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Immunology

Specific recognition of the product of a transferred major histocompatibility complex gene by cytotoxic T lymphocytes

(DNA-mediated gene transfer/*H-2L*^{d} gene)

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ABSTRACT **Mouse L cells transfected with a genomic clone containing the *H-2L*^{d} gene (8-5 cells) were shown to function as targets for H-2L^d-specific cytotoxic T lymphocytes (CTL). The CTL-mediated lysis of 8-5 cells was shown to be H-2L^d specific by the use of (*i*) CTL with restricted reactivity, (*ii*) unlabeled target inhibition, and (*iii*) monoclonal antibody inhibition. We also demonstrated that 8-5 cells could function as targets for antibody-plus-complement-mediated cell lysis. Specificity was confirmed by using H-2L^d-specific monoclonal antibodies. These experiments demonstrate that the gene products of a major histocompatibility complex genomic clone can be functionally expressed in a foreign cell and can mediate immunologically specific cellular interactions.**

Early interest in the major histocompatibility complex (MHC) derived from its role in the rejection of allografts, cell-mediated cytotoxic responses, and mixed lymphocyte reactions. It was subsequently learned that such "unrestricted" allogeneic reactions are an exception among the array of immune responses; immune cellular collaboration and T-cell specificity are generally restricted by recognition of self MHC determinants (1, 2). Thus it has become of interest to determine not only the effects of manipulating alloimmune responses but also the effects of manipulating MHC determinants themselves.

Until now, immunogenetic analysis of the mouse MHC, *H-2*, has been dependent upon the occurrence of intra-*H-2* recombinants or MHC mutants as a source of MHC variant cells. Recently, however, the availability of cloned cDNA probes for MHC antigens has allowed the isolation of several appropriate genomic DNA clones from a BALB/c sperm DNA library (3). One genomic clone, 27.5, was shown to contain the *H-2L*^{d} gene by DNA-mediated gene transfer into mouse L cells (4). The ability to place foreign H-2 molecules on the surface of cells by means of transformation with cloned *H-2* genes provides a method of manipulating given *H-2* genes *in vitro* and studying the immune properties of their cell surface products after gene transfer.

A rigorous correlation between 27.5 gene transfer and the expression of H-2L^d molecules has been established by the reactivity of H-2L^d-specific monoclonal antibodies with the 27.5-transfected L-cell clone, 8-5 (4). In order to determine whether the H-2L^d molecule on 8-5 cells was expressed in a biologically relevant way, we evaluated whether the H-2L^d product could also be recognized by cytotoxic T lymphocytes (CTL). Here we describe the recognition of the transferred gene product by *H-2*^{d} immune CTLs and provide further evidence that this product is H-2L^d.

MATERIALS AND METHODS

Mice. C-*H-2*^{dm2} mice were generously supplied by Roger Melvold (Northwestern University). All other mice were bred and housed in the immunogenetics mouse colony at the University of Southern California Medical School.

Cell Lines. Two cell lines derived from C3H mouse L cells (*H-2*^{k}) were used. Ltk$^+$ cells were obtained by transfection of thymidine kinase (TK)-negative (Ltk$^-$) cells with the cloned herpesvirus *tk* gene; 8-5 cells were obtained by cotransformation of Ltk$^-$ cells with the herpesvirus *tk* gene and genomic clone 27.5 containing the *H-2L*^{d} gene (4). Transformants were selected and cloned in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum and hypoxanthine/aminopterine/thymidine (HAT medium). Both 8-5 and Ltk$^+$ were maintained in HAT medium as adherent cell lines in 75-cm^2 tissue culture flasks. Before use as targets, cells were removed from the plastic by incubation with 0.02% trypsin at 37°C for 3–5 min. Trypsin activity was terminated by the addition of medium containing 10% fetal calf serum. The mastocytoma P815 (H-2^d) was maintained in RPMI-1640 with 10% fetal calf serum.

