

# ALLOSPECIFIC CYTOLYTIC T LYMPHOCYTES RECOGNIZE CONFORMATIONAL DETERMINANTS ON HYBRID MOUSE TRANSPLANTATION ANTIGENS

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Major histocompatibility complex (MHC)<sup>1</sup>-encoded gene products are intimately involved in a variety of immune responses associated with both antigen recognition and alloreactivity. The class I molecules are 45,000 mol wt membrane glycoproteins associated with a 12,000 mol wt protein,  $\beta_2$ -microglobulin. These proteins are composed of three external domains ( $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ), a transmembrane portion, and an intracytoplasmic carboxyl terminus. The first two external domains are extremely polymorphic (1). These polymorphic regions are considered integral in immune response gene control and MHC restriction. A more precise understanding of the structure-function relationships of the MHC class I molecules is critical to understanding immune recognition. Several different approaches have been used to investigate the topographic and functional locations of these polymorphic determinants using cytotoxic T cells (CTL) and alloantibodies. These include: (a) the examination of in vivo (2-4) and in vitro (5, 6) derived class I mutants; (b) differential blocking of CTL activity with anti-H-2 monoclonal antibodies (mAb) (7); and (c) most recently, gene transfer studies, in which native (8-10) or exon-shuffled MHC genes were transfected into L cells (11, 12-14). Gene transfer studies (11, 12), in which H-2L<sup>d</sup> and H-2D<sup>d</sup> genes have been split, exon-shuffled, recloned in bacteria and, finally, transfected into L cells, have shown that the specificities recognized by most mAb are determined by the  $\alpha_1$  and/or  $\alpha_2$  domains of these products, although occasional mAb recognize epitopes associated with the  $\alpha_3$  domain. By comparison, all CTL clones and bulk cultures analyzed to date react with epitopes associated with the  $\alpha_1$  and/or  $\alpha_2$  domains (13). Recently, we reported the generation of new hybrid class I constructs in which either the  $\alpha_1$  and  $\alpha_2$  domains of K<sup>b</sup> and D<sup>b</sup> had been exchanged as a unit or in which the  $\alpha_1$  domains of K<sup>b</sup> and D<sup>b</sup> had been exchanged (11). These new hybrid molecules are illustrated in Fig. 1. Analysis of these K<sup>b</sup>/D<sup>b</sup> hybrids using alloreactive and influenza-specific CTL suggested that CTL recognition of conformational determinants was dependent

<sup>1</sup> *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; FMF, flow microfluorometry; HBSS, Hanks' balanced salt solution; IFN, interferon; IL-2, interleukin 2; mAb, monoclonal antibody; MHC, major histocompatibility complex; tk, thymidine kinase; Ltk<sup>-</sup>, thymidine kinase-deficient mouse L cells.

on both the  $\alpha_1$  and  $\alpha_2$  domains. However, a number of mAb recognized the hybrid constructs as well as they recognized the native molecules; these mAb may represent a minor population of domain-specific allorecipients. In the present study we examined the recognition of the  $K^b/D^b$  hybrids with CTL generated against  $K^b$  or  $D^b$  by cells from H-2<sup>b</sup> mutant mice, which have few amino acid changes in either the  $\alpha_1$  or  $\alpha_2$  domains of  $K^b$  or  $D^b$ . These populations might be expected to react with determinants expressed on single MHC domains. In addition, CTL clones were examined as an approach to identify  $\alpha_1$  or  $\alpha_2$  domain-specific T cell populations. We demonstrate that the exchange of  $\alpha_1$  domains between  $K^b$  and  $D^b$  generates new antigenic determinants that can be recognized by CTL.

### Materials and Methods

**Animals.** C57BL/10 (B10), C3H/HeJ, B6.CH-2<sup>bm1</sup>, and B6.CH-2<sup>bm3</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.CH-2<sup>bm6</sup>, B6.CH-2<sup>bm10</sup>, B6.CH-2<sup>bm11</sup>, and B6.CH-2<sup>bm8</sup> mice were kindly provided as breeding pairs by Dr. R. J. Melvold (Chicago, IL) and bred in our colony. B6.CM-2<sup>bm13</sup> and B6.CH-2<sup>bm14</sup> mice were kindly provided by Dr. I. McKenzie (Melbourne, Australia) and bred in our colony.

**mAb.** The mAb and their known specificities have been described previously (15, 16). mAb EH-144, provided by Dr. T. V. Rajan (Bronx, NY), was derived from a C57BL/10-immunized BALB/c mouse, is specific for H-2K<sup>b</sup>, and crossreacts on H-2P<sup>r</sup>.

**Interferon Treatment.** Partially purified mouse fibroblast  $\gamma$  interferon (IFN- $\gamma$ ) (sp act,  $5 \times 10^7$  U/mg protein) was a gift from Dr. Suzanna Serrate (Department of Pathology, U. S. Uniformed Health Services). L cells were cultured as monolayers in 75-cm<sup>2</sup> tissue culture flasks with 10,000 U of IFN- $\gamma$ . After 24 h the cells were harvested and prepared for flow microfluorometry (FMF) analysis or as target cells for lysis by CTL.

