B cell activation. As a result, transgenic anti-CD8.2 antibodies bind to the CD8⁺ T cells but they did not induce their elimination. Furthermore, we observed the preferential expression of a limited subset of endogenous light chains with the transgenic μ chain. This suggests a positive or negative selection for particular heavy and light chain combinations in B lymphocytes.

Tolerance to self is one of the key properties of the immune system. To explain the generation and the expansion of self-reactive B lymphocytes, several regulatory mechanisms have been either postulated (1–3) or documented. Self-reactive clones were shown to be clonally deleted (4–6), to be functionally inactivated by clonal anergy (7–12), to be suppressed (13, 14), and to be controlled by virtue of anti-idiotypic network interactions (3).

The low frequency of self-reactive clones in healthy animals with a wide range of autoantibody affinities hampered the *in vivo* analysis of the origin and fate of autoreactive B lymphocytes. These restrictions were overcome by creating transgenic mice whose B cells express autoreactive antibodies of defined affinity (5, 6, 8–12). In the absence of the autoantigen, the transgenic receptors are expressed by nearly all B lymphocytes of these mice. Their fate in the autoreactive situation was analyzed by introducing the autoantigen in matings to appropriate mouse strains. Several recent investigations have been carried out analyzing the reaction of self-reactive B cells to either soluble (6, 8–12) or membrane-bound (5, 6) autoantigens. In these studies, the B cell repertoire of the autoreactive transgenic mice was either nearly monospecific due to the presence of transgenic heavy and light chains (5, 6, 8–12) or showed a high degree of permissiveness in the light chain usage in mice transgenic for the autoreactive heavy chain (12).

We chose as autoantigenic determinant an epitope present on the CD8 α chain expressed on the surface of T cells. Two alleles, CD8.1 and CD8.2, of the CD8 α chain are found in mice. They differ by the exchange of a single amino acid: a methionine residue located in the V-like domain of the CD8.2 α chain is replaced by valine in the CD8.1 α chain (15). Only the CD8.2 allelic form is recognized by a mAb produced by

the hybridoma line 19/178 (16) from which we cloned the antibody genes. To allow a certain degree of variability within the B cell repertoire, we introduced only the functionally rearranged anti-CD8 μ heavy chain into the germline of mice (17). The selection of the anti-CD8.2 self-specificity was then allowed by the combination of the transgenic anti-CD8.2 μ chain with endogenous light chains. To study B lymphocyte tolerance, we introduced the anti-CD8.2 μ transgene into mice expressing either the CD8.1 or the CD8.2 allele. We then examined the expression of the transgene by B lymphocytes, the tolerance status of these mice, the contribution of a functional T cell compartment, the role of membrane immunoglobulin expression, and the influence of polyclonal B cell activation on the tolerization of autoreactive B cells.

Materials and Methods

Mice. C57BL/6 and CD8.1⁺ congenic β 16 strains were kept and bred in the animal breeding facility under specific pathogen-free conditions. The production of the transgenic lines M4, M12, M8Y, M42, and M85 is described (17). The lines were backcrossed to C57BL/6 and β 16.

Antibodies. mAbs to mouse IgM, M41 specific for C μ 3, (18), RS3.1 for IgM^a (19), MB86 for IgM^b, 19/178 for the CD8.2 α chain (16), and RA3.3A1 for B220 (20) were conjugated with FITC (Research Organics, Cleveland, OH) or biotin (longarm biotin; Zymed, San Francisco, CA), according to Hardy (21). FITC-, biotin-, or PE-labeled anti-mouse CD8 (53-6.7) and anti-mouse CD4 (GK1.5) were purchased from Becton Dickinson & Co. (Mountain View, CA); labeled anti-mouse IgG2A sera were from Southern Biotechnology Associates (Heidelberg, FRG). The concentration of IgM^a in mouse serum was determined by ELISA. 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with RS3.1 at 10 μ g/ml in PBS and blocked with 1% BSA.

They were further incubated with serial dilutions of serum or supernatant and an appropriate standard of mouse IgM^a and developed with a phosphatase-coupled goat-anti mouse IgM serum (Southern Biotechnology Associates) and *p*-nitrophenyl phosphate (Sigma Chemical Co., Munich, FRG) as substrate. Plates were washed three times in between each step with PBS, 0.05% Tween 20, 0.1% azide in order to reduce background.

Complement-dependent Cell Lysis. Thymocytes from CD8.2⁺ C57BL/6 or CD8.1⁺ β16 mice were incubated (10⁶/ml, 80–90% CD8⁺) with serial dilutions of culture supernatants of transfectants or of mouse serum in a total volume of 50 μl together with 1/8 volume of Low Tox-M rabbit complement (Cedarlane Labs Ltd., Hornby, ON) at 4°C for 30 min. Dead cells were identified by inclusion staining using trypan blue (Sigma Chemical Co.). If >50% of the CD8.2⁺ target cells were lysed, the sera were scored as positive for containing anti-CD8.2 antibodies. Unspecific background lysis was observed at ~10%.

Flow Cytometry. Flow cytometry was performed as described (17). Briefly, 10⁵ to 10⁶ viable cells were incubated in a total volume of 20 μl with PE-, biotin-, or FITC-coupled antibodies at 4°C for 20 min in PBS buffer containing 3% FCS, 0.1% sodium azide. They were counterstained if required with PE-Streptavidin complex (Southern Biotechnology Associates). Dead cells were identified with propidium iodide and excluded from the analysis. All analyses were performed using a FACScan[®] flow cytometer (Becton Dickinson & Co.), with FACScan[®] and LYSYS software programs.

Isolation of Hybridomas and Analysis of Antibody Specificities. Hybridomas were obtained after fusion of spleen cells with the HAT-sensitive Ig-negative myeloma line X63Ag8.0 after 2 wk of culture in HAT-supplemented Iscove's medium (Gibco Laboratories, Karlsruhe, FRG). Cells were either in vitro stimulated with 10 μg/ml LPS EH100 (Sigma Chemical Co.) before the fusion or in vivo by injecting 5 μg of purified LPS (EH100, kindly provided by Chris Galanos, MPI, Freiburg) directly into the spleen of mice. Spleen cells were then isolated 4 d later and fused to X63Ag8.0 using PEG 1500 (Boehringer Mannheim Corp., Mannheim, FRG). IgM^a-positive clones were identified by ELISA. CD8.2-specific clones were identified by screening supernatants first in a cell ELISA followed by flow cytometric analysis using supernatants of positive clones. Alternatively, supernatants were directly screened by flow cytometry. For the cell ELISA, supernatants were incubated in 96-well U-bottomed microtiter plates in a total volume of 100 μl for 1 h at 4°C with 10⁵ CD8.2⁺ and CD8.1⁺ thymocytes, respectively. Cells were washed three times in PBS, 3% FCS, and bound IgM^a molecules were detected by incubating them with peroxidase-coupled goat anti-mouse IgM antiserum (Southern Biotechnology). Excess antibodies were removed by washing the cells three times in PBS, 3% FCS. Anti-CD8.2 binding was visualized using 2 mM 2,2'-azino-di-[3-ethylbenzthiazolin-sulfonate] (ABTS; Boehringer Mannheim Corp.) in 100 mM citric acid buffer containing 0.03% H₂O₂, pH 4.6. The green color reaction was stopped using azide and the A₄₁₀ was measured using an ELISA reader. Positive clones were identified by comparing the reaction of CD8.2⁺ vs. CD8.1⁺ thymocytes. Their CD8.2 specificity was further corroborated by FACS[®] analysis. Supernatants were incubated with CD8.2⁺ and CD8.1⁺ thymocytes, respectively (see Fig. 3). Specific binding was visualized using FITC- or PE-labeled goat anti-mouse serum (Southern Biotechnology). The binding properties of some of the CD8.2-specific supernatants were further tested for their capacity to inhibit the CD8.2-specific binding of 19/178 using flow cytometry. The actual number of IgM^a-positive hybrids was calculated from dilution plates using the Poisson distribution.