Monoclonal Antibodies and Alloantisera. Monoclonal antibodies 28-14-8S and 30-5-7S were supplied as ascites and were the generous gift of David Sachs (National Institutes of Health). They recognize specificities H-2.64 and H-2.65, respectively (5). With H-2^d mice, monoclonal 28-14-8S detects both H-2L^d and H-2R^d molecules, whereas monoclonal 30-5-7S is reactive with H-2L^d exclusively (6). Monoclonals 4-9.4, 5-7.1, 6-27.10, 7-16.20, 7-16.32, and 7-30.2 were produced in our laboratory according to published techniques (7) and will be described in detail elsewhere. Monoclonal 4-9.4 originated from a B10.P anti-B10.Q immunization and has a haplotype distribution pattern corresponding to specificity H-2.65 (reacts with H-2^{d,q}; does not react with H-2^{b,k,f,p,r,s,j,dm2}). That 4-9.4 reacts with BALB/c but not C-*H-2*^{dm2} cells indicates that it detects H-2L^d or H-2R^d molecules or both; the results described in this manuscript indicate that it recognizes at least H-2L^d products. Monoclonals 5-7.1, 6-27.10, 7-16.32, and 7-30.2 were selected as controls on the basis of their reactivity with *H-2K*^{d}- or *H-2K*^{k}-encoded determinants and their lack of activity with *H-2D* region-encoded products.

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Abbreviations: Con A, concanavalin A; CTL, cytotoxic T lymphocyte; HAT, hypoxanthine/aminopterin/thymidine; MHC, major histocompatibility complex; TK, thymidine kinase.

The C-*H*-2^{dm2} anti-BALB/c antiserum used here was obtained from Ted Hansen (Merck Sharp & Dohme, Rahway, NJ). It has been tested on a panel of *H*-2 haplotypes and found to exhibit weak cytotoxic activity (titer, 1:10) against H-2.64$^+$, H-2.65$^-$ strains and stronger activity (titer, 1:160) against H-2.64$^+$, H-2.65$^+$ strains. No activity was detected against H-2.64$^-$, H-2.65$^-$ strains. All other antisera used were produced and characterized in our laboratory by established techniques (8). The serological reagents used and their specificities are described in Table 1.

Serology. A complement-dependent cytotoxicity assay (9) was used to detect foreign H-2^d specificities on the surface of 8-5 cells. Selected rabbit serum was used as a complement source. Mesenteric lymph node cells were isolated after sedimentation with Lympholyte M (Cederlane Laboratories). Ltk$^+$ and 8-5 cells were used directly after brief trypsinization and washing. Complement control cytotoxicity was always <10% with lymph node or Ltk$^+$ targets. The 8-5 cells were more sensitive to killing by complement, with backgrounds of <25%.

***In Vitro* Sensitization.** Alloreactive cytotoxic T cells were generated by standard techniques (10). Briefly, spleen cells were depleted of erythrocytes, washed, and added to 25-cm^2 tissue culture flasks at a concentration of 2.5×10^7 responders and 2.5×10^7 irradiated [2,000 rads (1 rad = 0.01 gray)] stimulators in 20 ml of Click's medium supplemented with 1% normal mouse serum. Sensitization flasks were cultured upright in humidified 7.5% CO_2/92.5% air at 37°C for 5 days.

Chromium Release Assay. Effector cells or control cells (responders cultured with syngeneic stimulators) derived from 5-day sensitization cultures were mixed with 1×10^4 ^{51}Cr-labeled target cells in triplicate in round-bottomed microtiter plates containing a total volume of 0.2 ml. Effector/target ratios ranged from 100:1 to 6:1. Target cells were labeled by incubation with sodium [^{51}Cr]chromate (200–400 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) for 1–2 hr at 37°C. Target cells consisted of Ltk$^+$ cells, 8-5 cells, P815 cells, or concanavalin A (Con A)-induced lymphoblasts derived from normal mice of various haplotypes. Con A blasts were prepared by incubating splenic lymphocytes for 3 days at 37°C in RPMI medium supplemented with antibiotics, 10% fetal calf serum, and Con A at 2 μg/ml. Plates containing effectors and labeled targets were centrifuged at 60 × *g* for 3 min and incubated at 37°C for 4 hr. Supernatants were harvested by using the Titertek supernatant harvesting system (Flow Laboratories, McLean, VA). Maximal releasable ^{51}Cr was determined by distilled water lysis of an equal number of target cells. Spontaneous release was determined by incubation of target cells with medium alone. Percentage chromium release was calculated as:

$$\frac{\text{experimental cpm} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

In all cases, chromium release with control effector cells was <5% and is not shown.

Unlabeled Target Inhibition. Unlabeled Con A blasts (blockers) were added at the beginning of the chromium release assay in blocker/target ratios ranging from 20:1 to 2.5:1; the number of target cells was held constant. Percentage inhibition was calculated as:

$$1 - \frac{\%\text{ Cr release with blockers}}{\%\text{ Cr release without blockers}} \times 100.$$

Antisera/Monoclonal Antibody Inhibition. Various antisera or monoclonal reagents were added at the initiation of the chromium release assay in the dilutions shown in Fig. 5. Percentage inhibition was calculated as:

$$1 - \frac{\%\text{ Cr release in the presence of antibody}}{\%\text{ Cr release in the absence of antibody}} \times 100.$$

Table 1. Reagents used and their relevant activities

Antiserum or monoclonal antibody	Relevant H-2^d or H-2^k molecules detected	Serologic specificity
(A×B10)F_1 anti-B10.D2	K^d	H-2.31 (and others)
C-*H*-2^{dm2} anti-BALB/c	L^d and R^d	H-2.64 and H-2.65
7-16.20	None	H-2.37
7-16.32	K^k	H-2.1
7-30.2	K^d	Not assigned†
5-7.1	K^d	Not assigned†
6-27.10	K^d	Not assigned†
4-9.4	L^d (and R^d?)	H-2.65
28-14-8S*	L^d and R^d	H-2.64
30-5-7S*	L^d	H-2.65

* These reagents have been characterized by Ozato *et al.* (5) and Hansen *et al.* (6).

† Monoclonals 7-30.2, 5-7.1, and 6-27.10 recognize previously undefined specificities shared by K^d molecules.

RESULTS

Cell-Mediated Cytotoxicity. Goodenow *et al.* (4) described the successful transfer of a genomic DNA clone, 27.5, containing the gene encoding the BALB/c H-2L^d molecule (11) into mouse L cells. One of the clones (8-5) was shown to express H-2L^d molecules virtually indistinguishable from spleen H-2L^d molecules by two-dimensional gel electrophoretic analysis and at levels comparable to endogenous H-2K^k molecules. The fact that the H-2L^d molecules are serologically detectable on 8-5 cells indicates that they are expressed on the cell surface. In the following experiments, we applied functional criteria to assess the capacity of this antigen to mediate cell–cell interactions.

In the first experiment, alloreactive CTLs were generated in the combinations C3H anti-BALB/c (H-2^d specific) and BALB/c anti-C3H (H-2^k specific). These CTLs were then tested for their ability to kill ^{51}Cr-labeled P815 (H-2^d) cells, Ltk$^-$ cells transformed with the herpesvirus *tk* gene only (Ltk$^+$ cells), or Ltk$^-$ cells cotransformed with the *tk* gene and genomic clone 27.5 containing the H-2L^d gene (8-5 cells). Although P815 cells were lysed only by H-2^d-specific effectors and Ltk$^+$, by H-2^k-specific effectors, 8-5 cells were killed efficiently by both H-2^k- and H-2^d-specific effector cells (Fig. 1). Thus, 8-5 cells appear to express an *H*-2^d gene product (presumably H-2L^d) as well as endogenous H-2^k molecules capable of functioning as target antigens for CTLs.

