EPITOPES ON H-2D\textsuperscript{d} SOMATIC CELL MUTANTS RECOGNIZED BY CYTOTOXIC T CELLS* 

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Several studies have demonstrated the importance of the class I molecules of the murine H-2 major histocompatibility complex (MHC)\textsuperscript{1} as the target antigens for allogeneic cytotoxic T lymphocytes (CTL) and also as molecules that restrict CTL responses to viral-infected or chemically modified syngeneic target cells (1, 2). How these class I molecules are involved in the lytic specificity of CTL remains an unanswered question. Recent approaches to this question have used H-2 mutant mouse strains (3), biochemical characterization and amino acid sequencing of class I antigens (4, 5), and most recently gene transfer studies (6–8).

In this study we approach this question with the use of somatic cell mutants that express altered H-2 molecules (9–11). These cell lines, selected for the absence of a particular serologically defined determinant on a class I molecule, permit a precise correlation between molecular structure and function. We show that several mutant cell lines, isolated by selection against the expression of a serologically defined epitope which maps to the C2 domain of the H-2D\textsuperscript{d} molecule, also exhibit changes in epitopes recognized by both allogeneic CTL and CTL specific for hapten-modified self-determinants.

Materials and Methods 

Antibodies. The monoclonal antibodies to H-2 antigens listed in Table I have been described elsewhere (12, 13). They were kindly given to us by Drs. K. Ozato (National Institute of Child Health and Human Development, Bethesda, MD) and T. Hansen (Merck Institute, Rahway, NJ). In addition to the D\textsuperscript{d} antibodies we also had available several L\textsuperscript{d} and K\textsuperscript{d} monocloned antibodies. All of the hybridoma cell lines secreting the monoclonal antibodies were grown in the peritoneal cavities of (BALB/c × C3H/HeJ) F\textsubscript{1}
mice to generate ascitic fluid, and all antibodies had titers of greater than 1:10,000 on the ACCb cell line.

Cell Lines. The parental cell line ACCb from which the mutants were derived was produced by transformation of (BALB/c × BALB.B10) F1 neonatal bone marrow cells with Abelson virus, as described by Rosenberg and Baltimore (14). To ensure homogeneity of the starting population, the transformed line was cloned twice on soft agar (0.325% wt/vol Sea Plaque Agarose, FMC Corp., Rockland, ME) before use. These cells are grown in suspension culture in RPMI/FBS, ie. RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island NY) and 5 × 10^-5 M β-mercaptoethanol and have a doubling time of ~12 h. CTLL-A2, a cell line cytotoxic for H-2Dd targets, was derived from peritoneal exudate cells of C57B1/6 mice immunized with the BALB/c sarcoma Meth A and was cloned by limiting dilution in the presence of IL2 (15). This line has been maintained in culture for nearly 3 yr in IL2-conditioned media without antigen restimulation. The cloned CTL line, D.FL.12, which is specific for fluorescein isothiocyanate (FITC) in association with H-2Dd, was derived by Dr. Werner Haas at the Basel Institute for Immunology (16). This line was subcloned several times (by D. B. Wilson) and maintained in RPMI/FBS supplemented (15% vol/vol) with supernatant from concanavalin A (Con A)-stimulated rat spleen cells.

Derivation of Mutant Cell Lines. Mutagenesis and isolation of mutant cell lines was performed as previously described (17). ACCb cells were incubated with 600 µg/ml of ethylmethane sulfonate (Sigma Chemical Co., St. Louis, MO) or 2 mM nitrosoethylurea (Sigma Chemical Co.) 7 d before selection. The selection was performed by incubating 5 × 10^5 cells with an optimal amount (1:100 dilution of ascites fluid) of monoclonal H-2 antibody and a 1:3 dilution of rabbit complement in a total volume of 300 µl. After 3 h of incubation at 37°C, the cells were cloned on soft agar (0.325% wt/vol) and after 7 d individual clones were picked and their reactivity with several monoclonal H-2 antibodies was tested by microcytotoxicity.

Flow Microfluorometric Analysis of H-2 Antigen Expression. For immunofluorescence staining 10^6 cells in 100 µl of RPMI/FBS were incubated for 30 min on ice with an optimal amount (10 µl) of monoclonal anti H-2Dd antibody that had been conjugated with FITC using standard procedures (18). After being stained, the cells were washed and the fluorescence of 20,000 viable cells was measured on a FACS II instrument (Becton Dickinson FACS systems, Mountain View, CA).

