Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class I $\alpha 3$ domain: CD8 and the T-cell receptor recognize the same class I molecule

(H-2 mutations/major histocompatibility complex)

JANET M. CONNOLLY^{*†}, TED H. HANSEN^{*}, AMIE L. INGOLD[‡], AND TERRY A. POTTER[‡]

^{*}Department of Genetics, Washington University School of Medicine, Saint Louis, MO 63110; and [‡]Division of Basic Immunology, Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

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ABSTRACT The CD8 molecule on class I-reactive cytotoxic T lymphocytes (CTLs) is believed to function as a coreceptor along with the $\alpha\beta$ T-cell receptor. Whereas the $\alpha\beta$ T-cell receptor recognizes polymorphic residues in the $\alpha 1/\alpha 2$ domains of the class I molecule, the CD8 molecule is believed to recognize monomorphic class I residues. Our previous experiments suggested that residue 227 in the $\alpha 3$ domain of major histocompatibility complex class I molecules contributes to the determinant recognized by CD8. By using a panel of site-directed mutants of H-2D^d, this observation has been extended herein. Our findings indicate that for recognition by CD8-dependent CTLs, residue 227 must be either glutamic acid or aspartic acid and cannot be either basic or uncharged. However, the recognition by CD8-independent CTLs is unaffected by any of the substitutions at position 227 of H-2D^d. Similarly, alterations of other charged residues at positions 222, 223, and 229 have an analogous effect to substitution at residue 227, whereas substitutions at residues 192 and 232 do not affect the reactivity of CD8-dependent or CD8-independent CTLs. In addition, mutant H-2D^d molecules that are not recognized by CD8-dependent CTLs are unable to stimulate a primary CTL response, yet they can stimulate a secondary CD8-independent H-2D^d-specific CTL response. These findings suggest that CD8 recognition is obligatory for the priming of class I-dependent CTL responses. Since endogenous class I molecules were expressed by all of the transfected cell lines, these findings provide direct genetic evidence that CD8 and the $\alpha\beta$ T-cell receptor must interact with the same class I molecule.

CD8 and CD4 are present on mutually exclusive populations of peripheral T cells and are involved in the differential recognition of major histocompatibility complex (MHC) class I and class II antigens, respectively. This discrimination is apparently initiated during thymic maturation at which time CD4 and CD8 function in both positive and negative selection of the $\alpha\beta$ T-cell receptor (TCR) (1–4). A direct role for CD8 in the function of mature T cells derives from antibody-blocking experiments (5, 6) and gene-transfection studies (7, 8) that have established that the CD8 molecule facilitates interaction of T lymphocytes with MHC class I molecules. The interaction of CD8 with class I molecules has also been demonstrated by the ability of CHO cells transfected with the CD8 gene to form conjugates with class I-positive, but not class I-negative cells (9). It has been proposed that a critical step in T-cell activation results from the physical association between CD8 and the TCR induced by their simultaneous binding to the same MHC class I molecules (10). A similar model has been proposed for CD4 (11). Because of the importance of the CD8–class I molecule interaction, we have

focused on the definition of the region of the class I molecule that interacts with CD8 by using a panel of site-directed mutant class I molecules. Substitution of lysine for glutamic acid at residue 227 of MHC class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes (CTLs) (12, 13). This observation suggested that residue 227 and possibly other residues in the $\alpha 3$ domain contribute to the determinant recognized by CD8. Consistent with this hypothesis is a report (14) that substitution of valine for alanine at residue 245 (approximately 10 Å from residue 227) prevents conjugate formation between cells expressing the mutant molecule and CD8-positive CHO cells. Whereas this other study employed cells expressing the transfected CD8 gene product at levels much higher than normal to obtain binding independent of TCR recognition, our study involves $\alpha\beta$ TCR recognition of antigen and recognition by CD8 expressed at normal levels.

In the present study the constraints on residue 227, as well as other residues in this region, necessary to maintain reactivity with CD8-dependent CTL, were examined. In addition the effect of the Glu-227 → Lys mutation on the stimulation of primary (CD8-dependent) and secondary (CD8-independent) CTL responses was analyzed to further assess the functional significance of the CD8–class I molecule interaction.

MATERIALS AND METHODS

Mice. BALB/c-H-2^{dm2} (K^d, D^d) and [C3H.OL (K^d, D^k) × BALB.K (K^k, D^k)] F₁ mice were bred in the animal facility of Donald C. Shreffler, Washington University School of Medicine.

