INVOLVEMENT OF *H-2L* GENE PRODUCTS IN VIRUS-IMMUNE T-CELL RECOGNITION

Evidence for an *H-2L*-Restricted T-Cell Response*

BY WILLIAM E. BIDDISON,‡ TED H. HANSEN,§ ROBERT B. LEVY,‖ AND PETER C. DOHERTY

From The Wistar Institute of Anatomy and Biology, Philadelphia, Pa. 19104, and the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Recent experiments have identified a new H-2- like cell-surface glycoprotein which is determined by the *D* region of the murine major histocompatibility complex. This product, designated L (previously called D′) (1), is distinct from the K and D molecules which bear the H-2 private specificities detected by cocapping (2) and immunoprecipitation (3) techniques. Because D and L are encoded by genes which have yet to be separated by recombination, both remain markers for the *D* region. However, it was shown that a mutant mouse strain, BALB/c-$H\text{-}2^{db}$ (4), had lost the L (but not the D) antigenic specificities from the cell surface (1, 5). In studies of the BALB/c-$H\text{-}2^{db}$ strain and the coisogenic strain BALB/c, it was demonstrated that H-2L alloantigens elicit rapid skin graft rejection (4) and a strong primary in vitro cytotoxic T-cell response (6). Therefore, *H-2L* products appear to function as major transplantation antigens.

Blanden et al. (7) have recently reported that BALB/c-$H\text{-}2^{db}$ mice, in contrast to wild-type BALB/c, failed to generate a T-cell response to ectromelia virus in association with products of the *D* region, whereas BALB/c-$H\text{-}2^{db}$ ectromelia-infected target cells could be lysed by *D* region-compatible ectromelia-immune T cells. The absence of *H-2L* gene products was thus accompanied by a failure of stimulation, but not of recognition at the level of cytotoxic effector function. This situation does not apply for reactivity to minor transplantation antigens (7) or to trinitrophenol (TNP),[1, 2] and is unique for all the *H-2* mutant strains employed in previous virus studies (8–10). The present study investigates: (*a*) whether the requirement for H-2L specificities in stimulation of ectromelia virus-immune T cells specific for *D* region products is unique, or also applies to other viruses; and (*b*) whether virus-immune T cells recognize viral determinants in association with *H-2L* as well as *H-2D* gene products.

Materials and Methods

Mice. BALB/c-$H\text{-}2^{db}$ and BALB/ckh ($H\text{-}2^{d}$) mice were bred from stock which was kindly provided by Doctors Henry Kohn and Roger Melvold (Harvard Medical School and Shields

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[1] Abbreviations used in this paper: SDS, sodium dodecyl sulfate; TNP, trinitrophenyl.

[2] R. B. Levy, G. M. Shearer, and T. H. Hansen. 1978. Properties of H-2L locus products in allogeneic and *H-2* restricted, trinitrophenyl-specific cytotoxic responses. *J. Immunol.* In press.

Warren Lab, Boston, Mass.). BALB.K (H-2^k) mice were a gift from Dr. Kenneth Blank (Wistar Institute, Philadelphia, Penn.). D2.GD(H-2^{g2}), A.TH(H-2^{t2}), C3H.OH(H-2^{02}), and C3H.OL(H-2^{01}) were bred at the National Institutes of Health. B10.BR(H-2^k) were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Viruses and Immunizations. The influenza type A viruses HK[A/Hong Kong/8/68-X-31(H3N2)] and PR8[A/Puerto Rico/8/34(HONI)] were supplied and grown in embryonated eggs as previously described (11). Mice were immunized in vivo by intraperitoneal inoculation with 200–300 hemagglutination units of virus in alloantoic fluid and spleen cells obtained 5 days later for assay. In vitro induction of secondary influenza-immune T cells was performed by incubation of spleen cells from 4 to 16 wk-primed mice with influenza virus (2–4 HAU/10^7 cells, 37°C, 1 h) and culturing for 5 days in RPMI-1640 medium supplemented with 10% vol/vol fetal bovine serum, 0.01 mM nonessential amino acids, 0.1 mM sodium pyruvate, 0.03% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Grand Island Biologicals, Grand Island, N. Y.).

The WR strain of vaccinia virus (obtained from Dr. R. M. Zinkernagel, Scripps Clinic and Research Foundation, La Jolla, Calif.) was grown in mouse L cells as described by Joklik (12). Mice were immunized by intravenous inoculation of 5×10^6 plaque-forming units of virus and spleen cells harvested 6 days later for assay.