Labeling and Immunoprecipitation of H-2 Antigens. Labeling of cell surface molecules with ^125I was performed by the lactoperoxidase-catalyzed reaction in the presence of hydrogen peroxide (19). The day before, the cells were split into two aliquots, each with 3 × 10^6 cells in 20 ml RPMI/FBS. Tunicamycin (Sigma Chemical Co.) was added to one of the aliquots at a concentration of 2 µg/ml. 20 h later the cells were harvested and washed twice in PBS, and then 2 mCi of ^125I, 25 µl lactoperoxidase (2 mg/ml; Sigma Chemical Co.) and 25 µl of 0.05% hydrogen peroxide were added, mixed thoroughly, and allowed to react at room temperature for 5 min, when an additional 25 µl of hydrogen peroxide was added. After a further incubation period of 5 min the cells were washed twice in PBS and then the membranes were solubilized in 0.5% (vol/vol) NP-40. The membrane extracts were then precleared by overnight incubation at 4°C in the presence of rabbit anti-mouse immunoglobulin and sheep anti-rabbit immunoglobulin. After removal of the precipitates, the supernatants were incubated with monocular anti-H-2 antibody and goat anti-mouse immunoglobulin, overnight at 4°C. The precipitates were washed three times and then resuspended in either 9 M urea (for isoelectrofocusing) or SDS buffer (for SDS-PAGE). Immediately before loading on the gels, the samples were boiled for 2 min in the presence of 5% (vol/vol) β-mercaptoethanol.

Biochemical Analysis of Immunoprecipitates. (a) SDS-PAGE was performed in slab gels consisting of a 2-cm 3.5% acrylamide stacking gel and a 10-cm 10% acrylamide resolving gel as described by Laemmli (20). Electrophoresis was performed under a constant current of 30 mA until the dye front was 0.5 cm from the bottom of the gel. (b) Isoelectrofocusing was also performed in slab gels consisting of 9 M urea, 2.5% NP-40, and 5% ampholines pH 5–7 (LKB, Corp.) as described by O'Farrell (21). The samples were loaded at the
alkaline end of the gel (0.02 N NaOH) and ran towards the acid end (0.085% H$_3$PO$_4$) for 24 h at 200 V (22).

After drying, X-ray film (Kodak XAR-5) was exposed to the gel at room temperature without intensifying screens for a period of 10–30 d.

Generation of Allogeneic Effector Cells. Cytotoxic cells to H-2D$^d$ antigens were generated in (C3H.OH × BALB.B10) F, mice using spleen cells from the H-21.d loss mutant BALB/c H-2$^{d_{\text{H}}}$ as stimulators. For generation of secondary effector cells the mice were given an injection of 2 × 10$^7$ BALB/c H-2$^{d_{\text{H}}}$ spleen cells s.c. 4 wk before establishing the mixed lymphocyte reaction (MLR). Primary or secondary MLR's were performed in 24-well dishes, with each well receiving 5 × 10$^6$ responder (C3H.OH × BALB.B10) F, spleen cells and 5 × 10$^6$ irradiated (2,000 Rads) BALB/c H-2$^{d_{\text{H}}}$ spleen cells in a total volume of 2 ml RPMI/FBS. After 5 d of culture at 37°C the effector cells were harvested, washed, and tested on $^{51}$Cr-labeled target cells.

Assays of Cell-Mediated Cytotoxicity. Cell-mediated cytotoxicity by allogeneic effector cells and the cloned cell lines was assessed by release of $^{51}$Cr (New England Nuclear, Boston, MA) from labeled target cells. Target cells conjugated with FITC were prepared as described by Haas et al. (16). Target cells, either ACCb and mutants or 48-h Con A–induced spleen cell blasts, were labeled by incubation of 10$^6$ cells in 500 µl RPMI/FBS with 100 µCi $^{51}$Cr for 60 min at 37°C. After three washes target cells were adjusted to 10$^6$/ml and 100 µl was added to each well of a 96-well V-bottom tray. After addition of 100 µl of effector cells at effector/target ratios indicated in the tables the cells were pelleted and incubated for 4 h at 37°C. The percentage cytotoxicity was determined after counting the radioactivity in 100 µl of supernatant and after comparison to the maximum and the spontaneous release, using the formula:

\[
\% \text{ Specific Release} = \frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100.
\]

Each value is given as the mean of triplicate determinations.

Results

Isolation of H-2D$^d$ Structural Mutant Cell Lines. Selections, on ACCb cells that had been treated with a chemical mutagen, were performed with two monoclonal antibodies to H-2D$^d$, 34-4-21 and 34-2-12. Although these antibodies have identical reactivity with inbred mouse strains (both recognizing D$^d$ private specificities), the two antibodies do not inhibit each other in competitive binding studies (Potter, unpublished observation), suggesting that they react with different D$^d$ epitopes. From two selections using the 34-2-12 antibody as the selecting agent, 36 individual clones were screened (Table I): 11 failed to express any of the H-2D$^d$ antigens tested, i.e. they were K$^d$ L$^d$ D$^{d-}$; 6 expressed K$^d$ but not L$^d$ or D$^d$, i.e. they were K$^{d+}$ L$^{d-}$ D$^{d-}$; 9 failed to express any detectable D$^d$ but were still K$^{d+}$ L$^{d+}$; and 3 clones reacted with K$^d$ and L$^d$ antibodies, and with 34-4-21 but not with the selecting antibody 34-2-12. These latter three clones are referred to as H-2D$^d$ structural mutants, as they apparently have an altered D$^d$ molecule and are designated by their selecting antibody ACCb 34-3-12$^{-1}$, ACCb 34-2-12$^{-3}$ and ACCb 34-2-12$^{-4}$. For easier reading these three mutant cell lines will be referred to in the text as 2.12$^{-1}$, 2.12$^{-3}$, and 2.12$^{-4}$, respectively.

