

## Ia MOLECULE-ASSOCIATED SELECTIVITY IN T CELL RECOGNITION OF A 23-AMINO-ACID PEPTIDE OF LYSOZYME

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Immune response (Ir) gene effects in mice are seen as differences in T cell responsiveness to the same antigen among inbred strains. McDevitt et al. (1) found differences in responsiveness to several antigens in inbred mouse strains to be correlated with differences in the extremely polymorphic MHC, particularly in the I region within the MHC. The I region encodes the Ia molecules, which play key functions in antigen-induced activation of T cells, and there are strong reasons to believe that the clonally expressed receptor on T cells recognizes both the antigen and the Ia molecule (2-4). Also, largely through the use of hematopoietic chimeras constructed between MHC disparate strains, it has become evident that the Ia molecules can influence the expression of the T cell specificity repertoire, presumably in the absence of extrinsic antigens (5). How the Ia molecules influence T cell responsiveness leading to Ir gene effects has been a major question (reviewed in reference 6). One view, referred to as the "determinant selection" hypothesis, emphasizes the selective role of Ia molecules after introduction of antigen, and suggests that among T cell precursors there is strong selection for those clones specific for certain antigen/Ia combinations. A second hypothesis, referred to as the "holes-in-the-repertoire" hypothesis, emphasizes the role of Ia molecules before introduction of extrinsic antigens in establishment of the T cell repertoire, which is strongly biased towards recognition of self Ia molecules. This selection process could fortuitously select only certain clones specific for a given antigen/Ia combination, leading to the observed limited responsiveness.

Both hypotheses thus place limits on the function of the Ia molecules. Since these limits are detected as differences in responsiveness to certain antigens, it has been assumed that these antigens present a limited array of antigenic determinants. The Ir gene question is then reduced to one of understanding how the antigenic determinants are recognized in association with a particular

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Ia molecule and how this recognition varies as a function of the structure of different Ia molecules.

Our earlier studies (7, 8) on the antigenic determinants recognized by B6 T cells specific for lysozyme peptide 74–96 and restricted by the A<sup>b</sup> Ia molecule had shown the existence of two major determinants within this 23-amino-acid (23-aa)<sup>1</sup> peptide. Using a panel of synthetic peptides of varying lengths, these determinants were defined as being contained within peptides 74–90 and 81–96. The availability of this large panel of synthetic peptides, which unambiguously allowed us to define the antigenic specificity of T cells to a high degree of precision, prompted us to ask if T cell recognition of this lysozyme peptide 74–96 in the context of different Ia molecules would show any differences in determinant specificity. If so, these differences could then be attributed to functional differences among the structurally distinct Ia molecules.

In this study, we describe the specificity profiles of T cells restricted by either the A<sup>k</sup> or E<sup>k</sup> Ia molecules, and specific for determinants within the 23 aa peptide (aa 74–96) of lysozyme. A strict correlation was observed between the minimal peptide determinants and the Ia molecule restricting recognition. Moreover, sharp differences were observed in the boundaries of determinant regions recognized within the same 23-aa peptide by A<sup>k</sup>- or E<sup>k</sup>-restricted cells when compared with A<sup>b</sup>-restricted T cells. These results show the strict Ia-dependent selectivity in the choice of T cell determinants and are discussed in relationship to models of repertoire acquisition.

## Materials and Methods

### *Mice*

B10.A/SgSnJ (B10.A), CBA/J (CBA), and C57BL/6J (B6) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and were bred in our animal facilities. B10.A(4R) mice were originally a gift from Dr. J. Frelinger, then at the University of Southern California. Mice of either sex were used at 3–6 mo of age.

### *Antigens*

The peptides shown in Table I were synthesized by an improved solid-phase technique described earlier (7). The purity of the peptides was tested using HPLC on either C4 or C18 reverse-phase columns. All peptides eluted as a single major peak, integrated as containing 90–95% of material with UV absorbance at 214 nm.

### *Immunization*

Mice were immunized in the hind foot pads with 7 nmol of peptide emulsified with CFA. Popliteal lymph node cells (LNC) were obtained 10 d later, and they were used in antigen-induced proliferation assays or as a source of T cells to generate long-term lines.

### *T Cell Lines and Hybrids*

Continuously growing T cell lines were generated from immunized mice according to the method of Kimoto and Fathman (9). These were maintained and cloned by limiting dilution as described (10). T cell hybrids were obtained by fusing the established T cell lines with the drug-sensitive thymoma BW5147, as described (2). Briefly,  $3\text{--}10 \times 10^6$  T cells were fused to an equal number of BW5147 cells by using 50% PEG 1450 (J. T. Baker Chemical Co., Phillipsburg, NJ). The frequency of growing hybrids in HAT (100

<sup>1</sup> Abbreviations used in this paper: HEL, hen egg-white lysozyme; 23-aa, 23-amino-acid peptide; LNC, lymph node cells.

TABLE I  
Peptides Used in This Study

Peptide	Sequence																						
	75	80	85	90	95																		
74-96*	N	L	C	N	I	P	C	S	A	L	L	S	S	D	I	T	A	S	V	N	C	A	K
74-90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
74-86	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
74-82	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
77-96																							
81-96																							
85-96																							
87-96																							
81-93																							

\* All peptides were prepared by solid-phase synthesis. Numbers refer to the amino acid residues of hen egg-white lysozyme sequence.

$\mu$ M hypoxanthine, 0.4  $\mu$ M aminopterin, and 16  $\mu$ M thymidine) selection medium was  $\sim$ 1 in  $10^5$  T cells. The growing hybrids were screened for functional specificity as described below. Specific hybrids were then subcloned by limiting dilution.