Target Cells and Cytotoxicity Assays. L cells (C3H, H-2^k), P815 mastocytoma cells (DBA/2, H-2^d), NA neuroblastoma cells (A/J, H-2^a), and B10.A(5R) SV, an SV40 virus-transformed cell line of kidney fibroblasts from B10.A(5R) (H-2^i) mice, respectively, (obtained from Dr. Barbara Knowles, Wistar Institute, Philadelphia, Penn.), were maintained in tissue culture. Lipopolysaccharide (LPS)-induced splenic lymphoblasts were prepared, labeled with ^{51}Cr, and infected with influenza virus as described by Zweerink et al. (13). Influenza virus infection of ^{51}Cr-labeled tumor target cells was performed as described by Effros et al. (11) and vaccinia virus infection according to Doherty et al. (14). Results of cytotoxicity assays are reported as mean percent specific lysis of quadruplicate or triplicate determinations (11).

Immunoprecipitation Analyses. Radiolabeling, solubilization, precipitation, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were all performed by previously described methods (15).

Alloimmunizations. Alloantisera were raised by primary immunization with a skin graft followed by weekly injections of 2×10^7 live lymphoid cells. The titer was assessed in a two-stage microcytotoxicity assay using rabbit serum as a source of complement (16). Anti-D antiserum [(B10.AKM × A.BY) anti-A.AL)] and anti-L antiserum (BALB/c-H-2^{db} anti-BALB/c) had titers on BALB/c cells of 1:512 and 1:128, respectively. These alloantisera detect specificities on two distinct cell-surface glycoproteins as revealed by immunoprecipitation analysis (Fig. 1).

Alloimmune cytotoxic T cells were generated by 5-day in vitro sensitization as previously described (6).

Results

BALB/c-H-2^{db} and various control strains of mice were inoculated with influenza or vaccinia viruses and their spleen cells were assayed for cytotoxic activity against virus-infected *K* and/or *D* region-compatible target cells. No absolute discrimination was seen in the capacity of influenza- or vaccinia-specific BALB/c and BALB/c-H-2^{db} lymphocytes to mediate lysis of virus-infected *D* region-compatible NA (K^k-D^d) or B10.A(5R) (K^b-D^d) target cells (Tables I and II). The vaccinia-immune T-cell populations from BALB/c-H-2^{db} mice tended to be less effective, but there was no evidence of a total failure of responsiveness (Table II). In addition, secondary influenza-immune BALB/c-H-2^{db} T-cells generated by in vitro immunization of primed spleen cells were also able to specifically lyse *D* region-compatible A.TH virus-infected lymphoblasts (Table III). These results imply that L antigens are not required for the generation of *D* region-restricted influenza- and vaccinia-immune T cells.

To determine if H-$2L$ gene products are recognized by virus-immune T cells, anti-