Using the 34-4-21 antibody as the selecting agent we were unable to obtain any structural mutants with a reciprocal phenotype to that described above (i.e. 34-4-21$^{-}$ 34-2-12$^{+}$), from ACCb in three independent selections (Table I). The phenotypes of 125 independent clones from selections using 34-4-21 were 79 K$^{d+}$ L$^{d-}$ D$^{d-}$; 21 K$^{d+}$ L$^{d+}$ D$^{d-}$; 25 K$^{d+}$ L$^{d+}$ D$^{d+}$. Selections from two other H-2$^d$
Table I

| Selecting antibody* | No. of clones | Phenotype |
|---------------------|---------------|-----------|
| 34-2-12             | 11            | K⁺ L⁺ D⁺ |
|                     | 6             | K⁺ L⁺ D⁺ |
|                     | 9             | K⁺ L⁺ D⁺ |
|                     | 3             | K⁺ L⁺ D⁺ |
| 34-4-21             | 79            | K⁺ L⁺ D⁺ |
|                     | 21            | K⁺ L⁺ D⁺ |
|                     | 25            | K⁺ L⁺ D⁺ |

* Both of these antibodies, 34-2-12 and 34-4-21, react with D⁴ private specificities.

Other mutants derived from ACCb and used in experiments described herein include: ACCb H-2d⁻, a cell line that does not express any detectable H-2K⁰ H-2D⁰ or H-2L⁰; ACCb H-2D⁻, which does not express any detectable H-2D⁴ but is H-2K⁺ H-2L⁺ and H-2b⁺; ACCb H-2b⁻, which is a H-2b⁻ H-2d⁺ mutant and ACCb 34-2-12⁻⁻ H-2b⁺ (referred to as 2.12⁺⁻⁻ in text), which is a H-2b⁻ mutant isolated from the 2.12⁻⁻ mutant. The H-2 hemizygous mutants from ACCb and from the 2.12⁻⁻ mutant cell line were derived by selection using the Kb antibody Y-3, which was a kind gift of Dr. S. Nathenson.

Serological Analysis of H-2D⁴ Structural Mutants. In addition to 34-2-12 and 34-4-21 several other monoclonal antibodies react only with H-2D when tested on strains of the H-2d haplotype. The reactivity of these antibodies with the H-2D⁴ structural mutants was determined by cytotoxicity testing. Two of the antibodies, 28-8-6 and 34-5-8, gave a weak cross-reactivity with the H-2b⁰ haplotype and therefore these antibodies had to be tested on H-2b⁻ (i.e. hemizygous) mutants isolated after a second selection with H-2b antibodies on the mutants.

The results presented in Table II show that the mutants 2.12⁻⁻ and 2.12⁻⁻ react with all of the D⁴ antibodies except for the selecting antibody 34-2-12. The mutant 2.12⁻⁻ does not react with several D⁴ antibodies—15-1-5, 25-3-21, and 34-2-12—but retains the determinant recognized by 34-4-21. The loss of only one of the tested serological determinants of the H-2D⁴ molecule in the 2.12⁻⁻ and 2.12⁻⁻ cell lines suggests that the effect of the mutation in these cells was more localized than the mutation in the 2.12⁻⁻ cells.

Analysis of the reactivity of the anti-D⁴ monoclonal antibodies was also examined by immunofluorescence measured on the FACS. Testing with the 34-2-12 antibody confirmed that the three mutants 2.12⁻⁻, 2.12⁻⁻, and 2.12⁻⁻ were in fact 34-2-12⁻⁻ (Fig. 1). The reactivity of the 34-4-21 antibody conjugated with FITC was only slightly less with 2.12⁻⁻, compared with the parental ACCb line (Fig. 1). Although the reactivity of 34-4-21 with 2.12⁻⁻ was significantly reduced, the reaction was still greater than with a cell line ACCb H-2D³⁻⁻, which completely fails to express any D⁴ specificities (Fig. 1). The level of reactivity of 34-
TABLE II
Reactivity of Monoclonal Anti-H-2Dd Antibodies on ACCb and Dd Mutants

