The Relation Between Major Histocompatibility Complex (MHC) Restriction and the Capacity of Ia to Bind Immunogenic Peptides

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The capacity of purified I-A^d, I-E^d, I-A^k, and I-E^k to bind to protein derived peptides that have been previously reported to be T cell immunogens has been examined. For each of the 12 peptides studied strong binding to the relevant Ia restriction element was observed. All the peptides bound more than one Ia molecule; however, for 11 of 12 peptides, the dominant binding was to the restriction element, whereas in one instance the dominant binding was to a nonrestriction element. When the peptides were used to inhibit the presentation of antigen by prefixed accessory cells to T cells, an excellent correlation was found between the capacity of a peptide to inhibit the binding of an antigen to purified Ia and the capacity of the peptide to inhibit accessory cell presentation of the antigen. Thus, the binding of peptide to purified Ia is immunologically relevant, and Ia seems to be the only saturable molecule on the surface of the accessory cell involved in antigen presentation. Inhibition analysis also indicated that all peptides restricted to a particular Ia molecule competitively inhibited one another, suggesting that each Ia restriction element has a single binding site for antigen. Cross-linking of labeled peptides to Ia followed by electrophoretic analysis and autoradiography suggested that this single binding site is made up of portions of both α and β chains of Ia.

IN GENERAL, PROTEIN ANTIGENS ARE PHYSICALLY ALTERED (processed) by accessory cells as a prerequisite to T cell recognition (*1, 2*). Studies with proteolytic digests of native protein antigens have revealed several peptides that without further alteration can be presented to appropriately primed antigen-specific, major histocompatibility (MHC)–restricted T cells by previously fixed accessory cells (*3*) or by Ia containing planar membranes (*4*). These peptides have subsequently been synthesized and used to study the interaction between Ia and "processed" antigen. T cells of the helper subset only recognize protein antigens in the context of Ia molecules on accessory cells. The exact nature of the control of T helper cell responses, thus exerted by the immune response genes (Ir) through their gene product, the Ia molecules, is still a matter of controversy. One version of the so-called determinant selection hypothesis proposes that the function of Ia is to specifically bind some, but not all, peptide antigens created during antigen processing, thereby selecting the determinants that are to be presented by the accessory cells to T cells (*5, 6*). The concept that Ia and antigen interact specifically prior to being recognized by the T cells, is supported by the observation that antigens can compete with one another at the level of antigen presentation by accessory cells (*7*). Equilibrium dialysis has been used to study directly the interaction between Ia and immunogenic peptides. Babbitt *et al.* demonstrated that the hen egg lysozyme (HEL) peptide 46 to 61 [HEL(46–61)] bound to I-A^k, but not to I-A^d (*8*). This binding correlated with the finding that T cells specific for HEL(46–61) from high-responder H-2^k mice are restricted by I-A^k, whereas H-2^d mice are low responders to HEL. Subsequently, our laboratory demonstrated that the chicken ovalbumin (Ova) peptide 323 to 339, which is immunogenic to H-2^d mice and restricted to I-A^d, and nonimmunogenic in H-2^k mice, bound to I-A^d, but not to I-E^d, I-A^k, or I-E^k (*9, 10*). Thus, for HEL(46–61) and Ova(323–339), a correlation between antigen-Ia interaction and MHC restriction was demonstrated, suggesting that determinant selection is an important aspect of Ir gene control of T cell responses. In our present study, we have analyzed the capacity of I-A^d, I-E^d, I-A^k, and I-E^k to bind an additional ten peptides so that we could evaluate the generality of the correlation between binding to and restriction by Ia molecules and then could also ascertain whether such binding is responsible for the capacity of certain peptides to inhibit antigen presentation to T cells.

Binding of labeled peptides to Ia. The 12 peptides that we selected for examination are shown in Table 1, together with their primary sequence and their reported MHC-restriction (*8, 11–24*). In some, but not all, cases the responder status of both H-2^d and H-2^k mice was studied.