To test rigorously the specificity of CTL killing on 8-5, effector cells specific for either K end molecules or D end molecules were produced by using various intra-*H*-2 recombinant strains. B10.D2 targets (H-2^d) were effectively killed by both K^d- and D^d/L^d- as well as whole H-2^d-specific effectors (Fig. 2). In contrast, 8-5 cells were recognized by CTL specific for D^d/L^d products but not K^d products. None of the H-2^d-specific effectors were cytotoxic for Ltk$^+$.

Because there are no known recombinants between D and L, it was not possible to distinguish between D- and L-specific killing in the previous experiments. However, there exists a BALB/c mutant strain, C-*H*-2^{dm2}, that appears to be a loss mutant for expression of H-2L and H-2R molecules but has retained the H-2D molecule (6). By generating C-*H*-2^{dm2} anti-BALB/c effectors it is possible to produce a detectable L^d/R^d-specific CTL response (12). The results of this experiment are shown in Fig. 3. Significant killing was seen when C-*H*-2^{dm2}

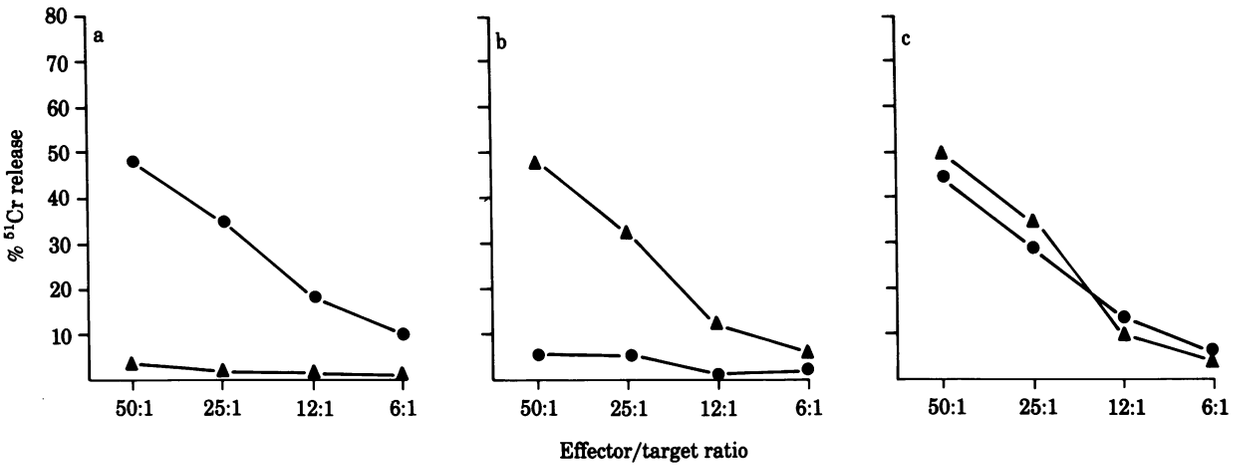

FIG. 1. Ability of 8-5 cells to serve as targets for H-2^d-specific CTLs. C3H anti-BALB/c effectors (●) or BALB/c anti-C3H effectors (▲) were assayed on P815 (*a*), Ltk$^+$ (*b*), or 8-5 (*c*) targets in a 4-hr ^{51}Cr release assay.

anti-BALB/c effectors were tested on both B10.A and 8-5 targets. However, no cytotoxicity was observed on Ltk$^+$ or B10.OH targets. This demonstrates the recognition of L^d molecules on 8-5 cells by a restricted population of effector cells.