**Construction of Hybrid H-2 Genes and Transformation of Mouse L Cell Fibroblasts.** The construction of the four hybrid class I genes was described previously (11). Briefly, the  $K^b$  and  $D^b$  second exons were isolated from the whole genes as 330 and 390 base pair (bp) fragments, respectively, and inserted directly into the heterologous gene from which the second exon was deleted. The exchange of promoter and exons 1, 2, and 3 between  $K^b$  and  $D^b$  was performed by, first, isolating these regions as 1,800 and 1,850 bp fragments, respectively. These fragments were relegated to  $K^b$  and  $D^b$  3' gene fragments. Ligated DNA were introduced into CaCl<sub>2</sub>-treated *Escherichia coli* DH1 or HB101 cells (17). Colonies were picked and constructs were identified by restriction enzyme digests on plasmid mini-preps. DNA for transformation of thymidine kinase (tk)-deficient mouse L cells (H-2<sup>b</sup>) (Ltk<sup>-</sup>) was isolated from plasmid maxi-preps. Exon exchange and correct orientation of insertion was confirmed by restriction mapping.

Ltk<sup>-</sup> cells were cotransformed with the  $K^b$ ,  $D^b$ , or one of four  $K^b/D^b$  hybrid genes and a plasmid containing the tk gene, by CaPO<sub>4</sub> coprecipitation (18). Cloned cell lines expressing high levels of  $K^b$ ,  $D^b$ , and hybrid  $K^b/D^b$  antigens were selected for further study (Fig. 1). The L cell line expressing the normal  $D^b$  product as well as some of the L cells expressing hybrid MHC molecules were unstable, as seen by a bimodal fluorescence intensity distribution (data not shown). Therefore, for the CTL studies, several lines were recloned before use.

**Immunofluorescence Staining and FMF.** Cell surface antigen expression by L cells was studied by FMF analysis as previously described (19), using a fluorescence-activated cell sorter (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA) interfaced to a PDP 11/34 computer (Digital Equipment Corporation, Marlboro, MA). Monolayer cultures of L cells were harvested by trypsinization and washed three times in Hanks' balanced salt solution (HBSS), 0.1% sodium azide, and 10% fetal calf serum (FCS). 10<sup>6</sup> cells were incubated with 25  $\mu$ l of culture supernatant or ascitic fluid (1:100) of mAb. After incubation with mAb at 4°C for 45 min, the cells were washed and stained with 25  $\mu$ l of fluorescein-conjugated coat F(ab')<sub>2</sub> anti-mouse IgG (Cappel Laboratories, Cochran-

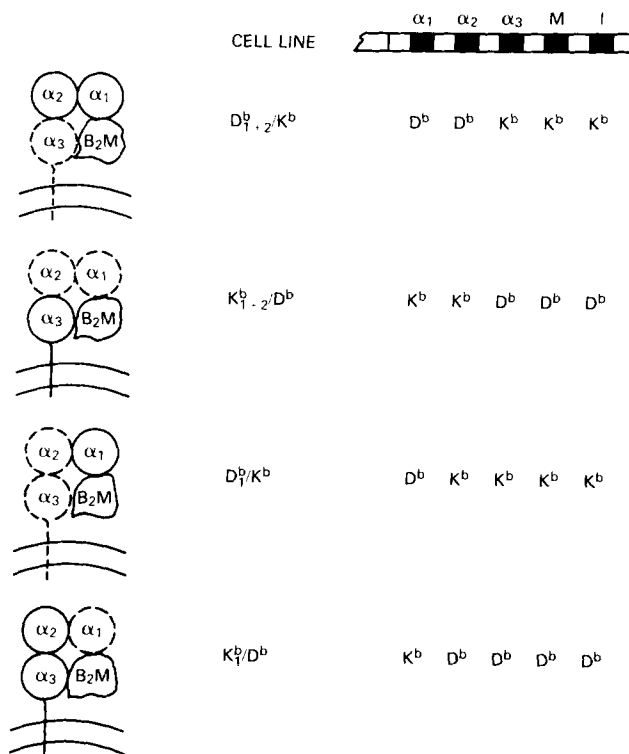


FIGURE 1. Cell lines expressing hybrid MHC gene products are illustrated. Genes encoding the three external domains are designated  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ , while  $M$  and  $I$  represent the transmembrane portion and intracytoplasmic portion of the molecule, respectively.

ville, PA). Fluorescence intensity was assessed by FMF, analyzing  $2-5 \times 10^4$  stained cells using logarithmic amplification. Fluorescence data are depicted as the percent median fluorescence relative to the L cell expressing the native MHC molecule.

**Generation of  $K^b$ - and  $D^b$ -specific CTL Populations.** Primary and secondary CTL populations were generated as previously described (6). Briefly,  $5 \times 10^6$  responder cells were mixed with  $2-4 \times 10^6$  irradiated (2,000 rad) stimulator cells in 2 ml final volume complete media (RPMI 1640 supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, glutamine, penicillin-streptomycin, and 2-mercaptoethanol). In some experiments, irradiated (10,000 rad) L cells were used as stimulator cells at concentrations ranging from  $5 \times 10^3$  to  $10^5$  cells per 2-ml cultures. In some studies responder cells were obtained from animals primed in vivo with the transfected L cells ( $10^7$  cells, intraperitoneally). The mixed lymphocyte cultures were incubated at  $37^\circ\text{C}$  in 10%  $\text{CO}_2/90\%$  humidified air for 5 d. After 5 d of culture, the effector cells were harvested, resuspended in complete media, counted, and then either replated as a secondary culture at  $2 \times 10^6$  responder cells and  $5 \times 10^6$  stimulator cells in 2 ml complete media or assayed for CTL activity in a  $^{51}\text{Cr}$  release assay. Secondary cultures were supplemented with 10% interleukin 2 (IL-2)-containing, lectin-free, concanavalin A (Con A) supernatant as previously described (5).