#### Specificity Assay

*Antigen-primed LNC.*  $4 \times 10^5$  LNC were cultured in 0.2 ml medium alone (Click's medium or RPMI 1640 supplemented with 2 mM glutamine, 1 mM pyruvate, 50  $\mu$ M 2-ME, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.5% normal mouse serum) or with indicated concentrations of antigens in 96-well culture dishes for 5 d. Antigen-induced proliferation was assessed by the incorporation of 1  $\mu$ Ci [ $^3$ H]thymidine during the last 18 h of culture.

*Long-term T-cell lines.*  $10^4$  Ficoll-purified T cells were cultured with  $5 \times 10^5$  irradiated (3,000 rad) or mitomycin c-treated (50  $\mu$ g/ml for 30 min at 37°C; Sigma Chemical Co., St. Louis, MO) spleen cells or Ia-expressing L cell transfectants ( $10^5$  cells) under conditions identical to those for lymph node cell cultures. Transfectant L cells CA14.11.14 and CA36.1.3 expressing the A<sup>k</sup> and E<sup>k</sup> molecules, respectively, were kindly provided by Dr. Bernard Malissen (Institut National de la Santé et de la Recherche Médicale, Marseille, France), and have been described (11, 12). Medium used was RPMI 1640 supplemented as above, except that 10% FCS (Lot 109617; Irvine Scientific, Santa Ana, CA) was used instead of normal mouse serum. Cells were cultured for a total of 3 d and [ $^3$ H]thymidine incorporation (1  $\mu$ Ci/culture) was assessed during the last 18 h.

*T cell hybrids.* Specificity of T cell hybrids was assessed by culturing  $10^5$  T cells with  $5 \times 10^5$  mitomycin c-treated spleen cells with or without antigen. IL-2 was estimated in 24-h supernatants by using a secondary culture with the IL-2-dependent cell line HT-2, as described (2). Briefly, 0.05 ml of culture supernatants were further incubated with  $5 \times 10^3$  HT-2 cells for 24 h in a total volume of 0.1 ml complete RPMI medium. Incorporation of 1  $\mu$ Ci [ $^3$ H]thymidine was assessed during the last 4 h of culture.

## Results

*Responsiveness of Ia<sup>k</sup> Haplotype Mice to Lysozyme Peptide 74-96.* Our earlier studies on the hen egg-white lysozyme (HEL)-specific T cell response of B6 mice had identified peptide 74-96 as a region recognized by T cells (10). Furthermore, using a panel of synthetic peptides (Table I), we had narrowed two major determinants available for recognition within this 23-aa region to peptides 81-96 and 74-90 (7, 8). To determine if mouse strains expressing different Ia molecules could respond to this region, we immunized B10.A or CBA mice

TABLE II  
*Peptide 74-96 Immunized Ia<sup>k</sup> Mice Respond to Both Peptides 74-86 and 85-96*

Strain	Cells	[ <sup>3</sup> H]Thymidine incorporation per culture* (cpm × 10 <sup>-3</sup> ± SE)			
		Medium <sup>‡</sup>	74-96	74-86	85-96
CBA	LNC	8.4 ± 0.5	49.4 ± 1.4	34.2 ± 1.6	46.2 ± 2.7
B10.A	LNC	0.6 ± 0.4	21.7 ± 1.2	13.6 ± 0.3	20.3 ± 4.7
CBA	KO1T	1.3 ± 0.8	56.7 ± 4.4	52.0 ± 3.3	11.7 ± 0.4
B10.A	AO1T	1.6 ± 0.2	76.2 ± 3.0	72.8 ± 4.1	4.8 ± 1.9
B10.A	AO2T	0.6 ± 0.1	12.2 ± 0.6	7.1 ± 0.9	13.3 ± 1.5
B10.A	AO3T	3.3 ± 1.0	184.5 ± 5.8	15.0 ± 2.3	203.1 ± 8.9

\* 5 × 10<sup>5</sup> LNC from mice primed 10 d earlier with 7 nmol of peptide 74-96 in CFA, or 10<sup>4</sup> T cells of long-term bulk T cell lines (KO1T, AO1T, AO2T, AO3T) and 5 × 10<sup>5</sup> irradiated syngeneic spleen cells were cultured with 7 μM of the indicated peptides or medium alone. Cultures were incubated for a total of 5 d for LNC or for 3 d for T cell lines. Incorporation of 1 μCi [<sup>3</sup>H]-thymidine was assessed during the last 18 h of the culture period.

<sup>‡</sup> Background incorporation with medium alone; has been subtracted from values in other columns.

(expressing both A<sup>k</sup> and E<sup>k</sup> molecules) with peptide 74-96 in CFA. The antigenic specificity of the induced T cells was tested in a lymph node proliferation assay.

Table II shows the results obtained with both freshly obtained primed lymph node cells and four long-term bulk T cell lines independently derived from either CBA or B10.A mice. In all cases, mice responded vigorously to the immunogen (peptide 74-96) in vitro, showing that similar to B6 mice (H-2<sup>b</sup> haplotype), these strains of mice elicit a T cell response to this antigen. On the other hand, when the same T cell populations were tested with the two shorter peptides, 74-86 and 85-96, to which B6 mice are unresponsive (8), we saw significant proliferation, suggesting that at least two T cell determinants were present within peptide 74-96 for mice expressing A<sup>k</sup> and E<sup>k</sup> molecules. The results showed that in many cases, bulk T cell lines were strongly biased towards recognition of one or the other of the short peptides. Another noteworthy point is that the sum of the responses to peptides 74-86 and 85-96 exceeds the response to the immunogen, peptide 74-96, indicating that it is difficult to determine clonal composition of the lines from such proliferation results. Since these two 13- and 12-aa peptides share only two amino acid residues, 85 and 86, and minimal T cell determinants can lie in the range of 7-9 amino acids, it was very likely that responsiveness to peptides 74-86 and 85-96 represented recognition of two distinct determinants contained within peptide 74-96.