Isoelectric Focusing (IEF) and Western Blotting. For IEF-PAGE 7.0% polyacrylamide gels, including 7 M urea and ampholines ranging from pH 5–7, pH 7–9, and pH 3–10 (Pharmacia Fine Chemicals, Freiburg, FRG), were used. Fresh supernatants (2–4 μl) from overnight cultures of hybridomas were reduced in 9 M urea and 10% β-mercaptoethanol at 37°C for 30 min and then applied onto the gel. Vertical gels were run at 700 V for 16 h. The proteins were transferred electrophoretically to nitrocellulose filters (Schleicher and Schüll, Dassel, FRG). κ light chains were identified using horseradish peroxidase (HRPO)¹-labeled goat anti-mouse κ chain antibodies (Southern Biotechnology) at ~250 ng/ml. Bound HRPO-antibody complexes were visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Corp., Braunschweig, FRG).

Cell-mediated Lysis Assay. K46 (22) and anti-CD8.2 μ/κ-transfected K46pp target cells were labeled with 200 μCi ⁵¹Cr-Na₂CrO₄ (DuPont Co., Wilmington, DE) in 400 μl culture medium followed by four wash steps to remove unincorporated radioactivity. As cytotoxic effector cells, we used the H2b⁺, CD8.2⁺ lines CL96 specific for a P815 antigen presented with H2K^d (23), T112-3.2 (TNP specific, H2K^b restricted), and LD26.10.3-3 (H2D^d specific), kindly provided by Bodo Ortman (MPI, Freiburg). 2 × 10⁵ target cells/well were incubated with or without 0.5% PHA in 96-well plates (NUNC). Effector cells were added in triplicates at different dilutions to a total volume of 200 μl. Samples were incubated for 4 h at 37°C in 7% CO₂. Cells were pelleted by centrifugation (1,200 rpm, 10 min), and released. ⁵¹Cr was analyzed in 100 μl of their supernatants using a gamma counter. Specific lysis was calculated as: percent specific lysis = 100 × (experimental release – spontaneous release)/(detergent release – spontaneous release).

DNA Constructs. The anti-CD8.2 μ chain containing plasmid pCμ19E was described (17). It was used for transfection experiments and it also served as a source for the isolation of the fragments used to generate the transgenic mice. The anti-CD8.2 κ light chain was cloned according to standard protocols (24) from a genomic library made with partially Sau3A-digested and size-selected (10–20 kb) 19/178 DNA ligated to BamHI/EcoRI double-digested EMBL3 DNA (Amersham Corp.). Ligations were packaged in vitro (GigaPack gold; Stratagene, Heidelberg, FRG) and plated on the *Escherichia coli* strain Q359 (*supE*, *hsdR*, φ80^r, P2). κ-positive clones were identified by plaque screening using a 0.9-kb HindIII-XbaI intron fragment of the mouse κ gene as a probe. They were further subcloned into the vector p72hyg, a derivative of the plasmid pSP72 (Promega Corp., Heidelberg, FRG) into which we inserted a hygromycin resistance gene controlled by HSV-tk promoter and polyadenylation sequences in order to allow expression of hygromycin resistance in murine cell lines. The clone, named κ19, contains the anti-CD8.2 κ gene including the 5' and the 3' κ enhancer. It is based on the vector p72hyg and was used in all further experiments.

Transfections. The myeloma line X63Ag8.0 was transfected using lipofection (25) and Lipofectin (Gibco Laboratories, Grand Island, NY). For cotransfection of pCμ19E (G418^R) and κ19 (Hyg^R), equal amounts (20 μg) of supercoiled plasmid DNA were used. Positive transfectants (X63/αCD8.2) were double selected in Iscove's modified DMEM containing 10% FCS (Gibco Laboratories) supplemented with 1 mg/ml G418 (Gibco Laboratories) and 400 μg/ml hygromycin (Sigma Chemical Co.). The B cell lymphoma line K46 was cotransfected with both plasmids by electroporation according

¹ Abbreviation used in this paper: HRPO, horseradish peroxidase.

to a modified protocol (26) using serum-free Iscove's medium instead of buffer and a capacitor set at 450 μ F charged with 250 V. Positive clones (K46pp) were selected with 0.8 mg/ml G418 (Gibco Laboratories) and 300 μ g/ml hygromycin. Their specificity for CD8.2 was tested by analyzing supernatants using flow cytometry.

Results

Expression of the Transgenes. We cloned the heavy and light chain antibody genes from the anti-CD8.2 hybridoma line 19/178 (16). The V region of the anti-CD8.2 heavy chain gene was linked to the c_{μ} constant region and used to generate two different sets of transgenic mice (17). The first set contains an Ig μ transgene ($\mu 19^{sm}$) encoding the membrane (μ^m) and the secreted (μ^s) form of the μ heavy chain (Fig. 1 A, lines M4, M12, and M8Y). The transgenic μ chain is expressed in B cells and forms in combination with endogenous light chain IgM^a molecules. Using allotype-specific antibodies, they can be distinguished from the endogenous IgM^b molecules that are found in the C57BL/6 and $\beta 16$ strains. The tolerization of B lymphocytes requires the surface expression of autoreactive Igs in order to ensure signal transduction via the Ig antigen receptor complex (27, 28). Since the B cells of the $\mu 19^{sm}$ mice express the anti-CD8.2 μ heavy chain in combination with endogenous light chains on their surface (Fig. 2; Table 1), they were used to analyze the role of surface Ig globulin antigen receptor complex. The second set ($\mu 19^s$) has integrated into its germline a truncated version of the μ gene (17) lacking both transmembrane exons $C_{\mu}5$ and $C_{\mu}6$ (Fig. 1 B, lines M42 and M85). As a result, only the secreted form (μ^s) of the transgenic μ chain can be expressed (Fig. 2; Table 1). B cells that can only produce the secreted form of anti-CD8 autoanti-

body molecules should not form a target for the tolerizing mechanism. The $\mu 19^s$ mice were used to test this prediction.

Tolerance in Mice Expressing Surface IgM; No Tolerance in Mice Expressing only Secreted IgM Antibodies. The combination of an appropriate light chain with the transgenic μ chain should eventually result in anti-CD8.2 IgM^a antibodies. In transgenic mice with a CD8.2 background, we expected such autoreactive cells to be deleted or to be inactivated. To follow this process, we analyzed tolerance induction in $\mu 19^{sm}$ mice directly in vivo for all B cells as well as in vitro at a single cell level.

For the in vivo analysis, we tested the presence of anti-CD8.2 IgM^a antibodies in sera of $\mu 19^{sm}$ mice in a complement-dependent cell lysis assay and by flow cytometry (Table 1). In the presence of the CD8.2 antigen, i.e., in a C57BL/6 background, we were unable to detect anti-CD8.2 antibodies as reflected by the lack of cytolytic activity against CD8.2⁺ target cells. In the absence of the CD8.2 antigen, i.e., in a CD8.1⁺ syngeneic $\beta 16$ background, almost all tested sera showed cytolytic activities against CD8.2⁺ thymocytes. The specificity of the IgM^a antibodies for CD8.2 was further verified by a flow cytometric test (Table 1). In $\mu 19^{sm}$ with a CD8.2⁺ C57BL/6 background, we were unable to detect anti-CD8.2 serum antibodies. This could be due to their complete adsorption to CD8.2 molecules present on the surface of T cells. This, however, is unlikely, since we found IgM^a anti-CD8.2 antibodies in the sera of 6 of 16 $\mu 19^s$ mice with a CD8.2⁺ C57BL/6 background, (Table 1). The failure to detect anti-CD8.2 antibodies in the presence of the CD8.2 antigen suggests that B lymphocytes of $\mu 19^{sm}$ transgenic mice are tolerized.