| Monoclonal antibodies | 34-4-21 Dd | 34-2-12 Dd | 15-1-5 Dd H:2b | 23-5-21 Dd | 28-8-6 Dd H:2b | 34-5-8 Dd H:2b |
|-----------------------|------------|------------|----------------|------------|----------------|----------------|
| ACCb                  | +          | +          | +              | +          | +              | +              |
| ACCb 34-2-12^{-1}     | +          | -          | +              | +          | NT             | NT             |
| ACCb 34-2-12^{-3}     | +          | -          | -              | -          | NT             | NT             |
| ACCb 34-2-12^{-4}     | +          | -          | +              | +          | NT             | NT             |
| ACCb 34-2-12^{-4} H:2b^{-} | +     | -          | +              | +          | +              | +              |
| ACCb H:2b^{-}         | +          | +          | +              | +          | +              | +              |
| ACCb H:2b^{-}         | -          | -          | -              | -          | NT             | NT             |

Reactivity assessed by microcytotoxicity using the monoclonal antibodies at an optimum concentration (1/200) and rabbit complement at a final dilution of (1/12).
+ denotes >99% of cells dead after 60 min incubation.
- denotes <5% specific kill after the same period.
NT, not tested.

FIGURE 1. Reactivity of 34-2-21 and 34-2-12 antibodies with ACCb and Dd mutants detected on the FACs. The 34-4-21 antibody was directly conjugated with FITC, whereas the binding of the 34-2-12 antibody was detected with FITC-conjugated goat anti-mouse immunoglobulin antibody. The target cells for each profile are as labeled.
4-21 with 2.12<sup>−3</sup> was very similar to that obtained with 2.12<sup>−1</sup> (profile not shown). Although the level of reactivity of 34-4-21 with 2.12<sup>−4</sup> was slightly less than with ACCb, the hemizygous variant 2.12<sup>−4b−</sup> expressed an increased amount of detectable H-2D<sup>d</sup> compared with ACCb (profile not shown). Overall, these observations confirm that the mutants are 34-2-12<sup>−</sup> 34-4-21<sup>−</sup>, however, the mutations have resulted in a variable reduction of detectable H-2D<sup>d</sup>.

**SDS-PAGE of H-2D<sup>d</sup> Molecules.** The molecular weight of D<sup>d</sup> from 2.12<sup>−4b−</sup> was compared with that of the unselected D<sup>d</sup> molecule precipitated from ACCb H-2<sup>b−</sup>. The cells were labeled with 11<sup>25I</sup>, lysates were prepared, and the solubilized membrane extract was precleared with anti-immunoglobulin. The H-2D<sup>d</sup> molecules were precipitated using the monoclonal antibody 34-5-8 and goat anti-mouse immunoglobulin. Electrophoresis in SDS-PAGE using a 10% acrylamide resolving gel showed that the D<sup>d</sup> molecule precipitated from 2.12<sup>−4b−</sup>; (Fig. 2, lane B) ran faster than the D<sup>d</sup> molecule precipitated from ACCb H-2<sup>b−</sup> using either 34-5-8 (Fig. 2, lane A) or 34-2-12 (Fig. 2, lane C). This reduction in molecular weight was estimated to be on the order of 2,000–3,000 daltons and was also apparent when both cell lines were grown in the presence of an inhibitor of glycosylation, tunicamycin (data not shown), strongly suggesting that the reduction in molecular weight was not due to an absence of one of the carbohydrate chains in the D<sup>d</sup> molecule from 2.12<sup>−4b−</sup>.

**Isoelectrofocusing Comparison of H-2D<sup>d</sup> Molecules.** The H-2D<sup>d</sup> molecules immu-

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**Figure 2.** SDS-PAGE of molecules precipitated from ACCb H-2<sup>b−</sup> denoted P, parental-type D<sup>d</sup>, and ACCb 34-2-12<sup>−4</sup> H-2<sup>d−</sup> denoted M, mutant D<sup>d</sup>. Membrane preparations labeled with 11<sup>25I</sup> were precipitated with D<sup>d</sup> antibodies 34-5-8, 34-2-12 or the K<sup>d</sup> antibody 11-4-1. Molecules in the 40–45,000-dalton (K = 10<sup>9</sup>) range are represented by bands approximately in the middle of the gel.
noprecipitated from ACCb H-2\textsuperscript{b−} and 2.12\textsuperscript{-b−} were also compared on isoelectrofocusing slab gels with a pH gradient from pH 7–pH 5. The molecule precipitated by 34-5-8 from ACCb H-2\textsuperscript{b−} had at least three strong bands in the middle of the gel (Fig. 3, lane A). The H-2D\textsuperscript{d} molecule precipitated by 34-5-8 from 2.12\textsuperscript{-b−} also had three principal bands (Fig. 3, lane C) however, relative to those of the precipitate from ACCb H-2\textsuperscript{b−}; these bands were shifted to the alkaline end. The isoelectrofocusing data therefore confirms the observation obtained by SDS-PAGE that the H-2D\textsuperscript{d} molecule expressed on 2.12\textsuperscript{-b−} is biochemically different from the H-2D\textsuperscript{d} molecule expressed on ACCb H-2\textsuperscript{b−}.