*Minimal Peptide Determinants Recognized by Peptide 74-96-specific T Cell Clones.* The responsiveness of heterogeneous T cell populations to peptides 74-86 and 85-96 suggested that at least some T cell clones within such populations recognized determinants contained within these two peptides. However, it was possible that there were other clones, which while recognizing the 23-aa peptide 74-96 did not recognize these short sequences, but required additional amino acid residues for generation of a complete determinant. To test this possibility, we cloned the bulk T cell lines and tested the responsiveness of individual clones to both the 23-aa peptide 74-96 and the two short peptides, 74-86 and 85-96. Representative clones shown in Table III show that each clone tested was specific for peptide 74-96, plus either one of the two short

TABLE III  
*Minimal Peptide Determinants Recognized by Peptide 74-96-reactive Clones*

Clone	[ <sup>3</sup> H]Thymidine incorporation per culture* (cpm × 10 <sup>-5</sup> )			
	Medium	74-96	74-86	85-96
AO1T.1.1	0.5 ± 0.1	<u>24.6 ± 3.8</u>	<u>40.5 ± 2.6</u>	4.6 ± 4.0
.1.3	4.1 ± 2.2	<u>29.0 ± 0.1</u>	<u>16.4 ± 0.9</u>	0.4 ± 0.8
.2.4	4.0 ± 0.1	<u>19.3 ± 2.1</u>	<u>16.9 ± 2.8</u>	2.9 ± 1.5
KO1T.1.8	4.1 ± 3.5	<u>38.7 ± 1.3</u>	<u>40.1 ± 3.8</u>	0.1 ± 0.6
.2.10	5.1 ± 1.1	<u>147.8 ± 7.4</u>	<u>126.7 ± 11.3</u>	3.9 ± 4.0
.2.8	3.6 ± 2.5	<u>138.5 ± 4.6</u>	<u>187.6 ± 3.7</u>	1.9 ± 0.8
AO2T.1.1	1.5 ± 0.5	<u>141.4 ± 14.0</u>	3.2 ± 5.2	<u>133.4 ± 4.6</u>
.1.3	1.5 ± 4.8	<u>147.0 ± 4.8</u>	2.4 ± 1.8	<u>79.3 ± 1.1</u>
.1.5	3.2 ± 0.6	<u>109.1 ± 7.5</u>	0.7 ± 1.3	<u>91.3 ± 5.2</u>
AO3T.1.1	2.6 ± 0.8	<u>66.4 ± 2.8</u>	0.9 ± 0.5	<u>63.3 ± 4.8</u>
.4.3	8.8 ± 2.5	<u>108.3 ± 9.8</u>	4.1 ± 0.3	<u>128.1 ± 5.5</u>
.4.5	4.9 ± 1.8	<u>38.9 ± 3.6</u>	2.3 ± 4.3	<u>66.7 ± 1.6</u>

\* 10<sup>4</sup> T cells, purified over Ficoll-Hypaque, were cultured with 5 × 10<sup>5</sup> irradiated or mitomycin c-treated B10.A spleen cells in the presence of 7 μM concentration of the indicated peptides or medium alone. Values significantly above those with medium alone are underlined. Other details are the same as in the footnote to Table II.

peptides. While considerable variations were noted in the relative proportion of peptide 74-86- or 85-96-reactive clones isolated from each of the bulk lines, each of the 55 clones analyzed from four independently derived bulk T cell lines was specific for either peptide 74-86 or peptide 85-96. This shows that peptides 74-86 and 85-96 represent two distinct and major, if not the only, determinants recognized by A<sup>k</sup>/E<sup>k</sup>-restricted T cells. It is worth noting here that this specificity of I-A<sup>k</sup>-restricted T cells for determinants contained within peptides 74-86 and 85-96 is very different from that observed earlier (7, 8) for I-A<sup>b</sup>-restricted B6 T cells immunized with the same 74-96 peptide. The B6 T cells were found to recognize either peptide 74-90 or peptide 81-96 and had completely failed to recognize peptide 74-86 or peptide 85-96 in the same dose range.

*Correlation between the Minimal Peptide Determinants and the Ia Molecule Restricting Recognition.* I-A<sup>k</sup> haplotype mice express both A<sup>k</sup> and E<sup>k</sup> molecules on the surface of APCs and antigen recognition by T cells can be restricted by either one of these two Ia molecules. To determine which of these two molecules restricted antigen recognition by the T cell clones, we assayed the antigen-induced proliferative response of these clones in the presence of APC from either the B10.A strain, which expresses both the A<sup>k</sup> and E<sup>k</sup> molecules, or the B10.A(4R) strain, which expresses only the A<sup>k</sup> molecule. As data in Table IV (Exp. 1) show, all the clones that recognized peptide 74-86 proliferated in the presence of antigen and APC from both strains, showing that antigen recognition by these clones was restricted by the A<sup>k</sup> molecule.

