INTRODUCTION OF H-2D^d DETERMINANTS INTO THE H-2L^d ANTIGEN BY SITE-DIRECTED MUTAGENESIS

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A hallmark of MHC class I antigens is their high degree of polymorphism, which is thought to have been generated by gene conversion and subsequent selection (1). The MHC-restricted T cell recognition of foreign antigens depends on the polymorphic portions of MHC antigens. A mismatch in polymorphic MHC class I loci within a species evokes unusually high levels of alloreactive T cells (2, 3) and complex antibody responses (4). Though there are a number of hypotheses, the basis of this vigorous alloresponsiveness within a species has not been fully elucidated (5).

In recent years, efforts have been made to define the sites within an MHC class I antigen that are responsible for eliciting alloreactivity and H-2 restriction. Studies of in vivo–generated H-2K^b mutants (1) and recombinant MHC class I genes engineered in vitro (6–13) have revealed that the first (α-1) and second (α-2) external domains of the molecule, but not the third (α-3) domain, are responsible for alloreactivity and MHC restriction. It has been shown (6–13) that alloantibodies can recognize determinants within a single domain independent of adjacent domains, while most CTL recognize conformational determinants dependent on the interaction of the α-1 and α-2 domains.

Unlike other antigenic molecules in which positions of epitopes are well defined (14–16), the precise localization of allodeterminants of an MHC class I antigen has only begun (1, 17, 18). Previously, we postulated (10) that the region of amino acids 63–73 of the α-1 domain of the H-2D^d (D^d) antigen forms alloantigenic sites to which mAbs specific for the D^d antigen react. This was based on a comparison of the primary amino acid sequences of H-2 antigens and their predicted secondary structures. The role of this region of the MHC molecule in controlling CTL epitopes has been unexplored, largely because all of the in vivo–derived H-2 mutant and HLA subtype molecules that have been characterized biochemically do not have alterations between residues 63 and 73 (13). To test

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whether this portion of the molecule controls antigenic sites recognized by both mAbs and CTL, we have undertaken sequential site-directed mutagenesis of the L^d gene to generate amino acid sequences identical to those of the D^d antigen in the region from positions 63–73. We show that after mutation at residues 63, 65, and 66 and again after a further mutation at residue 70, the L^d antigen gains D^d-specific determinants recognized by both alloreactive antibodies and CTL. Although these mutations lose some L^d epitopes detected by allo CTL, serological L^d specificities are retained. Furthermore, the mutation in these positions results in an almost complete loss of reactivity to L^d-restricted anti–vesicular stomatitis virus (VSV)[1] CTL.

Materials and Methods

Generation of Mutant Ld Genes. The mutant L^d antigens (Table I) were created by sequential site-directed mutagenesis of the wild-type L^d gene. In four separate cycles of mutagenesis the codons at positions 63, 65 and 66, 70, and finally 73 were changed to those of the D^d amino acids. The procedure was essentially the same as previously described (9, 19–21). The four mutagenic oligodeoxyribonucleotides (Table I) were synthesized on a Vega Coder 300 (Vega Biotechnologies, Tucson, AZ) by the phosphoramidite method (22) and purified by HPLC. Each oligomer directed one to three nucleotide substitutions, resulting in one or two amino acid changes (Table I). A 1.9-kb Xba I fragment of the L^d gene (containing the first three exons) subcloned into an M13 vector was used as a template for mutagenesis. After annealing the mutagenic oligomer to the template, dsDNA was generated by primer extension with the Klenow fragment of DNA polymerase I and DNA ligase. Phage preparations obtained after transformation of *Escherichia coli* JM109 were then spotted onto nitrocellulose and screened by differential hybridization at increasing temperatures with ^{32}P–end-labeled mutagenic oligomer. The template used for each successive cycle of mutagenesis was that generated in the previous step. After reinserting the mutated Xba I fragment back into the remaining portion of the wild-type L^d gene, restriction analysis and dideoxy sequencing (23) were performed for final verification of mutagenesis.

Expression of Mutant Genes in Mouse L Cells. The three mutant L^d genes and the wild-type L^d and D^d genes were introduced into thymidine kinase–deficient mouse L cells by the calcium phosphate coprecipitation method, using the herpes simplex thymidine kinase gene as a selectable marker (20, 21). Transformed cells were screened with specific anti-L^d mAb 28-14-8 (24). Clones expressing high levels of the mutant genes were propagated for further characterization. Cells expressing the wild-type L^d (20, 21), D^d (12, 25), and $Q10^d/L^d$ gene (26, 27) have been described.

mAbs and Cytofluorography. The L^d- and D^d-specific mAbs have been previously characterized for their strain distribution (10, 28, 29) as well as domain specificity by using L^d/D^d (30–32), D^d/D^p (12), and D^d/K^d (33) recombinants (see Tables II and III). Cytofluorography was carried out as previously described (10).

Generation and Assay of Alloreactive CTL. CTL clones have been generated from limiting dilution cultures as previously described (34). Anti-$Q10^d$ CTL clones were generated by priming (C3H × B6.K1)F_1 mice intraperitoneally with 10^7 L cells transfected with the $Q10^d/L^d$ gene followed by in vitro stimulation using the same transfected L cells as stimulators. After 8 d of culture, the cells were placed in limiting dilution to derive clones. CTL clones were tested against L cell targets in a 4-h ^{51}Cr-release assay at a 25:1 and 5:1 E/T ratio. Only data for the 25:1 ratio are shown.

