

## SPECIES-RESTRICTED INTERACTIONS BETWEEN CD8 AND THE $\alpha 3$ DOMAIN OF CLASS I INFLUENCE THE MAGNITUDE OF THE XENOGENEIC RESPONSE

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T cells are biased towards recognition of antigen presented in the context of molecules encoded by the MHC. This is attributable to the specificity of both the clonotypic TCR and the accessory molecules CD4 and CD8. The CD4/CD8 phenotype of T cells appears to closely correlate with the class of MHC molecule recognized (1-7). CD4<sup>+</sup> T cells demonstrate specificity for class II molecules, whereas CD8<sup>+</sup> T cells are specific for class I molecules. Various approaches have indicated that CD8 enhances the response of class I-specific T cells (8-10), and there is now good evidence to support the hypothesis that one important feature of CD8 enhancement is a physical interaction between CD8 and class I (11-14). In contrast to the TCR, which interacts with the highly polymorphic  $\alpha 1$  and  $\alpha 2$  domains of the class I molecule, CD8 appears to function by interacting with relatively nonpolymorphic residues within the  $\alpha 3$  domain of class I (15-17).

There is a strong species preference with regard to T cell recognition of MHC molecules (18-23). Thus, murine T cells recognize murine MHC molecules more efficiently than they recognize human MHC molecules. For this reason, one strategy used to analyze MHC recognition by T cells has been to examine the basis for poor recognition of xenogeneic MHC molecules (23). In this report such a strategy has been used to investigate the role of the  $\alpha 3$  domain of class I in T cell activation and antigen recognition. We have compared murine CTL recognition of the human class I molecule, HLA-A2 (A2)<sup>1</sup>, with recognition of the A2/H-2K<sup>b</sup> (A2/K<sup>b</sup>) molecule, a chimeric form of A2 that contains the  $\alpha 1$  and  $\alpha 2$  domains of A2 and the  $\alpha 3$ , transmembrane and cytoplasmic regions of K<sup>b</sup>. Using a series of A2-specific murine CTL clones induced by stimulation with human cells expressing the A2 molecule, it was observed that the majority of such CTL clones more efficiently lysed target cells that expressed the chimeric A2/K<sup>b</sup> molecule than target cells expressing the native A2 molecule. Such heteroclytic recognition correlated with sensitivity to inhibition by anti-CD8 antibody, suggesting that enhanced recognition of A2/K<sup>b</sup> was attributable to the preferential interaction of murine CD8 with the  $\alpha 3$  domain of a species-matched class I molecule. Further evidence supporting this view was obtained by studying activation of CTL precursors (CTLp). Human cells bearing the

<sup>1</sup> Abbreviations used in this paper: A2, HLA-A2;  $\beta 2m$ ,  $\beta 2$ -microglobulin; CTLp, CTL precursor; K<sup>b</sup>, H-2K<sup>b</sup>.

A2/K<sup>b</sup> molecule, but not human cells bearing the A2 molecule, were able to stimulate an A2-specific response. These results suggest that murine CD8 preferentially interacts with the  $\alpha 3$  domain of a species-matched class I molecule and that the reduced capacity of CD8 to function across species barriers is at least in part responsible for poor xenoreactivity.

### Materials and Methods

**Animals.** B10.D2, C57BL/6, and (C57BL/6  $\times$  B10.D2)F<sub>1</sub> mice were obtained from the breeding colony of the Research Institute of Scripps Clinic and were used between 6 and 20 wk of age.

**Cell Lines.** Human cell lines used in these studies included the A2-expressing, EBV-transformed B lymphoblastoid lines LG-2 and JY, and the A2<sup>-</sup> T cell leukemia line, Jurkat. The murine cell lines used included the DBA/2 mastocytoma P815 (H-2<sup>d</sup>) and the C57BL/6 thymoma EL4 (H-2<sup>b</sup>).