Testing of Anti-H-2D\textsuperscript{d} Cytotoxic Lymphocytes on the H-2D\textsuperscript{d} Mutants. The ability of the altered H-2D\textsuperscript{d} molecules to serve as a target in anti-D\textsuperscript{d} allogeneic and hapten-modified self cytotoxicity was tested using as effectors both cloned cytotoxic T cell lines, and killer cells generated in primary and secondary allogeneic re-

![Figure 3. Isofocusing of D\textsuperscript{d} molecules precipitated using D\textsuperscript{d} monoclonal antibodies 34-5-8 (lanes A–D) or 34-2-12 (lanes E–H). Membrane preparations were from ACCb H-2\textsuperscript{b−} (lanes A and E), ACCb 34-2-12 H-2\textsuperscript{b−} (lanes C and G), ACCb H-2\textsuperscript{b−} cells cultured in the presence of tunicamycin (lanes B and F) and ACCb 34-2-12 H-2\textsuperscript{b−} cells cultured in the presence of tunicamycin (lanes D and H).](image-url)
sponses.

(a) Cloned CTL Line Specific for H-2D\textsuperscript{d}. The cytotoxicity of the anti-D\textsuperscript{d} CTL clone, CTLL-A2, for ACCb and the D\textsuperscript{d} mutants is presented in Table III. This line was cytotoxic for ACCb (54-56% specific lysis) but did not kill the 2.12\textsuperscript{-4} (9%) or 2.12\textsuperscript{-4b-} (3%) mutants. The level of cytolytic activity of CTLL-A2 on the other mutants, 2.12\textsuperscript{-3} (32-45%) and 2.12\textsuperscript{-5} (39%), was reduced compared with ACCb target cells (Table III).

The effect of monoclonal anti-H-2D\textsuperscript{d} antibodies on the cytotoxicity of CTLL-A2 for ACCb and 2.12\textsuperscript{-1} target cells was also investigated. The results (Fig. 4) show that addition of either 34-2-12 or 34-4-21 significantly inhibited the cytotoxicity of CTLL-A2 for ACCb. For 2.12\textsuperscript{-1} target cells only 34-4-21 and not 34-2-12 inhibited the cytotoxicity of CTLL-A2 for these cells (Fig. 4). The failure of 34-2-12 to block the killing of 2.12\textsuperscript{-1} by CTLL-A2 is consistent with the serological findings that this target cell lacks the epitope recognized by 34-2-12. The blocking of cytotoxicity by both 34-2-12 and 34-4-21 antibodies would suggest that antiserum blocking studies using antibodies to the target cell are very prone to steric inhibition and therefore do not elucidate the fine specificity of the epitope being recognized by cytotoxic cells.

Overall the cytotoxic reactivity of CTLL-A2 on ACCb and the H-2D\textsuperscript{d} mutants demonstrates that CTLL-A2 recognizes an epitope different from that recognized by the 34-2-12 antibody, however, this epitope has been affected by the mutation in the 2.12\textsuperscript{-4b-} cells.

(b) Cytotoxicity of Primary and Secondary Allogeneic CTL Populations. Cytotoxic T cells specific for H-2D\textsuperscript{d} antigens were generated in a 5-d mixed lymphocyte reaction using (C3H.OH X BALB.B10) F\textsubscript{1} responder spleen cells and irradiated BALB/c H-2\textsuperscript{d2} spleen cells as stimulators. Cells stimulated in this manner were cytotoxic for ACCb with 29-38% specific release (Table IV) compared with <3% for the H-2\textsuperscript{d4} variant from ACCb (Table IV). Surprisingly, in both experiments all three H-2D\textsuperscript{d} mutants were only very weakly lysed, with the possible exception of an 18% specific kill of 2.12\textsuperscript{-3} in experiment 1 (Table IV). These findings suggest that the vast majority of the killer cells from a (C3H.OH X BALB.B10) F\textsubscript{1} anti-BALB/c H-2\textsuperscript{d2} primary response are directed against epitopes affected by the mutations in 2.12\textsuperscript{-1}, 2.12\textsuperscript{-3}, and 2.12\textsuperscript{-4}. The finding that

| Target | Specific lysis |
|--------|---------------|
|        | Experiment 1  | Experiment 2 |
| ACCb   | 54            | 56            |
| ACCb 34-2-12\textsuperscript{-1} | 45            | 32            |
| ACCb 34-2-12\textsuperscript{-3} | NT            | 39            |
| ACCb 34-2-12\textsuperscript{-4} | 9             | NT            |
| ACCb 34-21-12\textsuperscript{-4}H-2\textsuperscript{d4} | NT            | 3             |
| ACCb H-2\textsuperscript{d2} | NT            | 55            |
| ACCb H-2D\textsuperscript{d6} | 6             | 4             |

The effector to target cell ratio was 10:1. NT, not tested.
Figure 4. Blocking of the cytotoxic reactivity of CTLL-A2 by the addition of 50 μl doubling dilutions of D\(^d\) antibodies 34-2-12 (left-hand panels) or 34-4-21 (right-hand panels) to the reaction mixture. The target cells were ACCb (top panels) and ACCb 34-2-12\(^{-1}\) (bottom panels). The level of kill in the absence of antibody is indicated in the top left corner of each panel.