**$^{51}\text{Cr}$  Release Assay.** Target cells were radiolabeled by incubating  $2-4 \times 10^6$  tumor cells with  $250 \mu\text{Ci}$  of  $\text{Na}^{51}\text{Cr}$  in 0.5 ml of FCS.  $25 \mu\text{l}$  of radiolabeled target cells were added to effector cells in a total volume of  $225 \mu\text{l}$  in U-bottomed microtiter wells. After 1-2 h, target cells were washed three times in HBSS, counted, and resuspended in complete media. The plates were incubated for 5 h at  $37^\circ\text{C}$ . Supernatants were harvested using the Titertek supernatant collection system (Skatron, Inc., Sterling, VA). The percent specific lysis was calculated as follows: percent specific lysis = [(experimental release (cpm)

– spontaneous release)/(total release (cpm) – spontaneous release)]  $\times$  100, where total release represents cpm released after incubation with 0.1 N HCl.

*CTL Clones.* Cloned CTL were produced by methods described in detail elsewhere (5). Briefly, cells from 2° or 3° one-way mixed lymphocyte cultures were cloned by limiting dilution at one cell per well in the presence of  $5 \times 10^5$  spleen cells (3,300 rad) in 5–10% of a 50:50 mixture of lectin-free Con A supernatant and phorbol myristate acetate-induced EL-4 factor in flat-bottomed, 96-well microtiter plates. After 7–10 d, growth-positive wells were screened for cytotoxicity in a 4 h  $^{51}\text{Cr}$  release assay. All clones used in these studies had been subcloned at 0.3 cells per well with a 60–85% cloning efficiency.

## Results

*Examination of Polymorphic Determinants Using CTL Derived Across H-2<sup>b</sup> Mutant Anti-H-2<sup>b</sup> Combinations.* Cytotoxic T cells generated against class I antigens have been shown to be highly heterogeneous by clonal analysis (3, 5) and by the ability of mAb that react with different epitope clusters to differentially inhibit CTL activity (7). However, in some instances, certain mAb specific for one domain inhibit the majority of CTL. These results suggested that CTL react with specificities determined by the conformation of the whole H-2 molecule, not solely the  $\alpha_1$  or  $\alpha_2$  domains. Several investigators (2, 3) have shown that some H-2K<sup>b</sup> mutant anti-K<sup>b</sup>-specific CTL recognize fewer allodeterminants than conventional allo-CTL populations. In some cases, the determinants recognized appeared to be influenced by both the  $\alpha_1$  and  $\alpha_2$  domains (3). However, unique domain-specific structures may also be recognized. With this in mind, alloreactive bulk CTL and CTL clones were generated across a variety of H-2K<sup>b</sup> mutant strain combinations. In some combinations, such as the bm8 and bm3 anti-K<sup>b</sup>, the amino acid differences between the mutants and the wild-type K<sup>b</sup> molecule resided in the  $\alpha_1$  domain. In contrast, the bm10 and bm1 mice differed from K<sup>b</sup> in amino acids localized to the  $\alpha_2$  domain. Using a series of primary, secondary, and cloned CTL populations, we detected no lysis of the L cells expressing the hybrid H-2 antigens in which the  $\alpha_1$  domains of K<sup>b</sup> and D<sup>b</sup> are exchanged (Fig. 2 and Table I). Clearly, the CTL examined in Fig. 2, *top* are specific for H-2K<sup>b</sup>, since they lyse K<sup>b</sup>- but not D<sup>b</sup>-transfected L cells. However, no lysis was detected of either the K<sup>b</sup>/D<sup>b</sup>- or D<sup>b</sup>/K<sup>b</sup>-transfected L cells. Alloreactive CTL were also generated using H-2D<sup>b</sup> mutant mice as responders (bm13 and bm14). Fig. 2, *bottom* illustrates the results of such experiments, in which D<sup>b</sup>-specific CTL lysed D<sup>b</sup> and D<sup>b</sup><sub>1+2</sub>/K<sup>b</sup> transformants but failed to lyse L cells expressing the hybrid genes K<sup>b</sup>/D<sup>b</sup> or D<sup>b</sup>/K<sup>b</sup>. As previously shown, all L cells were equally lysable, as shown by their susceptibility to anti-H-2<sup>k</sup> CTL.

*Lack of Recognition of Hybrid Molecules With Exchanged  $\alpha_1$  Domains by Conventional Alloreactive CTL.* The present study, using a much larger panel of bulk culture and cloned CTL, confirmed the earlier result (11) that the hybrids K<sup>b</sup>/D<sup>b</sup> and D<sup>b</sup>/K<sup>b</sup> are not recognized by K<sup>b</sup>- or D<sup>b</sup>-allospecific CTL although the L cells were equally lysable by anti-H-2<sup>k</sup> effector CTL (Table I). One reason for the lack of lysis could have been the lower level of expression of the hybrid molecules by the L cell transfectants (Table II). To address this possibility, L cell transfectants were cultured for 24 h with IFN- $\gamma$ , which increases MHC expression in transfected L cells (20). However, even under conditions in which the levels

