Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6186–6190, August 1990
Immunology

Positive selection of transgenic receptor-bearing thymocytes by K^b antigen is altered by K^b mutations that involve peptide binding

(T-cell receptor/K^{bm} mutants/tolerance/alloreactivity/major histocompatibility complex restriction)

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Communicated by Herman N. Eisen, May 24, 1990

ABSTRACT **A specific interaction between the class I major histocompatibility complex molecule K^b and thymocytes expressing the antigen receptor from the cytolytic T lymphocyte 2C enhances maturation of T cells of the CD8 lineage in transgenic mice. By analyzing transgenic mice backcrossed to K^{bm} mutant strains of mice, we have identified five bm mutations of the K^b antigen-encoding gene that alter the positive selection of thymocytes induced by K^b antigen. Compared with K^b, K^{bm10} and K^{bm1} did not induce significant maturation of 2C T-cell receptor-bearing thymocytes, and K^{bm8} antigen positively selected for transgenic thymocytes only weakly. Altering residue 77 of K^b molecule from aspartic acid to serine made K^{bm3} and K^{bm11} allogeneic targets for the 2C antigen receptor and caused deletion of transgenic thymocytes. This deletion spared T cells that expressed low levels of CD8, a result differing from the total deletion of CD8-bearing T cells seen in mice that expressed the original target alloantigen L^d. This evidence indicates that (*i*) self-peptides bound to thymic major histocompatibility complex molecules can influence the positive selection of thymocytes and (*ii*) thymocytes with apparently weak interaction with self-major histocompatibility complex antigens can escape clonal deletion.**

Mature T cells display properties that suggest subjection during thymic development to both positive- and negative-selective events, which involve interactions between their $\alpha\beta$ antigen receptors and self-major histocompatibility complex (MHC) molecules (1). These properties are as follows: (*i*) T cells are tolerant to self (2). Several systems have convincingly demonstrated that clonal deletion of autoreactive thymocytes is largely responsible for the ability of mature T cells to distinguish self from nonself (3–8). (*ii*) T cells preferentially recognize antigens as peptide fragments presented by self- rather than non-self-MHC molecules (9–14). (*iii*) T cells display a marked propensity to recognize foreign MHC molecules, a phenomenon termed alloreactivity (1). Demonstration of these properties has prompted investigation of how engagement of the $\alpha\beta$ T-cell receptor (TCR) can initiate both death of autoreactive thymocytes and increased maturation of thymocytes appropriate for recognition of foreign antigen–self-MHC complexes.

We (7, 15) and others (16–19) have recently described TCR transgenic model systems that have provided evidence for the positive selection of thymocytes capable of interacting with self-MHC molecules. In a previous report (7), we showed that maturation of thymocytes bearing the TCR from the cytolytic T-cell clone 2C was significantly enhanced by expression of $H\text{-}2^b$ gene products. The clone 2C was originally isolated from an $H\text{-}2^b$ anti-$H\text{-}2^d$ mixed-lymphocyte culture (20). We now demonstrate that the K^b class I MHC molecule is the $H\text{-}2^b$ gene product responsible for positive selection of the 2C TCR. By backcrossing transgenic mice into six separate bm mutant haplotypes, which differ at defined residues in the K^b molecule (21), we have examined specific structural requirements for positive selection and identified a single-residue substitution in the K^b molecule, Asp-77 → Ser, which leads to negative selection. Our results argue strongly for a model in which self-peptides bound to class I MHC molecules directly influence positive selection.

EXPERIMENTAL PROCEDURES

Mice. Mice were analyzed for transgene transmission and H-2 haplotype by Southern blot analysis of tail DNA, as described (15). Haplotypes of mice backcrossed to bm mutant strains (The Jackson Laboratory), particularly bm8 mutant, were analyzed by polymerase chain reaction amplification of portions of exons 2 and 3 of the K^b antigen-encoding gene. Amplified DNA was either directly sequenced or probed with labeled oligonucleotides specific for either wild-type or mutant sequences. Haplotypes were confirmed by staining immunoglobulin-negative splenocytes with a panel of anti-class I monoclonal antibodies, including Y25 (22), 5F1.2 (23), B8-24-3 (24), and K9-178 (25), used to distinguish among mutant K^b molecules.