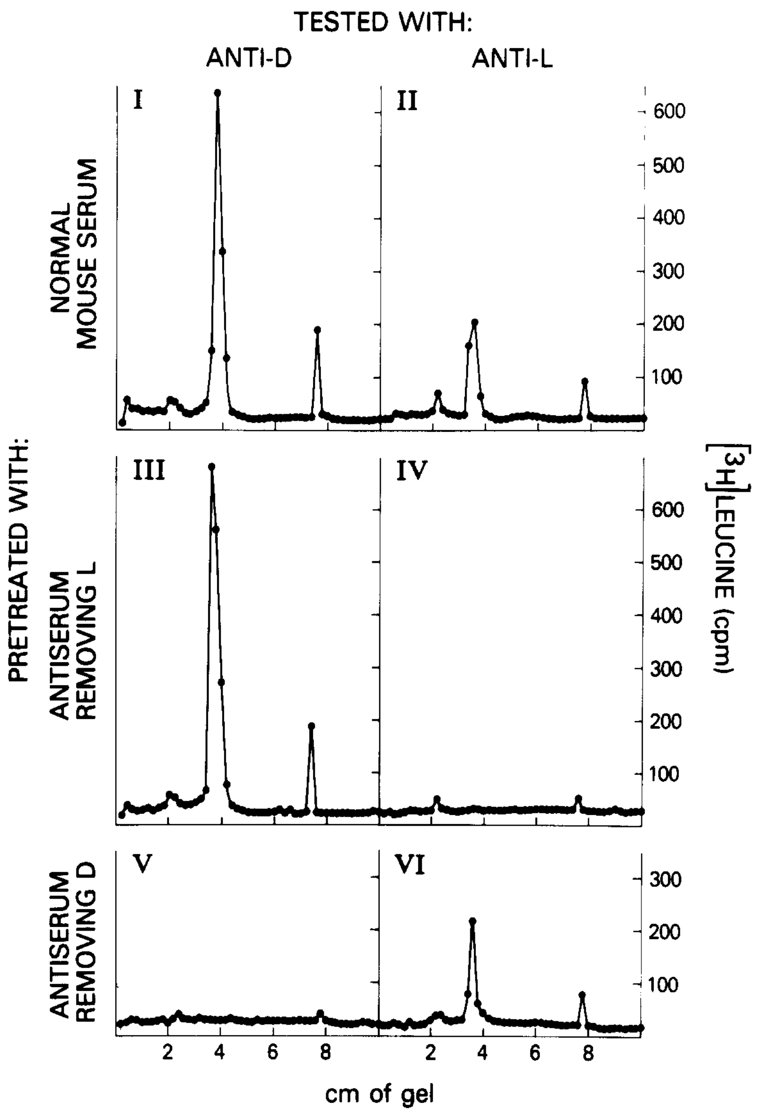

FIG. 1. Electrophoretic patterns of BALB/c antigen sequentially precipitated with antisera against the D and L molecules. [^{3}H]leucine-labeled antigen was pretreated by precipitation with the reagent indicated on the left and the supernates from this first precipitation step were then tested for residual reactivity with the reagent indicated above the panels.The test precipitates were solubilized with SDS and subjected to disk electrophoresis on 10% polyacrylamide gels. Using this system, H-2 molecules (45,000 mol wt) migrate to ≅4 cm and a nonspecific running front appears at 8 cm of gel. The top two panels indicate that both the anti-D and the anti-L serum precipitate BALB/c molecules with an apparent mol wt of 45,000 daltons. The middle two panels show that although pretreatment with antiserum to L completely removed L molecules (panel IV) the molecules detected by the anti-D serum were unaffected (compare panels I and III). Reciprocally, when the molecules detected by the anti-D serum were removed (panels V and VI), the molecules detected by the anti-L were unaffected (compare panels II and VI). Therefore, these results demonstrate that the anti-L (BALB/c-*H-2^{db}* anti-BALB/c) and the anti-D (anti-H-2.4) react with independent populations of molecules.

TABLE I

Cytotoxic Activity Against Influenza Virus-Infected Cells

Exp no.	HK-immune spleen cells	Effector cell: target cell	Percent-specific lysis of target cells*							
			NA(kd)‡		B10.A(5R)SV(bd)		L(kk)		P815(dd)	
			HK	N	HK	N	HK	N	HK	N
1	BALB/c	25:1	10.7	0.9	11.0	2.0	0.7	1.2	19.0	1.6
		50:1	20.7	2.2	21.1	2.6	3.7	1.9	27.2	1.7
		100:1	35.8	3.4	30.0	1.3	3.4	3.0	37.8	1.9
	BALB/c-H-2^{db}	25:1	10.3	1.9	11.1	4.3	2.1	1.4	15.7	1.3
		50:1	18.5	3.8	15.7	5.9	4.7	4.0	27.5	1.4
		100:1	37.4	7.2	24.1	5.9	9.5	4.5	41.6	2.0
	BALB.K	25:1	32.3	1.4	4.2	2.4	45.0	0.2	4.2	1.6
		50:1	45.1	2.0	4.0	1.6	57.3	−0.1	4.6	1.1
		100:1	58.1	5.0	7.1	4.2	68.3	0.9	5.3	2.7
2	BALB/c	25:1	12.3	−1.7			0	0.6	45.4	−0.1
		50:1	23.2	−1.4			2.0	1.7	66.2	−0.7
		100:1	33.6	0.5			2.1	4.8	75.0	11.1
	BALB/c-H-2^{db}	25:1	4.4	−0.6			−0.3	−1.8	28.6	3.3
		50:1	11.8	−1.7			1.7	−0.6	46.0	5.3
		100:1	23.3	0.7			1.3	1.5	65.0	1.2
	B10.BR	25:1	33.1	−2.1			44.2	−2.4	−3.5	2.0
		50:1	46.4	−1.6			56.1	−1.0	−3.5	4.4
		100:1	55.1	−0.7			59.7	0.9	0.3	0.8

* Target cells were either infected with HK influenza virus or uninfected (N). SEM was always <10% of the mean. Cytotoxicity assays were run for 12 h.

‡ Letters in parenthesis indicate *H-2K* and *D* region haplotypes of target cells.