Unlabeled Target Inhibitions. Further specificity mapping was performed by using the technique of unlabeled target inhibition. With this approach, CTL lysis of a ^{51}Cr-labeled target cell can be inhibited competitively when unlabeled cells expressing the same target antigens are added in the assay. This technique has the advantage of avoiding potential ambiguities resulting from the use of different populations of effector cells. In this experiment, only one population of effector cells was used, B10.K anti-B10.D2. Because this combination produces a mixture of CTL with specificity for H-2K^d, -D^d, or -L^d, they can be used to lyse either B10.OH targets (due to the expression of K^d) or 8-5 target cells (presumably due to expression of L^d). B10.K anti-B10.D2 effector cells were assayed on B10.OH or 8-5 target cells at a constant effector/target ratio of 50:1, with varying numbers of unlabeled blockers. B10.A(18R) and A.AL, both of which express H-2L^d and -R^d but not -K^d were effective inhibitors of 8-5 lysis but not B10.OH lysis (Fig. 4). In contrast, B10.OH cells (K^d, D^k) did not inhibit the lysis of 8-5 cells but did inhibit B10.OH lysis. B10.D2 cells (K^d, D^d) were effective inhibitors for both targets. These results also demonstrate that the target antigens being recognized on 8-5 cells, even by whole H-2-specific effectors, map to the *D* region of the *H-2* complex.

Complement-Mediated Cytotoxicity. H-2L^d molecules on the surface of 8-5 cells have been identified by monoclonal antibody-mediated binding of ^{125}I-labeled protein A (4). The results shown in Table 2 indicate that the presence of 8-5 cell-associated H-2L^d determinants can also be detected by complement-mediated cytotoxicity. Confirming the earlier results, 8-5 cells were efficiently lysed by the H-2L^d-reactive monoclonals 28-14-8S and 30-5-7S whereas Ltk$^+$ cells were not killed. In addition, a third H-2L^d-reactive monoclonal, 4-9.4, was selectively reactive with clone 8-5. Three monoclonals with confirmed H-2K^d activity did not kill either 8-5 cells or Ltk$^+$ cells whereas an H-2K^k-reactive reagent, 7-16.32, effectively lysed both targets. The 8-5 transformants and normal BALB/c spleen cells express similar levels of H-2L^d as determined by binding studies (4). The present results, indicating that all three H-2L^d-reactive monoclonals have similar cytotoxic titers on 8-5 and BALB/c spleen cells, support this conclusion.

Antibody Blocking of CTL. Finally, we investigated the blockade of CTL lysis of 8-5 cells by antibodies to H-2L^d molecules. B10.K anti-B10.D2 effector cells were assayed on B10.OH or 8-5 targets in the continuous presence of varying dilutions of antisera or monoclonal antibodies. An H-2L^d-spe-