None of the clones that were reactive to peptide 85-96 in the presence of B10.A APC responded in the presence of APC from B10.A(4R) strain (data not shown). This suggested that these clones required the E<sup>k</sup> molecule, expressed by murine B10.A APC but not B10.A(4R) APC, for antigen recognition. Since there is no mouse strain available that expresses only the E<sup>k</sup> molecule, to prove that the E<sup>k</sup> molecule was the restricting element for these T cells, we used the

TABLE IV  
Ia Restriction of Peptide 74-96-specific Clones

Exp.	Clone	Peptide specificity	[ <sup>3</sup> H]Thymidine incorporation per culture* (cpm × 10 <sup>-3</sup> )			
			APC = B10.A (A <sup>k</sup> ,E <sup>k</sup> )		APC = B10.A(4R) (A <sup>k</sup> )	
			Medium	74-96	Medium	74-96
1	AO1T.1.1	74-86	0.5	<u>38.4</u>	0.5	<u>27.2</u>
	.1.3	74-86	6.5	<u>86.4</u>	4.4	<u>60.5</u>
	.2.4	74-86	0.2	<u>14.8</u>	2.3	<u>20.3</u>
	KO1T.1.8	74-86	7.1	<u>260.3</u>	2.3	<u>114.8</u>
	.2.10	74-86	1.7	<u>155.0</u>	6.3	<u>182.8</u>
	.2.8	74-86	2.3	<u>230.8</u>	4.8	<u>162.9</u>
2	AO2T.1.1	85-96	2.2	1.4	1.3	<u>21.9</u>
	.1.3	85-96	2.5	1.7	2.9	<u>33.1</u>
	AO3T.4.3	85-96	4.6	5.6	5.9	<u>177.0</u>
	.4.5	85-96	9.5	3.8	5.3	<u>33.9</u>
	KO1T.2.8	74-86	12.2	<u>28.8</u>	5.8	5.6

\* Culture conditions for Exp. 1 were identical to those described in the footnote to Table II. T cell clones were incubated with APC bearing both A<sup>k</sup> and E<sup>k</sup> (B10.A) or only A<sup>k</sup> (B10.A(4R)). In Exp. 2, 10<sup>4</sup> T cells were incubated with 10<sup>5</sup> mitomycin c-treated transfectant L cells in the presence of 7 μM peptide 74-96 or with medium alone. The transfectant L cells express either A<sup>k</sup> (CA 14.11.14) or E<sup>k</sup> (CA 36.1.3). Incorporation of 1 μCi [<sup>3</sup>H]thymidine was assessed during the last 18 h of a 3-d culture.

recently available Ia-expressing L cell transfectants that can function as APC (11, 12). In such cells, the expression of the Ia molecules is controlled by the appropriate genes chosen for transfection, making it possible to obtain cells that express only the A<sup>k</sup> or the E<sup>k</sup> molecule. The results in Exp. 2 of Table IV show that an antigen-induced proliferative response of clones recognizing peptide 85-96 could only be obtained in the presence of L cell transfectant CA 36.1.3 which expresses the E<sup>k</sup> molecule, and not in the presence of transfectant cells CA 14.11.14 which express the A<sup>k</sup> molecule. As a reciprocal control, clone KO1T.2.8, which recognizes peptide 74-96 in the context of the A<sup>k</sup> molecule, responded in the presence of CA 14.11.14 cells but not CA 36.1.3 cells. This experiment conclusively shows that responsiveness of clones specific for peptide 85-96 is restricted by the E<sup>k</sup> molecule.

This strict correlation between the minimal peptide determinants and the Ia molecule restricting recognition shows the Ia-dependent selectivity in T cell recognition of antigenic determinants on peptide 74-96. These results also illustrate the phenomenon of Ir gene effects in the sense that within the Ia<sup>k</sup>-restricted T cell repertoire, the antigen/Ia combinations such as peptide 74-86/A<sup>k</sup> and peptide 85-96/E<sup>k</sup> are permissible, whereas the reciprocal combinations of peptide 74-86/E<sup>k</sup> and peptide 85-96/A<sup>k</sup> appear to remain unrecognized.

TABLE V  
*Minimal Peptide/Ia Associations Are Maintained in Mice Immunized  
 With the Individual Peptide Determinants*

Peptide immunogen	T cells <sup>‡</sup>	[ <sup>3</sup> H]Thymidine incorporation per culture* (Δcpm × 10 <sup>-3</sup> ± SE)		
		Peptide 74-96 plus		
		Medium	10.2.16 (αA <sup>k</sup> ) <sup>§</sup>	14.4.4S (αE <sup>k</sup> )
74-86	LNC	29.6 ± 2.2	2.5 ± 1.2	27.1 ± 0.8
74-86	AO3N	73.5 ± 6.0	0.8 ± 1.5	68.9 ± 2.7
85-96	LNC	68.9 ± 3.2	65.6 ± 7.6	0.9 ± 0.7
85-96	AO2C	46.2 ± 4.2	43.6 ± 1.9	0.8 ± 0.1

\* Culture conditions were identical to those described in the footnote to Table II.

<sup>‡</sup> LNC were obtained from B10.A mice immunized 10 d earlier with 7 nmol of peptide in CFA. AO3N and AO2C are long-term bulk lines derived from similarly immunized mice.

<sup>§</sup> mAbs 10.2.16 (culture supernatant) and 14.4.4S (ascites fluid) used at a final dilution of 1:80 and 1:2,000, respectively, were present for the duration of culture.

*How Strict Is the Correlation between the Minimal Peptide Determinants and the Ia Molecules Restricting Recognition?* While the experiments described showed that there was a clear preference for recognition of peptide determinants 74–86 or 85–96 in the context of A<sup>k</sup> or E<sup>k</sup> molecules, respectively, it could not be concluded that this correlation was absolute. For example, it could be argued that the observed clonal specificities represented the dominant antigen/Ia interactions because the experiments tested the responsiveness of mice expressing both the A<sup>k</sup> and E<sup>k</sup> molecules with an immunogen (peptide 74–96) that consisted of two distinct determinants (amino acid 74–86 and 85–96). Thus, competition between the peptide determinants for available restriction sites on the Ia molecules could have favored only strong associations; weaker interactions (e.g., peptide 74–86/E<sup>k</sup> or 85–96/A<sup>k</sup>) would lie below the detection limits of the assays.