Flow Cytometry. Flow cytometry was performed by using a Becton Dickinson fluorescence-activated cell sorter (FACS) model 440 essentially as described (7, 15). First-step reagents used were phycoerythrin-conjugated GK1.5 (rat anti-mouse CD4, Becton Dickinson), fluorescein isothiocyanate-conjugated 53-6.72 (rat anti-mouse CD8, Becton Dickinson), and biotinylated 1B2 (26). Second-step reagents used were avidin–fluorescein isothiocyanate (Becton Dickinson) and streptavidin–phycoerythrin (Chromoprobe, Redwood City, CA). For thymocyte contour plots, 100,000 events were analyzed, and contours were drawn at 10, 30, 90, 270, and 810 events. Events and contour levels were halved for splenocyte contour plots.

Proliferation Assay. Responder splenocytes (10^5) were stimulated in triplicate in 200 μl in a 96-well plate for 3 days in the presence of recombinant interleukin 2 and 5×10^5 irradiated spleen-cell stimulators. Because of the high $1B2^+CD8^+$ frequency, proliferative responses to stimulators were assayed by measuring incorporation of [^{3}H]thymidine during a 4-hr treatment (pulse). This brief pulse minimized the contribution of alloreactivity to class II MHC present in some combinations.

Cytolytic Assay. Responder splenocytes from 5-day primary mixed-lymphocyte cultures, in the presence of recombinant interleukin 2, were purified over Ficoll/Hypaque and

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Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter.

were assayed for lytic activity in triplicate at 25:1 effector/target ratio against 10^4 Con A lymphoblast targets in a 3-hr ^{51}Cr release assay and in duplicate against 5×10^3 L-cell targets in a 6-hr ^{51}Cr release assay.

RESULTS

K^b Molecule Positively Selects the 2C TCR. K^b was initially identified as the H-2^b gene product responsible for positive selection of 2C TCR-bearing thymocytes by analyses of transgenic mice backcrossed to B10.MBR and B10.AKM inbred mice (27). These two strains of mice differ only in the K region of the H-2 complex; B10.MBR mice express the gene for K^b, whereas B10.AKM mice express the gene for K^k. The monoclonal antibody 1B2 is an anticlonotypic reagent specific for the 2C TCR (26). In flow cytometric analyses of peripheral T cells from B10.MBR mice, $1B2^+CD8^+$ T cells were 31 ± 8% (n = 3) of immunoglobulin-negative spleen cells. In contrast, only 6 ± 1% (n = 4) of immunoglobulin-negative spleen cells from B10.AKM mice were $1B2^+CD8^+$. This result indicates that expression of the K^b molecule-encoding gene was responsible for enhanced maturation of thymocytes bearing the 2C TCR.

K^{bm10} and K^{bm1} Molecules Do Not Induce Enhanced Maturation of 2C TCR. K^{bm10} and K^{bm1} proteins contain changes to residues that can contact TCR directly and serve as potential ligands for processed antigens (28). In transgenic H-$2^{b/bm10}$ mice, expression of K^{bm10} protein did not alter positive selection of $1B2^+CD8^+$ thymocytes by K^b, whereas in matched transgenic H-2^{bm10} littermates, absence of K^b expression severely reduced the percentage of mature thymocytes (Fig. 1*A*). Thymocytes from H-$2^{b/bm10}$ mice expressed mature levels of the 2C TCR and had an expanded population of $CD4^-CD8^+$ thymocytes. In contrast, thymocytes from H-2^{bm10} mice were predominantly $CD4^+CD8^+$ and expressed lower levels of the 2C TCR. A similar pattern of thymocyte development was seen in transgenic H-$2^{b/bm1}$ and H-2^{bm1} littermates (Fig. 1*B*).