Our analysis of the serum antibody reactivities against CD8 surface molecules does not allow the differentiation between clonal deletion or inactivation of autoreactive B cells. In addition, anti-CD8.2 autoantibodies present in low amounts would not have been detected. We therefore investigated at a single cell level the proportion of CD8.2-specific B cells within the splenic B lymphocyte population. Hybridomas were generated from in vitro LPS-activated spleen cells derived from the transgenic lines and tested for the expression of the transgenic μ^s chain and for their anti-CD8.2 specificity (Table 2; Fig. 3, A and B). The expression of the transgenic μ chain before light chain rearrangement could have an influence on the selection of endogenous light chains by the B cells. We therefore also analyzed the frequency of CD8.2-specific hybridomas derived from unselected B cells by fusing spleen cells from C57BL/6 or $\beta 16$ mice to a transfectant of the myeloma line X63Ag8.0 expressing the transgenic anti-CD8.2 μ chain (X63/ $\mu 19$, fusion A). CD8.2-specific hybridomas were found in 1 of 200 hybridomas (1/100 to 1/480, fusion A). This result was then compared to the frequency of transgenic anti-CD8.2 hybridomas derived from CD8.1⁺ $\beta 16$ $\mu 19^{sm}$ spleen cells (Table 2, fusion B). Here, we also found in 1 of 200 (1/60 to 1/313) clones reconstitution of the original anti-CD8.2 specificity (fusion B). This comparison shows that the frequency for anti-CD8.2 antibodies was almost identical in both types of fusions using either transgenic B cells from CD8.1⁺ $\mu 19^{sm}$ mice (fusion B) or B cells

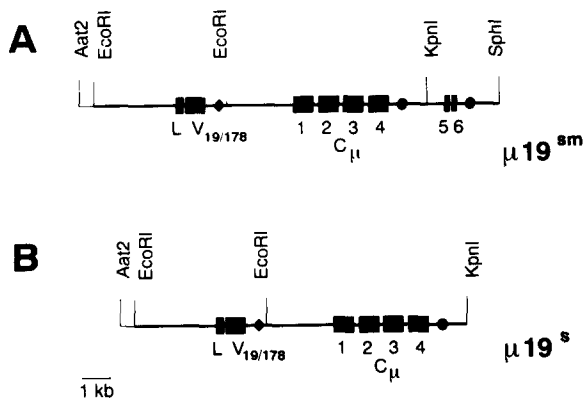


Figure 1. Map of the transgenes. The $\mu 19^{sm}$ transgenic lines M4, M12, and M8Y were generated by microinjecting nuclei of fertilized eggs with the Aat2-SphI fragment shown in A (17). The fragment carries the complete anti-CD8.2 μ chain. Line M8Y has the transgene integrated on its Y chromosome. The $\mu 19^s$ lines M42 and M85 carry the Aat2-KpnI fragment shown in B. Here, the transmembrane exons of the μ chain are lacking. It is therefore expressed only in the form of secreted IgM molecules. (Thin line) Plasmid (pUC19)-derived sequences; (thick line) murine genomic DNA. (■) Coding sequence of the L, V, $C_{\mu}1$, $C_{\mu}2$, $C_{\mu}3$, $C_{\mu}4$, $C_{\mu}5$, $C_{\mu}6$, exons, (●) polyadenylation sites, (◆) heavy chain intron enhancer.

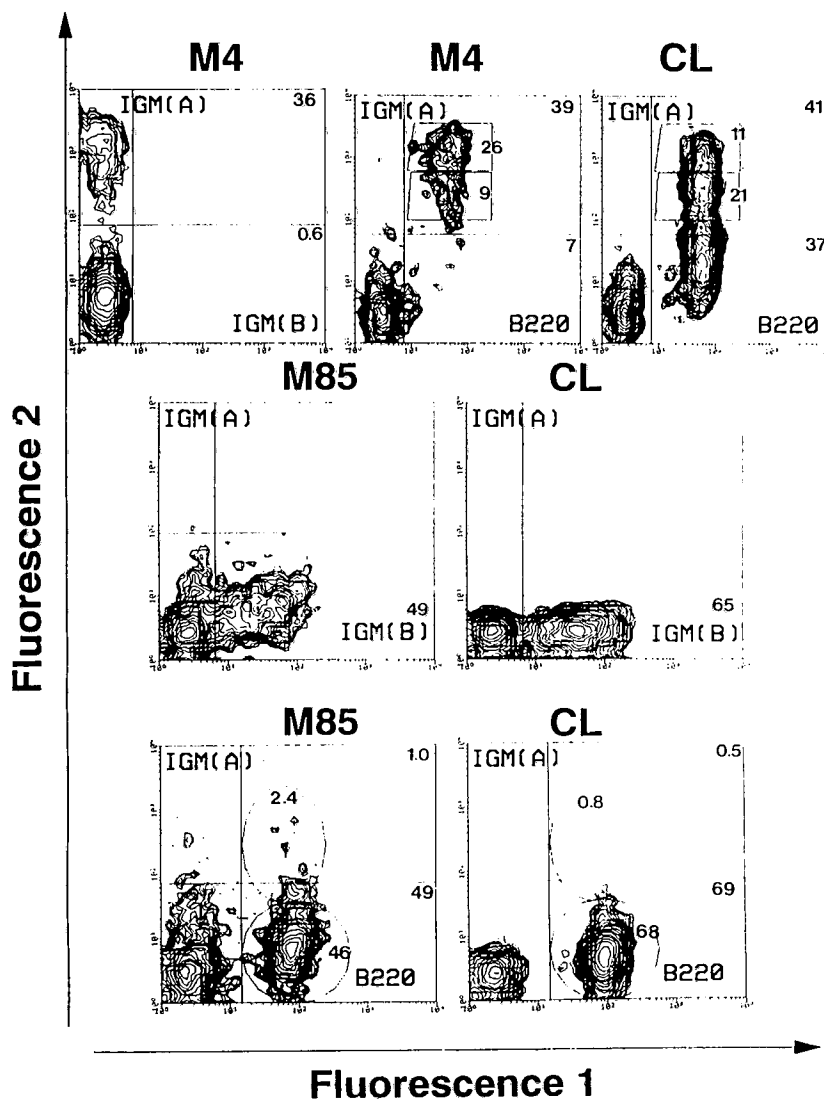


Figure 2. Expression of the transgenes in spleen cells. For flow cytometry, splenocytes were double stained with either RS3.1 (transgenic IgM^a in fluorescence 2; PE) and M86 (endogenous IgM^b molecules in fluorescence 1; FITC) or with RS3.1 and RA3.3A1 detecting the B cell marker B220 (fluorescence 1; FITC). Cells of a $\mu 19^{\text{sm}}$ transgenic line (M4) and of a $\mu 19^{\text{s}}$ transgenic line (M85) were compared to control mice (CL; all mice were 6–8 wk old). Flow cytometry was carried out as described in Materials and Methods using a large forward/side-ward scatter gate during acquisition and a small gate characteristic for B lymphocytes during analysis. The relative percentage of cells is given in each quadrant or region. (Top) M4 spleen cells (IgM^a-IgM^b+ C57BL/6 background) stained for IgM^a/IgM^b or IgM^a/B220 compared to an heterozygous IgM^a+ / IgM^b+ control mouse (CL) obtained from M4 \times (C57BL/6 \times BALB/c)F₁. The $\mu 19^{\text{sm}}$ transgene in M4 is expressed by most of the B220⁺ cells (26% IgM^ahiB220⁺) resulting in a strong allelic exclusion for endogenous IgM^b (36% IgM^a+, 0.6% IgM^b+). This is also reflected by a strong reduction in the IgM^ahiB220⁺ population (IgM^ahiB220⁺/IgM^alo/B220hi = 26/9 in M4; 11/21 in CL). The IgM^a-B220⁺ (37%) population represents B cells expressing the IgM^b allele (data not shown). (Middle and bottom) Comparison between M85 ($\mu 19^{\text{s}}$) and a control animal (CL; IgM^a-IgM^b+ in a C57BL/6 background). The product of the $\mu 19^{\text{s}}$ transgene lacks the C μ 5 and C μ 6 transmembrane exons (Fig. 1 B) preventing its membrane insertion. Due to the lack of allelic exclusion (49% IgM^b+ cells in M85), it is coexpressed with endogenous μ chains in the form of mixed molecules. They can be found on the surface of B cells using the IgM^b membrane domains as anchor (2.4% IgM^a+B220⁺ cells in M85).

from normal mice fused to the X63/ $\mu 19$ transfectant (fusion A). Therefore, the expression of the anti-CD8.2 μ chain in transgenic mice does not result in the increased formation of anti-CD8.2-specific B cells. Anti-CD8.1-specific hybridomas were not found when supernatants were tested for their ability to bind to CD8.1⁺ thymocytes.