To test this possibility, we carried out experiments where such putative competition effects could be minimized or eliminated. First, we immunized B10.A mice (A<sup>k</sup>, E<sup>k</sup>) with the individual peptide determinants amino acid 74–86 or 85–96 alone to allow detection of T cells specific for the apparently nonpermissive combinations: 74–86/E<sup>k</sup> or 85–96/A<sup>k</sup>. We tested both freshly obtained LNC from immunized mice (before they were subjected to in vitro selection) and long-term bulk T cell lines (to increase the sensitivity of detection). The association between the antigen and the Ia molecule restricting recognition was tested by using mAbs directed toward either one of the available Ia molecules. Data in Table V show that proliferative responses of T cell populations induced with peptide 74–86 could be inhibited only with anti-A<sup>k</sup> mAb 10.2.16, and there was no effect of anti-E<sup>k</sup> mAb 14.4.4S. The reciprocal inhibition pattern was obtained when cells were obtained from mice immunized with peptide 85–96 alone. Thus, under conditions where the other Ia molecule (A<sup>k</sup> or E<sup>k</sup>) was free to interact with the immunogenic peptides (85–96 or 74–86), we could not detect the recognition of the antigen/Ia combinations 74–86/E<sup>k</sup> or 85–96/A<sup>k</sup>.

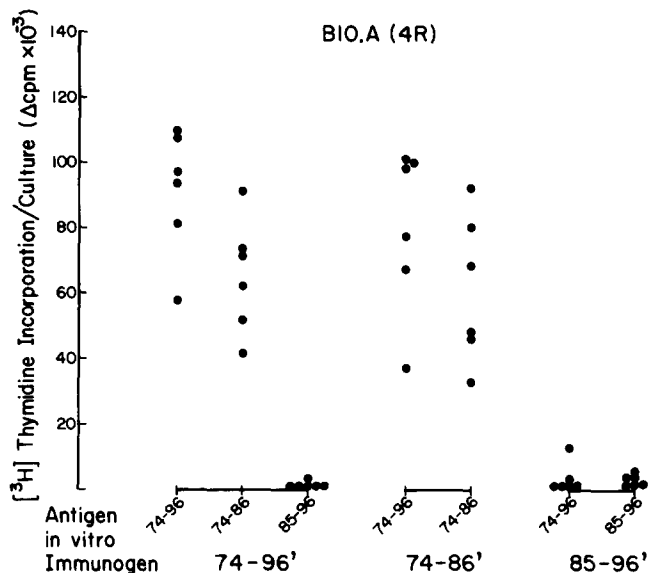


FIGURE 1. Proliferative response of LNC from B10.A(4R) ( $A^k$ ) mice immunized 10 d earlier with 7 nmol of the indicated peptides in CFA. Culture conditions were the same as those described in the footnote to Table II. Each point represents the mean incorporation of triplicate cultures set up with cells obtained from individual mice.

This experiment, however, left open the possibility that the mere existence of a strong peptide/Ia interaction, such as peptide 85–96 with the  $E^k$  molecule on the APC could preempt responsiveness to the same peptide in the context of the other  $A^k$  molecule. To test this possibility, we immunized B10.A(4R) mice which express the  $A^k$  but not the  $E^k$  molecule with either peptide 74–96, 74–86, or 85–96. Fig. 1 shows the results obtained with immunized mice, individually tested for responsiveness to these peptides in vitro. LNC from each of the mice immunized with either peptide 74–96 or 74–86 responded well to peptide 74–86. However, no significant response was observed to peptide 85–96 in all mice immunized with either 74–96 or 85–96 alone.

These experiments show the strict associations between peptides 74–86 or 85–96 with the  $A^k$  or  $E^k$  molecules, respectively. Table VI summarizes all of the results emphasizing this point. Among a total of six bulk T cell lines reactive to peptide 74–96, and 69 clones derived from these lines, we did not observe any exception to the rule. Note that the analysis includes T cell clones derived from bulk lines A03N and AO1C that were obtained from B10.A mice immunized with the individual peptide determinants 74–86 and 85–96, respectively.

*Differences between the Antigenic Determinant Recognized on Peptide 74–96-specific T cells Restricted by  $A^k$ ,  $E^k$ , or  $A^b$  Molecules.* This analysis of peptide 74–96-specific T cells restricted by either the  $A^k$  or  $E^k$  molecules showed that, dependent on the Ia molecule, the minimal peptide determinants recognized by these T cells were contained within peptides 74–86 or 85–96. Interestingly, sharp differences in determinant regions recognized were apparent when compared with  $A^b$ -restricted T cells obtained from B6 mice immunized with the same peptide 74–96 (7, 8). Figs. 2 and 3 illustrate this point by comparing the minimal



TABLE VI  
 Reactivity to Peptides 74-86 or 85-96 Is Uniquely Associated with A<sup>k</sup>  
 and E<sup>k</sup> Molecules, Respectively

Ia Molecule	Peptide	Bulk lines*	Clones
A <sup>k</sup>	74-86	AO1T (20), AO2T (3) KO1T (7), AO3T AO3N (8)	38
	85-96	None	0
E <sup>k</sup>	74-86	None	0
	85-96	AO1T (1), AO2T (11) AO3T (13), AO1C (6)	31

\* Association of minimal peptide specificity and Ia molecule in the bulk lines was tested by reactivity to peptides 74-86 or 85-96 in the presence of B10.A(4R) (A<sup>k</sup>), CA14.11.14 (A<sup>k</sup>), or CA36.1.3 (E<sup>k</sup>) APC, and also by the ability of anti-A<sup>k</sup> or E<sup>k</sup> mAbs to inhibit these proliferative responses. Numbers in parentheses show the number of clones tested from each bulk line.