Generation and Assay of VSV-specific CTL. (C57BL/6 × DBA/2)F_1 (BDF1) mice were inoculated with 4×10^7 PFU of VSV_{Ind}. 6–7 d later, 5×10^6 of their spleen cells were added to individual wells of 24-well plates together with 5×10^6 spleen cells treated with

[1] *Abbreviations used in this paper:* c, CTL-defined; s, serologically defined; VSV, vesicular stomatitis virus.

anti-Thy-1 + complement that had been cultured with 10 μg/ml of LPS for 2–3 d before infection with a temperature-sensitive mutant of VSV (ts_{G41}) at 4 PFU/cell. After 5 d the CTL were tested against infected and uninfected L cell targets, as described (35).

Results

Evaluation of Potential Alloantigenic Sites and Site-directed Mutagenesis. We have previously postulated that the region from amino acids 63 to 73 in the α-1 domain of the D^d antigen is involved in the generation of alloantigenic sites (10), based on the following lines of evidence: (*a*) A comparison of the amino acid sequences of murine and human MHC class I antigens shows that the region of greatest diversity is in the α-1 domain between amino acids 63 and 83; (*b*) according to the algorithm of Hopp and Woods (36) this region of the α-1 domain of D^d has the greatest predicted hydrophilicity. It has been shown (36) that the antigenic portions of proteins tend to be hydrophilic; and (*c*) some anti-D^d mAbs have patterns of crossreactivity to different H-2 antigens that correlate with amino acid substitutions in this region (10).

To evaluate the validity of this assessment we have used site-directed mutagenesis to sequentially replace amino acids 63, 65, 66, 70, and 73 in the α-1 domain of the L^d antigen with those of the D^d antigen (Table I). The amino acid sequences of the L^d and D^d antigen in this region, oligomers used for mutagenesis, as well as the DNA sequences of the D^d, L^d, and the resultant mutant genes are shown in Table I. The first mutant bears D^d-type amino acids at positions 63, 65, and 66 and is designated M66 (Table I A). Using mutant gene M66 as a template, oligomer LE2-4 was used to change glutamine to asparagine at position 70, creating gene M70. The final mutant, M73, was made by changing the codon at position 73 from that of tryptophan to serine using oligomer LE2-7. Each mutant DNA was sequenced (23) to confirm the expected nucleotide substitution (data not shown).

The hydrophilicity predictions for the native D^d, L^d, and the mutant antigens derived according to Hopp and Woods (36) are shown in Fig. 1. The region from positions 63 to 73 in the native L^d antigen is relatively neutral in its predicted hydrophilicity, while this region comprises the highest predicted local hydrophilicity in the D^d antigen (Fig. 1*a*). The three amino acid changes in antigen M66 result in a significant increase in its predicted hydrophilicity, while the subsequent mutation at position 70 has no effect (*b* and *c*). The final substitution at position 73 makes the mutant antigen almost identical to D^d in terms of predicted hydrophilicity (*c*).

Acquisition of D^d Serologically Defined (s) Epitopes by Mutations of the Ld Gene at Positions 63–73. We introduced the mutant genes into mouse L cells and expression of the introduced genes was monitored by binding of mAb 28-14-8. This mAb (28) reacts with the α-3 domain of the L^d antigen and its binding is therefore unlikely to be affected by mutations in the α-1 domain (30). L cells expressing the mutant antigens were tested with a panel of 41 anti-D^d mAb by cytofluorography. These mAbs do not react with L^d (10, 30). As seen in Table II, 12 of these antibodies react with determinants in the α-1 domain and seven are specific for the α-2 domain of the D^d antigen. The remaining 22 mAbs react with conformational determinants dependent on the interaction of the α-1 and

TABLE I

Amino Acid Sequences of Class I Molecules and Oligonucleotides Used for Mutagenesis

Sequence	Molecule	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	
A*	H-2L[d]	GLU	ARG	ILE	THR	GLN	ILE	ALA	GLY	LYS	GLY	GLN	GLU	TRP	PHE	ARG	
	H-2D[d]	·	·	GLU	·	ARG	ARG	·	·	·	ASN	·	·	SER	·	·	
	M66	·	·	GLU	·	ARG	ARG	·	·	·	·	·	·	·	·	·	
	M70	·	·	GLU	·	ARG	ARG	·	·	·	ASN	·	·	·	·	·	
	M73	·	·	GLU	·	ARG	ARG	·	·	·	ASN	·	·	SER	·	·	
	Q10[d]	·	·	GLU	·	ARG	ARG	·	·	·	ASN	·	·	SER	·	HIS	
						***											
B‡	LE2-1	ATA	ACC	CTC	GCC	CTC	TGC	GTC	TAG	CGG							
		59	60	61	62	63	64	65	66	67							
								*	**								
	LE2-3			CTC	GCC	CTC	TGC	GCC	TCC	CGG	TTC	CCG					
				61	62	63	64	65	66	67	68	69					
											* *						
	LE2-4								TTC	CCG	TTA	CTC	GTC	ACC	AAG	GCT	
									68	69	70	71	72	73	74	75	
														*			
	LE2-7									CCG	TTA	CTC	GTC	AGC	AAG	GCT	CAC
										69	70	71	72	73	74	75	76

Column headers 63, 65, 66, 70 and 73 are underlined in the original.

* Comparison of the amino acid sequences of the α-1 domains of the L[d], D[d], Q10[d], and three mutant L[d] antigens generated in this study. Mutations were introduced into the *L[d]* gene at the underlined residues with the D[d] amino acids.

‡ Sequences of the four oligomers used for site-directed mutagenesis with the corresponding amino acid positions indicated. The positions of mismatched nucleotides are indicated by an asterisk.