Eleven of the antigenic peptides listed in Table 1 were labeled with ^{125}I, and their capacity to bind to I-A^d, I-E^d, I-A^k, and I-E^k was examined (Table 2). Of the 11 different labeled peptides, 8 bound significantly better to the Ia molecules that serve as their restriction elements than to the other Ia molecules ($P < 0.02$). Thus,

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Ova(323–339) bound to I-A^d, influenza hemagglutinin peptide Ha(130–142) to I-A^d, HEL(46–61) to I-A^k, HEL(81–96) to I-E^k, Ha(111–122) to I-E^d, sperm whale myoglobin Myo(132–153) to I-E^d, pigeon cytochrome c peptide p Cytc(88–104) to I-E^k, and moth cytochrome c peptide m Cytc(88–103) to I-E^k. The extent of binding varied from 35 percent with HEL(46–61) to 1.3 percent for HEL(81–96). A ninth peptide, herpes simplex virus glycoprotein D HSV(8–23) bound preferentially, but not statistically significantly, to one of its restriction elements, I-E^d, but not to its other known restriction element, I-E^k. With the remaining two peptides there was no correlation between MHC restriction and binding. HEL(74–86) bound weakly to all four Ia molecules with no clearcut preference being observed, and lambda repressor protein λ-repr(12–26) bound preferentially to I-E^d, whereas it is restricted by I-A^d and I-E^k. These two restriction molecules did bind the λ-repr(12–26) peptide, although significantly less, than did I-E^d ($P < 0.01$).

Competitive inhibition of interactions between the ^{125}I-labeled peptide and Ia. The degree of binding of the labeled peptides to Ia in several instances was very low (<5 percent). There are at least two possible problems inherent in the direct binding assay that would decrease or abrogate the ability to detect the binding of a peptide to Ia. (i) Some peptides contain internal tyrosine and histidine residues that, upon iodination, might change the capacity of a peptide to bind to Ia. Similarly, some of the peptides, including HEL(74–86), HEL(81–96), and HSV(8–23), contain methionine and cysteine residues that might become oxidized in the course of iodination, thereby altering the capacity of the peptides to bind to Ia. (ii) The direct binding assay will only detect Ia-peptide interactions with a dissociation constant, K_D, of $\leqq 10^{-5} M$, whereas interactions with lower affinities will go undetected. Thus, in order to extend the findings obtained with the direct binding of labeled peptides to Ia, we decided to test the capacity of the 12 different peptides to inhibit the binding of selected, labeled peptides to Ia. Using the combinations of labeled peptides and Ia that in Table 2 showed sufficiently high binding to allow us to perform inhibition studies, we were able to examine the capacity of the four Ia molecules to bind the 12 different peptides. The use of unlabeled peptides as inhibitors eliminated the possibility of important structural alterations induced by the chloramine T iodination procedure and should, at high concentrations of inhibitor, allow detection of significantly lower affinity interactions ($K_D \approx 10^{-3} M$) than the direct binding assay. Representative data obtained over a dose range of inhibitor concentrations are shown in Fig. 1. The capacity of three different unlabeled peptides to inhibit the binding of ^{125}I-labeled HEL(46–61) to I-A^k is shown. The Ha(130–142) peptide was a strong inhibitor, giving 50 percent inhibition at 10 μ*M*, the HEL(74–86) was a weaker inhibitor giving 50 percent inhibition at 270 μ*M*, whereas the Myo(132–153) peptide was not inhibitory.