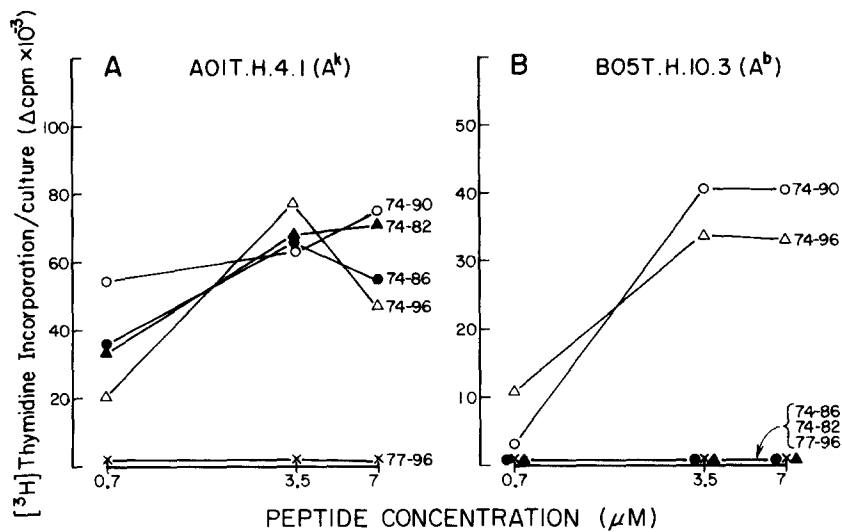


FIGURE 2. Minimal determinants recognized by peptide 74-96-induced, A<sup>k</sup>- and A<sup>b</sup>-restricted T hybrid clones.  $10^5$  T hybrid cells (A01T.H.4.1 or B05T.H.10.3) were cultured with either  $10^5$  CA-14.11.14 (A<sup>k</sup>) L cells or  $5 \times 10^5$  mitomycin c-treated B6 spleen cells, respectively, in the presence of indicated concentrations of peptides shown. IL-2 content in supernatants was assessed in secondary culture with IL-2-dependent HT-2 cells as described in Materials and Methods. Background incorporation in medium alone was  $0.8 \times 10^3$  (A01T.H.4.1) and  $1.6 \times 10^3$  cpm (B05T.H.10.3).

determinant specificities of four representative T cell clones obtained from either B10.A or B6 mice. As described earlier (8) and in this paper, T cells from these strains recognize two distinct determinants on peptide 74-96, which can be broadly categorized as being contained within the amino- or carboxy-terminal regions of this 23-aa peptide. Fig. 2 compares the minimal determinants recognized by T cell hybrids A01T.H.4.1 and B05T.H.10.3, which are restricted by

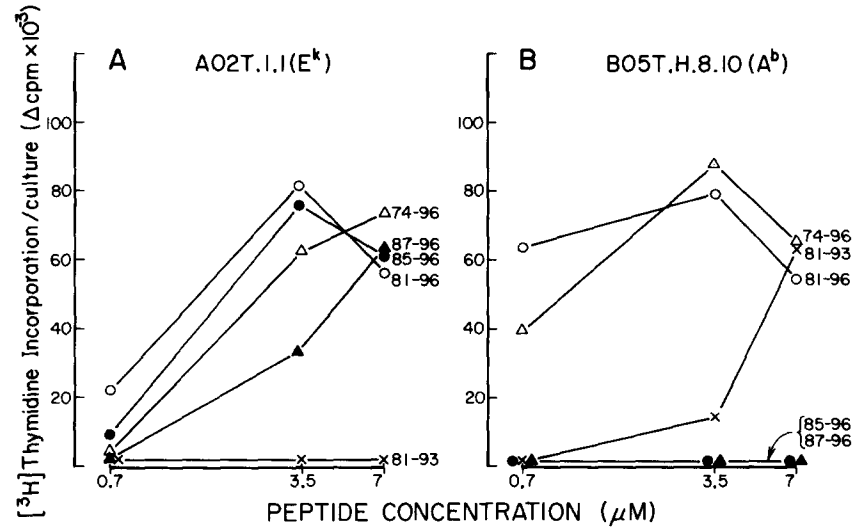


FIGURE 3. Minimal determinants recognized by peptide 74-96-induced  $E^k$ - and  $A^b$ -restricted T cells.  $10^4$  (A02T.1.1) or  $10^5$  (B05T.H.8.10) T cells were cultured with  $5 \times 10^5$  mitomycin *c*-treated B10.A or B6 spleen cells, respectively, in the presence of indicated concentrations of peptides shown.  $[^3\text{H}]$ Thymidine incorporation was assessed directly for long-term clone A02T.1.1 and in an IL-2 assay for hybrid clone B05T.H.8.10. Background cpm in medium-alone cultures was  $0.3 \times 10^3$  (A02T.1.1) and  $0.9 \times 10^3$  (B05T.H.8.10).

$A^k$  or  $A^b$  molecules, respectively. Each of these hybrids, obtained after fusion of the BW 5147 thymoma with T cell lines from mice immunized with peptide 74-96, recognizes the *N*-terminal region of this peptide, as evidenced by comparable reactivity to both peptides 74-96 and 74-90. Further elimination of four or eight *C*-terminal residues as in peptides 74-86 or 74-82 did not affect the reactivity of A01T.H.4.1 cells, but completely abrogated reactivity of B05T.H.10.3 cells. It is significant that B6 ( $A^b$ ) mice do not respond even when immunized with peptide 74-86 (8). This dramatic difference in the requirement of additional residues carboxy-terminal to residue 82 for  $A^b$ - vs.  $A^k$ -restricted T cell recognition of *N*-terminal region of peptide 74-96, illustrates the influence of polymorphic differences between the two Ia molecules on antigenic specificity. This is further emphasized by comparison of  $E^k$ - versus  $A^b$ -restricted T cell clones A02T.1.1 and B05T.H.8.10 recognizing the *C*-terminal region of the same peptide (Fig. 3). Reactivity of clone A02T.1.1 to peptides with varying *N*-termini reveals comparable reactivities to peptides 74-96, 81-96, and 85-96 (Fig. 3A). Peptide 87-96 showed lower but significant reactivity. In contrast,  $A^b$ -restricted hybrid clone B05T.H.8.10, induced by the same peptide 74-96 in B6 mice, while reactive to peptides 74-96 or 81-96, completely failed to recognize peptides 85-96 or 87-96 (B). A further discrimination in determinant size was noted with peptide 81-93. This peptide, while showing significant reactivity with  $A^b$ -restricted clone B05T.H.8.10, completely failed to react with  $E^k$ -restricted clone A02T.1.1, showing the relative importance of the three *C*-terminal residues 94-96 for  $E^k$ - but not  $A^b$ -restricted recognition of determinants contained within peptide 81-96. Thus, similar to  $A^b$ - vs.  $A^k$ -restricted recognition of the *N*-terminal region of peptide 74-96, dramatic differences were noted