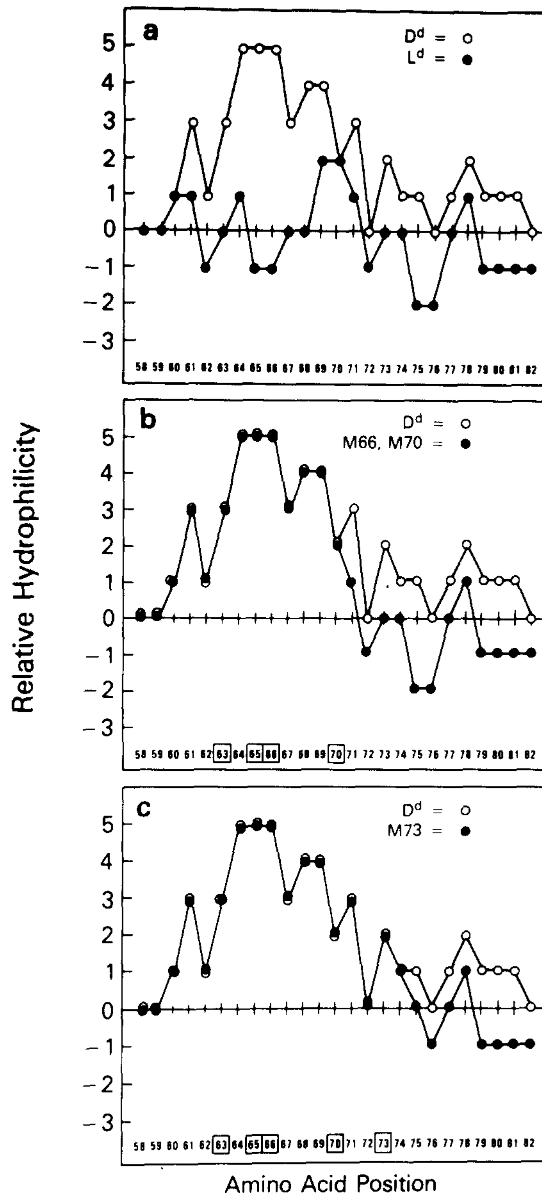

FIGURE 1. Predicted hydrophilicity indices of the mutant L^d antigens. The predictive procedure for locating hydrophilic portions of a protein developed by Hopp and Woods (36) was used for the native L^d (*a*), mutant M66, M70 (*b*), and M73 (*c*) in comparison with the native D^d antigen. Circled amino acid positions represent the site of substitution.

α-2 domains of the D^d antigen (12). Although the conformational nature of these determinants were demonstrated with D^d/D^p (12) and K^d/D^d recombinants (33), it was not revealed by D^d/L^d hybrid antigens (10), presumably due to greater homology of the D^d and L^d molecule.

As shown in Table II, replacement of the L^d residues at positions 63, 65, and 66 with D^d residues resulted in the binding of 11 anti-D^d-specific mAbs. Four of these mAbs are α-1 domain specific; the remainder recognize conformational

TABLE II
Binding of D^d-specific mAb to Mutant Ld Antigens

Antibody	Domain specificity	References	Antibody binding to transformed cells*			
			M66	M70	M73	D^d
T.O.107	α-1	10, 12	(0)	(0)	(0)	676
T.O.111		"	(3)	(0)	(0)	298
T.O.119		"	(0)	(0)	(0)	332
T.O.120		"	(8)	(0)	(0)	157
T.O.124		"	132	317	313	326
T.O.129		"	(19)	(13)	(1)	700
T.O.131*1		"	(16)	(1)	(1)	710
T.O.133*1		"	(15)	(3)	(0)	637
T.O.136*2		"	376	313	527	326
T.O.137		"	(6)	307	205	509
34-4-21*2		24, 30, 31	257	384	389	214
34-1-2		"	34	577	136	457
34-5-8	α-2	10, 12, 24, 31	(0)	(12)	(0)	567
T.O.112		10, 12, 32	(0)	(0)	(0)	610
T.O.113*3		"	(0)	(0)	(0)	710
T.O.114*3		"	(0)	(0)	(0)	712
T.O.117		"	(0)	(0)	(0)	533
T.O.121		"	(0)	(8)	(1)	624
T.O.123		"	(0)	(7)	(0)	580
23-5-21	α-1/α-2	28, 30	(3)	23	(4)	96
T.O.104		10, 12	(0)	(0)	(1)	125
T.O.108*4		"	(11)	274	138	376
T.O.109*4		"	(8)	267	68	306
T.O.110		"	65	395	125	329
T.O.115		"	(6)	(0)	(3)	94
T.O.116		"	(12)	(2)	(0)	149
T.O.118		"	(10)	257	179	392
T.O.122		"	(0)	44	(0)	289
T.O.125		10	(0)	(2)	(0)	43
T.O.126		10, 12	(5)	156	20	314
T.O.127		"	431	627	464	302
T.O.128		"	35	569	431	184
T.O.130		10	(15)	(4)	(0)	255
T.O.132		10, 12	49	439	341	386
T.O.134*5		"	113	431	277	421
T.O.138*6		"	(20)	(3)	(0)	195
T.O.139*6		"	(15)	(6)	(0)	185
T.O.141		"	(5)	(11)	(2)	160
T.O.142*6		"	(5)	(8)	(7)	89
T.O.144*5		"	413	601	414	316
T.O.145		"	258	440	(14)	181

* Cytofluorography was performed as described in Materials and Methods. Mean fluorescence values were determined on a linear scale and normalized by subtraction of background fluorescence obtained by negative control mAb, anti-IA^k (10, 12). Binding values >22 were scored positive. Values in parenthesis represent antibody binding scored negative. Asterisks with numbers represent mAb with the same specificity.

determinants influenced by both the α-1 and α-2 domains (10, 12, 31, 32). As judged by the crossreactivity of these mAbs toward different mouse strains (10, 24, 28, 29), as well as D^d/D^p (12) and D^d/K^d recombinant antigens, this represents a minimum of nine independent specificities (34-4-21 and T.O.136 are identical in specificity, so are T.O.134 and T.O.144, the remaining seven mAbs show unique reactivity). From these results it is apparent that residues 63, 65, and 66 are involved in creating multiple D^d s-specificities, although independent contributions of each amino acid to the epitope formation remain to be tested.