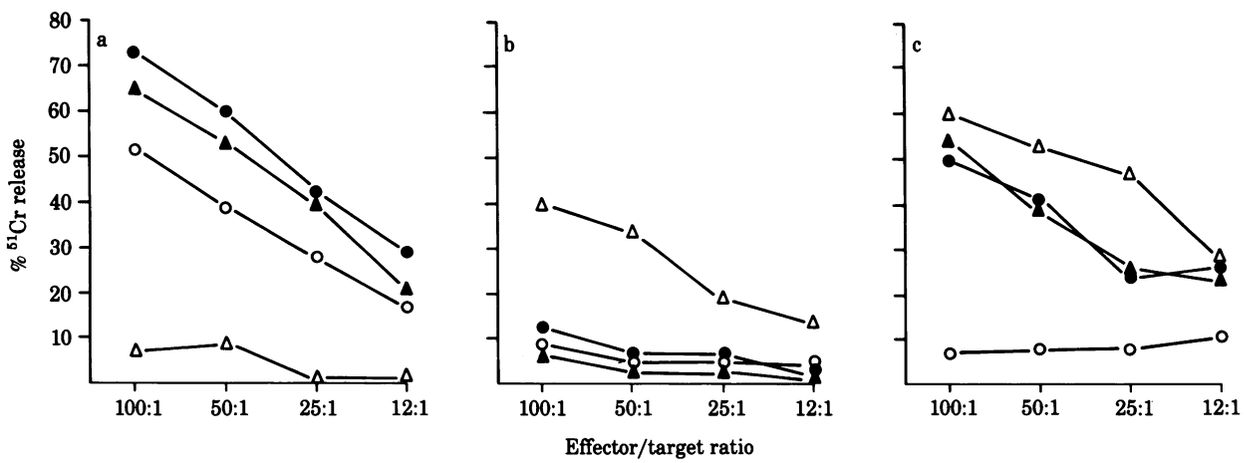

FIG. 2. Susceptibility of 8-5 targets to lysis by effector cells with restricted specificities. anti-H-2^d, B10.K anti-B10.D2 (●), anti-H-2D^d,L^d, B10.K anti-B10.A (▲), anti-H-2K^d, B10.A anti-B10.D2 (○), or anti-H-2^k, B10.D2 anti-B10.K (△) effector cells were assayed on B10.D2 (*a*), Ltk$^+$ (*b*), or 8-5 (*c*) targets.

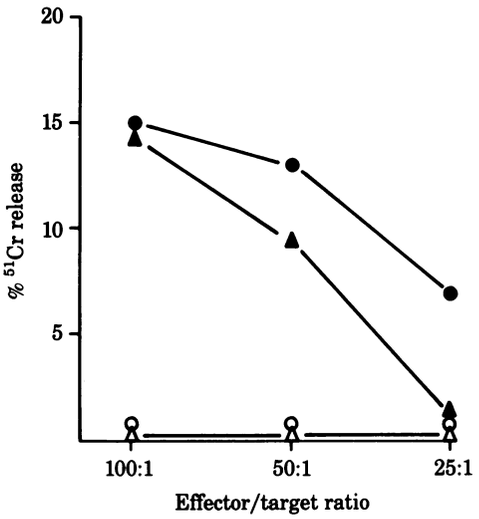

FIG. 3. Susceptibility of 8-5 cells to lysis by mutant anti-BALB/c effectors. C-*H*-2^{dm2} anti-BALB/c effectors were assayed on 8-5 (●), B10.A (K^k,D^d,L^d) (▲), Ltk$^+$ (K^k,D^k) (○), or B10.OH (K^d,D^k) (△) targets.

cific antiserum, C-*H*-2^{dm2} anti-BALB/c, and an H-2L^d-specific monoclonal, 28-14-8S, significantly inhibited lysis of 8-5 targets but no inhibition by these reagents was seen with B10.OH targets (Fig. 5). Interestingly, another H-2L^d-specific monoclonal, 4-9.4, failed to show significant inhibition of 8-5 lysis. Because 4-9.4 did show reactivity toward 8-5 cells in complement-dependent cytotoxicity assays (Table 2), its inability to block CTL recognition may reflect a specificity on the H-2L^d molecule not seen readily (or at least in high frequency) by the CTL.

DISCUSSION

We have provided direct evidence for the functional expression of a cell surface recognition molecule, H-2L^d, derived by DNA-mediated gene transfer. The H-2L molecule and the -K and -D molecules, are polymorphic 45,000-dalton glycoproteins that function as specific recognition structures for CTLs (1, 2). The data in this paper demonstrate that foreign H-2L^d molecules expressed by cells transformed with the *H-2L^d* gene are specifically recognized by CTLs and that this recognition triggers the sequence of events that results in lysis of 8-5 target cells.

Table 2. Cytotoxic activity of H-2L^d-specific monoclonals on 8-5 cells

Monoclonal*	Relevant activity	Reciprocal cytotoxic titer with various target cells†				
		BALB/c	H-2^{dm2}	B10.K	Ltk$^+$	8-5
5-7.1	K^d	80	160	—	—	—
7-30.2	K^d	80	80	—	—	—
6-27.10	K^d	640	ND	—	—	—
7-16.32	K^k	—	—	640	160	160
4-9.4	L^d	40	—	—	—	80
28-14-8S	L^d	2,048	—	ND	—	1,024
30-5-7S	L^d	16,384	ND	ND	—	16,384

* Monoclonals 28-14-8S and 30-5-7S were from ascites fluid; the remainder were from culture supernatants.
† BALB/c, C-*H*-2^{dm2}, and B10.K targets were mesenteric lymph node cells. —, No cytotoxicity above the complement background levels was detected; ND, not done.