Self-Peptides Influence Positive Selection of the 2C TCR. K^{bm8} contains residue substitutions in the floor of the peptide-binding cleft that are likely to influence T-cell recognition of K^b entirely by alterations in binding of peptide antigens (28). In transgenic H-2^{bm8} mice the percentage of mature $CD4^-CD8^+$ thymocytes, 7.3 ± 1.8% (n = 7), was substantially reduced relative to H-$2^{b/bm8}$ littermates, 28 ± 4.9% (n = 5), but not down to the levels seen in H-2^{bm10} mice, 2.0 ± 0.5% (n = 5), or H-2^{bm1} mice, 3.4 ± 0.8% (n = 5) (Fig. 1*C*). The decreased ability of K^{bm8} to positively select for $1B2^+$ thymocytes in H-2^{bm8} mice was also reflected in the reduced

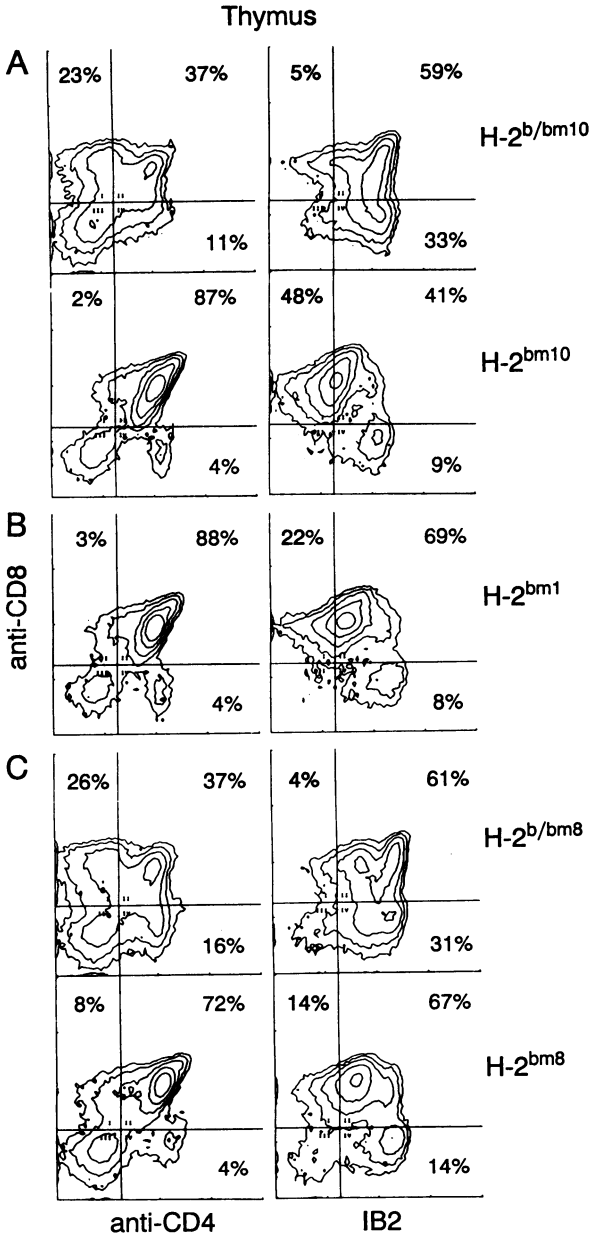

FIG. 1. K^{bm10}, K^{bm1}, and K^{bm8} mutants have diminished ability to induce maturation of $1B2^+CD8^+$ thymocytes. Representative FACS analyses of transgenic thymocytes from H-$2^{b/bm10}$ (n = 3) and H-2^{bm10} (n = 5) littermates (*A*), H-2^{bm1} (n = 5) mice (*B*), and H-$2^{b/bm8}$ (n = 5) and H-2^{bm8} (n = 7) (*C*) littermates.