L and anti-D alloantisera were tested for their ability to block the lysis of *D* region-compatible virus-infected NA (K^k-D^d) target cells by BALB/c and BALB/c-H-2^{db} influenza-immune T cells. The results in Table IV show that anti-L alloantiserum was consistently able to produce significant inhibition of cytotoxicity mediated by virus-immune T cells from BALB/c, but not BALB/c-H-2^{db}. The specificity of the inhibiting effect of anti-L alloantiserum was shown by its ability to block alloimmune effector cell activity directed against L antigens and the inability of this serum to block the activity of alloimmune effector cells sensitized to D antigens. Anti-D alloantiserum inhibited virus-immune cytotoxicity of both BALB/c and BALB/c-H-2^{db} T cells, but did not inhibit the activity of effectors directed against L alloantigens on the NA target cells.

Discussion

Although BALB/c-H-2^{db} mice do not display detectable cell-surface *H-2L* gene products, they are able to generate influenza- and vaccinia-immune cytotoxic T-cells which lyse *D* region-compatible target cells. However, these mice apparently do not make a similar response to ectromelia virus (7). These observations are particularly interesting because vaccinia- and ectromelia-immune cytotoxic T-cell responses are highly cross-reactive (17). Furthermore, these two pox viruses share many serologically defined determinants, detected by both virus neutralization and immunoprecipitation in gels (18, 19). Relatively minor differences between two very similar viruses, together with the presence or absence of a particular H-2 determinant (L), may thus determine

TABLE II

Cytotoxic Activity Against Vaccinia Virus-Infected Cells

Exp. no.	Vaccinia-immune spleen cells	Effector cell: target cell	Percent-specific lysis of target cells*							
			NA(kd)		B10.A(5R)SV(bd)		L(kk)		P815(dd)	
			VAC	N	VAC	N	VAC	N	VAC	N
1	BALB/c	25:1	8.4	2.0	17.0	0.6	3.6	2.0	27.9	0.5
		50:1	18.4	3.0	26.9	2.8	5.9	3.4	43.8	2.2
		100:1	32.1	9.1	36.0	3.3	8.1	7.2	63.2	1.4
	BALB/c-H-2^{db}	25:1	4.9	2.7	11.0	3.6	3.4	2.1	21.3	2.7
		50:1	10.5	5.4	16.8	5.9	7.0	4.2	27.6	3.8
		100:1	24.8	11.9	23.9	7.6	9.1	6.7	57.1	3.4
	BALB.K	25:1	31.9	1.1	4.7	4.9	48.8	1.7	3.4	4.4
		50:1	48.2	3.0	9.8	6.0	64.3	0.8	5.4	5.3
		100:1	57.0	9.8	15.5	6.8	70.9	2.4	9.7	7.1
2	BALB/c	25:1	12.1	−0.1	16.7	−5.5	−4.0	1.4	30.9	−5.9
		50:1	27.2	6.0	26.9	−4.7	0.3	6.2	36.3	−3.7
		100:1	41.1	13.6	38.4	−0.9	5.6	10.6	45.6	10.9
	BALB/c-H-2^{db}	25:1	10.2	5.1	7.6	2.4	1.5	5.6	28.1	4.8
		50:1	18.9	10.5	17.1	6.0	5.6	9.9	41.6	4.0
		100:1	39.0	16.1	25.0	8.0	15.0	14.9	46.3	15.6
	B10.BR	25:1	30.5	3.0	0.9	1.0	41.2	3.6	7.4	7.6
		50:1	41.2	5.9	3.2	2.3	51.7	8.7	11.9	13.9
		100:1	50.7	17.5	8.2	9.1	61.2	12.9	15.1	15.5

* Target cells were infected with vaccinia virus (VAC) or uninfected (N). SEM was always <20% of the mean. Cytotoxicity assays were run for 12 h.