The data on the capacity of all 12 peptides to inhibit four different Ia-peptide interactions are summarized in Table 3. Some of the interactions that gave a low percentage of binding when the labeled peptide was studied in the direct binding assay became very clearcut in the inhibition assay. Thus, the Ha(111–122) peptide, which in the direct binding assay only bound slightly, although significantly, to I-E^d, in the inhibition assay bound quite well to its restricting element, I-E^d. Similarly, HSV(8–23) peptide, which in the direct binding assay showed a weak interaction with its restriction element

Table 1. The MHC restriction and amino acid sequence of the peptides studied. The peptides were synthesized, with or without an added tyrosine for ^{125}I labeling (Applied Biosystems 430A peptide synthesizer). After removal of the α-amino-*tert*-butyloxycarbonyl (t-Boc) protecting group, the phenylacetamidomethyl resin-peptide was coupled with a fourfold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, asparagine, and glutamine), for 1 hour in dimethylformamide (DMF). After the synthesis was completed, the peptide was cleaved from the resin, and the protecting groups were removed by treatment with hydrogen fluoride (HF), dimethylsulfide, anisole, *p*-toluenethiol (9:0.4:0.4:0.2) under nitrogen for 1 hour at 0°C. The HF was evaporated under a stream of nitrogen, and the resin was washed with anhydrous ether. The peptide was extracted into 50 percent (v/v) acetic acid, lyophilized to dryness, desalted, and purified by reversed-phase high-performance liquid chromatography (HPLC). The purity of the peptides was substantiated by amino acid sequence and composition analysis. They were routinely >95 percent pure after HPLC. ^{125}I labeling of the tyrosinated peptides was done by the chloramine T method (*36*).

Peptide	Restriction to			Amino acid sequence
	Ia^d	Ia^k	Other	
Ova(323–339)	A^d	LR*		ISQAVHAAHAEINEAGR†
Ha(130–142)	A^d			HNTNGVTAACSHE
Myo(106–118)	A^d			FISEAIIHVLHSR
λepr(12–26)	A^d	E^k		LEDARRLKAIYEKKK
HEL(46–61)	LR	A^k		NTDGSTDYGILQINSR
HEL(74–86)	LR	A^k		NLCNIPCSALLSS
HEL(81–96)	LR	E^k	A^b	SALLSSDITASVNCAK
Ha(111–122)	E^d			FERFEIFPKESS
Myo(132–153)	E^d	LR		NKALELFRKDIAAKYK-ELGYQG
HSV(8–23)	E^d	E^k	$E^k_\alpha E^b_\beta$	SLKMADPNRFRGKDLP
p Cytc(88–104)	LR	E^k		KAERADLIAYLKQATAK
m Cytc(88–103)		E^k	$E^k_\alpha E^b_\beta$	ANERADLIAYLKQATK

*LR, low responder. †One-letter code for amino acid sequence is Ala (A); Arg (R); Asn (N); Asp (D); Cys (C); Gln (Q); Glu (E); Gly (G); His (H); Ile (I); Leu (L); Lys (K); Met (M); Phe (F); Pro (P); Ser (S); Thr (T); Trp (W); Tyr (Y); Val (V).

Table 2. The binding of radiolabeled peptides to Ia^d and Ia^k. Ia molecules were purified as previously described (*10*); 40 μ*M* Ia or an equal weight amount of gelatin was taken up in a mixture of 1 percent NP-40 and phosphate-buffered saline (PBS) containing phenylmethylsulfonyl fluoride (PMSF) (final concentration 1 m*M*), 1.10 phenanthroline (0.26 mg/ml), pepstatin A (50 μg/ml), iodoacetamide (IAA) (50 m*M*), EDTA (3 mg/ml), and NaN_3 (0.1 percent). ^{125}I-labeled peptide (0.2 to 0.6 μ*M*) was added and the mixture was incubated for 48 hours at room temperature to allow for equilibrium to be established (*10*). The Ia-peptide complexes were separated from free peptide by gel filtration on a Sephadex G-50 (Pharmacia) column (23 by 1.3 cm), the exception being the largest peptide in our selection, the myoglobin peptide Myo(132–153), which required a Sephadex G-100 column in order to obtain resolution of complexes from free peptide. The columns were eluted in 0.5 percent NP-40/PBS, collected in 1-ml fractions, and the radioactivity was measured. The fraction of peptide bound to Ia (α) relative to the total amount of offered peptide was calculated as α equal to the ratio of the peptide in the void volume to the total peptide recovered. The α was always corrected by subtracting the α value obtained with the gelatin control from the α value obtained with Ia. Throughout the article this is the α value reported for the gel filtration. Known MHC restrictions are underlined.