**Construction of the A2/K<sup>b</sup> Chimeric Gene.** The HLA-A2.1/H-2K<sup>b</sup> gene was constructed from a genomic clone of HLA-A2.1 (provided by Dr. Harry Orr [24]) and a genomic clone of K<sup>b</sup> (provided by Dr. Bruce Wallace [25]). A Hind III-Bgl II fragment of the genomic clone of HLA-A2.1, which contained the genetic information coding for the leader sequence,  $\alpha 1$  and  $\alpha 2$  domains of the HLA-A2.1 molecule, was ligated to a Bam HI-Bam HI fragment excised from the K<sup>b</sup> gene, which contained the  $\alpha 3$ , cytoplasmic, and transmembrane regions of the K<sup>b</sup> molecule. The A2/K<sup>b</sup> construct was purified and ligated into the Bam HI-Hind III sites of pSV2.

**Transfection.** pSV2 plasmids containing genomic clones of HLA-A2.1 (A2) or the chimeric construct A2/K<sup>b</sup> were cotransfected with the pSV2 neo plasmid containing the neomycin resistance gene into each of three cell lines: P815, EL-4, and Jurkat. The Jurkat cell line was also transfected with a pSV2 plasmid containing the neomycin resistance gene and genomic K<sup>b</sup>. Transfection was accomplished by electroporation using an X-Cell 450 transfection apparatus (Promega Biotec, Madison, WI), with a 50-msec discharge, constant voltage (400 mV), and capacitors charged to 800 mfd (EL4), 1,000 mfd (P815), or 1,450 mfd (Jurkat). For electroporation, 10<sup>7</sup> cells in 1 ml of PBS were mixed with 10  $\mu$ g of plasmid containing the appropriate class I gene and with 2  $\mu$ g of pSV2 neo plasmid in a 1-ml cuvette. Selection was initiated after 24 h by addition of 400  $\mu$ g/ml of G418 (Gibco Laboratories, Grand Island, NY) for EL-4 and P815, and 800  $\mu$ g/ml of G418 for Jurkat cells. Neomycin-resistant cells were subcloned and tested for cell surface expression of either A2 or A2/K<sup>b</sup> by flow cytometry. Transfected cell lines were maintained in the presence of 250  $\mu$ g/ml of G418 and periodically screened by flow cytometry to ensure stable expression of A2 or A2/K<sup>b</sup>.

**Detection of A2, A2/K<sup>b</sup>, or H-2 by Flow Cytometry.** Expression of A2, A2K<sup>b</sup>, and H-2 was detected using the A2-specific mAbs BB7.2 (26) or MA2.1 (27), the H-2D<sup>d</sup>-specific mAb 34-2-12S (28), or the H-2D<sup>b</sup>-specific mAb 28-14-4S (29). A biotinylated rat anti-mouse  $\kappa$  L chain (AMAC, Inc., Westbrook, ME) and phycoerythrin-conjugated streptavidin (Biomedica, Foster City, CA) was used to detect cells labeled with the class I-specific antibodies. Where stated, biotinylated mAb MA2.1 and phycoerythrin-conjugated streptavidin was used to detect expression of A2 or A2/K<sup>b</sup> molecules.

**Derivation of A2-specific CTL Clones.** CTL clones specific for A2 were generated by intraperitoneal immunization of a (C57BL/6  $\times$  B10.D2)F<sub>1</sub> mouse with 2  $\times$  10<sup>7</sup>,  $\gamma$ -irradiated (10,000 rad) LG-2 cells. Splenocytes from this primed animal (3  $\times$  10<sup>6</sup>/ml) were cultured in 2 ml of culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 50  $\mu$ M 2-ME, 2 mM L-glutamine, and 50  $\mu$ g/ml gentamicin, with  $\gamma$ -irradiated (10,000 rad) JY cells (7.5  $\times$  10<sup>4</sup> cells/ml) and  $\gamma$ -irradiated (3,000 rad) C57BL/6 feeder cells (3  $\times$  10<sup>6</sup> cells/ml). Cells from such cultures were cloned under limiting dilution conditions with JY stimulator cells and C57BL/6 feeder cells (used at the same concentrations as described for 2-ml cultures), in 0.2 ml culture medium supplemented with 10% con A-stimulated rat spleen cell supernatant (30). CTL clones were selected from responder cell dilutions such that <30% of the wells were positive for specific cytotoxicity of JY target cells on day 7.