among A<sup>b</sup>- vs. E<sup>k</sup>-restricted T cell recognition of the C-terminal region of the same antigen.

### Discussion

The ability to recognize discrete peptide/Ia combinations can be considered the current definition of Ir gene phenomena. Certain combinations appear to be permissive for certain haplotypes, whereas other combinations are not permissive, even when the animal is challenged directly with the peptide in question. Using a large panel of synthetic peptides to assay T cell specificity to lysozyme peptide 74–96, we determined that a strict correlation existed between the two minimal N- or C-terminal peptide determinants (amino acid 74–86 or 85–96) and the A<sup>k</sup> or E<sup>k</sup> Ia molecules restricting recognition. These results show the selectivity associated with Ia molecules in T cell recognition of determinants available on the same peptide 74–96. Furthermore, clear differences were found in the boundaries of each of the two N- or C-terminal determinant regions recognized by A<sup>b</sup>- vs. A<sup>k</sup>- or E<sup>k</sup>-restricted T cell clones. Thus, these results further emphasize the specificity of antigen/Ia interactions, as detected by the T cell receptor, not only in the usual sense of MHC restriction, but also as changes that occur in the antigenic determinants available on the same peptide.

One of the major difficulties in earlier analysis of Ir gene phenomena for multideterminant antigens such as random copolymers of a few amino acids, minor histocompatibility molecules, etc., has been the poor definition of antigenic determinants recognized by T cells specific for these antigens. Moreover, the influence of the Ir genes was characterized as either the presence or absence of a response to any determinant on the antigen. Although such results focused interest on the critical role of Ia molecules, it was clear that information on the precise molecular structure of the antigenic determinants would be required to distinguish between restrictions in T cell repertoire and determinant presentation. The use of protein antigens (insulins, myoglobins, lysozymes, cytochromes) of defined sequence and structure to study T cell antigen recognition overcame this limitation (13). It became possible to precisely identify the determinant regions involved and to systematically vary these regions by using available methods of peptide synthesis to study the consequence of these changes on recognition.

Coupled with the techniques of generating homogeneous populations of T cell clones, the above technique enabled Schwartz and his colleagues (14–16) to make a significant advance in understanding. They analyzed T cell recognition of analogs of the C-terminal peptide of pigeon cytochrome *c* in the context of closely related Ia molecules. It was found that stimulation of T cell clones in the presence of either of two Ia molecules varied both as a function of the Ia molecule expressed by the APCs and the structure of the antigenic analog. Thus, the Ia molecule could directly affect T cell recognition of the antigen, and was consistent with the response status of the strains involved. Furthermore, by using a panel of synthetic peptide analogs, Hansburg et al. (16) functionally delineated two classes of sites on the antigen, one affecting the ability to interact with the Ia molecule (agretope), and the other affecting binding to the T cell receptor (epitope). These studies gave rise to the concept that a T cell antigenic determi-

nant must possess both of these sites (4, 17). Moreover, there appeared to be an element of specificity in the interaction between the determinant and the Ia molecule, as had been predicted earlier (18, 19).

This notion of specificity in antigen/Ia interactions, together with the correlation found between the response status of the strains involved and the residues on the antigenic analogs affecting antigen/Ia interaction, was consistent with the determinant selection hypothesis of Ir gene defects. However, since the response to cytochrome peptides requires, and is restricted by the E molecule, and comparisons were made among strains that express E molecules closely related in structure, it was not clear what influence Ia molecules of widely differing structures might have on recognition of the same antigenic region. A recent study of H-2<sup>d</sup>-restricted T cell response to sperm whale myoglobin has indicated that the recognition of two distinct determinant regions is associated with either the A<sup>d</sup> or E<sup>d</sup> molecules, respectively (20). However, only one of the two determinants was localized to peptide aa 132–153, and the antigen/Ia associations were not rigorously tested by direct immunization with the individual peptide determinants.

Results presented here emphasize the generality of the notion of specificity in antigen/Ia interactions. The correlation observed between A<sup>k</sup>- vs. E<sup>k</sup>-restricted T cell clones and the minimal peptide sequences aa 74–86 vs. aa 85–96, showed that distinct determinants were recognized by these two sets of clones. This result suggests that only a circumscribed area on the same 23-aa peptide 74–96 can be used for antigen presentation with a single Ia molecule. The failure to detect T cells reactive to the reciprocal combinations of peptide 74–86/E<sup>k</sup> or peptide 85–96/A<sup>k</sup> indicates that these are not permissible and thus, are examples of Ir gene regulation.