A further amino acid substitution at position 70 resulted in the gain of reactivity of an additional seven anti-D^d mAbs. All of the mAbs reactive with M66 remained positive for M70, making a total of 18 anti-D^d-specific mAbs that react with this mutant antigen. Of the seven additional mAbs reacting with M70, one was α-1 domain specific (T.O.137). mAbs reactive with the α-1 domain or α-1/α-2 conformational determinants examined in this work represent 28 independent specificities. Thus, more than half of all the s-specificities recognized by these mAb are created at least in part by the stretch of amino acids from 63 to 70.

The final mutation was introduced at position 73 (M73 in Table II). This mutant antigen showed no evidence for the acquisition of new D^d determinants. All of the α-1 domain–specific mAbs retained reactivity toward this antigen. However, three mAbs (23-5-21, T.O.122, and T.O.145) that recognize conformational determinants lost their reactivity. In addition, the reactivity of mAb T.O.126 appeared to be greatly reduced (Table II). The results indicate that residue 73 does not play an important role in formation of the α-1 domain–specific s-epitopes for the mAbs studied here. The basis of the loss of reactivity of the three mAbs is not clear; possibly, the conformation created by residues 63–70 of the mutant α-1 domain and the α-2 domain of the L^d antigen is altered or destroyed by the change at position 73. As shown in Table II, none of the seven α-2 domain–specific mAbs became reactive toward any of the three mutant antigens. Consistent with these results, previous studies (12) have shown that the determinants recognized by these antibodies are independent of the α-1 domain.

Retention of L^d-specific and L^d/D^d-crossreactive s-Epitopes in the Mutant Antigens. Binding of mAbs specific for the L^d antigen and for shared determinants present in both the L^d and D^d antigens is shown in Table III. All six anti-L^d mAbs reactive with determinants in the α-1, α-2, or α-3 domains retained reactivity to all three mutant antigens. In addition, all eight mAbs crossreactive with the L^d and D^d antigens were positive with each of the mutants, as might have been expected.

Ability of Alloreactive CTL to React with Mutant Ld Molecules. Bulk-cultured and cloned anti-L^d and -D^d reactive CTL were tested against L cells expressing native L^d, native D^d, and the mutant molecules M66, M70, and M73. As seen in Fig. 2 (*right*), B10.D2(R103) (K^d, D^b) anti-BALB/c-H-2^{dm2} (K^d, D^d (anti-D^d) CTL react against D^d (K8-30) and M70-transfected L cells. Although weak lysis is observed against the other two mutant L^d molecules (M66, M73), these CTL also crossreact on the native L^d (W12). Therefore, the analysis of reactivity using CTL clones was more informative. The four CTL clones tested showed three different specificity patterns. Clones D.98.9 and D8.3 (not shown) recognized D^d only and did not detect L^d or any of the mutant antigens (Fig. 3, *left*; Table IV, II A).

TABLE III

Binding of anti-L^d, or L^d/D^d Crossreactive Antibodies to Mutant Ld Antigen

Antibody	Specificity	Reference	Antibody binding				
			W12	M66	M70	M73	D^d
28-14-8	L^d, α-3	28, 30	499	406	494	492	NT*
66-8-2	L^d, α-3	29, 32	102	119	178	199	NT
30-5-7	L^d, α-2	31	557	421	394	466	NT
23-10-1	L^d, α-2	30, 31	163	85	363	201	NT
66-3-5	L^d, α-2	29, 32	496	119	454	396	NT
64-3-7	L^d, α-1	20, 21	152	325	128	134	NT
28-11-5	L^d/D^d	30, 31	140	38	422	392	567
27-11-13	"	"	128	163	679	436	346
34-4-20	"	"	201	50	665	326	291
T.O.101	"	10, 12	30	12	118	170	83
T.O.102	"	"	161	30	713	307	527
T.O.103	"	"	241	140	222	132	536
T.O.105	"	"	234	40	764	438	424
T.O.106	"	"	60	53	778	138	321

Cytofluorography was performed as described in Materials and Methods. Fluorescence values were determined as in Table II.

* NT, not tested.

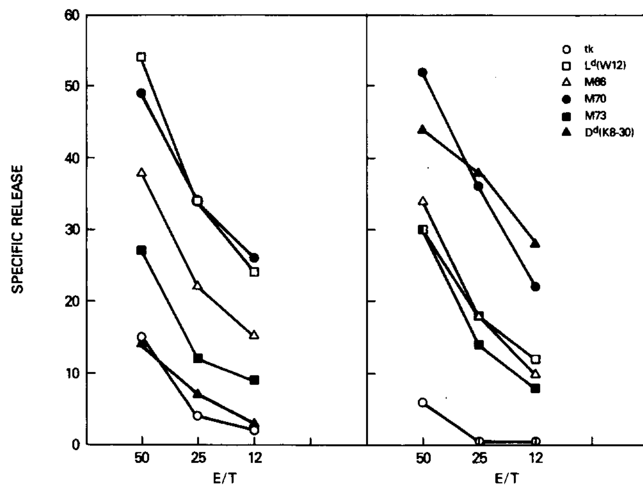

FIGURE 2. Lytic activity of BALB/c-H-2^{dm2} anti-BALB/c (anti-H-$2L^d$) (*left*) or B10.D2 (R103) anti-BALB/c-H-2^{dm2} (anti-H-$2D^d$) CTL (*right*). The CTL were generated in 5-d bulk culture and tested against L cells transfected with tk, H-$2L^d$ (W12), M66, M70, M73, and H-$2D^d$ (K8-30).