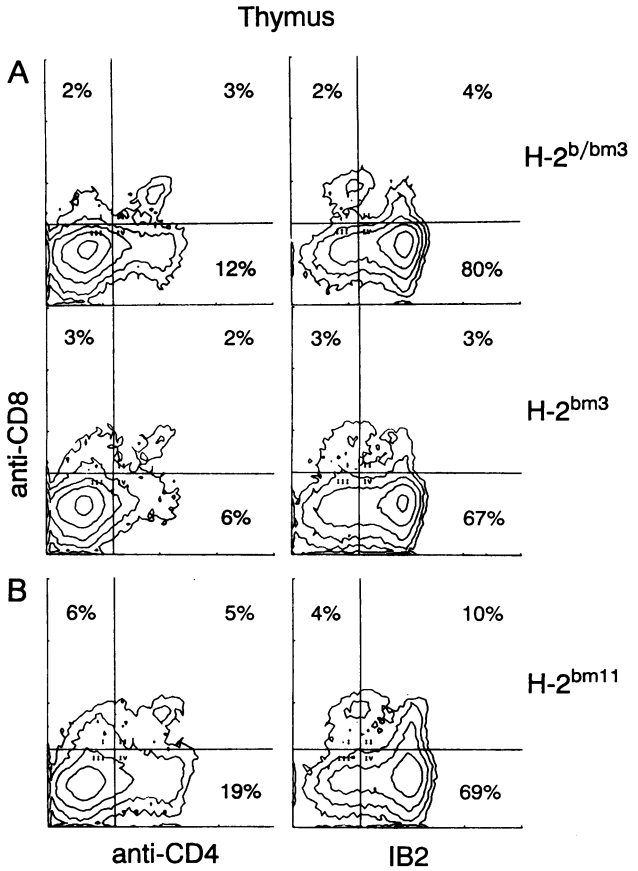

FIG. 2. K^{bm3} and K^{bm11} mutants induce deletion of $1B2^+CD4^+$-$CD8^+$ thymocytes. Representative FACS analyses of transgenic thymocytes from H-$2^{b/bm3}$ (n = 2) and H-2^{bm3} (n = 4) littermates (*A*) and H-2^{bm11} (n = 2) mice (*B*).

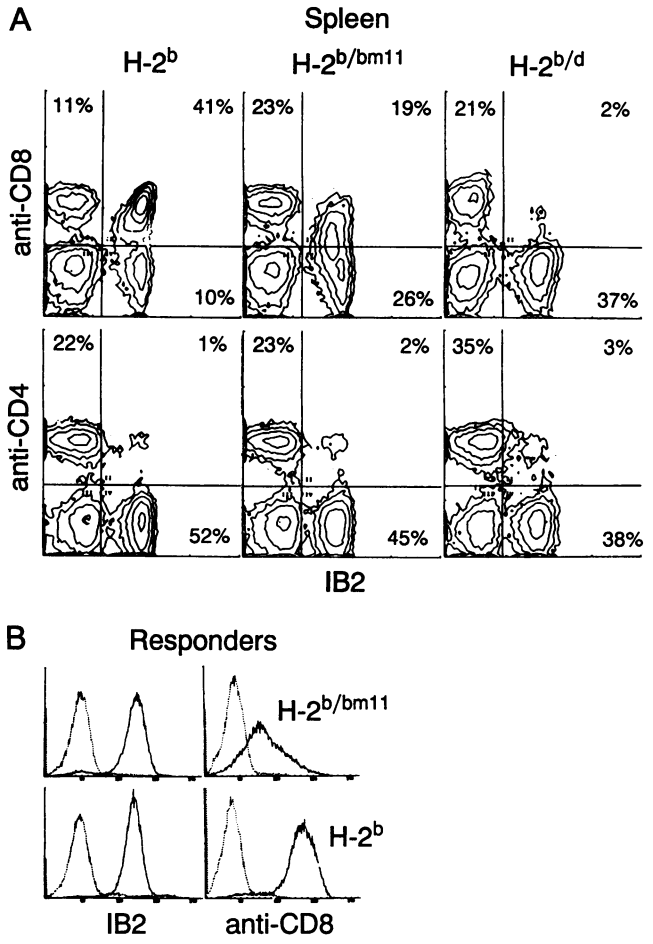

FIG. 3. Differential CD8 phenotypes of 1B2$^+$ spleen cells and responder cells from transgenic H-2^b, H-2$^{b/bm11}$, and H-2$^{b/d}$ mice. (*A*) Representative FACS analysis of immunoglobulin-minus splenocytes from transgenic H-2^b ($n > 60$), H-2$^{b/bm11}$ ($n = 6$), and H-2$^{b/d}$ ($n > 30$) mice. (*B*) Representative responder populations isolated after 5 days from transgenic H-2^b anti- and H-2$^{b/bm11}$ anti-BALB/c mixed-lymphocyte cultures. Staining controls are shown as dotted lines.

percentage of CD8$^+$ thymocytes expressing mature levels of the 2C TCR. In contrast, K^{bm7}, which also contains residue substitutions in the floor of the peptide-binding cleft, still positively selected as well as K^b (data not shown).