**Generation of an *In Vitro* Xenoresponse.** C57BL/6 spleen cells ( $6 \times 10^6$ ) were incubated with an equal number of irradiated (3,000 rad) syngeneic spleen cells and  $2 \times 10^5$  irradiated (10,000 rad) stimulator cells (Jurkat-A2 or Jurkat-A2/K<sup>b</sup>) in 2 ml of culture medium in wells of 24-well tissue culture plates. Cells from 12 wells were pooled on day 6, and varying dilutions were assayed for lysis of EL4 transfectants expressing A2 or A2/K<sup>b</sup>. On day 7 after primary stimulation, secondary stimulation was performed by expanding the contents of each primary culture well into two wells (2 ml each) and restimulating with the same number of transfected stimulators and syngeneic spleen cells as described above. CTL activity was assessed after 6 d of incubation.

**Cytotoxicity Assay.** The cytotoxicity assay was performed essentially as previously described (31). Briefly, varying numbers of effectors and  $10^4$  <sup>51</sup>Cr-labeled target cells were incubated for 4 h at 37°C in 0.2 ml of culture medium in U-bottomed 96-well plates (Costar, Cambridge, MA). Inhibition by anti-CD8 antibody was assessed by adding 20  $\mu$ l culture media or supernatant from the anti-CD8 hybridoma 3.168 (32) to effectors 30 min before addition of <sup>51</sup>Cr-labeled target cells. In control experiments, culture supernatants from the anti-H-2D<sup>d</sup> hybridoma 34-5-8S (28), the anti-H-2<sup>k</sup> hybridoma 12-2-2S (33), and the anti-CD4 hybridoma GK1.5 (34) did not affect cytotoxicity. Percent specific <sup>51</sup>Cr release was calculated as:  $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ .

## Results

To examine the role of the  $\alpha 3$  domain of class I in xenorecognition by murine CTL, the murine cell lines P815 and EL4 were transfected with the genes encoding either the A2 or the A2/K<sup>b</sup> molecule. Cloned cell lines expressing comparable levels of A2 or A2/K<sup>b</sup> were selected (Fig. 1). The level of surface expression of A2 or A2/K<sup>b</sup> molecules was  $\sim 50\%$  the level of expression of H-2D<sup>b</sup> molecules on the EL4 transfectants and 70% the level of expression of H-2D<sup>d</sup> molecules on the P815 transfectants (data not shown).

CTL clones specific for A2 were produced by immunizing a (C57BL/6  $\times$  B10.D2)F<sub>1</sub> mouse with the A2-bearing human B lymphoma, LG2, followed by in