The second pattern was displayed by clone D.88.42, which recognized D^d and the mutant molecules, M66 and M70, but did not react with M73 (Fig. 3, *center*; Table IV, II B). The third pattern was displayed by clone D98.36. This clone was derived from a CBA (K^k, D^k) anti-BALB/c-H-2^{dm2} culture and was found to react not only against D^d, but L^d as well. Not unexpectedly, this clone also reacts on all three mutant antigens (Fig. 3, *right*; Table IV, II C). This pattern was also evident in some of the anti-$Q10^d$ clones (see below), making it similar to the mAbs that crossreact on both L^d and D^d.

Bulk-cultured BALB/c-H-2^{dm2} anti-BALB/c (K^k, D^d, L^d) (anti-H-$2L^d$) CTL reacted against native L^d (W12) and not D^d (K8-30)-transfected L cells (Fig. 2,

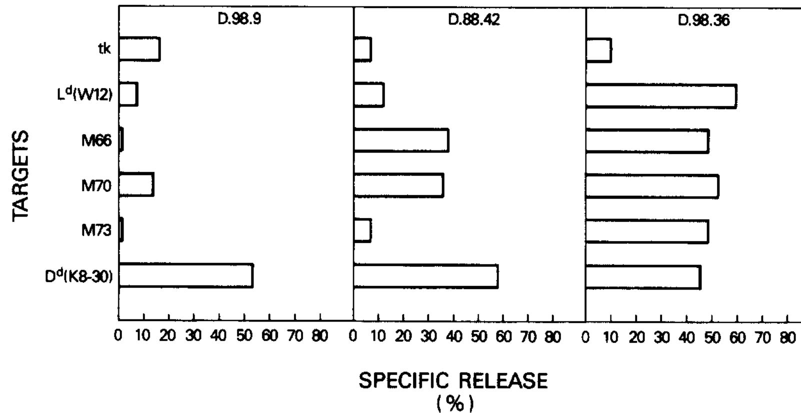

FIGURE 3. Lytic activity of CTL clones generated against H-2D^d. Clone D8.3 is from B10.D2 (R103) anti-BALB/c-H-2^{dm2} and the other three clones are from CBA anti-BALB/c-H-2^{dm2} cultures. CTL are tested against L cells transfected with tk, H-2L^d (W12), M66, M70, M73, and H-2D^d (K8-30). Data shown for E/T ratio of 1:25.

TABLE IV
Specificity Patterns of CTL Clones

Group*	Pattern	L^d	M66	M70	M73	D^d
I	A	+	+	+	+	−
Anti-L^d	B	+	−	−	−	−
	C	+	+	−	+	−
II	A	−	−	−	−	+
Anti-D^d	B	−	+	+	−	+
	C	+	+	+	+	+
III	A	−	−	+	+	−
Anti-Q10/L^d	B	+	±	+	+	±
	C	+	+	+	+	+

+, Strong reactivity; −, no reactivity; ±, low lytic activity relative to that observed on L^d targets.
* Group I, patterns A–C, and group II, patterns B and C, represented by single clones. Group II, pattern A and C, represented by two clones each. Group III, pattern A, represented by three clones, pattern B represented by five clones.

left). These effector cells also recognized L^d mutant molecules. M73 targets were lysed to the least extent, whereas M70 were recognized to an extent similar to native L^d.

This data was extended using CTL clones derived from cultures of anti-L^d CTL (Fig. 4, Table IV). The three clones with anti-L^d reactivity displayed three different specificity patterns. Clone L9.4 reacts with W12, M66, M70, and M73 to an equivalent extent, indicating that the L^d epitope it recognizes is not affected by changes in this region of the molecule. Clone L.13D.4C shows an opposite pattern of reactivity in that it recognizes L^d but does not lyse L cells expressing any of the mutant target molecules (Table IV, I B). Finally, clone L.13D.8A reacts with L^d and the mutant molecule M66 with changed amino acids at 63, 65, and 66 (Table IV, I C). Although it does not react on the M70 antigen, it reacts weakly on M73, suggesting that the loss of the epitope introduced by the cluster of changes at 63, 65, 66, and 70 is restored by changing residue 73.