K^{bm3} and K^{bm11} Induce Deletion of CD8^{hi} Cells Bearing 2C TCR. K^{bm3} and K^{bm11} contain residue substitutions that are structurally modeled as potential ligands for processed antigens (28). In contrast to the other mutant K^b molecules examined, K^{bm3} and K^{bm11} mutants affected the positive selection of 1B2$^+$ thymocytes seen in heterozygous mice expressing K^b molecule (Fig. 2*A*). This dominant effect was similar but not identical to the complete deletion of CD4$^+$CD8$^+$ already described for transgenic H-2$^{b/d}$ mice (7). In both H-2$^{b/bm3}$ and H-2^{bm3} mice, thymocytes expressed mature levels of the 2C TCR and were predominantly CD4$^-$CD8$^-$. Trailing populations of 1B2$^+$ thymocytes expressing low levels of CD4 and CD8 were also evident. A similar pattern of thymocyte development was seen in transgenic H-2^{bm11} mice (Fig. 2*B*).

CD8^{lo} Cells Bearing 2C TCR Respond to L^d but Not to K^{bm11}. The phenotype of 1B2$^+$ spleen cells from H-2$^{b/bm11}$ mice was consistent with deletion of 1B2$^+$CD8^{hi}, but not 1B2$^+$CD8^{lo}, T cells (Fig. 3*A*). To assess why 1B2$^+$CD8^{lo} T cells survived selection in H-2$^{b/bm11}$ mice but were absent in H-2$^{b/d}$ mice, proliferative and lytic responses of transgenic spleen cells were examined (Tables 1 and 2). Spleen cells from H-2^b mice proliferated vigorously in response to K^{bm11}, K^{bm3}, and L^d (B10.D2). Responder spleen cells from similar H-2^b anti-H-2^d mixed-lymphocyte cultures were greater than 95% 1B2$^+$-CD8$^+$ and could specifically lyse lymphoblast targets bearing K^{bm11} and L^d proteins. Recognition of K^{bm11} and K^{bm3} antigens by 1B2$^+$CD8$^+$ T cells appeared to result solely from the residue substitution Asp-77 → Ser. In a panel of transfected L cells (29), substitution at residue 77, but not at residue 89, of the K^{bm3} protein was sufficient to allow for specific lysis of target cells (Fig. 4).

Spleen cells from transgenic H-2$^{b/bm11}$ mice were unresponsive to K^{bm11} mutant, proliferated weakly in response to K^{bm3} mutant, and responded strongly to L^d. Because CD4$^-$CD8$^-$ T cells from H-2$^{b/d}$ mice have been shown (7) to be unresponsive to L^d, the 1B2$^+$ T cells that responded to L^d were almost certainly the additional CD4$^-$CD8^{lo} population. This interpretation was verified when 1B2$^+$CD8^{lo} T cells were found to be the predominant responder population isolated from H-2$^{b/bm11}$ anti-H-2^d mixed-lymphocyte cultures (Fig. 3*B*). These CD8^{lo} T cells, in contrast to their normal CD8$^+$ counterparts, distinguished between L^d and K^{bm11} alloantigens. Activated CD8^{lo} T cells preferentially lysed lymphoblast targets bearing L^d over those bearing K^{bm11}, suggesting that the 2C TCR recognized L^d alloantigen more efficiently than K^{bm11} alloantigen.