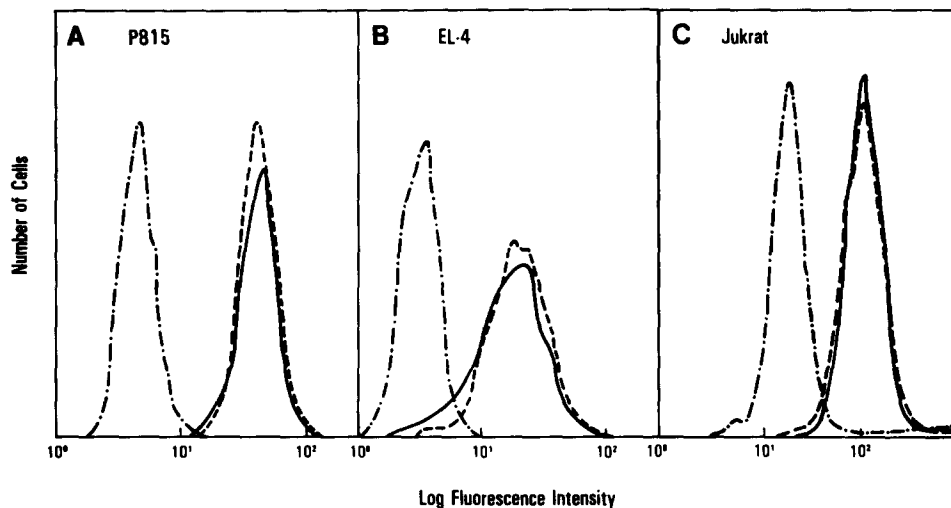


FIGURE 1. Expression of A2 or A2/K<sup>b</sup> by P815 (A), EL4 (B), or Jurkat (C) cell lines. Cell lines were transfected with either the neomycin resistance gene alone (---) or cotransfected with the gene for A2 (----) or A2/K<sup>b</sup> (—). Transfectants were analyzed by flow cytometry after staining with a biotinylated preparation of the HLA-A2-specific mAb, MA2.1, and avidin phycoerythrin.

vitro stimulation with a second A2-bearing B lymphoma, JY. Five CTL clones were derived from this CTL population. These were determined to be A2 specific by their capacity to lyse A2-bearing targets and the inhibition of this lysis by the HLA-A2.1-specific mAb, MA2.1 (data not shown). The A2-specific CTL clones were assessed for their capacity to lyse murine transfectants expressing the A2 or A2/K<sup>b</sup> gene product (Fig. 2). Clones C9 (Fig. 2, *A* and *D*) and C2 (Fig. 2, *B* and *E*) are representative of the specificity of four of the five clones tested. Each exhibited 5–10-fold greater recognition of A2/K<sup>b</sup> than A2 as determined by the number of effectors required to achieve the same level of target cell lysis. This was observed for recognition of both P815 (Fig. 2, *A–C*) and EL4 (Fig. 2, *D–F*) transfectants, indicating that enhanced recognition of A2/K<sup>b</sup> was not a property unique to a particular cell line. One CTL clone, D1 (Fig. 2, *C* and *F*), showed comparable recognition of A2 and A2/K<sup>b</sup> transfectants.

To assess the role of CD8 in recognition by these A2-specific CTL clones, the effect of anti-CD8 mAb on CTL lysis of P815 transfectants was examined (Fig. 3). In contrast to clone D1, CTL clones C2 and C9, which showed enhanced recognition of A2/K<sup>b</sup>, were highly susceptible to inhibition by anti-CD8 mAb. Failure to inhibit clone D1 indicated that the antibody preparation was not nonspecifically inhibiting CTL clones, and implied that lysis by clone D1 was CD8 independent. Inhibition