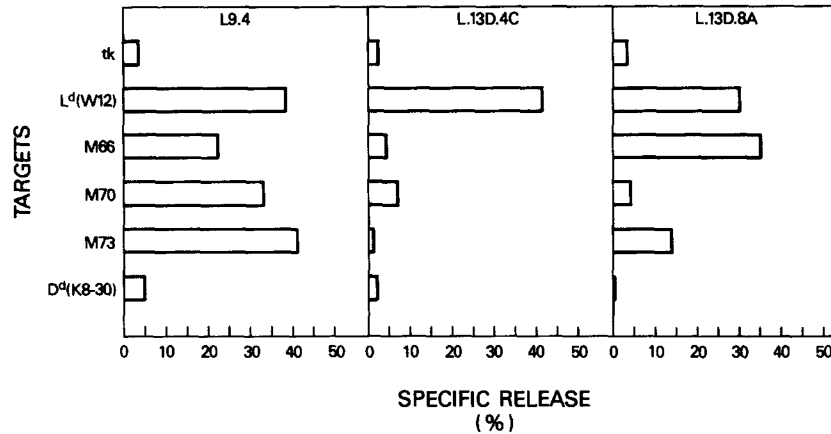

FIGURE 4. Lytic activity of CTL clones generated against H-2L^d. Clone L9.4 is from (C3H × BALB/c-H-2^{dm2})F$_1$ anti-BALB/c and clones L.13D.4C and L.13D.8A are from CBA anti-A.AL cultures. CTL are tested against L cells transfected with tk, H-2L^d (W12), M66, M70, M73, and H-2D^d (K8-30). Data shown for E/T ratio of 1:25.

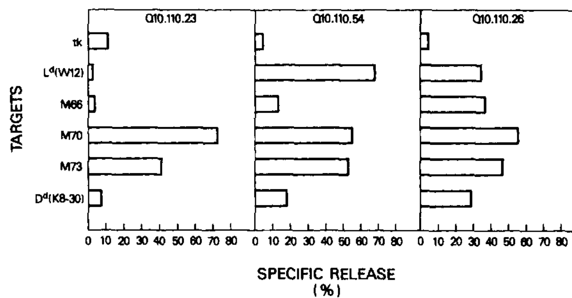

FIGURE 5. Lytic activity of CTL clones generated against Q10^d/L^d. Anti-Q10 CTL clones were generated in (C3H × B6.K1)F$_1$ mice (see Materials and Methods). CTL are tested against L cells transfected with tk, H-2L^d (W12), M66, M70, M73, and H-2D^d (K8-30). Although not shown in the figure, all clones lyse Q10^d/L^d-transfected L cells. Data shown for E/T ratio of 1:25.

We further investigated how mutation in this region of the H-2L^d molecule would affect CTL epitopes by using CTL clones generated against Q10^d. Q10^d is a class I molecule normally found in serum of most strains of mice at relatively high concentrations (37). Although Q10 has a mutant transmembrane domain which accounts for its secretion (38), it can be converted into an integral membrane protein by exon shuffling the gene with H-2L^d and transfecting the shuffled gene into L cells (25, 26). The resultant molecule is expressed on the membrane and bears the α-1 and α-2 domains of Q10^d together with the α-3 and COOH-terminal domains derived from L^d (this molecule is referred to as Q10^d/L^d). We have previously shown (27) that mice can generate a CTL response against Q10^d/L^d transfected L cells, indicating that secreted class I molecules do not induce CTL tolerance.

Since the amino acid sequence of Q10^d/L^d at position 61–75 closely resembles that of D^d and the mutants (Table I, A), we tested anti-Q10^d/L^d CTL clones against L^d, D^d, the three L^d mutants. Three different specificity patterns were observed. Pattern III A (Table IV) is represented by clone Q10.110.23, which does not react against either L^d or D^d but does react against M70 and M73 (Fig. 5, *left*). Pattern III B is represented by clone Q10.110.54, which reacts against L^d as well as L^d M70 and M73 (Fig. 5, *center*). These clones also display weak

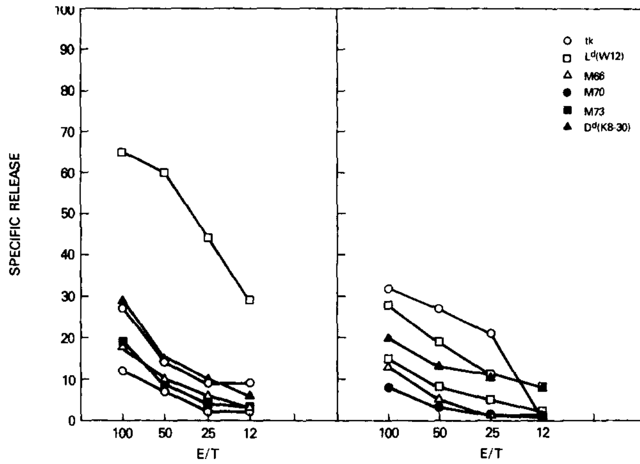

FIGURE 6. Lytic activity of in vitro-cultured CTL from (C57BL/6 × DBA/2)F_1 (BDF_1) mice primed with 4 × 10^7 PFU of VSV. The CTL were generated in 5-d bulk culture and tested against L cells transfected with tk, H-$2L^d$ (W12), M66, M70, M73, and H-$2D^d$ (K8-30). Lysis shown against VSV-infected targets (400 PFU/cell) in left panel and uninfected targets in right panel. Spontaneous release ranged from 11 to 32%.

reactivity against D^d and M66. Pattern III C, represented by clone Q10.110.26, reacts on L^d, D^d, and all three of the mutants (Fig. 5, *right*). Similar to pattern III B clones, the acquisition of reactivity is correlated with reactivity against M66. This last set of clones has a specificity similar to pattern II C clone D.98.36, which crossreacts on both D^d and L^d as well as all three mutants.

Taken together, the above data demonstrate that changes of amino acids in L^d between residues 63 and 73 can affect CTL epitopes. Further, by changing amino acid residues at only positions 63, 65, and 66 to the D^d type, the L^d molecule acquires a D^d CTL epitope, as detected by clone D.88.42.