This selectivity in the association of minimal determinants with the Ia molecules, however, does not by itself prove whether the influence of the A<sup>k</sup> or E<sup>k</sup> molecules was direct and exerted on the choice of antigenic determinants, or indirect, leading to skewing of the T cell repertoire available in these strains. We have elsewhere argued (7) against clonal deletional models of repertoire selection, based on the observation that considerable T cell diversity exists in the recognition of epitopes within a single peptide/Ia determinant such as amino acid 74–86/A<sup>k</sup> or 81–96/A<sup>b</sup>. This clonal diversity renders it very unlikely that deletion of appropriate clones (e.g., aa 74–86/E<sup>k</sup>) had occurred due to fortuitous cross-reaction with self antigens and/or MHC determinants during ontogeny. More recently, cloning and sequencing of distinct V<sub>β</sub> genes used by the T cell receptors of clones specific for different epitopes within aa 74–86/A<sup>k</sup> or aa 85–96/E<sup>k</sup> determinants has confirmed that these T cell clones are of independent origin (J. Kobori, N. Shastri, and L. Hood, manuscript in preparation). This rules out the possibility that the diverse specificity phenotypes could have arisen through somatic hypermutation events in a single or limited set of clones expressing a given combination of receptor genes. Therefore, it is possible that similar to the recently described difference among A<sup>k</sup> and A<sup>d</sup> molecules to bind lysozyme peptide 46–61 (21), peptides 74–86 and 85–96 may show differential ability to interact with A<sup>k</sup> and E<sup>k</sup> molecules, respectively. It should be noted though, that the difference in binding should be apparent only when the short peptides are

compared, and not peptide 74–96, since the latter is recognized in the context of both A<sup>k</sup> and E<sup>k</sup> molecules, but at different sites.

However, while the postulated selectivity in association of agretope sites on the antigen and the Ia molecule, considered together with the direct binding experiments of Babbitt et al. (21), appears an adequate explanation for the lack of clones specific for certain (e.g., 74–86/E<sup>k</sup> or 85–96/A<sup>k</sup>) determinants, this notion, in its simplest form, does not seem sufficient to explain why T cell clones restricted by a given Ia molecule showed clear distinctions in the boundaries of minimal determinants on peptide 74–96. For example, A<sup>b</sup>-restricted T cells recognizing aa 74–90 failed to recognize aa 74–86 (reference 8 and Fig. 2). On the other hand, all T cell clones restricted by the A<sup>k</sup> molecule were reactive to aa 74–86, and several of those tested (seven out of eight) could even react to aa 74–82. Similarly, A<sup>b</sup>-restricted T cells recognizing aa 81–96 failed to recognize aa 85–96 (reference 7 and Fig. 3), while all E<sup>k</sup>-restricted T cells were fully reactive to aa 85–96, and three out of six showed significant reactivity to aa 87–96. The C-terminal boundary of the determinant could be further distinguished by the reactivity to peptide 81–93, which was not evident in E<sup>k</sup>-restricted clones.

From previous results showing hierarchical use of sites on a protein antigen (22), we considered the possibility that direct challenge with certain nonimmunogenic peptides might reveal additional reactivities. However, we could not induce significant A<sup>b</sup>-restricted T cell responses in B6 mice by direct immunization with peptides 74–86 or 85–96 (8). Thus, A<sup>k</sup>, E<sup>k</sup>, or A<sup>b</sup> molecules can each interact with peptide 74–96, but clearly with a unique profile.

Specificities such as aa 74–86/A<sup>k</sup> or 85–96/E<sup>k</sup> proved to be dominant over alternatives such as aa 74–90<sup>+</sup>, 74–86<sup>-</sup>/A<sup>k</sup> or aa 81–96<sup>+</sup>, 85–96<sup>-</sup>/E<sup>k</sup>. This dominance was not simply a consequence of structural characteristics of peptide 74–96 such as  $\alpha$ -helicity or amphipathicity (23, 24) alone, because the latter, but not the former set of antigenic specificities are readily found among A<sup>b</sup>-restricted T cells. Rather, the explanation must invoke unique contributions of the distinct Ia molecules in determining the differences in the peptide structures available for T cell recognition. It still remains to be explored whether these distinctions in the minimal peptide/Ia determinants possible in a particular haplotype can be explained by conformational changes in the antigen and/or the Ia molecule induced by their association. Alternatively, these differences may relate to the still ill-characterized interactions postulated to occur between the T cell receptor with the Ia molecule (the "histope" as defined by Schwartz and coworkers [4, 17]). Since interaction with the Ia molecule alone can readily occur during the process of ontological selection of the self MHC biased T cell repertoire, it is possible that these may occur at a particular site on the Ia molecule. A strict definition of this site may then place constraints on how much of the antigen surface can be accommodated within the combining pocket of the T cell receptor.

### Summary

A<sup>k</sup>- or E<sup>k</sup>-restricted T cells, generated by immunization with a 23-amino-acid peptide of hen egg-white lysozyme (amino acid 74–96), showed a strict correlation between the minimal peptide determinant recognized and the Ia molecule restricting recognition. All A<sup>k</sup>-restricted clones obtained from six independently

derived lines recognized determinants contained within peptides 74–86, while E<sup>k</sup>-restricted clones recognized determinants within 85–96. This correlation was true whether B10.A mice (A<sup>k</sup>,E<sup>k</sup>) were immunized with peptide 74–96 or with each of the two smaller peptides (74–86 or 85–96). Furthermore a T cell response could be obtained to peptide 74–86, but not to peptide 85–96 in B10.A(4R) mice, which express only the A<sup>k</sup> molecule. Thus, an Ia molecule-associated selectivity exists in the choice of T cell determinants even within this small 23-amino-acid peptide antigen. Significant differences were noted, however, in the boundaries of the minimal peptide determinants recognized within peptide 74–96 by A<sup>k</sup>- or E<sup>k</sup>-restricted T cells, in comparison to those recognized by A<sup>b</sup>-restricted T cells. These results indicate that interaction of the same peptide with distinct Ia molecules results in recognition of unique aspects of the antigenic determinants by the T cell receptor.

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