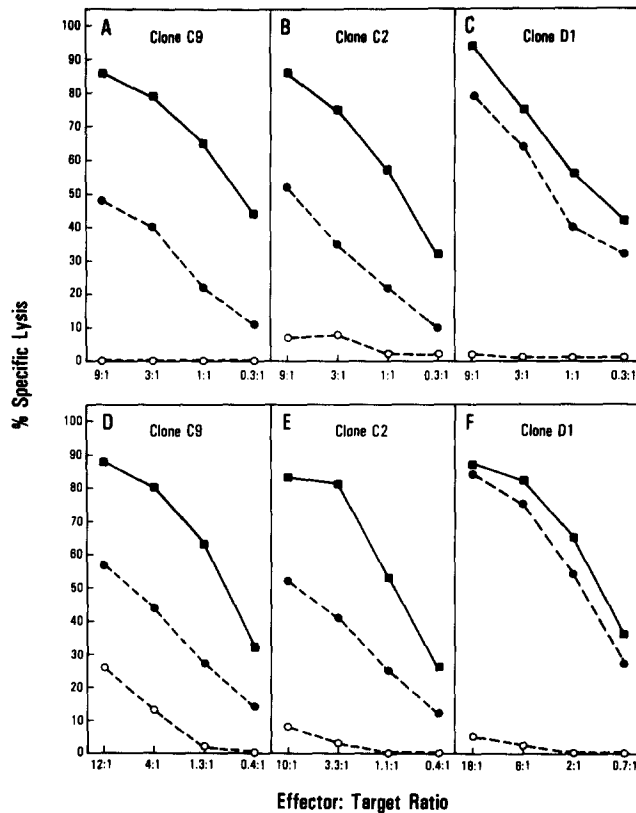


FIGURE 2. A2-specific murine CTL recognition of P815 or EL4 cells expressing A2 or A2/K<sup>b</sup>. A2-specific CTL clones C9 (*A* and *D*), C2 (*B* and *E*), and D1 (*C* and *F*) were examined for lysis of P815 (*A–C*) or EL4 (*D–F*) target cells transfected with the neomycin resistance gene alone (○) or cotransfected with the A2 gene (●) or the A2/K<sup>b</sup> gene (■).

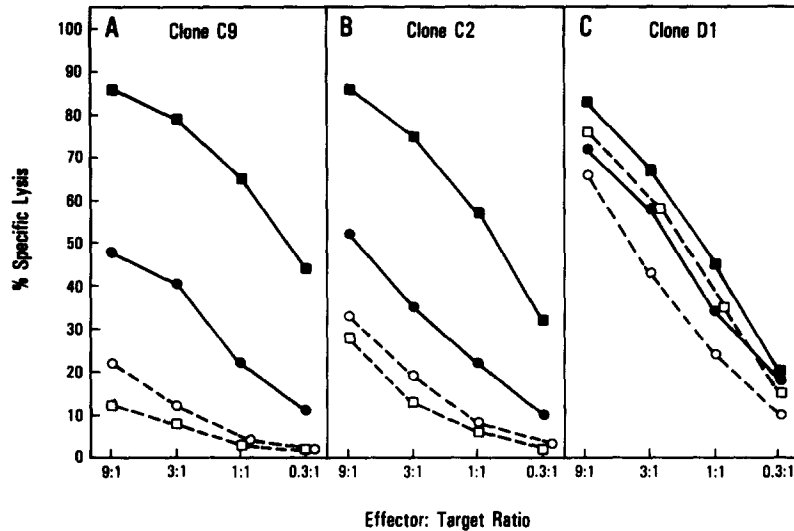


FIGURE 3. Inhibition of A2-specific CTL clones by anti-CD8 antibody. CTL clones C9, C2, and D1 (A-C, respectively) were incubated with culture media (—) or with anti-CD8 mAb, 3.168, (---) for 30 min before addition of  $^{51}\text{Cr}$ -labeled target cells. Target cells used were P815 cells transfected with either A2 (●) or A2/K<sup>b</sup> (■).

of clones C2 and C9, which showed enhanced recognition of A2/K<sup>b</sup>, suggested that a region in the  $\alpha 3$  domain of A2/K<sup>b</sup> was important for CD8 binding.