As was already expected from the analyses of the transgenic sera, mice expressing the antigenic CD8.2 allele proved to be tolerant. Among >6,700 tested hybridomas from the $\mu 19^{\text{sm}}$ lines M4, M12, and M8Y, none was found to be CD8.2 specific (fusion C). The >30-fold reduction of CD8.2-specific B cells suggests that in the presence of the antigenic CD8.2 α chain even rare splenic anti-CD8.2 B cells are efficiently counterselected. Some of our $\mu 19^{\text{sm}}$ transgenic lines like M4 and M12 suffer from a severe blockade in thymocyte development (17). Their maturation is arrested before the stage of CD4⁺8⁺ double-positive cells at the stage of IL-2R^{hi}HSA^{hi} thymocytes (our unpublished results). As a consequence, the more mature T cell populations in the thymus are highly reduced in size (10–60-fold). This leads to an ap-

proximately fivefold reduction of their splenic T cells. This T cell deficiency, however, does not influence the tolerization of B cells. A comparison between lines suffering from the thymocyte differentiation block (M4, M12) and a different $\mu 19^{\text{sm}}$ line with a normal T cell complement (M8Y) revealed no difference in the manifestation of B cell tolerance (Table 2, fusions C1–C6 and C9 for M4 and M12; fusions C7 and C8 for M8Y).

In contrast to the $\mu 19^{\text{sm}}$ lines (M4, M12, M8Y), some mice of the $\mu 19^{\text{s}}$ lines M42 and M85 were capable of synthesizing anti-CD8.2 serum antibodies even in the presence of the antigen (Table 1). Their majority, however, showed no detectable anti-CD8.2 activities in their sera. This difference between individual $\mu 19^{\text{sm}}$ mice may be due to the lacking allelic exclusion of endogenous heavy chain expression in μ^{s} transgenic mice (17, 29). In both $\mu 19^{\text{s}}$ lines, the B cells coexpress transgenic $\mu 19^{\text{s}}$ chains together with endogenous μ chains (17). As a consequence, in some animals the serum concentration of anti-CD8.2 antibodies may be below our detection limit. To test their tolerance status at

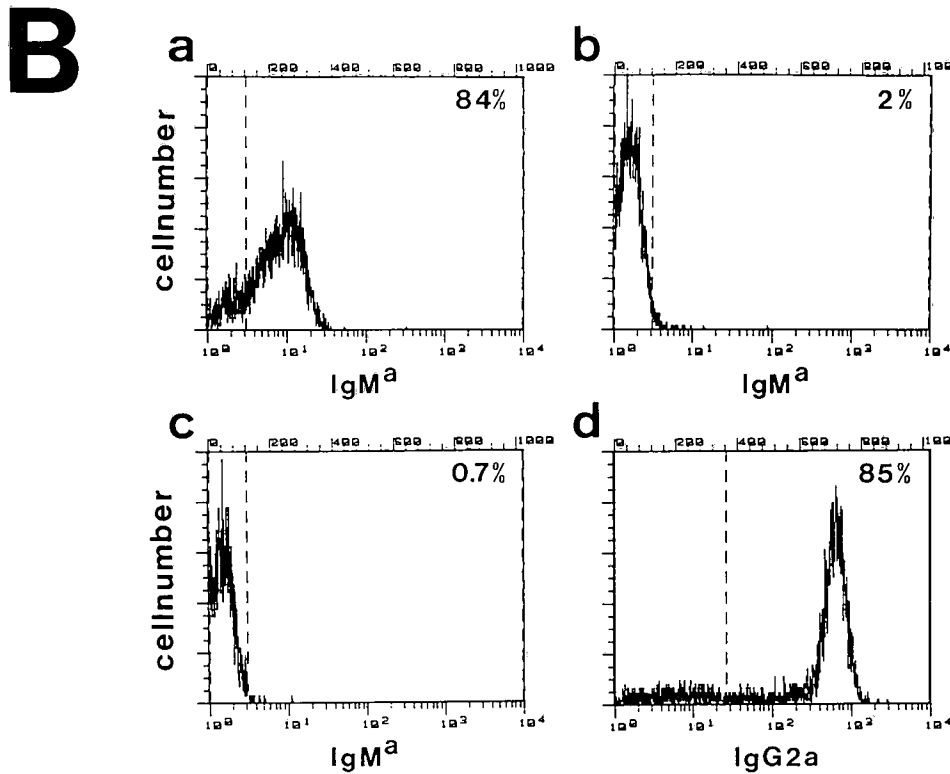
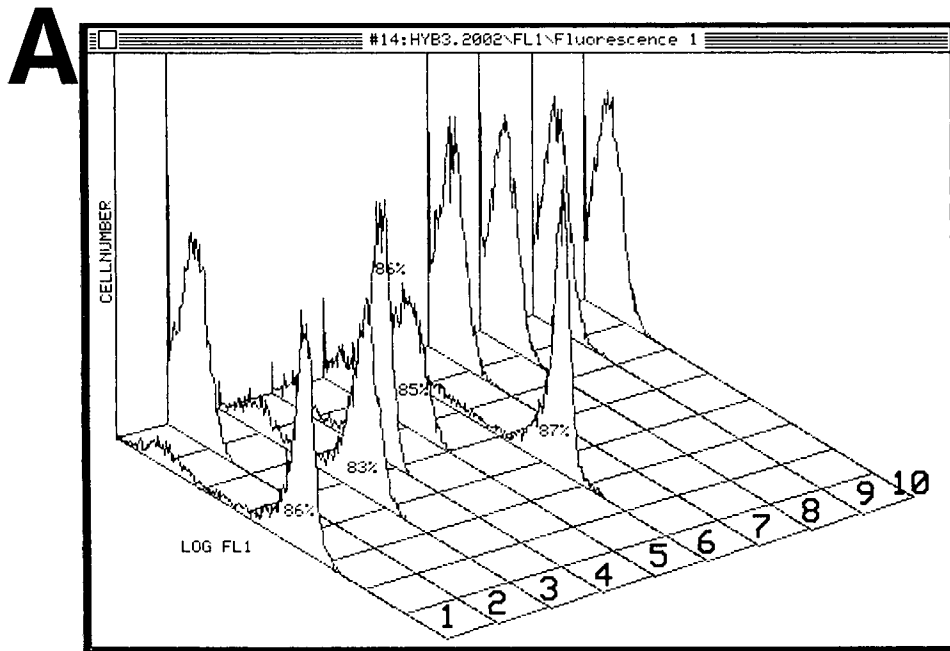


Figure 3. Flow cytometric analysis of hybridoma supernatants for CD8.2 specificity. (A) CD8.2⁺ thymocytes from C57BL/6 mice (lanes 1–5) and from the CD8.1⁺ congenic strain β 16 (lanes 6–10) were stained with the FITC-labeled CD8.2-specific IgG2A antibody 19/178 (lanes 1 and 7), with the FITC-labeled IgM^a-specific antibody RS3.1 (lane 2) and with the CD8-specific antibody 53-6.7 (lane 6) as controls. CD8.2-specific, IgM^a⁺ hybridoma supernatants of the fusions A (A49, lanes 3 and 8), D (D39, lanes 4 and 9), and B (B107, lanes 5 and 10) were first incubated with thymocytes and then counterstained with FITC-labeled RS3.1. The supernatants bind only to CD8.2⁺ but not to CD8.1⁺ thymocytes. (B) Thymocytes from CD8.2⁺ C57BL/6 mice were first incubated with B107 (see legend to A) supernatant and then counterstained with the IgM^a-specific FITC-labeled antibody RS3.1 (a). In b, thymocytes were preincubated with an excess of unlabeled 19/178, then with B107 supernatant, and counterstained with RS3.1. The preincubation with 19/178 inhibits binding of the B107 supernatant. FITC-labeled RS3.1 alone (c) does not bind to thymocytes. (d) Positive control for 19/178 binding: cells were first incubated with 19/178 and then counterstained with FITC-labeled goat anti-mouse γ 2a antibodies.

a single cell level, we therefore generated hybridomas from both μ 19^s lines using spleen cells that were activated with LPS before the fusion. CD8.2-specific clones were obtained in both lines at a frequency of 1/858 IgM^a-positive clones (Table 2, fusion D).