Table IV

| Target                  | Experiment 1: E/T ratio | Experiment 2: E/T ratio |
|-------------------------|-------------------------|-------------------------|
|                          | 10  | 5  | 30  | 15  | 6  |
| ACCb                    | 32  | 29 | 38  | 29  | 32 |
| ACCb 34-2-12\(^{-1}\)   | 5   | 5  | 0   | 1   | 0  |
| ACCb 34-2-12\(^{-2}\)   | 18  | 11 | 6   | 4   | 4  |
| ACCb 34-2-12\(^{-4}\) H-2\(^{bm2}\) | 5   | 1  | 8   | 8   | 5  |
| ACCb H-2\(^{bm2}\)      | 53  | 34 | 29  | 26  | 16 |
| ACCb H-2\(^{dm}\)       | 1   | 0  | 2   | 3   | 3  |

E/T, Effector/Target ratio.
The effectors were generated in a 5-d mixed lymphocyte reaction in the combination (C3H.OH x BALB.B10) F\(_1\) anti-BALB/c H-2\(^{bm2}\). The results expressed are specific lysis after a 4-h incubation with \(^{51}\)Cr-labeled target cells.

The H-2D\(^d\) mutant cells were only very marginally lysed by effector cells from a primary allogeneic response was unexpected, so we investigated the reactivity of killer cells from a secondary response on ACCb and the H-2D\(^d\) mutants. Anti-D\(^d\) CTL generated in a secondary response were highly cytotoxic for ACCb with a specific release between 90–95%, compared with -2 to 3% on ACCb H-2\(^{dm}\) cells (Table V). When these cells were tested on the H-2D\(^d\) mutants significant
levels of lysis were observed 2.12⁻¹ (21–31%), 2.12⁻³ (49–52%) and 2.12⁻ⁿ⁻⁻ (18–31%, Table V). The observation that the level of kill of the mutants was lower than that seen for ACCb cells was further investigated by cold target competition. As presented in Table VI, addition of excess unlabeled BALB/c H-2dm² cells completely inhibited cytotoxicity of the CTL population for labeled BALB/c H-2dm² cells. The effect was specific, as addition of C57BL/6 or ACCb H-2dm cells did not affect the level of kill. Although not as complete as the inhibition obtained with BALB/c H-2dm² cells, addition of unlabeled ACCb cells significantly reduced the killing of BALB/c H-2dm² cells. The effect of the addition of unlabeled mutant H-2Dk cells was less marked; addition of 2.12⁻³ produced the greatest reduction of cytotoxicity by the three mutants, whereas the addition of 2.12⁻¹ or 2.12⁻ⁿ⁻⁻ cells gave only a slight reduction in the level of cytotoxicity. For none of these three mutants was the reduction in cytotoxicity as great as that obtained with ACCb, suggesting that none of the mutants possessed all of the epitopes recognized by the heterogeneous population of
secondary cytotoxic cells.

(c) H-2D<sup>d</sup> Mutant Cells as Targets for a CTL Clone Specific for FITC. ACCb and the D<sup>d</sup> mutant cell lines were labeled with FITC and tested for their ability to be lysed by a cloned line of CTL, D.FL.12, which has lytic specificity for FITC-labeled target cells expressing the H-2D<sup>d</sup> molecule. The results (Fig. 5) showed that the H-2D<sup>d</sup> mutants 2.12<sup>-1</sup> and 2.12<sup>-4b</sup> were not killed by D.FL.12, 2.12<sup>-3</sup> was lysed to some extent, but not to the extent of the parental line ACCb or its hemizygous variant ACCb H-2<sup>b+</sup>. The results indicate that the H-2D<sup>d</sup> determinant(s) involved in restriction of anti-FITC T cell-mediated lysis has also been altered by the mutations that led to the serologically detected changes.