## CTL RECOGNITION OF HYBRID MHC MOLECULES

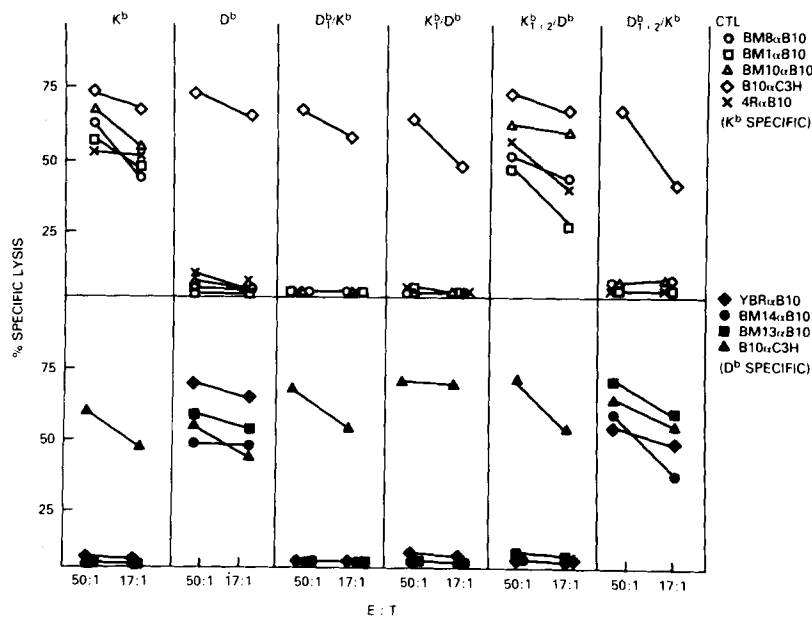


FIGURE 2. Alloreactive CTL were analyzed for reactivity on L cell transformants. CTL lines were derived by restimulation of responding cells for three cycles before assay.

of expression of hybrid H-2 were almost equivalent (>90% of control) to the L cells transfected with the native molecule, no lysis was detected by serological analysis (Table III). Therefore, it appears that the determinants recognized by the alloreactive CTL were altered by the exchange of the  $\alpha_1$  domains or that the CTL did not recognize isolated determinants of the  $\alpha_1$  or  $\alpha_2$  domains.

*Expression of Neoantigenic Determinants on Hybrid MHC L Cell Transformants.* Although the L cells expressing the hybrid MHC molecules appeared to be good targets for CTL lysis (i.e., anti-H-2<sup>k</sup> CTL lysed the L cells), it was not clear that the hybrid molecule was expressed by the L cell in sufficient quantity or in a conformation that could be recognized by CTL. To address this possibility, we attempted to generate CTL against the transformed L cells. If new antigenic determinants that were not present on H-2D<sup>b</sup> products had been created by the interaction of the  $\alpha_1$  and  $\alpha_2$  domains of D<sup>b</sup> and K<sup>b</sup>, respectively, then (H-2<sup>b</sup> × H-2<sup>k</sup>)F<sub>1</sub> CTL generated against the transformants might react with the new determinants. (C3H/HeJ × C57BL/10)F<sub>1</sub> spleen cells were stimulated against L cells expressing the hybrid gene D<sup>b</sup><sub>1</sub>/K<sup>b</sup>. In preliminary experiments (data not shown), stimulation with L cells resulted in nonspecific lysis of tk<sup>+</sup> L cell control. However, pretreatment of the responder cells with anti-L3T4 plus complement, or Sephadex G-10 depletion of responder macrophages (21) appeared to lower the nonspecific lysis. Therefore, we performed an experiment using responding cells that had undergone both treatments (Fig. 3A). It is clear from the results that F<sub>1</sub> cells stimulated with the D<sup>b</sup><sub>1</sub>/K<sup>b</sup> transformant generated CTL reactive against the D<sup>b</sup><sub>1</sub>/K<sup>b</sup> transformants. No lysis of K<sup>b</sup>-transfected L cells was seen. mAb

TABLE I  
Reactivity of  $K^b$ - and  $D^b$ -specific CTL Clones on Transformants

CTL Population	Targets (percent specific lysis)*			
	$K^b$	$D^b$	$K^b/D^b$	$D^b/K^b$
<b>3° Lines<sup>‡</sup></b>				
B10 anti-B10.BR <sup>§</sup> (anti- $K^bD^k$ )	32.3	36.4	42.3	66.8
B10.BR anti-B10 (anti- $K^bD^b$ )	44.6	22.4	-1.3	2.1
C3H/HeJ anti-C3H.KBR (anti- $K^b$ )	52.3	-1.6	2.8	3.7
B10.BR anti-B10.A(2R) (anti- $D^b$ )	1.6	42.9	0.1	-4.2
<b>CTL Clones<sup>¶</sup></b>				
C3H/H3J anti-C3H.KBR-1	41.3	1.9	-2.2	1.6
-3	32.6	4.6	-3.4	2.7
-5	48.4	3.8	0.1	-1.1
-13	18.1	-1.1	4.1	-1.3
-19	22.3	-1.9	-1.6	4.2
-22	29.4	6.6	3.2	2.7
BM10 anti-B10-5	44.8	1.3	2.6	4.1
-6	36.3	2.1	1.4	-2.6
-37	26.1	-1.8	-2.3	3.0
-38	44.7	3.7	4.3	-3.0
-42	24.8	1.0	1.6	1.4
BM8 anti-B10-5	32.6	-6.6	-4.8	-3.7
-13	46.2	1.3	1.7	-0.3

\* Percent specific lysis relative to medium.

<sup>‡</sup> Effector/target ratio, 33:1.

<sup>§</sup> The MHC haplotypes of the mouse strains used to generate the CTL were: C3H/HeJ,  $K^kI^kD^k$ ; C3H.KBR,  $k^bI^bD^k$ ; B10.BR,  $K^dI^dD^k$ ; B10.A(2R),  $K^kI^kD^b$ ; and B10,  $K^bI^bD^b$ .