Our hybridoma analysis suggested that the μ 19^s mice were not tolerant. Therefore, we were interested to determine whether it would be possible to increase the in vivo

levels of anti-CD8.2 antibodies. We activated their B cells polyclonally by injecting LPS either intravenously or directly into the spleen. After 4 d, their splenocytes were analyzed by flow cytometry for CD8⁺ T cells with IgM molecules bound to their surface. Three control littermates, six μ 19^s mice of the M42 line, seven μ 19sm mice with a CD8.2⁺ C57BL/6 background (four mice of the M4 line and three of the M8Y line), and two μ 19sm mice from the M4 line

Table 1. Expression of Transgenic μ Chains

Transgene line	Age	Cells/spleen	IgM ^a serum titer	Percent IgM ^{a+} cells/spleen	Anti-CD8.2 serum specificity in:	
					Complement lysis [*]	FACS [†]
	<i>wk</i>	$\times 10^7$	$\mu\text{g/ml}$			
$\mu 19^{\text{sm}}$						
M4 (B/6)	4–16	2.0 [§]	1–8	36	0/4	0/49
M4 ($\beta 16$)	8, 12	4.2 [†]	2, 7	32	4/4	20/23
M8Y (B/6)	10, 14	5.8 [†]	4, 6	28	ND	ND
$\mu 19^{\text{s}}$						
M42 and M85 (B/6)	4–13	5.5 ^{**}	3–14	2	1/9	6/16
Control	4–17	8.2 [‡]	0	0	ND	ND

* Anti-CD8.2 lytic activity in sera was tested as described in Materials and Methods using CD8.1⁺ and CD8.2⁺ thymocytes as target cells.

† Anti-CD8.2 antibodies present in sera were analysed by flow cytometry using CD8.1⁺ and CD8.2⁺ thymocytes as target cells as described in Materials and Methods.

§ Six mice were analyzed.

|| Number of positive/number of tested mice.

† Two mice were analyzed.

** Seven mice were analyzed.

‡ In total, 12 control littermates were analyzed.

with a CD8.1⁺ $\beta 16$ background were tested. All analyses gave very similar results, and the data of a representative analysis are shown in Fig. 4. All CD8⁺ splenic T cells of the $\mu 19^{\text{s}}$ line M42 had IgM^a molecules bound on their surface (Fig. 4 A, M42/BL6). The bound IgM was composed from hybrid molecules formed by transgenic (IgM^a, Fig. 4 A) and endogenous (IgM^b, Fig. 4 B) μ chains. This result supports our observation that $\mu 19^{\text{s}}$ mice are not tolerant. In the $\mu 19^{\text{sm}}$ line M4, we did not observe the induction of transgenic high affinity anti-CD8.2 antibodies after injection of LPS. This suggests that the tolerance state of the B cells in $\mu 19^{\text{sm}}$ mice cannot be broken by polyclonal B cell activation.

The Direct Interaction of Anti-CD8.2 Antibodies on the Surface of B Cells with CD8 on Cytotoxic T Cells Does Not Induce Cytolysis. A potential mechanism of eliminating the anti-CD8.2-reactive B cells would be their cytolysis. Direct interactions between the surface anti-CD8.2 antigen receptor on B cells and CD8 molecules on cytotoxic T cells could possibly bring both cell types into close contact, induce cytotoxic effector functions, and result in a killing reaction mediated by the CD8⁺ cytotoxic T cells. We tried to investigate this potential elimination mechanism by mimicking the reaction in vitro using a cell-mediated lympholysis assay. To obtain a B lymphocyte population that homogeneously expresses the anti-CD8.2 IgM^a antigen receptor on the membrane, we cotransfected the B lymphoma line line K46 (22) with vectors encoding the anti-CD8.2-specific μ and κ chain. An anti-CD8.2 IgM^a-positive K46 transfectant (K46pp) was labeled with ⁵¹Cr and coincubated with two different CD8.2⁺ CTL

lines (CL96 and T112.3-2). Cytotoxicity was then analyzed with varying effector to target cell ratios and compared to lectin (PHA)-mediated killing that can induce the approximation of CTL and target cells also in the absence of antigen. None of the tested cytotoxic T cell lines was able to lyse the transfectant in the absence of PHA (Fig. 5, A and B). As a control for the antigen-specific killing, we analyzed the cytotoxic activity of the H2D^d-specific line LD26.10.3-3 against the H2D^d MHC class I molecules expressed on the surface of the K46pp transfectant. Here, both antigen-specific and lectin-mediated killing was observed (Fig. 5 C). Interestingly, we found a slight reduction in ⁵¹Cr release for the K46pp transfectant by changing the E/T cell ratios and by varying the incubation time in all lectin-mediated killing assays as compared to the untransfected K46 controls. The abrogation of effector function has been reported before by blocking CD8 surface molecules using soluble 19/178 anti-CD8.2 antibodies (30). The resistance of K46 transfectants to killing suggests that also membrane-bound anti-CD8 antibodies may cause inhibition of the cytotoxic activity. In analogy, anti-CD8 B cells are probably not eliminated by cytotoxic activities of their target, the CD8⁺ T cells.

Light Chain Usage. Since the frequency of CD8.2-specific hybridomas was in the range of 1/200 to 1/860 and, in comparison to other immunoglobulin heavy chain transgenic lines (12, 29, 31), and A. Iglesias, unpublished results), rather low, we expected that the transgenic μ heavy chain would be able to combine only with a restricted subset of endogenous light chains to form CD8.2-specific antibodies. We therefore ex-

Table 2. Analysis of Hybridomas

Fusion	Spleen cells	Transgene	Myeloma partner	IgM ⁺ *	CD8.2 specific [†]	Anti-CD8.2 frequency
A	Control	None	X63/μ19			
A1 [§]	C57BL/6	None	X63/ μ 19	480	1	1/480
A2 [§]	C57BL/6	None	X63/ μ 19	2,040	5	1/408
A3 [§]	β 16	None	X63/ μ 19	1,300	13	1/100
A	C57BL/6,β16	None	X63/μ19	3,820	19	1/201
B	CD8.1⁺	μ19sm	X63Ag8.0			
B1	M4- β 16	μ 19 sm	X63Ag8.0	66	1	1/66
B2 [§]	M4- β 16	μ 19 sm	X63Ag8.0	500	4	1/125
B3 [§]	M4- β 16	μ 19 sm	X63Ag8.0	1,250	4	1/313
B	μ19sm-β16	μ19sm	X63Ag8.0	1,816	9	1/201
C	CD8.2⁺	μ19sm	X63Ag8.0			
C1 [§]	M4-C57BL/6	μ 19 sm	X63Ag8.0	1,100	0	
C2 [§]	M4-C57BL/6	μ 19 sm	X63Ag8.0	840	0	
C3 [§]	M12-C57BL/6	μ 19 sm	X63Ag8.0	540	0	
C4 [§]	M12-C57BL/6	μ 19 sm	X63Ag8.0	170	0	
C5 [§]	M12-C57BL/6	μ 19 sm	X63Ag8.0	1,000	0	
C6 [§]	M8Y-C57BL/6	μ 19 sm	X63Ag8.0	2,160	0	
C7 [§]	M8Y-C57BL/6	μ 19 sm	X63Ag8.0	360	0	
C8 [§]	M8Y-C57BL/6	μ 19 sm	X63Ag8.0	440	0	
C9	M4-C57BL/6	μ 19 sm	X63Ag8.0	148	0	
C	μ19sm-C57BL/6	μ19sm	X63Ag8.0	6,758	0	<1/6,758
D	CD8.2⁺	μ19^s	X63Ag8.0			
D1 [§]	M42-C57BL/6	μ 19 ^s	X63Ag8.0	980	2	1/440
D2 [§]	M85-C57BL/6	μ 19 ^s	X63Ag8.0	2,450	2	1/1,225
D	μ19^s-C57BL/6	μ19^s	X63Ag8.0	3,430	4	1/858

* Calculated by counting the IgM⁺ wells on dilution plates.

† Obtained by FACS[®] analysis using supernatants from IgM⁺ wells.