Antibodies. Monoclonal antibodies (mAbs) 34-2-12 and 34-5-8 are both IgG2a isotype derived from C3H mice (15) and have been shown (16) to react with the $\alpha 3$ and $\alpha 1/\alpha 2$ domains of H-2D^d, respectively. mAb 53-6.72 is a rat IgG2 that reacts with CD8 in all mouse strains (17).

Derivation of Cells Expressing Altered H-2D^d Molecules. Oligonucleotide-directed mutagenesis of the wild-type H-2D^d gene was performed as described (18). The mutant genes, cloned into the pSV2neo plasmid, were transfected into R8.15 by electroporation and selected in medium containing G418 antibiotics. Transfection of the mutant genes along with the pHSV106 plasmid into thymidine kinase-deficient L cells was performed by the modified calcium phosphate procedure (19) and selection in hypoxanthine/aminopterin/thymidine (HAT) medium. Transfectant clones were analyzed by flow microfluorometry for the expression of H-2D^d serological

determinants as described (12) and then maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum.

Generation of H-2D^d-Specific CTLs. The *in vitro* generation of CTLs was performed as described (12). Briefly, 7.5×10^6 (C3H.OL $\times$ BALB.K)F₁ responding spleen cells were co-cultured with 3.5×10^6 2000-R-irradiated (1 R = 0.258 mC/kg) BALB/c-H-2^{dm2} stimulating spleen cells or 1×10^5 10,000-R-irradiated L cells in 24-well Linbro trays (Flow Laboratories) containing 2 ml of RPMI 1640 medium (Mediatech, Washington) supplemented with 2 mM L-glutamine, 0.1 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin at 100 units/ml, streptomycin at 100 units/ml, 50 μ M 2-mercaptoethanol, and 10% fetal calf serum (HyClone). After 5 days at 37°C in 5% CO₂/95% air, effector cells were harvested, washed, and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum. For primary cultures, spleen cells from naive mice were used as the responding cell population whereas for secondary *in vitro* cultures the responding cells were obtained from mice immunized by skin grafting (tail skin to dorsal thorax) or by i.p. injection of spleen cells or L cells.

⁵¹Cr-Release Assay. CTL function was assessed in a standard ⁵¹Cr-release assay as described (12). Briefly, 100 μ l of effector cells in RPMI 1640 medium containing 10% fetal calf serum was added to the round-bottom wells of microtiter plates. For antibody-blocking studies, 50 μ l of anti-CD8 mAb was added to the effector cells for 15 min at 37°C. The anti-CD8 mAb was titrated and used at concentrations in excess of saturation. Approximately 1×10^4 target cells were added to a final volume of 200 μ l per well and the plates were incubated for 4 hr in a humidified atmosphere of 5% CO₂/95% air at 37°C. The plates were centrifuged and radioactivity in 100 μ l of supernatant was measured in a Searle automatic γ counter (Searle). The mean of triplicate samples was calculated and percent ⁵¹Cr release was determined by the following equation: % ⁵¹Cr release = $100 \times [(\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})]$, where experimental ⁵¹Cr release represents cpm from target cells mixed with effector cells, control ⁵¹Cr release represents cpm from target cells incubated in medium alone (spontaneous release), and maximum ⁵¹Cr release represents cpm from target cells exposed to 5% (vol/vol) Triton X-100.

RESULTS

Requirement for an Acidic Group at Residue 227 to Maintain Reactivity with CD8-Dependent CTLs. We have found (12) that a glutamic acid to lysine substitution at residue 227 of MHC class I molecules abrogated reactivity with CD8-dependent CTLs. To determine if the other charged amino acids would have a similar effect, aspartic acid, arginine, or histidine was substituted, by site-directed mutagenesis, at residue 227 of H-2D^d and cells expressing the transfected mutant genes were analyzed for recognition by anti-H-2D^d CTLs generated in primary (CD8-dependent) and secondary (CD8-independent) *in vitro* cultures. Primary CTLs are referred to as CD8-dependent because their lytic activity is completely inhibited by antibody to CD8. Secondary CTLs are referred to as CD8-independent, although in some assays their activity is partially inhibited by antibody to CD8. It is difficult to obtain CTLs that are completely CD8-independent unless CTLs are restimulated several times, mAbs to CD8 or to the $\alpha 3$ domain are included in the cultures, or both (12). As shown in Fig. 1 and summarized in Table 1, substitution of aspartic acid did not affect the reactivity with either CD8-dependent or CD8-independent CTLs. In contrast, substitution with the basic residues arginine or histidine abrogated the reactivity with CD8-dependent CTLs. To