An alternative explanation for these results could be that murine  $\beta_2$ -microglobulin ( $\beta_2\text{m}$ ) interacts differently with A2 than A2/K<sup>b</sup>, resulting in an unusual conformation for the A2 molecule as expressed by murine cells. To examine this point human cell transfectants were used to ensure human  $\beta_2\text{m}$  would be available for association with the A2 molecule. The human cell line Jurkat was transfected with plasmids containing the A2 or A2/K<sup>b</sup> genes, and clones showing comparable levels of surface expression were selected (Fig. 1 C). Jurkat transfectants were used as targets for lysis by clone C2 (Table I). Again, enhanced recognition was observed for transfectants expressing A2/K<sup>b</sup>. The view that  $\beta_2\text{m}$  associates similarly with A2 and A2/K<sup>b</sup> is also supported by examination of the x-ray crystallographic structure of

TABLE I  
Lysis of Jurkat Transfectants by A2-specific CTL Clone C2

E/T ratio	Target cells*		
	Jurkat-K <sup>b</sup>	Jurkat-A2	Jurkat-A2/K <sup>b</sup>
6.0	24 <sup>†</sup>	55	100
2.0	8	36	86
0.67	2	19	43
0.22	1	6	29
0.07	1	1	12

\* Jurkat-K<sup>b</sup> was used as a specificity control.

<sup>†</sup> Percent specific lysis.

the A2 molecule (35). All amino acid residues of A2 reported to interact with human  $\beta_2m$  are conserved in the A2/K<sup>b</sup> molecule.

The preceding results suggested that one reason xenogeneic responses are much weaker than allogeneic responses (18-22) may be related to a species-restricted CD8-class I interaction. Unlike allogeneic responses that are readily detectable by primary in vitro stimulation, an additional in vivo priming step is often required to detect the much weaker xenoreponse. To examine the significance of the CD8-class I interaction in the stimulation of primary CTL precursors specific for xenogeneic cells, Jurkat transfectants expressing A2 or A2/K<sup>b</sup> were compared as stimulators of C57BL/6 spleen cells. An in vitro primary CTL response to A2 could not be obtained using the human cell line expressing the A2 molecule (Fig. 4). In contrast, Jurkat transfectants expressing the A2/K<sup>b</sup> molecule were capable of inducing an A2-specific xenoreponse. Due to the high level of nonspecific lysis, primary cultures were restimulated in an attempt to reveal a weak response to the A2 transfectant. Again, only cultures stimulated with A2/K<sup>b</sup> transfected cells demonstrated A2-specific lysis.

### Discussion

The data presented in this report indicate that a majority of A2-specific murine CTL clones induced by stimulation with A2-expressing human stimulator cells showed enhanced recognition of target cells expressing the chimeric molecule A2/K<sup>b</sup>. Fur-

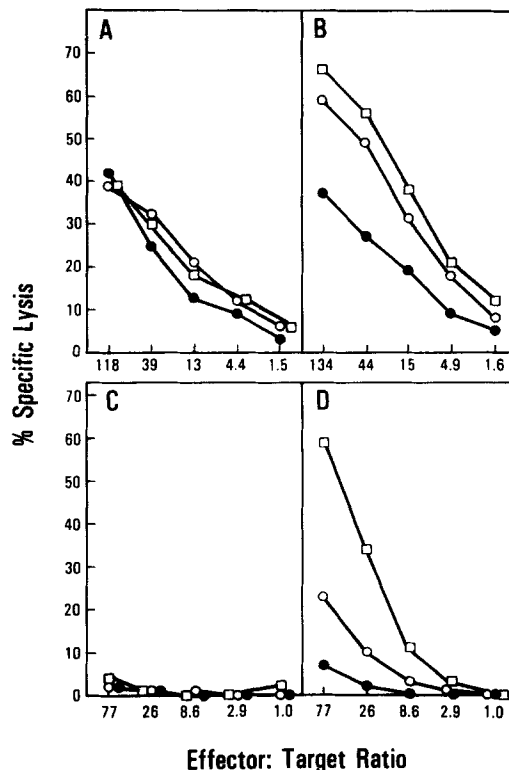


FIGURE 4. Primary and secondary in vitro response of C57BL/6 spleen cells to Jurkat cells transfected with either A2 or A2/K<sup>b</sup>. Naive C57BL/6 spleen cells were cultured for 6 d in vitro with irradiated Jurkat cells transfected with A2 (A) or A2/K<sup>b</sup> (B), and responses measured by lysis of EL4 cells transfected with the neomycin resistance gene alone (●) or cotransfected with A2 (○) or A2/K<sup>b</sup> (□). Primary cultures were restimulated in vitro with the same Jurkat transfectants as initially used. (C and D represent stimulation with A2 and A2/K<sup>b</sup> transfectants, respectively).