§ Spleen cells were stimulated for 3 d in vitro with 10 μ g/ml LPS.

|| Spleen cells were stimulated in vivo by injecting 5 μ g of LPS into the spleen.

amined the heterogeneity of these light chains by one-dimensional IEF in polyacrylamide gels. A representative result of this analysis is presented in Fig. 6. The κ light chains of the six different, CD8.2-specific, hybridomas derived from three independent fusions (fusions A, B, and D; compare with Table 2) show an identical migration pattern (Fig. 6A). This result, and the lack of any detectable microheterogeneity, suggest that these hybridomas produce one κ light chain that allows, in combination with the transgenic heavy chain, restoration of anti-CD8.2 specificity. Interestingly, the same specific light chain is also used in CD8.2-specific hybridomas obtained from unselected B cells fused to the X63/ μ 19 transfected myeloma line. Therefore, only one type of κ light chain, which has a different isoelectric point than the κ chain of the original anti-CD8.2 hybridoma 19/178, allows restoration of the

original CD8.2 specificity. We also analyzed the κ light chain usage in other hybridomas that did not restore the original anti-CD8.2 specificity. Comparing 31 IgM^s/ κ ⁺ hybridomas derived from 4 μ 19 CD8.1⁺ transgenic mice, we observed seven κ light chains with an identical migration pattern in the IEF-PAGE (Fig. 6B), four additional pairs with an identical but different migration behavior, and 20 other light chains each having an individual isoelectric point (data not shown). A comparable analysis of κ light chains derived from control hybridomas where normal spleen cells were fused with the X63/ μ 19 transfected myeloma did not reveal large groups with an identical IEF-PAGE migration pattern (Fig. 6C). Based on these results, we conclude that in transgenic hybridomas the μ chain is preferentially expressed with particular subsets of κ light chains.

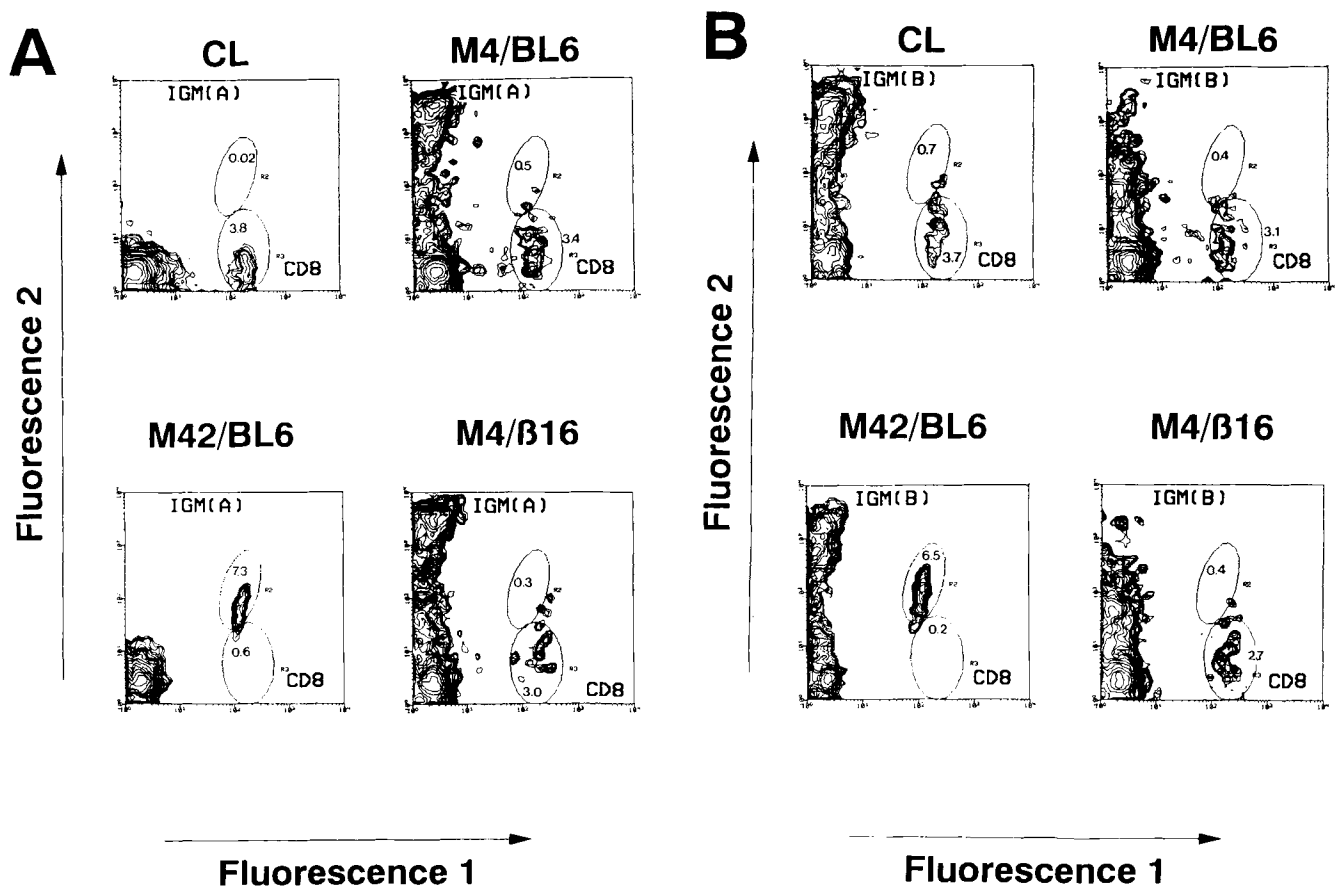


Figure 4. Polyclonal B cell activation induces anti-CD8.2 antibodies that bind to surface CD8 molecules in $\mu 19^s$ mice. Mice were injected intrasplenically with 5 μg of LPS. Spleen cells were isolated after 4 d and analyzed by flow cytometry. Lymphocytes were gated according to their forward/sideward scatter distribution. The percentage of the individual splenocyte subsets are given in each region. (A) RS3.1 (anti-IgM α , fluorescence 2; PE) and 53-6.7 (anti-CD8, fluorescence 1; FITC) double-stained cells from a control littermate (CL), the $\mu 19^{\text{sm}}$ line M4 (M4/BL6), the $\mu 19^s$ line M42 (M42/BL6), all in the CD8.2 $^+$ C57BL/6 background, and from M4 in the congenic CD8.1 $^+$ $\beta 16$ background (M4/ $\beta 16$). In contrast to all other lines, the CD8 $^+$ cells from M42 show a shift in fluorescence 2 (IgM α^+) from R3 to R2 due to the binding of transgenic antibodies to CD8 $^+$ T cells. (B) The same spleen cells double stained with MB86 for IgM β (fluorescence 2; PE) and 53-6.7 for CD8. In the $\mu 19^s$ line M42, the transgenic μ^s chains form mixed IgM molecules together with endogenous μ^b chains. Binding of CD8.2-specific antibodies to CD8 surface molecules leads to the shift of CD8 $^+$ T cells towards fluorescence 2.

Discussion

Tolerance Induction in $\mu 19^{\text{sm}}$ Mice. Tolerance may be imposed on B lymphocytes by several mechanisms (4, 5, 8, 12, 13, 32). Recent investigations demonstrated that tolerance induction can be mediated by clonal deletion as well as by clonal anergy (5–12). The induction can take place during early stages of B cell development when pre-B cells mature into sIg $^+$ small B cells (5, 6) as well as in mature B lymphocytes (9). In these transgenic mouse models, most B lymphocytes carry the autospecific s-Ig receptor molecule.