FIG. 1. H-2D^d-specific CTLs generated from primary and secondary *in vitro* cultures and tested on R8.15 cells expressing the wild-type H-2D^d gene (○) or site-directed mutant H-2D^d genes that possess other charged residues at position 227: aspartic acid, [Asp²²⁷]H-2D^{dm} (Δ); lysine, [Lys²²⁷]H-2D^{dm} (□); arginine, [Arg²²⁷]H-2D^{dm} (◇); and histidine, [His²²⁷]H-2D^{dm} (▽). The lysis of H-2D^d or [Asp²²⁷]H-2D^{dm} shown in A was completely inhibitable by antibody to CD8. The CD8-independent CTL lysis shown in B was obtained in the presence of antibody to CD8. Lysis of cells expressing H-2D^d was approximately 20% higher in the absence of antibody. Lysis of nontransfected R8.15 cells was not significantly different from zero. Lysis in the presence of a negative control antibody (rat IgG-2) was not different from lysis in the absence of antibody.

determine whether this loss of recognition was due to the absence of the negative charge or the presence of a positive charge, uncharged residues were also substituted at position 227. The uncharged polar residues, asparagine or tyrosine, or the nonpolar residues, alanine, leucine, proline, or phenylalanine, destroyed reactivity with CD8-dependent CTLs (Table 1). Therefore, the only substitution at position 227 that did not abrogate recognition by CD8-dependent CTL was aspartic acid, which is the acidic residue found at position 227 in all class I molecules (mouse, rat, and human) other than H-2D^d, which has glutamic acid at position 227 (compiled in ref. 20). Thus, a negatively charged residue at position 227 is required for CD8 recognition. However, none of the substitutions at position 227 affected recognition by CD8-independent CTLs, suggesting that the determinants in the $\alpha 1/\alpha 2$ domains of H-2D^d recognized by the $\alpha\beta$ TCR were unaltered. Therefore, it is likely that the determinant recognized by the CD8 molecule is being disrupted.

In addition to analyses with CTL populations, the effect of these substitutions on serologically defined determinants was assessed by flow microfluorometry. mAb 34-5-8, which recognizes the H-2D^d $\alpha 1/\alpha 2$ domains, reacted with all of the transfectants (Table 1) whereas the $\alpha 3$ domain mAb 34-2-12 (the mAb used to select the original lysine-substituted mutant [Lys²²⁷]H-2D^d) reacted only with the wild-type H-2D^d molecule and not with any of the mutant H-2D^d molecules. The failure of mAb 34-2-12 to react with the Asp²²⁷-substituted H-2D^d molecule ([Asp²²⁷]H-2D^d) is consistent with the observation that all class I molecules except H-2D^d molecules have aspartic acid at this position. It is likely that the mAb 34-2-12 binding site and the CD8 binding site in the $\alpha 3$ domain are overlapping and have different constraints. This is consistent with our earlier studies that demonstrated mAb 34-2-12 could be used to skew allorecognition of H-2D^d toward CD8-independency (12).

Substitution at Other Charged Residues Near Position 227 Also Abrogates CD8 Recognition. There are several other

Table 1. Effects of mutations at residue 227 of H-2D^d on serological expression and recognition by CTLs

Amino acid	Serological expression		Recognition by CTLs	
	34-5-8	34-2-12	CD8-dependent	CD8-independent
Glu	128	114	+	+
Asp	146	3	+	+
Lys	135	3	—	+
Arg	145	1	—	+
His	149	0	—	+
Asp*	140	3	—	+
Tyr	157	1	—	+
Ala	135	0	—	+
Leu	133	1	—	+
Pro*	122	9	—	+
Phe*	145	7	—	+

After transfection of the mutant *H-2D^d* genes, the transformants were stained with mAb 34-5-8 (H-2D^d $\alpha 1/\alpha 2$) or mAb 34-2-12 (H-2D^d $\alpha 3$) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin as described (12) and analyzed on an Epics with a logarithmic amplifier. The results are expressed as the shift in mean fluorescence channel number from samples stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin only. The reactivity of CTLs with the transfectants was assessed. For CD8-dependent CTL recognition, anti-H-2D^d CTLs generated in a primary *in vitro* response that are inhibited by the addition of mAb to CD8 (see Fig. 1) are measured. For CD8-independent CTL recognition, anti-H-2D^d CTLs generated during secondary *in vitro* responses after skin-graft priming are measured. The reactivity of these CTLs was either not inhibited or weakly inhibited by the addition of mAb to CD8. +, Reactivity comparable to that from cells transfected with the wild-type *H-2D^d* gene assayed in the presence of antibody to CD8; —, reactivity indistinguishable from nontransfected cells. The CTL results reported in this table are the summary of several independent experiments. The wild-type H-2D^d and the mutant [Lys²²⁷]H-2D^{dm} have been compared in numerous experiments. All of the other mutant H-2D^d molecules were tested between three and six times.