thermore, CTL clones showing enhanced recognition of A2/K<sup>b</sup> were sensitive to inhibition by anti-CD8 antibody. Taken together, these data suggest that murine CD8 interacts more efficiently with class I molecules that contain a species-matched  $\alpha 3$  domain.

These data are in agreement with results presented in previous reports that have demonstrated enhanced T cell recognition of epitopes on the  $\alpha 1$  and  $\alpha 2$  domains when species-matched interactions between CD8 and the  $\alpha 3$  domain of class I were permitted (36, 37). In these reports, human CTL specific for A2 demonstrated less efficient killing of target cells expressing the A2/K<sup>b</sup> molecule (36), while murine CTL specific for the K<sup>b</sup> molecule showed reduced killing of target cells expressing the K<sup>b</sup>/A2 molecule (37). In these previous studies, CTL were generated against the molecule for which they showed greater reactivity, raising the possibility (favored by the authors [36]) that reduced recognition of molecules with substituted  $\alpha 3$  domains was due to conformational changes in the  $\alpha 1$  and  $\alpha 2$  domains. In contrast, the results presented in this report utilize CTL induced to A2. Heteroclytic recognition of the chimeric A2/K<sup>b</sup> molecule strongly decreases the likelihood that conformational effects on the  $\alpha 1$  and  $\alpha 2$  domains were responsible for our findings. Furthermore, none of the CTL clones examined in this study appeared to detect conformational differences in the  $\alpha 1$  and  $\alpha 2$  domains of A2/K<sup>b</sup> as compared with A2.

The significance of species-specific interaction between CD8 and  $\alpha 3$  was also apparent at the level of stimulation of CTL precursors. Whereas a murine A2-specific primary in vitro CTL response to human cell transfectants expressing the A2/K<sup>b</sup> molecule was detected, no response was stimulated by the same cell type expressing comparable levels of A2. This implies that properties of the  $\alpha 3$  domain are also important for T cell activation. It is well established that xenogeneic responses are much weaker than allogeneic responses (18–23). A previous study suggested that the structural basis for poor recognition of human MHC molecules by murine T cells derives from an inability of the murine TCR repertoire to recognize the  $\alpha 1$  and  $\alpha 2$  domains of HLA molecules (23). No evidence was found to indicate that substitution of a murine  $\alpha 3$  domain into a human class I molecule could alter the frequency of activation of CTL precursors. There are a number of important differences in experimental protocol that could account for this reported lack of contribution of the  $\alpha 3$  domain to xenogeneic T cell activation. First, in the previous study, recognition of B7 and B7/L<sup>d</sup> was compared. It is possible that, due to species polymorphism within the  $\alpha 3$  domain, either the  $\alpha 3$  domain of B7 is recognized with higher affinity than that of A2 by murine CD8, or the  $\alpha 3$  domain of L<sup>d</sup> is not recognized as well by murine CD8 as is that of K<sup>b</sup>. Second, the previous study examined the response to HLA class I molecules expressed by murine transfectants, whereas we have compared stimulation by transfectants of the human cell line, Jurkat. Murine transfectants may provide species-specific accessory interactions that obviate the need for an efficient CD8 interaction with the class I molecule recognized by the TCR. Supporting this conclusion we have detected weak xenogeneic CTL responses to murine but not human A2-transfected cell lines (unpublished observations). Finally, exogenously produced IL-2 was not provided in our cultures. Perhaps the presence of high levels of IL-2 facilitates activation of CTLp, resulting in less dependence on optimal CD8–class I interactions.