<sup>¶</sup> Effector/target ratio, 2:1.

TABLE II  
Binding of Anti-H-2 mAb to L Cells Transfected With Hybrid MHC Genes

mAb	Antibody binding (percent fluorescence)* hybrid molecules						Domain specificity
	$K^b$	$K^{I+2}/D^b$	$K^b/D^b$	$D^b/K^b$	$D^{I+2}/K^b$	$D^b$	
20-8-4	100	116	46	0	0	0	$K^b \alpha_1$
EH-144	100	108	72	0	0	0	$K^b \alpha_1$
B8-3-24	100	130	32	0	0	0	$K^b \alpha_1$
5F1	100	122	0	61	0	0	$K^b \alpha_2$
28-13-3	100	136	0	63	0	0	$K^b \alpha_2$
34-4-20	100	106	0	27	0	0	$K^b \alpha_2$
K9-178	100	118	0	25	0	0	$K^b \alpha_2$
K9-136	100	142	0	0	0	0	$K^b \alpha_1/\alpha_2$
B22-249.1	0	0	71	0	98	100	$D^b \alpha_2$
28-11-5	0	0	0	30	96	100	$D^b \alpha_1$
H141-31	0	0	0	15	79	100	$D^b \alpha_1$
27-11-13	26	32	13	43	94	100	$K^bD^b \alpha_1$
28-14-8	0	117	70	0	0	100	$D^b \alpha_3$
H141-29	0	0	0	0	103	100	$D^b \alpha_1/\alpha_2$

\* Values normalized relative to binding of native molecule.

TABLE III  
Effect of IFN- $\gamma$  on H-2 Expression and CTL Recognition

	Percent lysis					
	Target cells (effector/target, 20:1)					
	D <sup>b</sup>	D <sup>b</sup> I*	D <sup>b</sup> <sub>1</sub> /K <sup>b</sup>	D <sup>b</sup> <sub>1</sub> /K <sup>b</sup> I	K <sup>b</sup> <sub>1</sub> /D <sup>b</sup>	K <sup>b</sup> <sub>1</sub> /D <sup>b</sup> I
BM3 anti-B10 (anti-K <sup>b</sup> )	1.5	-1.8	5.5	-2.9	-10.1	4.1
BR anti-B10 (anti-K <sup>b</sup> D <sup>b</sup> )	30.9	21.2	-1.7	-4.2	-3.1	-1.2
B10 anti-B10.A(2R) (anti-D <sup>b</sup> )	60.1	73.7	5.6	-5.9	4.2	1.2
BM14 anti-B10 (anti-D <sup>b</sup> )	51.1	31.9	-3.4	2.4	-5.0	-0.9
B10 anti-BR (anti-K <sup>b</sup> D <sup>b</sup> )	32.2	21.8	46.7	33.6	38.1	40.7
	Fluorescence intensity <sup>‡</sup> (mV <sup>-1</sup> )					
	925.1	2167.5	ND	ND	329.8	863.1

\* IFN- $\gamma$  treated as described in Materials and Methods.

<sup>‡</sup> Cell lines were stained with 28-14-8 mAb. ND, not determined.

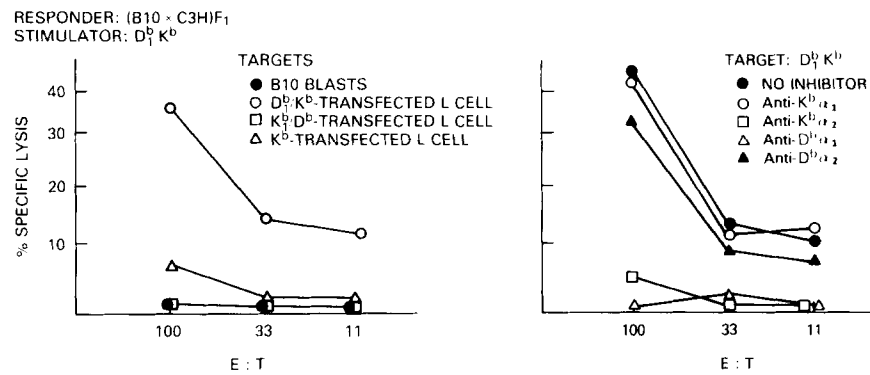


FIGURE 3. Generation of CTL derived against neodeterminants expressed on hybrid class I antigens. F<sub>1</sub> mice were primed in vivo with 10<sup>7</sup> L cell transformants 1 wk before in vitro assay. Mab (28-11-5 and H141-11 for D<sup>b</sup>  $\alpha_1$ ; 5F1 and 28-13-3 for K<sup>b</sup>  $\alpha_2$ ; 20-8-4 and EH-144 for K<sup>b</sup>  $\alpha_1$ ; and B22-249.1 for D<sup>b</sup>  $\alpha_1$ ) were incubated with target before the addition of effectors (B).

blocking, using anti-D<sup>b</sup> or -K<sup>b</sup> antibodies specific for the  $\alpha_1$  or  $\alpha_2$  domain of H-2, confirmed the specificity of the CTL generated (Fig. 3B). Either anti-D<sup>b</sup> $\alpha_1$ - or anti-K<sup>b</sup> $\alpha_2$ -specific mAb blocked CTL function at the effector phase. These antibodies had no effect on the lysis of the same L cell transfectants by anti-H-2<sup>k</sup> CTL (data not shown).