By introducing only the μ heavy chain gene of an antibody with anti-CD8.2 specificity into the germline of mice, we chose a slightly different approach that allows the selection of endogenous light chains during B lymphocyte maturation (17). The original antibody is specific for a single epitope of a membrane-bound protein antigen and recognizes specifically the CD8.2 allele encoded by the CD8 α chain (16). To analyze the B cell tolerance status of our transgenic

mice, we made use of the allelic differences between CD8.1 and CD8.2. We expected to find anti-CD8.2 antibodies at low frequencies resulting from the combination of the transgenic heavy chain with appropriate endogenous light chains. As a control, we generated hybridomas from fusions between normal spleen cells and a $\mu 19^{\text{sm}}$ heavy chain transfectant (X63/ $\mu 19$) of the myeloma line X63Ag8.0. 1 in 200 κ light chains was found to restore the original anti-CD8.2 specificity (Table 2). A similar result was obtained with hybridomas generated from $\mu 19^{\text{sm}}$ mice in a CD8.1 $^+$ $\beta 16$ background. In the absence of the antigenic CD8.2 α chain, 1 in 200 hybridomas produced anti-CD8.2 antibodies using the transgenic μ chain. This finding was also reflected by the presence of transgenic CD8.2-specific serum antibodies. In mice expressing the antigenic CD8.2 α chain, we found no anti-CD8.2 serum antibodies nor were we able to detect CD8.2-specific hybridomas among the 6,700 clones analyzed from

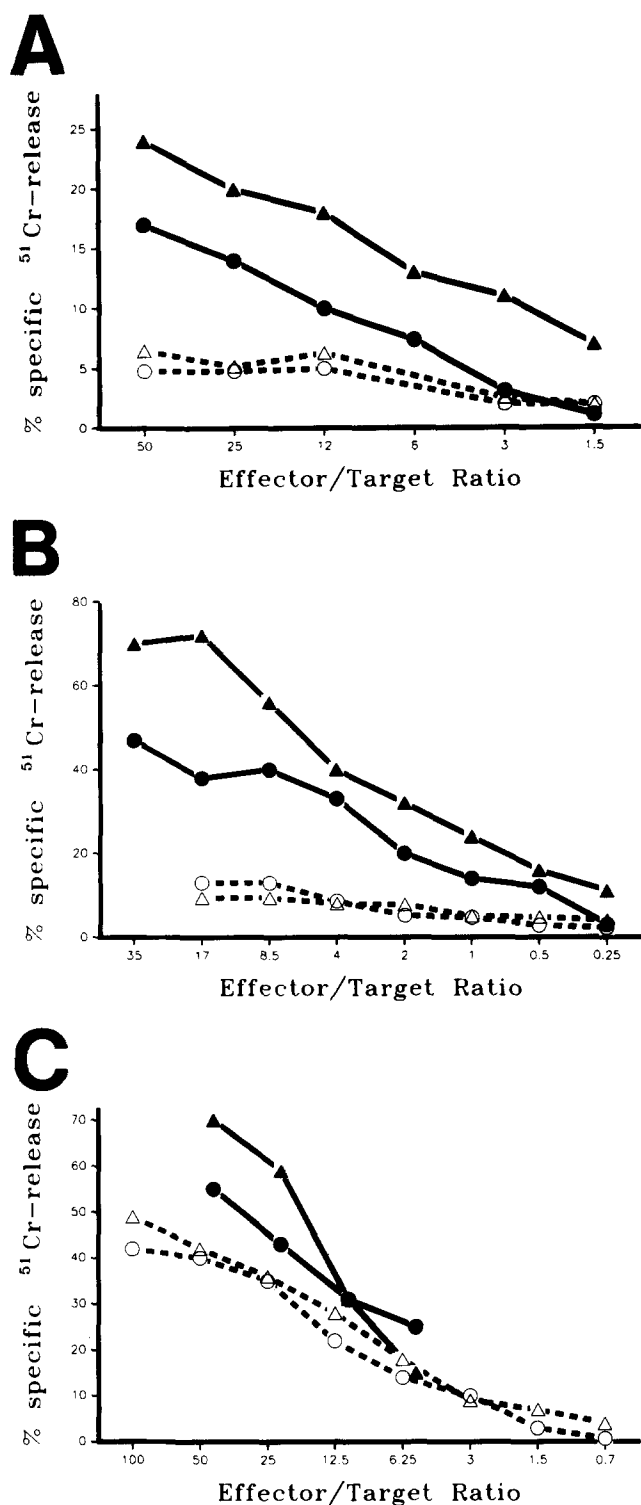


Figure 5. Binding to CD8.2 does not induce cytotoxicity of anti-CD8.2-specific B lymphoma cells. The H2d+ B lymphoma line K46 was transfected with the anti-CD8.2 μ and κ chains (K46pp [Δ and \blacktriangle]) and compared to the parental K46 line (\circ and \bullet) in a cell-mediated lympholysis assay as described in the Materials and Methods. The following CTL were used: (A) CL96, H2Kd-restricted anti-P815; (B) T112.3-2, H2Kb-restricted anti-TNP; (C) LD26.10.3-3, anti-H2Dd. K46pp and K46 target cells were labeled with ^{51}Cr and incubated at different ratios with the CTL effector cells without (dashed lines) or with the addition of the lectin PHA (solid lines). Specific killing was only observed for LD26 recognizing H2Dd.

the $\mu 19^{\text{sm}}$ transgenic mice. These data show that in a CD8.2+ background, the $\mu 19^{\text{sm}}$ mice are tolerant. At present, we cannot conclusively distinguish between clonal anergy and clonal deletion of the autoreactive B cells. Since our detection assay involves the generation of hybridomas from transgenic spleen cells that were polyclonally activated by LPS stimulation before the fusion, LPS refractile cells would have escaped our detection. For anergic B cells, it previously has been shown that within the first 3 d of stimulation with LPS they may respond only poorly in terms of Ig secretion although they are capable of proliferation (11, 33). An elongation of the incubation period for another 4 d results in almost equivalent clonal response as compared to controls. Using μ heavy chain transgenic mice with an anti-ssDNA specificity, Erikson et al. (12) were able to demonstrate that anergized B cells could be efficiently used to generate ssDNA-specific transgenic hybridoma lines after in vivo LPS treatment. For $\mu 19^{\text{sm}}$ transgenic mice, in vivo LPS treatment does result in neither a detectable synthesis of high affinity anti-CD8.2 serum antibodies nor in the generation of anti-CD8.2 hybridomas (Table 2; Fig. 4). Using Ig transgenic mice as a model for B cell tolerance, there seems to be a difference between monovalent and repetitive self-antigens as tolerogens. Monovalent self-antigens, like soluble proteins, seem to induce clonal anergy (8–11, 28, 32, 33) or seem to have no tolerizing capacity (6, 34), whereas repetitive self-antigens, which also can be presented in the form of membrane-bound molecules, appear to result in clonal deletion of autoreactive B cells (5, 6, 33, 34). Since the CD8 molecule is expressed as a membrane protein, our results suggest a deleting mechanism acting on the anti-CD8.2 B cells in the presence of the antigenic CD8.2 α chain. This conclusion is also supported by other systems where anergized B cells were shown to be rescued in hybridomas (12).

We also asked whether the immune system would be able to suppress the expression of transgenic autoantibodies when the transmembrane exons of the μ transgene were deleted. The resulting construct allows expression only of the secreted form of the transgenic μ chain. Thus, in the presence of the CD8.2 antigen, the B cells should not be a target for tolerance induction. Searching for anti-CD8.2 serum antibodies, we were unable to find them in the majority of the tested $\mu 19^{\text{s}}$ mice. This observation is most probably due to the lack of allelic exclusion in transgenic μ^{s} lines (17, 29), which leads to coexpression of transgenic and endogenous μ chains in B cells. As a consequence, in some mice the avidity of such hybrid anti-CD8.2 IgM^{ab} antibodies may have been lowered. They may have, therefore, remained undetected. However, our analysis of their tolerance status at a single cell level proved them to be nontolerant: hybridomas generated from spleen cells of M42 and M85 mice revealed in both cases anti-CD8.2-specific clones (1/858 hybridomas; Table 2). Their frequency is about fourfold lower if compared to the $\mu 19^{\text{sm}}$ lines. Again, this lower anti-CD8.2 antibody frequency may be ascribed to the problem of detecting hybrid anti-CD8.2 antibodies. The nontolerance of $\mu 19^{\text{s}}$ mice was further supported by in vivo stimulation of their splenocytes by LPS injection. This led to an increase of the secreted anti-CD8.2