Ability of H-$2L^d$ Mutant Molecules to Restrict Anti-VSV CTL. The CTL response against VSV in the H-2^d haplotype is restricted by L^d and not D^d (35, 39). Since bulk-cultured alloreactive anti-L^d CTL lyse M66 and M73 targets and kill M70 cells equivalently to native L^d (Fig. 2), we wished to determine whether anti-VSV L^d-restricted CTL would also react against the same mutant molecules. The data in Fig. 6 show that these viral-specific CTL fail to recognize these mutant molecules. Thus, most of the VSV-restricting epitopes on the L^d molecule appear to be affected by changes in this region of the α-1 domain and therefore differ from a large portion of the alloantigenic CTL epitopes, as detected in bulk cultures.

Discussion

We have used site-directed mutagenesis to localize allodeterminants in the α-1 domain of the D^d antigen, based on the hypothesis that the portion of the D^d antigen formed by residues 63–73 is important for generating s-alloantigenic epitopes. Specific mutations were introduced into the L^d gene to replace amino acids of this region with those of the D^d type. Localization of a number of s- and CTL-defined (c)-epitopes in the mutant antigens was made possible by the availability of a large number of mAbs and CTL clones against the D^d or L^d antigen representing unique specificities.

We show first that changing the three amino acids at positions 63, 65, and 66 results in a gain of nine D^d s-specificities. Secondly, we found that the additional

amino acid substitution at residue 70 confers an unexpectedly large number of D^d s determinants. A total of 15 D^d s-specificities were found in mutant M70, which is more than half of the total number of s-determinants in the α-1 or α-1/α-2 domain of the D^d molecule studied here. No additional s-epitopes were revealed by the subsequent mutation at position 73, indicating that this position is not crucial in creating epitopes recognized by the mAbs used. It can be concluded that the area surrounding amino acids 63–70 serves as an "immunodominant" region of the D^d molecule that produces multiple s-alloantigenic sites. This immunodominant area appears to be involved in generating both α-1-specific and α-1/α-2-conformational s-epitopes.

In agreement with our results, there have been several reports demonstrating that synthetic peptides corresponding to this area of MHC class I antigens are capable of producing antibodies that react specifically with the original antigens. Walker et al. (40) used a peptide corresponding to residues 61–83 of HLA-B7, and Ways et al. (41) used one derived from residues 56–69 of the HLA-A2 and B-17 antigens. Singh et al. (42) demonstrated similar results with an octapeptide corresponding to residues 61–69 of the H-$2K^b$ antigen. All these investigators were able to show specific reactivity of the antibodies to the original MHC class I antigen in either native or denatured form. Consistent with our observations, studies on D^d/K^d intradomain recombinants have mapped the epitopes for mAbs T.O 124, T.O 127, T.O 136, T.O 144, and 34.2.21 into the region between residues 57 and 65 of the D^d antigen (33).

The pattern of reactivity of anti-D^d CTL against the L^d mutant molecules is similar to that observed with anti-D^d mAbs. Bulk-cultured anti-D^d CTL demonstrated reactivity against the L^d mutant molecule M70. Although some low level of reactivity against M66 and M73 may have been present in these cultures, it could not be detected, since there was weak crossreactivity observed against wild-type L^d. Anti-D^d CTL clone (D.88.42) reacted against the L^d mutant molecules M66 and M70. Thus, we demonstrate for the first time the generation of a CTL epitope by transferring a specific amino acid sequence into a class I molecule. This D^d c-epitope is similar to s-epitope(s) in that they were lost when amino acid 73 was changed from tryptophan to serine. It should be noted that this c-epitope is likely to be dependent on the structure of the α-2 domain, since D^d c-epitopes are readily detectable in D^d/L^d (α-1/α-2) but not in a D^d/$Q7^d$ domain-shuffled molecule (11, 26). As expected, not all D^d c-determinants are controlled by this region, as evidenced by the lack of reactivity of two anti-D^d CTL clones against any of the mutant L^d molecules (Table IV, II A).

The importance of this region was further revealed by the reactivity of anti-$Q10^d$ CTL clones. We previously reported (27) that anti-$Q10^d$ CTL crossreact with H-2^d antigens. The sequence of $Q10^d$ between residues 63 and 73 is identical with H-$2D^d$ (Table I) (43, 44). Some of these clones crossreact on M70 and M73 but fail to recognize D^d (Table IV, III A). This suggests that the presence of the $Q10^d$ 61–73 amino acid sequence in the context of L^d rather than D^d is more permissive for the expression of the crossreactive epitope. Anti-Q10 clones that crossreact on D^d (Table IV, III, B and C) also acquire reactivity on M66, suggesting that the c-epitope(s) recognized on D^d crossreacts with a determinant on L^d, which is present only when D^d amino acids are found at residues 63, 65,

and 66. The introduction of additional D^d or $Q10^d$-like sequences into the L^d molecule (M70 and M73) further enhances the reactivity above that seen with M66 or the wild-type D^d. Although the sequence of $Q10^d$ is identical to H-$2D^d$ between residues 63–73, most of the anti-$Q10^d$ CTL clones (Table IV, III, B and C) preferentially crossreact on L^d rather than D^d. Further, the reactivity of group III$clones is affected by changes in amino acids in the α-2 domain (Mann, D. W., and J. Forman, unpublished observations). Thus, it is likely that these clones recognize conformational epitopes dependent on the interactions of both α-1 and α-2 domains.

Although amino acid sequences from 63 to 70 control both s- and c-epitopes in the D^d molecule, the same region of the L^d molecule exhibits an apparent dichotomy in the expression of s- and c-epitopes. None of anti-L^d mAbs lose reactivity to any of the mutants tested here. That most of anti-L^d mAb to date are α-2 and α-3 domain specific may indicate that this region (and thus the α-1 domain) of the L^d molecule does not have major serological alloepitopes. On the other hand, reactivity of bulk-cultured anti-L^d CTL is affected by the mutations, as evidenced by reduced reactivity against M66 and M73. More conclusively, two of three anti-L^d CTL clones lose their reactivity to some or all mutants, demonstrating the involvement of this region for c-allospecificity.