*Alloreactive CTL From Bulk Culture and CTL Clones Do Not Recognize Polymorphic Determinants on the  $\alpha_3$  Domain of the Hybrid MHC Molecules.* We previously reported (11) that allogeneic CTL generated against K<sup>b</sup> and D<sup>b</sup> locus products efficiently lyse K<sup>b</sup><sub>1+2</sub>/D<sup>b</sup> and D<sup>b</sup><sub>1+2</sub>/K<sup>b</sup>-transfected L cells, respectively. These results suggested that the majority of CTL recognize polymorphic regions determined by the  $\alpha_1$  and  $\alpha_2$  domains. It was possible, however, that a minority of cloned CTL populations recognize the  $\alpha_3$  domain. We have tested here >20 K<sup>b</sup>-specific CTL clones derived in different alloreactive combinations on the L cell transfectants. All CTL that react with K<sup>b</sup>-transfected but not D<sup>b</sup>-transfected L cells recognized polymorphic sites localized to the  $\alpha_1/\alpha_2$  domains (Table IV).

TABLE IV  
 Reactivity of  $K^b$ -specific CTL Clones on L Cell Transformants

CTL Clone*	Percent specific lysis <sup>‡</sup>			
	Target (effector/target, 33:1)			
	$K^b$	$D^b$	$K_{1+2}^b/D^b$	$D_{1+2}^b/K^b$
C3H/HeJ anti-C3H.KBR-1	24.3	-0.7	29.1	0.1
-3	46.4	3.8	36.3	4.0
-5	23.3	-1.8	26.4	2.1
-13	37.1	-0.2	31.9	-1.6
-19	26.8	-6.1	21.3	-1.4
-22	42.9	3.3	44.9	3.8
B10.GD anti-B10-3	50.2	1.8	62.3	-1.0
-9	19.9	4.6	23.3	1.4
-10	31.3	-2.2	21.6	2.3

\* Each clone examined at least three times. For simplicity, data from different experiments have been compiled into a single table.

<sup>‡</sup> Percent specific lysis of each clone relative to medium.

In addition, limiting dilution analysis of  $K^b$ -specific CTL showed no  $\alpha_3$  recognition (data not shown). Thus, apparently no CTL were detected that recognized the  $\alpha_3$  domain; in contrast, mAb specific for this domain have been defined (10, 11).

*Localization of Serological Determinants on Mouse Hybrid MHC Molecules.* Previously (11), transformed L cell lines expressing  $K^b$ ,  $D^b$ , or the hybrid class I molecules resulting from exon exchanges were analyzed by radioimmunoassay to map the mAb-binding specificity. However, fluorescence analysis suggested that some of the transformant's lines were composed of mixed populations expressing different levels of H-2 antigens. This result could explain the lower binding of some mAb to the transfectants expressing the hybrid MHC antigens. Therefore, several of the L cell lines were recloned, and transformant clones that exhibited unimodal fluorescence profiles were reexamined to confirm the binding specificity of several of the anti-H-2<sup>b</sup> mAb previously studied and to determine the binding specificity of five additional anti-H-2<sup>b</sup> mAb. In accordance with previous results (11), the majority of the mAb bound to polymorphic sites determined by the  $\alpha_1$  and/or  $\alpha_2$  domains (Table II). Most of the  $K^b$ -specific mAb bound the L cell line expressing the  $K_{1+2}^b/D^b$  hybrid molecule to a greater extent than the  $K^b$ , due to more MHC antigen on the cell surface (data not shown). As previously reported (11), one mAb, 28-14-8, demonstrated significant binding to L cells transfected with  $D^b$  or  $K_{1+2}^b/D^b$ , mapping its reactivity to the  $\alpha_3$  domain.

The binding of the mAb was next analyzed on the  $\alpha_1$  hybrid gene transformants ( $K_1^b/D^b$  and  $D_1^b/K^b$ ). Binding of all mAb to L cell transformants with H-2 genes in which the  $\alpha_1$  domains were exchanged was significantly lower than binding to L cells expressing the native molecule (Table II). Since the binding of 28-14-8, an  $\alpha_3$ -specific anti- $D^b$  mAb, was unaffected by substituting  $K^b$  in both the  $\alpha_1$  and  $\alpha_2$  domains (Table II) but was significantly lower on the  $K_1^b/D^b$  transfectants (70% of control), it seemed likely that this transfected L cell expressed fewer



hybrid molecules on the cell surface. Scatchard analysis using Fab of 28-14-8 ( $\alpha_3$  specific) showed somewhat fewer hybrid MHC molecules on the  $K_I^b/D^b$  cells compared with the L cells transfected with either the native molecule ( $D^b$ ) or  $K_{I+2}^b/D^b$  (Table V). In addition, the binding of mAb 20-8-4, specific for the  $\alpha_1$  domain of  $K^b$ , showed a lower binding affinity, suggesting a conformational alteration of this molecule as well (Table V). However, with the exception of mAb K9-136 and 27-11-13, all the mAb binding could be mapped to either the  $\alpha_1$  or  $\alpha_2$  domains of  $K^b$  and the hierarchy of reactivity among the mAb was similar to that reported previously (11). 27-11-13 was found to bind  $K^b$  and  $D^b$ , in contrast to previous findings (15). However, the titer on  $K^b$  was much greater than  $D^b$ . Since 27-11-13 bound  $K_I^b/D^b$  better than the  $D_I^b/K^b$  transfectant, this mAb has been tentatively mapped to the  $\alpha_1$  domain. Therefore, as previously reported (11), the majority of  $K^b$ -specific mAb are directed at the polymorphic sites determined by the  $\alpha_1$  and/or  $\alpha_2$  domains. The interaction of these two domains appears critical for optimal binding; the conformation of domains 1 and 2 in the  $K_I^b/D^b$  and  $D_I^b/K^b$  hybrids seem to differ from that in the parental.