Table 2. Lytic specificity of T cells from transgenic mice after stimulation with H-2^d alloantigens

	Specific ^{51}Cr release, %				
	B10.D2		bm11		dm1
Responder	−1B2	+1B2	−1B2	+1B2	−1B2
H-2^b	33 ± 3	0 ± 0	43 ± 0	2 ± 0	0 ± 1
H-2$^{b/bm11}$	41 ± 1	9 ± 1	10 ± 1	NT	−1 ± 0
Control	64 ± 4	63 ± 2	1 ± 0	NT	61 ± 3

Splenocytes from the responder mice in Table 1 were stimulated for 5 days with irradiated BALB/c splenocytes in the presence of recombinant interleukin 2. 1B2 was used at 10 μg/ml to block lytic activity. Representative phenotypes of H-2^b and H-2$^{b/bm11}$ responders are shown in Fig. 3*B*.

DISCUSSION

The 2C TCR transgenic model encompasses at least five distinct phenotypic patterns of T-cell selection in response to K^b molecule, mutant K^b molecules, and L^d, the original target alloantigen. These phenotypic patterns include positive se-

Table 1. Specificity of proliferation of T cells from transgenic mice as measured by [^{3}H]thymidine incorporation

	Spleen cell stimulation, cpm × 10^{-2}					
Responder	dm1	B10.D2	B6	bm1	bm3	bm11
H-2^b	11 ± 1	1000 ± 30	16 ± 1	12 ± 1	440 ± 0	730 ± 50
H-2$^{b/bm11}$	13 ± 2	370 ± 20	13 ± 1	13 ± 2	29 ± 1	14 ± 0
Control	12 ± 2	12 ± 3	8 ± 1	10 ± 3	7 ± 1	4 ± 1

Control splenocytes were isolated from a nontransgenic H-2$^{b/bm11}$ littermate of the transgenic H-2$^{b/bm11}$ mouse. Spleen cell stimulators were prepared from C57BL/10, B10.D2, B10.D2-H-2^{dm1}, C57BL/6, B6.C-H-2^{bm1}, C57BL/6-H-2^{bm3}, and B6.C-H-2^{bm11} strains of mice (The Jackson Laboratory).

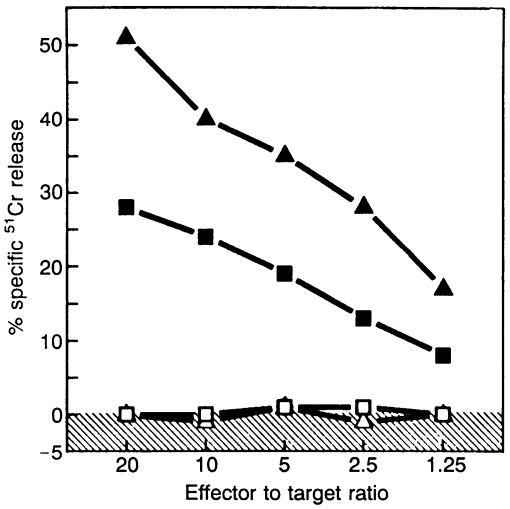

FIG. 4. Recognition of K^{bm3} and K^{bm11} mutants by the 2C TCR results from residue substitution Asp-77 → Ser. Target cells were as follows: ▲, LTK/K^{bm3}; ■, LTK/K^{bm3-77}; □, LTK/K^{bm3-89}; △, LTK/K^{b}. L-cell transfectants LTK/K^{bm3} and LTK/K^{b} express, respectively, chimeric genes composed of exons 1–3 of the K^{bm3} and K^{b}-encoding genes with exons 4–8 of the L^{d}-encoding gene (29). LTK/K^{bm3-77} and LTK/K^{bm3-89} are derivatives of LTK/K^{bm3} and correspond, respectively, to individual substitutions of residues 77 and 89 of the K^{bm3} mutant (29). $1B2^{+}CD8^{+}$ T cells from transgenic H-2^{b} anti-BALB/c mixed-lymphocyte cultures were used as responders. Blocking with 1B2 completely inhibited lysis of LTK/K^{bm3} and LTK/K^{bm3-77} by $1B2^{+}CD8^{+}$ T cells (data not shown). Under parallel assay conditions at a 10:1 effector-to-target cell ratio, the clone CTL 3 (30) caused a 61%, 51%, 72%, and 69% specific release of ^{51}Cr from LTK/K^{bm3}, LTK/K^{bm3-77}, LTK/K^{bm3-89}, and LTK/K^{b} targets, respectively.