Discussion

Recent studies have provided major insights into the structure and genetic organization of H-2 class I molecules. An important tool for an understanding

![Graph](image)

**Figure 5.** Cytotoxicity of D.FL.12 for ACCb and D<sup>d</sup> mutant cell lines. The target cells were conjugated with FITC as described by Haas et al. (16) before testing. (●) FITC-conjugated cells. (○) Nonconjugated cells.
of H-2 structure-function relationships has been the mutant strains of mice isolated by Melvold, Kohn, Egorov, and Bailey (23–25). Comparisons of mutant and wild type phenotypes have been made in many functional systems, including histogeneic studies of allogeneic determinants recognized by T cells in vivo or in vitro (26), investigation of the T cell repertoire to allogeneic determinants (27), and characterization of determinants restricting viral (28, 29) or minor histocompatibility antigen (30) responses. In addition to the H-2 mutants isolated in vivo, mutants in H-2 class 1 molecules can also be isolated from somatic cells in culture (9, 10). The latter procedure makes it possible to isolate cells that have lost the ability to express a particular H-2 determinant.

We have isolated three cell lines expressing mutant D^d molecules and have characterized the mutant molecule serologically, biochemically, and in assays investigating the functional role of D^d both as a target for CTL and as a molecule restricting responses to hapten-modified self-determinants. The effect of the mutation in the three cell lines is summarized in Table VII. In addition, biochemical analysis demonstrated that the mutant D^d molecule from the 2.12^-b^- mutant cell line is ~2,000–3,000 daltons smaller than normal D^d and as this difference was also seen in cells treated with tunicamycin, the reduction in size is apparently not due to the absence of a carbohydrate chain. Analysis by isoelectrofocusing confirmed that the H-2D^d molecule expressed on this cell line is structurally altered.

**Relationship between Serologically Defined Determinants and Determinants Recognized by Allogeneic CTL.** The major finding from these studies is that the mutant cell lines selected for by serological alterations also show changes in epitopes recognized by T cells (Table VII). The failure of CTLL-A2 to react with 2.12^-d^- demonstrated that the mutation that produced the nonexpression of the 34-2-12 serological specificity by this cell line also altered the epitope recognized by CTTL-A2. However, the cytotoxicity of CTLL-A2 for the two other 34-2-12^- mutants, suggested that the CTL clone recognizes a determinant different from the serological determinant. It was striking that the two mutants, 2.12^-1^- and 2.12^-4^, which have a similar serological profile demonstrated such a contrast in their susceptibility to cytotoxicity by CTLL-A2. It should be pointed out that

| Cell line | ACCb 34-2-12^-1 | ACCb 34-2-12^-2 | ACCb 34-2-12^-3 | ACCb 34-2-12^-4 |
|-----------|-----------------|-----------------|-----------------|-----------------|
| Nonreactive D^d antibodies | Nil 34-2-12 | 34-2-12 | 34-2-12 |
| | 15-1-5 | 23-5-21 |
| Sensitivity to killer cells | CTLL-A2 | +++ | ++ | ++ | - |
| | 1^- MLR | +++ | - | + | - |
| | 2^- MLR | +++ | + | ++ | + |
| | D.FL.12 (anti-FITC) | +++ | - | + | - |

The level of lysis, represented by -, +, ++, +++ is designated using the level seen for ACCb as +++.
the failure of CTLL-A2 to kill $2.12^{-4}$ was not because these targets were resistant to T cell killing, because anti-K$^b$ or anti-L$^d$ killer cells readily killed these cells (data not shown). In addition, the analysis by immunofluorescence of the expression of H-2D$^d$ on these cells together with the observation that $2.12^{-1}$ and $2.12^{-5}$ cells were killed by CTLL-A2, demonstrated that the inability of CTLL-A2 to kill these targets was not caused by a quantitative reduction in the amount of H-2D$^d$ expressed on the mutant cells.

In our efforts to further correlate serologically defined and CTL-defined determinants, we investigated the inhibition of the cytotoxicity of CTLL-A2 for ACCb by the addition of monoclonal antibodies. It was found that the binding of any monoclonal to the target molecule inhibited the CTL. This was the case even for antibodies such as 34-2-12 and 34-4-21, which bind to separate determinants on the D$^d$ molecule and in competitive binding experiments do not inhibit the binding of each other (Potter, unpublished observations). This implies that antibody blocking studies of the epitopes recognized by T cells are affected by steric constraints, and it is therefore difficult from antisera blocking studies to make conclusions about the fine specificity of the target epitope.