*Mutant gene expressed only in L cells. Other mutant genes were expressed in both L cells and R8.15 cells.

charged residues in the vicinity of residue 227 and these amino acids are conserved within the class I molecules sequenced thus far. In particular, positions 222, 223, and 229 are always acidic and position 232 is also acidic in all class I molecules with the exception of Q10. We substituted lysine for glutamic acid at these four positions and, in addition, changed histidine to aspartic acid at residue 192. None of these changes affected the expression of the molecule as determined by the ability to bind mAb 34-5-8 (Table 2) except for substitution at position 222, which slightly reduced expression. The only substitution, other than residue 227, which eliminated binding of the $\alpha 3$ domain mAb 34-2-12 was that at residue 229. When used as targets for CD8-dependent CTLs the substitutions at residues 222, 223, and 229 eliminated reactivity, whereas the substitutions at residues 192 and 232 had no effect. None of the substitutions affected the reactivity with CD8-independent CTLs (Table 2), again suggesting that the class I $\alpha 1/\alpha 2$ domains were unaltered. This result extends the CD8 recognition site of the $\alpha 3$ domain to include, as a minimum, residues 222–229 and disruption of the negative charge at residues 222, 223, 227, or 229 is sufficient to abrogate recognition by CD8.

The [Lys²²⁷]H-2D^{dm} Mutant Molecule Can Stimulate CD8-Independent, but Not CD8-Dependent CTLs *in Vitro*. Since substitution of residue 227 affected recognition by CD8-dependent but not CD8-independent H-2D^d-specific CTLs, we wanted to determine if the mutant molecules could stimulate CD8-independent CTLs to H-2D^d. The mutant [Lys²²⁷]H-2D^{dm} molecule failed to stimulate a CD8-dependent primary CTL response when assayed on cells

Table 2. Effects of substitutions in the $\alpha 3$ domain of H-2D^d on serological expression and recognition by CTLs

Substitution	Serological expression		Recognition by CTLs	
	34-5-8	34-2-12	CD8-dependent	CD8-independent
D ^d Glu-227*	128	114	+	+
Glu-227 → Lys*	135	3	—	+
Glu-222 → Lys†	99	53	—	+
Glu-223 → Lys†	156	93	—	+
Glu-229 → Lys†	166	4	—	+
Glu-232 → Lys†	148	99	+	+
His-192 → Asp†	130	93	+	+

For legend, see Table 1. D^d, wild-type H-2D^d.

*Mutant genes transfected in R8.15 and L cells.

†Mutant genes transfected in L cells only.

expressing either the mutant [Lys²²⁷]H-2D^{dm} or wild-type H-2D^d molecule (ref. 12 and Fig. 2A). Therefore, secondary conformational effects on the $\alpha 1/\alpha 2$ domains do not account for the lack of recognition by CD8-dependent CTLs since [Lys²²⁷]H-2D^{dm} would be expected to stimulate a response specific for [Lys²²⁷]H-2D^{dm}. We then also attempted to generate a CTL response against the [Lys²²⁷]H-2D^{dm} molecule by *in vivo* priming F₁ mice with transfected L cells (three injections at 4-week intervals). The primed spleen cells were then stimulated *in vitro* with L cells transfected with the wild-type or mutant H-2D^d molecules and the resulting CTL populations were assayed on the R8.15 transfectants, which serve as better targets than L cells. If CD8-independent precursors are present but in fewer numbers than the CD8-