None of the mutant molecules were recognized by bulk-cultured, L^d-restricted anti-VSV CTL. Since we have shown that alterations in this segment can have dramatic effects on c-epitopes, we may assume that this region makes up the major portion of VSV-restricting epitopes. Alternatively, this part of the molecule may play a role in the postulated binding of virus peptide fragments to class I molecules (44). The importance of the α-1 domain of L^d in controlling VSV-restricting epitopes was previously noted by Murre et al. (31), who showed that a D^d/L^d (α-1/α-2) hybrid molecule failed to react with restricted anti-VSV but not antiinfluenza CTL.

What is the structural basis of the gain and loss of D^d and L^d epitopes that were observed upon mutations at positions 63–73? As originally conceived, increased hydrophilicity may explain s-epitope changes. High local hydrophilicity has been proposed (36) to correlate with antigenic determinants seen by antibodies. The local hydrophilicity is expected to expose the area to the surface, thus making it more accessible to antibodies in solution. Several examples that support this proposal have been reported (15, 36). The replacement of the L^d amino acids at position 63, 65, and 66 with those of the D^d type increases predicted hydrophilicity of this region substantially, which may accord with the gain of multiple s-epitopes. The paucity of s-determinants on this region of the L^d antigen might be consistent with the role of hydrophilicity for s-epitope formation, as this area of L^d is low in hydrophilicity. However, the additional epitope gain found in M70 cannot be explained by this mechanism, since no hydrophilicity change is expected to occur by this substitution. Furthermore, the significance of hydrophilicity has not been implicated so far for c-epitopes. Recently, Geysen et al. (16) proposed an alternative hypothesis for the sites of antibody binding to an antigen. These authors examined stereochemical properties of the myohemerythrin antigen, based on crystallographic analysis of the molecule, and tested antibodies to the peptide homologues of the antigen. These authors found that the most frequently recognized sites tend to have high local mobility and

convex surface potential. Poorly recognized sites are characterized as tightly packed, least mobile regions. No correlation was indicated between hydrophilicity and antibody binding sites in this study. This is an attractive hypothesis, as it may explain the role of residue 70 in creating epitopes. However, in the absence of detailed crystallographic information (45), it is not possible to evaluate this model for MHC class I antigens at present.

DeLisi and Berzofsky (14) have proposed a model for an antigenic structure that is specifically recognized by T cells, distinct from that detected by antibodies. According to this hypothesis, T cells recognize lower order structures that exhibit amphipathic properties, as opposed to tertiary structures seen by antibodies. The region encompassing residues 63–73 of the D^d molecule is clustered with charged amino acids interspersed by neutral ones, which might create a structural environment favoring an amphipathic tendency. That this region of the L^d molecule also forms c-antigenic sites may be relevant to amphipathic properties created by other elements, such as periodic hydrophilicity (14).

It is possible that structural requirements for s- and c-epitopes are distinct. If so, this region of the D^d molecule may possess structural features necessary for creating both s- and c-epitopes.

Summary

We used site-directed mutagenesis to localize serologically defined (s) and CTL (c)-defined alloantigenic determinants to discrete amino acid sequences of a murine MHC class I antigen. Based on the prediction that amino acid position 63–73 of the H-$2D^d$ antigen forms s-allodeterminants, the *H-2L*^{d} gene was mutated in a sequential fashion to replace codons for amino acid positions 63, 65, 66, 70, and 73 with those of the H-$2D^d$ amino acids. Epitopes of the mutant antigens expressed in L-cells were examined by the binding of a series of mAbs specific for the H-$2D^d$ antigen. The mutant antigen M66 had substitutions at residues 63, 65, and 66, and resulted in the acquisition of a number of H-$2D^d$-specific s-epitopes. Mutant M70 had an additional substitution at residue 70, which led to the gain of multiple additional H-$2D^d$ s-epitopes. Together, more than half of all the relevant H-$2D^d$ s-epitopes were mapped into amino acid position 63–70 of the H-$2D^d$ molecule, which was expressed in the mutant *H-2L*^{d} gene. The final mutation at residue 73 (M73) caused no new epitope gains, rather, a few D^d s-epitopes acquired by the preceding mutations were lost. All of the H-$2L^d$-specific s-determinants were retained in the mutant molecules, as were H-$2D^d$ s-determinants specific for the α-2 or α-3 domains. Changes of these residues affected c-determinants defined by CTL. Anti-H-$2D^d$ CTL cultures and an anti-H-$2D^d$ CTL clone recognized the mutant H-$2L^d$ molecules, M66 and M70. Some CTL clones generated against the $Q10^d$ molecule, which has an identical sequence to H-$2D^d$ between residues 61 and 73, failed to recognize native H-$2D^d$ or L^d but did crossreact with mutant L^d. While bulk-cultured anti-H-$2L^d$ CTL cultures reacted strongly against M73, bulk-cultured H-$2L^d$ restricted anti–vesicular stomatitis virus CTL did not. Finally, at the clonal level two of three anti-H-$2L^d$ CTL clones lost reactivity with some or all of these mutant molecules. From these results we conclude that a stretch of amino acids

from position 63 to 70 of the α-1 domain controls major s- and c-antigenic sites on the H-2D^d antigen and c-sites on H-2L^d antigen.

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