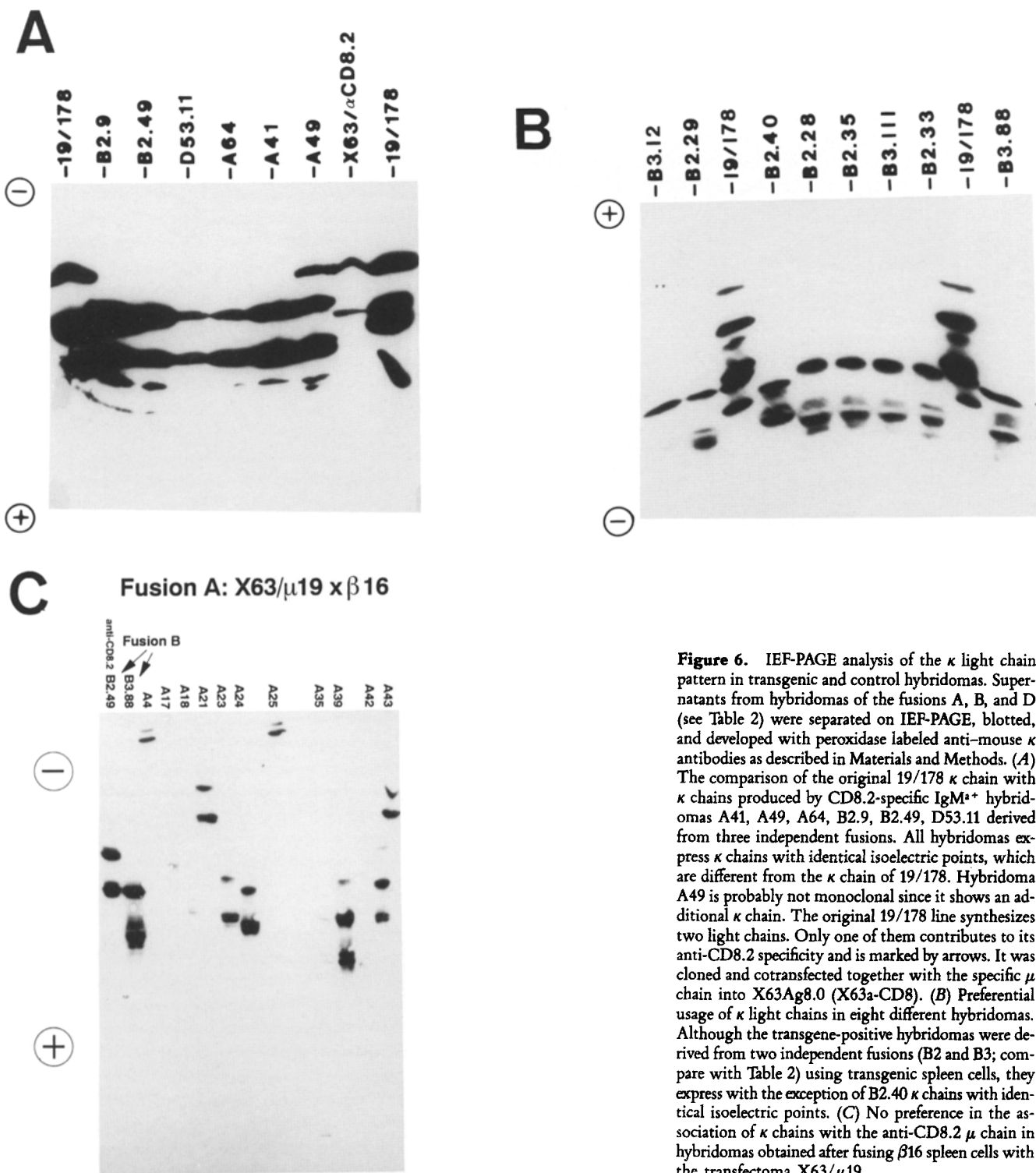


Figure 6. IEF-PAGE analysis of the κ light chain pattern in transgenic and control hybridomas. Supernatants from hybridomas of the fusions A, B, and D (see Table 2) were separated on IEF-PAGE, blotted, and developed with peroxidase labeled anti-mouse κ antibodies as described in Materials and Methods. (A) The comparison of the original 19/178 κ chain with κ chains produced by CD8.2-specific IgM^{a+} hybridomas A41, A49, A64, B2.9, B2.49, D53.11 derived from three independent fusions. All hybridomas express κ chains with identical isoelectric points, which are different from the κ chain of 19/178. Hybridoma A49 is probably not monoclonal since it shows an additional κ chain. The original 19/178 line synthesizes two light chains. Only one of them contributes to its anti-CD8.2 specificity and is marked by arrows. It was cloned and cotransfected together with the specific μ chain into X63Ag8.0 (X63a-CD8). (B) Preferential usage of κ light chains in eight different hybridomas. Although the transgene-positive hybridomas were derived from two independent fusions (B2 and B3; compare with Table 2) using transgenic spleen cells, they express with the exception of B2.40 κ chains with identical isoelectric points. (C) No preference in the association of κ chains with the anti-CD8.2 μ chain in hybridomas obtained after fusing β 16 spleen cells with the transfectoma X63/ μ 19.

antibodies, which were found to be bound to the CD8 surface molecules of all CD8⁺ T cells (Fig. 4). The binding did not eliminate the CD8⁺ T cells, as it was observed when the original anti-CD8.2 IgG2A antibody 19/178 (35) recognizing the same epitope was injected into mice. Since many

inbred mouse strains have very low levels of complement activity (36) and because injected IgM anti-T cell antibodies are much less efficient in depleting T lymphocytes than many IgG antibodies (37), the in vivo synthesized transgenic anti-CD8.2 IgM^a antibodies may simply bind to the CD8 sur-

face molecules without affecting the size of CD8⁺ T cell population. For the $\mu 19^{\text{am}}$ line M4, we did not observe the synthesis of high affinity anti-CD8.2 antibodies after LPS injection. We cannot, however, rule out an increase in transgenic low affinity anti-CD8 antibodies. Those may be either present at low concentrations or they may have been competed out by the labeled high affinity anti-CD8 antibodies during FACS[®] analysis. In the case of the $\mu 19^{\text{a}}$ mice, their presence may have been obscured by the expression of high affinity anti-CD8.2 antibodies.

Role of Cytotoxic T Cells. Tolerance induction of B cells has been demonstrated to occur both at early stages of B lymphocyte development during the transition from pre-B to B cells (6) as well as for mature B cells (9). One might envisage that B cells autoreactive for self-determinants expressed on cytotoxic T cells are controlled by CTL effector functions. Here, we could demonstrate that tolerance is efficiently imposed on B cells even if their T cell compartment is largely deficient. We also could show that the surface expression of the anti-CD8.2 Ig receptor on the B lymphoma line K46 does not mediate specific killing in vitro. Both results argue against a CTL-dependent control of the autospecific anti-CD8.2 B cells.

Light Chain Selection. Comparing hybridomas from normal and from transgenic spleen cells, we found with similar frequencies only one light chain that was able to complement for the original anti-CD8.2 specificity (Fig. 6 A). This contrasts observations made with other Ig μ heavy chain transgenic mice. There, the usage of the original light chain (κ_{167} for anti-phosphoryl-choline specificity [38]) was found to be

increased. This expansion of B cells with selected light chains was interpreted to be due to idiotypic interactions and/or to exposure to antigens during B lymphocyte maturation. In analogy, we found that 20% of the transgenic hybridomas used κ light chains with identical isoelectric points that were different from the anti-CD8.2-specific light chains. This contrasts with our results obtained for hybridomas derived from normal spleen cells fused to the transfectoma X63/ $\mu 19$. There we found a large variety of κ light chains with unique isoelectric points that were associated with the μ heavy chain. This shows, that the anti-CD8.2 μ chain is able to combine with many different light chains. A similar result was found in other hybridomas for artificial heavy/light chain combinations (39). Interactions of heavy and light chains are known to vary widely in their pairing affinity, and preferential heavy/light chain associations are well documented in competition experiments (40). In analogy, many combinations of endogenous light chains with the transgenic μ chain in vivo could also have low pairing affinities. This could prevent their selection into the pool of peripheral B cells. In addition, other mechanisms could lead to the preferential expression of light chain subsets together with a given heavy chain. Similar to T cells, B cells could be negatively or positively selected or the selective expansion of particular B cell subsets could be driven by antigenic challenge, the size of the available light chain repertoire, and/or on idiotypic interactions. These processes, which should occur after light chain rearrangement in pre-B cells, are now subject of further investigations.

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