FIG. 2. [Lys²²⁷]H-2D^{dm} stimulates CD8-independent but not CD8-dependent CTLs. Spleen cells were stimulated *in vitro* with L cells expressing wild-type H-2D^d (Δ) or with L cells expressing the mutant [Lys²²⁷]H-2D^{dm} ($\circ$). Data in A and D were obtained from unprimed animals, data in B and E were from animals primed three times *in vivo* with L cells expressing either H-2D^d or [Lys²²⁷]H-2D^{dm} and restimulated with the same cell type, and data in C and F were from animals primed once *in vivo* with wild-type H-2D^d [BALB/c H-2^{dm2} (dm2) spleen]. The target cells in A–C were R8.15 cells expressing the wild-type H-2D^d and the target cells in D–F were R8.15 cells expressing the mutant [Lys²²⁷]H-2D^{dm}. The addition of mAb to CD8 during the 4-hr CTL assay is indicated by $\blacktriangle$ or $\bullet$.

dependent precursors, then the mutant molecule should be able to prime these CD8-independent precursors *in vivo* and they should then be detectable *in vitro*. It is interesting that cells expressing the [Lys²²⁷]H-2D^{dm} molecule were unable to stimulate a response against either [Lys²²⁷]H-2D^{dm} or wild-type H-2D^d, even after *in vivo* priming, whereas the control wild-type H-2D^d molecule stimulated an H-2D^d-specific secondary response *in vitro* after *in vivo* priming (Fig. 2B). If instead, F₁ mice were primed *in vivo* with dm2 spleen cells (wild-type H-2D^d), then cells expressing [Lys²²⁷]H-2D^{dm} stimulated an *in vitro* secondary H-2D^d-specific response that was CD8-independent (Fig. 2B). In addition, several other mutant molecules were tested for the ability to stimulate CD8-dependent primary and CD8-independent secondary CTLs. All of the mutant molecules that were transfected into both L cells and R8.15 cells (see Table 1) were used for this analysis. Only those molecules recognized by CD8-dependent CTLs (wild-type H-2D^d and [Asp²²⁷]H-2D^{dm}) could stimulate CD8-dependent CTLs when used as the antigen. However, all of the mutant molecules tested stimulated CD8-independent H-2D^d-specific CTLs (data not shown). These data suggest that the TCR interaction with the $\alpha 1/\alpha 2$ domains alone is insufficient to prime a T-cell response even though CD8-independent CTLs are capable of effective interaction with the class I molecule. Therefore, the simultaneous interaction between CD8 and the class I $\alpha 3$ domain must also be required as a necessary first step in the generation of both CD8-dependent and CD8-independent CTLs.

DISCUSSION

It is generally accepted that CD4 and CD8 bind to MHC class II and class I molecules, respectively. This concept is strongly supported by experiments demonstrating the ability of liposomes expressing CD8 (21), CHO cells expressing CD8 (9), or CVI cells expressing CD4 (22) to form conjugates with cells expressing the appropriate class of MHC molecule. The biological consequences of these events include a presumed increased affinity between the effector and target cell, receptor redistribution (23), and transmission of biochemical signals (24, 25). Initially, exon-shuffling experiments provided evidence that the $\alpha 1$ and $\alpha 2$ domains of the MHC class I molecules express the allele-specific determinants recognized by the $\alpha\beta$ TCR (26–28). In addition, determination of the x-ray crystallographic structure of a human class I molecule (29, 30) provided compelling evidence that the $\alpha\beta$ TCR recognizes peptides bound within a cleft between the α helices of the $\alpha 1$ and $\alpha 2$ domains. In contrast, relatively little is known about the determinant recognized by CD8. We have reported (12) that a lysine for glutamic acid substitution at residue 227 of the H-2D^d $\alpha 3$ domain abrogates reactivity of CD8-dependent but not CD8-independent CTLs. This finding raised the possibility that this region contributes to the determinant recognized by CD8. The finding that an alanine to valine substitution at residue 245, which is within 10 Å of residue 227, destroys the ability of CHO cells expressing CD8 to form conjugates with cells expressing class I molecules (14) further supports this hypothesis.

The results presented in this paper extend these findings and demonstrate that residue 227 must be either glutamic acid or aspartic acid to serve as a target for CD8-dependent CTLs. Among MHC class I genes whose sequence has been determined, residue 227 is always either glutamic acid or aspartic acid. Therefore, in the exon-shuffling experiments involving the $\alpha 3$ domain, the acidic nature of this residue would have been maintained. In addition to residue 227, we also examined the effect of altering other residues in this region. In the crystallographic structure of HLA-A2, residues 219–227 traverse the molecule between two β sheets, one from residue 210 to residue 219 and the other from residue 229 to residue

238. From residue 219 to residue 235 there are several positions including residues 222, 223, 227, 229, and 232, at which all MHC class I molecules have an acidic residue. We found that changing residue 222, 223, 227, or 229 from glutamic acid to lysine abrogates reactivity with CD8-dependent CTLs. A similar substitution at residue 232 had no effect on recognition by CD8-dependent or CD8-independent CTLs. Other investigators suggested (31) that residue 192 affects the reactivity with CD8-dependent CTLs; however, we did not observe any alterations in CTL reactivity with the H-2D^d molecules substituted at this position. Thus our studies implicate the residues at positions 222, 223, 227, and 229 of the class I molecule as imperative for CD8 recognition.