TABLE III

Secondary Influenza-Immune Effector-Cell Activity on Virus-Infected H-2K or H-2D Region-Compatible Lymphoblasts

HK-immune spleen cells‡	Effector cell: target cell	Percent-specific lysis ± SEM of target cells*					
		D2.GD (db)		A.TH (sd)		B10.BR (kk)	
		HK	N	HK	N	HK	N
BALB/c	1:1	14.9 ± 1.5	−0.2 ± 0.6	10.7 ± 1.7	−2.5 ± 0.6	2.3 ± 2.1	5.6 ± 3.4
	5:1	27.8 ± 0.8	0.1 ± 0.6	22.1 ± 2.2	−0.6 ± 0.8	6.1 ± 2.8	8.7 ± 2.5
	20:1	37.0 ± 1.0	2.7 ± 0.7	35.3 ± 3.0	−1.2 ± 0.7	11.1 ± 2.6	11.1 ± 2.1
BALB/c-H-2^{db}	1:1	19.2 ± 2.5	2.6 ± 1.5	6.3 ± 1.8	1.1 ± 0.6	1.0 ± 3.2	1.6 ± 2.1
	5:1	34.2 ± 1.1	1.2 ± 0.8	17.1 ± 1.7	2.3 ± 0.8	2.0 ± 2.7	3.6 ± 2.4
	20:1	38.0 ± 1.5	0.6 ± 0.5	29.8 ± 3.1	2.1 ± 1.2	4.1 ± 3.0	6.3 ± 2.1
B10.BR	1:1	−0.1 ± 0.8	0.3 ± 0.5	−3.4 ± 1.7	−1.7 ± 0.8	9.8 ± 2.3	0.7 ± 1.6
	5:1	1.4 ± 0.8	1.3 ± 0.9	−2.3 ± 1.6	0.1 ± 0.9	23.7 ± 2.2	3.3 ± 3.5
	20:1	3.5 ± 0.7	4.4 ± 1.1	1.5 ± 1.6	3.9 ± 1.1	27.8 ± 2.3	0.9 ± 1.8

* Target cells were LPS-induced splenic lymphoblasts. Cytotoxicity assay was run for 3 h.

‡ Spleen cells from mice primed 7 wk previously with PR8 were restimulated in vitro with HK virus.

the extent to which an animal can respond. Perhaps this reflects a phenomenon similar to that described for Friend leukemia virus by Bubbers et al. (20), where presence or absence of a cytotoxic T-cell response is correlated with whether or not a particular H-2K or H-2D component is incorporated into the virus particle.

Antisera-blocking experiments indicated that BALB/c mice possess a subset of

TABLE IV

Ability of Anti-H-2L Alloantisera to Block Influenza-Immune Cytotoxicity on D Region-Compatible Target Cells

	Exp. no.	HK-immune effector cells‡	Blocking Antisera*			
			None	Normal mouse	Anti-H-2L	Anti-H-2D
HK-infected NA-target cells	1	BALB/c	25.8 ± 3.5§	30.7 ± 2.4	17.5 ± 1.1	13.8 ± 1.6
					$P < 0.01$‖	$P < 0.01$
		BALB/c-H-2^{db}	15.5 ± 1.2	16.0 ± 1.2	13.9 ± 0.9	3.4 ± 1.3
					$P > 0.2$	$P < 0.005$
	2	BALB/c	26.8 ± 2.7	36.2 ± 3.1	17.4 ± 1.8	24.4 ± 3.0
					$P < 0.008$	$P < 0.05$
		BALB/c-H-2^{db}	15.0 ± 1.2	21.6 ± 2.6	17.9 ± 2.1	9.0 ± 2.3
					$P > 0.3$	$P < 0.03$
	3	BALB/c	29.8 ± 1.5	31.6 ± 1.9	13.5 ± 1.7	nd¶
					$P < 0.005$	
		Alloimmune effector cells				
Uninfected NA-target cells	1	Anti-H-2L**	31.1 ± 2.6	29.4 ± 2.7	11.7 ± 2.4	25.6 ± 3.7
					$P < 0.01$	$P > 0.5$
		Anti-H-2D‡‡	18.9 ± 1.3	12.3 ± 3.2	12.0 ± 1.1	−4.4 ± 2.2
					$P > 0.9$	$P < 0.005$
	2	Anti-H-2L	16.3 ± 2.6	17.4 ± 2.4	3.3 ± 1.9	14.4 ± 1.3
					$P < 0.02$	$P > 0.3$
		Anti-H-2D	25.7 ± 2.1	34.2 ± 6.1	29.4 ± 3.1	2.2 ± 2.0
					$P > 0.5$	$P < 0.009$
	3	Anti-H-2L	24.7 ± 2.8	22.1 ± 2.0	5.2 ± 1.2	nd
					$P < 0.01$	
		Anti-H-2D	27.8 ± 1.2	23.7 ± 2.5	22.3 ± 1.6	nd
					$P > 0.5$	

* 10 μl (Exp. 1) or 5 μl (Exp. 2 and 3) of each serum were added to 10^4 target cells.

‡ See Table III.

§ Percent specific lysis ± SEM of triplicate determinations in a 3–4 h assay.