lection (K^{b} and K^{bm7}), weak positive selection (K^{bm8}), absence of positive selection (K^{bm1} and K^{bm10}), negative selection of $CD8^{hi}$ cells (K^{bm3} and K^{bm11}), and negative selection of $CD8^{hi}$ and $CD8^{lo}$ cells (L^{d}). A specific interaction between the 2C TCR and the K^{b} class I MHC molecule induces maturation of $CD4^{+}CD8^{+}$ thymocytes into $CD4^{-}CD8^{+}$ T cells. This selection event is analogous to recognition of antigen by mature T cells (31) in its requirement for multiple contacts with the antigen-presenting face of K^{b} and its capacity for modulation by self-peptides bound to K^{b}, as evidenced by the alterations in selection achieved by K^{bm8}, K^{bm3}, and K^{bm11} mutants.

The reduced ability of K^{bm8} mutant to induce maturation of thymocytes bearing the 2C TCR indicates that the self-peptides bound to self-MHC can directly influence positive selection of thymocytes (32). The recent determination of the structure of a second class I human MHC molecule (33), HLA-Aw68, demonstrates that residue substitutions in the β-pleated sheets forming the floor of the antigen-binding cleft can locally alter the shape of the binding cleft without significantly altering the α-helices thought to contact TCR directly (34). Thus, altered recognition of K^{bm8} molecule by the 2C TCR during thymocyte development is likely to result solely from changes related to bound peptides. Experiments in which radiation chimeras were used have also recently shown that self-peptides influence positive selection (35).

The identification of K^{bm3} and K^{bm11} molecules as allogeneic targets for the 2C TCR defines discrete structural differences that span the developmental distinction between positive selection of a self-restricted thymocyte and deletion of a self-reactive thymocyte. Based upon the structural model of K^{bm3} and the molecular dissection of the lytic response of $1B2^{+}$ T cells to K^{bm3} mutant, Asp-77 → Ser is the critical residue substitution, shared by K^{bm3} and K^{bm11} mutants, that shifts recognition of K^{b} from positive to negative selection. Modeling studies indicate that residue 77 is a potential ligand for processed antigens and probably does not interact directly with TCR (28). Substitution of a charged for a neutral amino acid at position 77 may modulate recognition of K^{b} molecule, either by inducing an antigenic conformation of self-peptides normally bound to K^{b} or by allowing antigenic self-peptides to bind to K^{bm3} and K^{bm11} molecules.

In H-$2^{b/bm11}$ transgenic mice we observed deletion of $CD8^{hi}$, but not $CD8^{lo}$, T cells, analogous to the deletion of autospecific T cells seen in B6.2.16 TCR transgenic mice (6, 8). These $CD8^{lo}$ cells were still capable of specific lysis of target cells bearing L^{d}. Given the demonstrated ability of CD8 to modulate antigen recognition (36), we interpret these findings as reflecting the synergistic interaction between CD8 and TCR in determining the overall avidity of T cells for target alloantigens. Thus, 2C TCR-bearing cells expressing a normal level of CD8 had an overall avidity for K^{bm11} mutant high enough to produce measurable functional responses, whereas T cells expressing a lower level of CD8 could not respond to K^{bm11} mutant and, thus, could survive deletion in H-$2^{b/bm11}$ mice. These results suggest that thymocytes with lower avidity for self-MHC molecules, as well as those with no avidity for self-MHC molecules, are spared deletion in the thymus.

A single, positively selecting MHC gene product has been identified in TCR transgenic mice for both antigen-specific class I- and II-restricted TCRs (17–18) and the alloreactive class I TCR described here. These results suggest that alloreactive and MHC-restricted T cells may share a common developmental origin and support the view (37) that alloreactivity, the phenomenon underlying allograft rejection, results from the positive selection of thymocytes capable of engaging self-MHC molecules.

We thank Donald Shreffler and his laboratory for generous support in maintaining mice, Don Wiley and Ted Jardetzky for helpful discussion, Gary Elliott for FACS analysis, and Ulrich Hämmerling, Ted Hansen, Charles Janeway, and Linda Sherman for gifts of ascites and hybridomas.

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