This conclusion would obviously not be justified if the lack of CD8-dependent recognition of the above mutants resulted from secondary effects of their respective $\alpha 3$ mutations on the $\alpha\beta$ TCR binding site. Five lines of evidence indicate that the conformation of the $\alpha 1/\alpha 2$ domains was not affected by each of the $\alpha 3$ mutations studied here. (i) Each $\alpha 3$ mutant molecule was detected by mAbs to determinants in the $\alpha 1/\alpha 2$ domains (Tables 1 and 2 and unpublished data). (ii) All the $\alpha 3$ mutant molecules studied here were clearly recognized by CD8-independent CTLs (Fig. 1, Tables 1 and 2). (iii) Each of the $\alpha 3$ mutant molecules that was not recognized by CD8-dependent CTLs was unable to elicit a primary CTL response. If the $\alpha 3$ mutation affected $\alpha 1/\alpha 2$ conformation, then one would expect these mutants to stimulate a primary response specific for the respective mutant molecule tested. (iv) The conjugate experiments reported by Salter *et al.* (14) were independent of TCR yet a mutation in the $\alpha 3$ domain affected binding. (v) The distance separating these $\alpha 3$ mutants (e.g., position 227) from the $\alpha\beta$ TCR binding site is greater than 50 Å (29, 30), making it unlikely to result in secondary conformational changes. Thus the location of the CD8 binding site around position 227 of the class I molecule would be ideal for providing sufficient steric separation from the $\alpha\beta$ TCR binding site. However, our data do not exclude the possibility that other $\alpha 3$ substitutions—for example, those introduced during the construction of interspecies chimeric class I genes—can affect epitopes recognized by the $\alpha\beta$ TCR (32). In addition, it should be noted that since [Lys²²⁷]H-2D^{dm} molecules are β_2 -microglobulin-associated (ref. 18; data not shown) and since none of the mutations we tested are known β_2 -microglobulin association sites (29), lack of association with β_2 -microglobulin cannot explain the failure of these mutant D^d molecules to be recognized by CD8-dependent CTLs.

Primary *in vitro* CTL responses are strongly CD8-dependent, whereas secondary responses favor generation of CD8-independent CTLs (12, 33). A possible explanation for this observation is that CD8-dependent and CD8-independent CTL populations arise from separate precursors that express $\alpha\beta$ TCRs of disparate affinities and further that the higher-affinity (CD8-independent) CTLs are initially fewer in number and are preferentially expanded with repeated stimulation. The inability of the mutant H-2D^{dm} molecules to stimulate H-2D^d-specific CTLs unless animals were first primed with wild-type H-2D^d indicates that the CD8–class I interaction is a prerequisite for the development of either a CD8-dependent or a CD8-independent response. Even after three *in vivo* and one *in vitro* stimulation with [Lys²²⁷]H-2D^{dm}, no detectable H-2D^d-specific CTL response was measured. Thus there was no evidence in these mice for the expansion of high-affinity (CD8-independent) CTL clones in the absence of a CD8-dependent response. Therefore, the stimulation of CD8-independent CTLs requires CD8-dependent CTLs. An additional finding relevant to the mode of action of CD8 is that all of the transfected cells express endogenous class I molecules (K^k, D^k for the L cells vs. D^b for the R8.15 cells) at levels equivalent to that expressed by the

nontransfected parent line (data not shown). This endogenous class I molecule is presumably capable of CD8 recognition, yet no lysis of mutant H-2D^{dm} targets was observed. If CD8 functions as an adhesion molecule for low-affinity $\alpha\beta$ TCRs then these endogenous class I molecules should have circumvented the CD8-recognition defect of the H-2D^{dm} mutant molecules. Thus, the failure of CD8-dependent CTLs to recognize these $\alpha 3$ mutants provides direct genetic evidence that CD8 must function as a coreceptor with the $\alpha\beta$ TCR for recognition of the same class I molecule.

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