‖ P values determined by Student's *t* test which compares experimental lysis relative to lysis in presence of normal mouse serum.

¶ Not determined.

** Effector cells are BALB/c-H-2^{db} anti-BALB/c.

‡‡ Effector cells are C3H.OL (Exp. 1 and 2) or C3H.OH (Exp. 3) anti-BALB/c-H-2^{db}.

influenza-immune T-cells which recognize *H-2L* gene products, and that products of the *H-2L* locus can thus operate in an analogous manner to *H-2K/D* gene products in virus-immune T-cell recognition. This conclusion is based on the specificity of the anti-L alloantiserum used in this study. As shown by immunoprecipitation studies (Fig. 1 and reference 21), the anti-L serum did not precipitate molecules which bear the *K* or *D* region private specificities. The inability to block alloimmune T-cells directed against H-2D alloantigens further confirmed the lack of anti-D reactivity in the anti-L alloantiserum. Thus, the inhibitory effect of the antiserum is most likely due to anti-L antibodies because of the specific inhibition of cytotoxicity and the lack of reactivity with H-2D molecules by immunoprecipitation analysis. Although it could be argued that the inhibitory effect of anti-L alloantiserum on virus-immune T cells is due solely to a steric inhibition of H-2D specificites on the virus-infected target cell membrane, the inability of anti-L to inhibit lysis mediated by virus-immune BALB/c-$H\text{-}2^{db}$ T cells on these targets makes such an explanation untenable.

The results, which show that influenza- and vaccinia-immune BALB/c-$H\text{-}2^{db}$ mice are able to lyse *D* region-compatible wild-type target cells, are in contrast to those obtained using another *D* region mutant, B10.D2-$H\text{-}2^{da}$ (22). Zinkernagel and Klein (23) observed that lymphocytic choriomeningitis virus- and vaccinia virus-immune B10.D2-$H\text{-}2^{da}$ T cells were unable to kill *D* region-compatible wild-type targets, a result which we have also observed with influenza virus (data not shown). These findings imply that B10.D2-$H\text{-}2^{da}$, unlike BALB/c-$H\text{-}2^{db}$, lack virus-immune T cells which recognize both wild type L and D molecules. Consistent with this notion are recent findings by McKenzie et al.[3] which demonstrated that B10.D2-$H\text{-}2^{da}$ mice possess mutations which affect both the L and D molecules.

Although influenza-immune T cells apparently recognize H-2L specificities, current studies using the same alloantisera and mouse strains indicate that there is no *H-2L*-restricted component in the T-cell response to trinitrophenyl (TNP)-modified cells.[2] Blanden and Kees (24) recently reported that T cells generated against ectromelia virus and minor histocompatibility antigens do not recognize H-2L specificities. Thus, T cells appear to possess a more selective repertoire of responses associated with L than with K or D antigens. The mechanisms which produce this selectivity of responsiveness are not known, but an altered self model may most easily explain these observations: the minimum requirement for the generation of *H-2*-restricted T-cell responses is the physical association of foreign antigens with H-2*K, D,* or *L*-encoded self structures. Because TNP most probably is covalently linked to L molecules, the failure to demonstrate a TNP-specific *H-2L*-restricted T-cell response would seem to argue against the concept that formation of an altered self structure is the only requirement for generation of *H-2*-restricted cytotoxic responses. Perhaps this result may be due to a failure to generate the *H-2L*-restricted anti-TNP repertoire during differentiation in the thymus (25, 26).

Summary

The *H-2L* locus is closely linked to *H-2D* and codes for antigenic specificities present on a 45,000 mol wt glycoprotein that is distinct from the molecule which bears the *D* region private specificity. It was found that BALB/c-$H\text{-}2^{db}$ mice, which lack detectable cell-surface *H-2L* gene products, were able to generate influenza- and vaccinia-immune cytotoxic T cells which lyse *D* region-compatible target cells, although they have been reported to be incapable of making a similar response to ectromelia virus (7). Thus, the lack of H-2L antigenic specificities does not produce a general loss of responsiveness for other viruses even when a highly cross-reactive pox virus (vaccinia) was studied.

Antisera-blocking experiments utilizing sera specific for either L or D molecules indicated that BALB/c mice generate influenza virus-immune cytotoxic T-cell subsets which independently recognize *H-2L* and *H-2D* gene products in association with viral antigens. These results are the first indication that products of the *H-2L* locus can operate analogously to *H-2K/D* gene products in virus-immune T-cell recognition.

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