### Discussion

In recent years biochemical and genetic analysis have been used to examine the structure of the H-2 class I molecule. The  $\alpha_3$  domain of class I molecules interacts with  $\beta_2$ -microglobulin, while the  $\alpha_1$  and  $\alpha_2$  domains appear to express the polymorphic sites involved in allorecognition and MHC restriction of responses to foreign antigens by T cells (1). However, the precise localization of the amino acid sequences recognized by T cells or alloantibodies have not yet been determined. It is these polymorphic sites that appear to be recognized during an immune response. The advent of gene splicing techniques has allowed the construction of hybrid H-2 class I genes. These new genes can then be expressed as novel class I molecules after transformation into mouse fibroblasts. Initial studies of allostereic determinants expressed by these L cells has been hindered by the heterogeneity of bulk CTL generated across a fully allogeneic class I difference. Mouse strains exist with mutations restricted to the first and second domains (1) and may be useful to study limited MHC differences (3). Previous

TABLE V  
*Analysis of Binding of Anti-H-2 mAb to L Cell Transformants*

Cell line	mAb (Fab)	$R^*$ (molecules/ cell $\times 10^4$ )	$K^\ddagger$ ( $M^{-1} \times 10^{-8}$ )
$D^b$	28-14-8	$8.7 \pm 0.6$	$2.2 \pm 0.1$
$K_{I+2}^b/D^b$	28-14-8	$10.1 \pm 0.9$	$2.3 \pm 0.2$
	20-8-4	$9.4 \pm 0.7$	$6.2 \pm 0.5$
$K_I^b/D^b$	28-14-8	$4.1 \pm 0.2$	$2.0 \pm 0.1$
	20-8-4	$3.3 \pm 0.4$	$.73 \pm 0.08$

\* The number of antibody molecules bound on a cell at saturation ( $R$ ) and the binding affinity ( $K$ ) were determined as described (24) by fitting data to the equation:  $r = RK \cdot (Ab) / 1 + K(Ab)$ , where  $r$  is the number of antibody molecules bound per cell,  $(Ab)$  is the free antibody concentration, and  $R$  is a measure of the average number of antigenic determinants per cell.

analysis (5) of these mutant MHC molecules has provided insights into the structure/function relationship of class I molecules. Using H-2K<sup>b</sup> mutant mice, it has been possible to examine the allodeterminants recognized by T cell populations and to compare them with allodeterminants revealed by anti-H-2 antibodies. Because of these advantages, the H-2K<sup>b</sup> and D<sup>b</sup> genes were isolated, the external  $\alpha_1$  and  $\alpha_2$  domains exchanged, and the new class I genes transfected into mouse L cells.

We have confirmed in this study that CTL and alloantibodies recognize L cell lines transfected with H-2K<sup>b</sup> and D<sup>b</sup> genes to the same extent as they recognize native proteins on splenocytes. However, no alloreactive CTL could be identified that recognize the L cells expressing hybrid molecules in which the  $\alpha_1$  domains of K<sup>b</sup> and D<sup>b</sup> were exchanged. This lack of lysis was observed using CTL generated from H-2K<sup>b</sup> and D<sup>b</sup> mutant mice in response to wild-type H-2K<sup>b</sup> and H-2D<sup>b</sup> antigens. Since a number of these mutants involve amino acid changes in only a single domain, it might have been expected that these CTL would be predominantly specific for determinants localized to a single MHC domain (2, 3). However, studies using the K<sup>b</sup> mutants have shown that the amino acid changes in one domain often alter the CTL recognition of another domain (2, 3). Therefore, the failure of these CTL to react with the hybrid gene products is consistent with these previous results. One concern in the present study was the reduced binding of an  $\alpha_3$ -specific mAb to L cells transformed with the hybrid molecule due to significantly less H-2 antigen on the cell surface. To rule out the possibility that these cells expressed too few MHC molecules to allow lysis by the CTL, the L cells were cultured with IFN- $\gamma$ , which augmented MHC expression two to threefold. Even under these conditions the transformed L cells were not lysed by CTL. Another concern was that the inability to lyse the transformants was due simply to an altered cell surface expression of the hybrid molecules. Perhaps the class I molecule does not associate with other cell interaction molecules required for CTL recognition or perhaps the tertiary structure was completely destroyed. This latter possibility seems unlikely since the binding to the hybrid molecules by several mAb was unaltered and one hybrid molecule (D<sup>b</sup>/K<sup>b</sup>) was able to act as a target for CTL generated against it. In most experiments, *in vivo* priming was required, suggesting that the CTL precursor frequency for the hybrid molecule was significantly lower than that seen for conventional class I antigens.

These findings are in contrast to those recently reported by Murre et al. (12), in which determinants localized to the  $\alpha_1$  or  $\alpha_2$  exons from the H-2L<sup>d</sup> and H-2D<sup>d</sup> MHC genes were shown to function as targets for CTL recognition. The reason for this disparity is not clear but may represent differences in the genes chosen for the exon exchanges. Perhaps in the L<sup>d</sup>/D<sup>d</sup> system, more allodeterminants are conserved in the hybrid molecules.