The specificity of the CTL response against 8-5 cells was verified independently by using *D* region limited killers, unlabeled target inhibition, and monoclonal antibody inhibition. The *D* region of the *H*-2^d haplotype appears to code for at least four molecules—D, L, R, and M (6, 13). Because there are no recombinants among these genes, it was not possible to distinguish among D-, L-, R-, and M-specific killing by using D-restricted killers or unlabeled target inhibition although these experiments localized the specificity to the *D* region. However, further specificity was achieved by using the loss mutant C-*H*-2^{dm2}, which fails to express both H-2L^d and H-2R^d molecules (6). C-*H*-2^{dm2} anti-BALB/c effectors specifically lysed 8-5 cells, thus narrowing the specificity down to the H-2L^d or R^d molecule. Finally, monoclonal antibody inhibition experiments showed that two H-2L^d specific monoclonals could almost completely block CTL recognition and lysis of 8-5 cells, thereby demonstrating that the relevant CTL target antigen on 8-5 cells is, in fact, the H-2L^d molecule. In no case was H-2^d-specific killing detected on Ltk$^+$ cells, indicating that the transfection

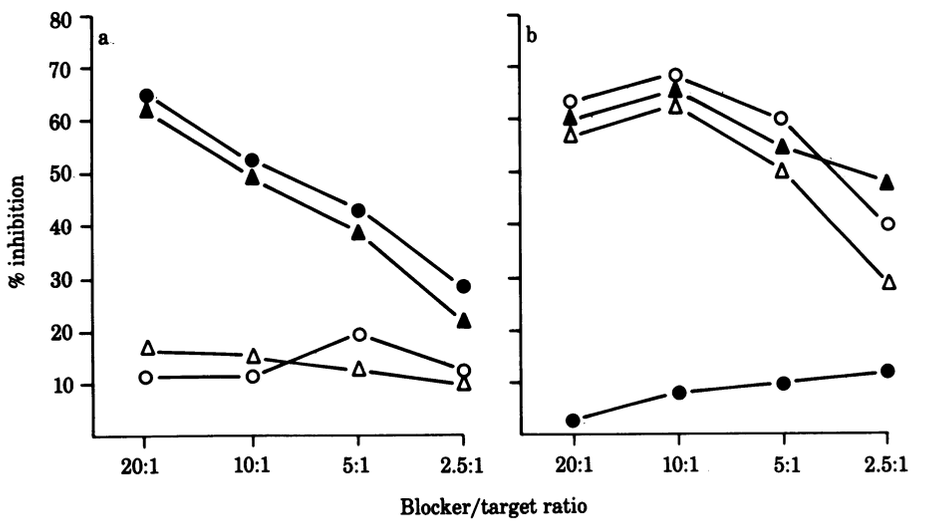

FIG. 4. Unlabeled target inhibition of H-2^d-specific CTL on 8-5 targets. B10.K anti-B10.D2 effectors were assayed on B10.OH (K^d, D^k) (*a*) or 8-5 (*b*) targets at an effector/target ratio of 50:1 in the presence or absence of various numbers of unlabeled Con A blasts derived from B10.OH (●), B10.D2 (▲), B10.A(18R) (K^k,D^d,L^d) (○), or A.AL (K^k,D^d,L^d) (△). Percentage lysis of targets in the absence of blockers was 46% for B10.OH targets and 40% for 8-5 targets.