Using recombinant D$^d$-L$^d$ genes, recent studies have mapped the D$^d$ serological specificities to particular domains (31). For the antibodies used in this study, 34-4-21, 34-5-8, and 23-5-21 react with specificities in the N or C1 domains, whereas the determinant recognized by 34-2-12 is in the C2 domain. In apparent conflict with our findings, that in mutants selected by using an antibody 34-2-12 which recognizes a determinant in the C2 domain there are also changes to the determinants recognized by allogeneic CTL, is a recent observation that using recombinant D$^d$-L$^d$ genes, all of the determinants recognized by allogeneic CTLs map exclusively to the N and C1 domains (32). The failure to detect any allogeneic CTL determinants in the C2 domain using the recombinant genes was consistent with amino acid DNA sequencing data, which demonstrated that the greatest homology between MHC class I molecules of different loci and from different haplotypes, was located in the C2 domain. At present we cannot resolve the differences between our data and that obtained using the recombinant genes, but if there are no determinants recognized by allogeneic CTL in the C2 domain then presumably the mutation in the mutants that we have isolated must have affected T cell–recognized determinants in N or C1 while having no effect on the serologically defined epitopes in these domains. An alternative explanation could be that there are some epitopes formed by interactions between the C2 and C1 domains; such determinations might not be generated using recombinant genes in which the C1 and C2 domains come from different sources. It is interesting to note that the mouse strain B10 H-2$^{ae1}$, which has altered D$^d$ and L$^d$ molecules, shows a similar D$^d$ phenotype to $2.12^{-4}$ in that it reacts with all monoclonal D$^d$ antibodies except 34-2-12 (Dr. T. Hansen, personal communication) and it is not killed by the anti-D$^d$ CTL line CTLL-A2 (Palladino and Potter, unpublished observations).

Comparison of Epitopes Recognized by Allogeneic CTL and Epitopes Involved in Anti-Modified-self Responses. The finding that a CTL specific for FITC, D.FL.12, did not react with $2.12^{-1}$ and $2.12^{-5}b^-$, and reacted only weakly with $2.12^{-5}$ demonstrated that the mutations had affected the D$^d$ epitope(s) that restricts the anti-
FITC cytotoxic response. As the patterns of reactivity of the CTLL-A2 and D.FL.12 CTL cell lines were different from each other, these two cell lines must recognize different D\(^d\) epitopes. These findings therefore suggest that it is possible to express a H-2 class I molecule that has epitopes recognized by allogeneic CTL but does not function as a restricting element to certain antigens.

**Comparison of H-2 Somatic Cell Mutants and H-2 Mutant Mice.** Although the D\(^d\) mutants were selected for by alterations in serologically defined determinants, there were also alterations in the determinants recognized by T cells. However, in the H-2K\(^b\) mutant mouse strains, which were selected for by changes in T cell-defined determinants (skin graft rejection), there are relatively few established alterations in the expression of H-2K\(^b\) determinants defined by monoclonal antibodies or alloantisera (33). Studies on the allogeneic determinants expressed on H-2K\(^b\) have demonstrated that there are many different or overlapping, epitopes recognized by T cells, whereas our data showed that mutants isolated by their inability to express a serologically defined determinant had accompanying alterations in epitopes recognized by T cells. One possible interpretation of these findings is that in comparison to K\(^b\) there are fewer epitopes on D\(^d\) that are recognized by T cells. An alternate explanation is that the mutations that we have observed in the H-2D\(^d\) mutants have altered the conformation of the H-2D\(^d\) molecules in a manner such that they are not recognized by T cells with anti-H-2D\(^d\) specificity, while the reactivity with the monoclonal antibodies has remained unaffected. The H-2D\(^d\) mutant molecule that we characterized biochemically had a substantial reduction in size compared with D\(^d\) molecules from unselected cells. Although it is likely that this reduction did significantly affect the conformation of the molecule, all of the monoclonal anti-D\(^d\) antibodies, with the exception of the selecting antibody, still reacted with the altered molecule.

**Summary**

We have generated several cell lines that express an altered H-2D\(^d\) molecule. These cell lines, which were selected for by the failure to express the serological specificity reacting with the monoclonal antibody 34-2-12, have also undergone alterations in epitopes recognized by CTL. One of the mutants, 2.12\(^-4\) was not killed by an allogeneic anti-D\(^d\) CTL line, CTLL-A2, even though this line was cytotoxic for the parental cell line and two other 34-2-12\(^-\) mutant lines. Two of the 34-2-12\(^-\) mutant lines had an identical serological profile using other monoclonal D\(^d\) antibodies, however these two mutants differed markedly in their susceptibility to cytotoxicity by CTLL-A2. In addition to the determinants recognized by allogeneic CTL we also examined the effect of the mutation on the determinants involved in restricting the anti-FITC modified-self-cytotoxic response. An anti-FITC-D\(^d\) CTL line did not react with two of the mutants and reacted only weakly with the other mutant, demonstrating not only that the D\(^d\) epitopes recognized by this cell line and the allogeneic CTL were different, but also that it is possible for a H-2 class I molecule to express epitopes recognized by allogeneic CTL but not epitopes that function as restricting elements to certain antigens. The observation that both T cell- and B cell-defined determinants were altered in these mutant cell lines is in contrast to the findings, with the mutant mouse strains which were selected for by changes in T cell-defined
determinants, which show few, if any, alterations to serological specificities. Characterization of T cell–recognized epitopes expressed on serologically selected somatic cell variants may therefore prove to be most useful for the study of structure-function relationships of H-2 class I molecules.

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