In this study, CTL clones were examined for  $\alpha_3$  recognition, since mAb specific for this domain have been defined (11, 14). To date, no allospecific CTL clones generated across a conventional class I (a total of 23) or H-2<sup>bm</sup> (a total of 19) mutant difference has been shown to recognize determinants encoded by the  $\alpha_3$  domain. These findings are consistent with the localization of most of the amino acid sequence polymorphism for class I antigens to the  $\alpha_1$  and  $\alpha_2$  domains (1).

However, the mechanism underlying the failure of alloreactive CTL to recognize the  $\alpha_3$  domain is unclear. One possibility is that the repertoires of T and B cells differ so that T cells fail to recognize determinants expressed by the  $\alpha_3$  domain. These differences may alternatively reflect steric considerations. Since CTL are much larger than antibodies, the allogeneic sites within the  $\alpha_3$  domain of the native MHC molecule may be inaccessible to CTL. Currently, studies are underway to examine CTL and antibody recognition of L cells transfected with truncated genes and expressing only the  $\alpha_3$  domain on the cell surface. Finally, it is possible that class I-specific CTL express a different repertoire than mAb since they recognize native MHC molecules, while activation of antibody-producing B cells may require recognition of processed antigen and accessory function by class I-specific helper cells (22). Therefore, studies designed to examine the recognition of the hybrid MHC molecules by class I-specific T helper cells are currently being pursued.

In the present study there appeared to be a difference between antibody and CTL recognition of hybrid MHC molecules in which the  $\alpha_1$  domain of  $K^b$  and  $D^b$  had been exchanged. Most of the mAb bound to the cells expressing the hybrid molecules. However, no alloreactive CTL recognized these hybrids. Several hypotheses can be postulated to explain this difference: (a) the combining site of the T cell receptor is larger than that of antibodies, and T cells are therefore more sensitive to perturbations of the MHC molecules. (b) The affinities of CTL are significantly lower than those of antibodies and thus more susceptible to changes in conformation. This hypothesis is the least complex, since it would not be necessary to invoke different repertoires for alloreactive T cells and antibodies, but this possibility can only be addressed once the relative affinities of T and B cell receptors are known. (c) Perhaps the most attractive hypothesis is that the T and B cell receptors have different expressed repertoires. Recent molecular analysis (23) of the T cell receptor genes suggests that the T and B cell receptors would appear to be encoded by different genes. The difference in T cell and antibody repertoires may reflect the fact that T cells recognize different portions of the MHC molecule than do antibodies. For example, CTL may recognize an amino acid sequence located at the junction of the  $\alpha_1$  and  $\alpha_2$  domains where some polymorphism has been shown (1). Although this hypothesis is consistent with other studies suggesting that T and B cells recognize distinct antigenic determinants (5, 6), the folding of the MHC domains and the location of the MHC hypervariable regions to other areas of the molecule might argue against allorecognition of this part of the molecule. An alternative hypothesis that would lead to differences in the T cell and antibody repertoires was recently proposed by Gress and Hodes (24). In their studies, CTL recognition of H-2K<sup>b</sup> appeared to be MHC restricted. The MHC restriction appeared to be intramolecular, in that recognition of H-2K<sup>bm6</sup> by alloreactive CTL required maturation of the CTL precursors in an H-2K<sup>b</sup> environment. It is possible that the generation of the hybrid MHC molecules results in a loss of the restriction elements required for CTL but not antibody recognition.

In any case, it would appear that the CTL target structures are influenced to a greater degree by interactions between the  $\alpha_1$  and  $\alpha_2$  domains than are the

structures recognized by antibodies. This difference may provide an insight into the expressed repertoires of different immune populations.

### Summary

Alloreactive cytolytic T cell (CTL) lines and clones have been used to identify the sites of polymorphism of antigens of the major histocompatibility complex (MHC). Specific CTL were generated against wild-type H-2<sup>b</sup> products by cells from H-2<sup>b</sup> mutant mice that had one or a few amino acid changes in either the  $\alpha_1$  or  $\alpha_2$  domains of the K<sup>b</sup> or D<sup>b</sup> class I molecules. These CTL populations, which might be expected to react with determinants expressed on single MHC domains, were examined for lytic activity on L cells expressing newly constructed hybrid class I molecules. Transformed cell lines expressing native class I molecules or hybrid class I molecules in which the  $\alpha_1$  and  $\alpha_2$  domains of H-2K<sup>b</sup> had been substituted by those domains of H-2D<sup>b</sup> were lysed by H-2D<sup>b</sup>-specific CTL. Similarly, all H-2K<sup>b</sup>-specific CTL recognized hybrid molecules in which the  $\alpha_1$  and  $\alpha_2$  domains of H-2K<sup>b</sup> were inserted into the H-2D<sup>b</sup> molecule. In contrast, exchange of the  $\alpha_1$  domains of H-2K<sup>b</sup> and H-2D<sup>b</sup> resulted in a total loss of recognition by K<sup>b</sup> and D<sup>b</sup>-specific CTL. These results suggest that the allostereic determinants recognized by H-2 mutant CTL are influenced by interactions between the  $\alpha_1$  and  $\alpha_2$  domains, findings similar to those seen using conventional alloreactive T cells (11). These results were compared to the binding of alloreactive mAbs, including 5 new mAbs specific for the K<sup>b</sup> molecules. Finally, it was shown that primary and secondary CTL responses could be generated by direct sensitization against hybrid class I molecules, demonstrating that these molecules express neoantigenic determinants recognized by alloreactive CTL.

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