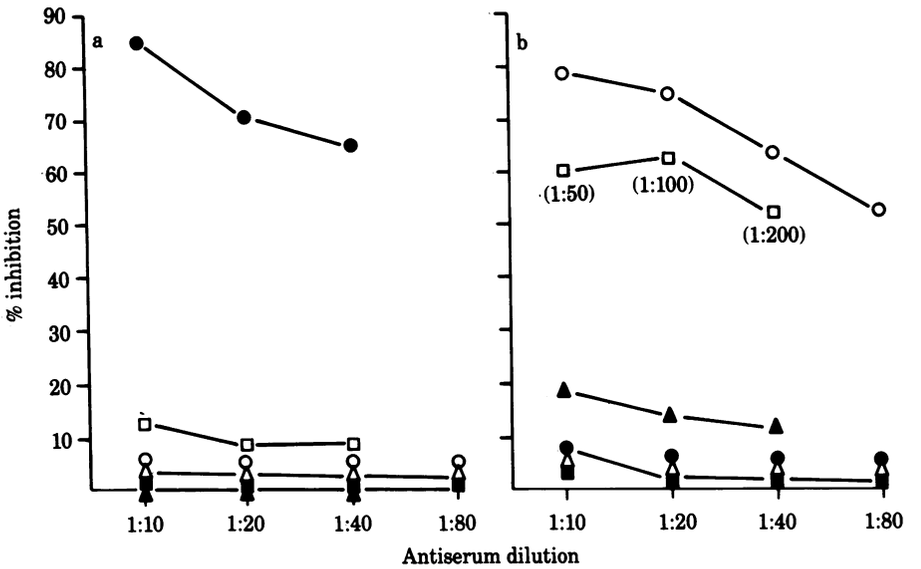

FIG. 5. Antisera/monoclonal antibody inhibition of H-2^d-specific effector cells. B10.K anti-B10.D2 effector cells were assayed on B10.OH (*a*) or 8-5 (*b*) targets at an effector/target ratio of 50:1, in the presence or absence of varying dilutions of (A×B10)F_1 anti-B10.D2 (●), 28-14-8S (□), C-*H-2*^{dm2} anti-BALB/c (○), 7-16.20 (△), 7-16.32 (■), and 4-9.4 (▲). Percentage lysis of targets in the absence of antisera was 46% for B10.OH and 40% for 8-5 targets.

procedure itself did not result in expression of H-2^d specificities.

We also demonstrated that H-2L^d molecules expressed on 8-5 cells can serve as targets for antibody-dependent complement-mediated lysis. Three H-2L^d-specific monoclonals were selectively cytotoxic on 8-5 cells but were unreactive with Ltk$^+$ cells. One monoclonal reagent, 4-9.4, was specifically cytotoxic for 8-5 cells but failed to block significantly their lysis by CTLs, even at a concentration well above its cytotoxic titer. This may reflect a serologic specificity on the L^d molecule not efficiently recognized by CTLs. Alternatively, the concentration of 4-9.4 antibody used may not have been sufficient to bind with enough of the available H-2L^d molecules to block their recognition by CTLs completely. Monoclonal antibodies with similar CTL blocking properties have been reported by Weyand *et al.* (14) for other MHC antigens.

The successful transfer and functional expression of a cloned gene coding for cell-surface recognition molecules provides a powerful approach for study of the structure–function relationships of these molecules. Thus, it is now technically possible to produce, by *in vitro* recombination and site-directed mutagenesis, molecules that have precisely defined alterations in the sequence. With these novel H-2 molecules, it will be possible to evaluate the effects of these alterations on the functional expression of the gene products. By using these techniques, H-2 molecules with known sequence deletions or substitutions of entire domains or of single amino acids can be produced. It then will be possible to map the areas of the H-2 molecules that are responsible for CTL recognition. It will also be possible to determine if H-2 molecules, like immunoglobulins, have domains specialized for different biological functions.

Finally, the technique should prove useful for the identification of genes coding for cell-surface products with similar structures and for the determination of their function. By using monoclonal antibodies and MHC-specific T-cell clones to analyze transformants expressing different cloned genes, it will be possible to assess the true diversity of functional MHC molecules and to probe the fine structure of the *H-2* gene complex.

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