It has been argued that CD8 increases the avidity of T cell–target cell interaction

by binding to class I molecules on the target cell (11-14). Despite the expression of high levels of endogenous murine class I molecules by A2-expressing murine transfectants, murine CTL clones preferentially recognized A2/K<sup>b</sup>-expressing murine transfectants. Failure of endogenous class I molecules to compensate for the apparently weak interaction of CD8 with A2 is consistent with the view that for optimal function, CD8 must interact with the same class I molecule as is recognized by the TCR (16, 17, 38). This may be due to an increase in avidity of the TCR for the MHC molecule by formation of a trimolecular complex with CD8, or to enhanced signal transduction as a result of crosslinking CD8 and the TCR complex (39-41).

In a recent report, binding between cells expressing high levels of human CD8 and cells expressing high levels of class I molecules was used to monitor CD8-class I interactions (17). Salter et al. (17) reported that binding occurred between cells expressing human CD8 and cells expressing various human or murine class I molecules. Detection of a CD8 interaction with class I molecules of a different species is not necessarily inconsistent with the results presented in the present report for the following reasons. First, it is possible that murine CD8 interacts less efficiently with human class I than does human CD8 with murine class I. Second, there is significant polymorphism within the  $\alpha 3$  domain of class I molecules that may affect the interaction of CD8 with individual class I molecules. Finally, the binding assay used by Salter et al. (17) utilized cells that express extraordinarily high amounts of CD8 and class I. At such high levels it may not be possible to monitor subtle differences in affinity between CD8 and class I molecules as may exist between species. Such an affinity difference may be more readily demonstrable in a functional assay.

Residues 227 and 245 of class I molecules have been implicated in the binding of murine and human CD8, respectively (15-17). As murine CD8 appears to interact more efficiently with A2/K<sup>b</sup> than with A2, examination of the differences between the  $\alpha 3$  domains of these molecules may identify other residues important for CD8 binding. Comparison of the amino acid sequence of the  $\alpha 3$  domains of A2 and K<sup>b</sup> reveals three major regions of difference. These regions span residues 193-199, 219-225, and 251-256, and are found within three turns in the membrane proximal region of the molecule. It may be predicted that a region that demonstrates species specificity in its interaction with CD8 would be relatively conserved within a species, yet vary between species. Comparison of the  $\alpha 3$  variable regions for species conservation identifies regions spanning residues 219-225 and 251-256. Of interest, residues 219-225 are spatially close to residues 227 and 245, which have been reported to be involved in CD8 binding (15-17). Future experiments will attempt to further localize the residues that are important for CD8 interaction with class I.

### Summary

As compared with the vigorous T cell response normally observed against allogeneic MHC molecules, T cells recognize xenogeneic MHC molecules poorly. To define structural features of the MHC molecule important for such species-specific recognition, HLA-A2(A2)-specific murine CTL were examined for their recognition of transfected cell lines expressing the class I molecules A2 or A2/H-2K<sup>b</sup>(A2/K<sup>b</sup>). A2/K<sup>b</sup> is a chimeric molecule consisting of the  $\alpha 1$  and  $\alpha 2$  domains of A2 and the  $\alpha 3$ , transmembrane, and cytoplasmic regions of K<sup>b</sup>. The majority of CTL clones showed enhanced recognition of transfected cell lines expressing this chimeric mole-



cule. Enhanced recognition was shown to correlate with sensitivity of the CTL clones to inhibition by anti-CD8 antibody. These results suggested that CD8 may interact with class I in a species-specific manner, and that suboptimal CD8 interaction with the  $\alpha 3$  domain of xenogeneic molecules may be an important contribution to poor xenoreactivity. This conclusion was supported by the capacity of A2/K<sup>b</sup>, but not A2 human cell transfectants, to induce a primary in vitro CTL xenoreponse specific for A2.

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