Labeled peptide	Percentage of total offered peptide bound to 40 μ*M* of			
	A^d	E^d	A^k	E^k
Ova(323–339)	11.8*	0.1	0.2	0.1
Ha(130–142)	18.9*	0.6	7.1	0.3
λ-repr(12–26)	1.6	8.9*	0.3	2.3
HEL(46–61)	−0.1	−0.5	35.2*	0.5
HEL(74–86)	2.0	2.3	2.9	1.7
HEL(81–96)	0.4	0.2	0.7	1.1*
Ha(111–122)	0.2	2.8*	0.2	0.3
Myo(132–153)	0.8	6.3*	0.5	0.7
HSV(8–23)	0.1	2.1	0.1	0.5
p Cytc(88–104)	0.6	1.2	1.7	8.7*
m Cytc(88–103)	0.1	1.0	1.7	5.3*

*The asterisk indicates the binding of a peptide to a particular Ia molecule analyzed by a one-tailed *t* test with correction for unequal variances (*37*), and Bonferroni corrections for multiple comparisons, was significantly greater ($P < 0.05$) than to the other three Ia molecules tested.

$I\text{-}E^d$, bound very well to $I\text{-}E^d$ in the inhibition assay. The binding of HSV(8–23) to its other restriction element, $I\text{-}E^k$, was still questionable. In the inhibition assay the λ-repr(12–26) bound to both of its restricting elements, $I\text{-}A^d$ and $I\text{-}E^k$, along with binding to $I\text{-}E^d$ which was also evident in the direct binding assay. The HEL(74–86) peptide, which is $I\text{-}A^k$ restricted and which showed no preferential binding in the direct binding assay, bound quite well to both $I\text{-}A^d$ and $I\text{-}A^k$ in the inhibition assay. The HEL(81–96) peptide, which is $I\text{-}E^k$ restricted, bound very strongly to $I\text{-}E^k$ in the inhibition assay.

Combining the direct binding data with the inhibition of binding data, we can conclude that, for every one of the 12 peptides that we have studied, a specific interaction with the restriction Ia molecule is demonstrable. These bindings were saturable and specific in that addition of unlabeled peptide led to a dose-dependent inhibition of the binding of labeled peptide to Ia; in no case did one peptide bind to all four Ia molecules examined; and every Ia molecule was capable of binding some, but not all, peptides.

In addition to binding to their restriction elements, each of the peptides examined bound to some degree to one or two other Ia molecules that were not evident in the direct binding assay and that have not been previously reported to serve as restriction elements for the presentation of that particular peptide. In most cases (11 of the 12 peptides) these alternative bindings were weaker than bindings to the relevant restriction element, whereas in one instance—that of λ-repr(12–26)—it was stronger. These other bindings tended to display a pattern in which a peptide that bound to I-A (or I-E) of one haplotype was prone to bind to the same Ia molecule of the other haplotype. Thus, Ova(323–339), Ha(130–142), Myo(106–118), HEL(46–61), and HEL(74–86) showed "A-ness" in their binding pattern, whereas Ha(111–122), Myo(132–153), HSV(8–23), and m Cytc(88–103) showed "E-ness" in their binding patterns, suggesting that the peptide combining site on, for example, $I\text{-}A^d$ is more closely related to the combining site on $I\text{-}A^k$ than to the combining site on $I\text{-}E^d$ or $I\text{-}E^k$. The biologic significance of these interactions with other Ia molecules is unclear. Some of these peptides have not been tested for immunogenicity in both $H\text{-}2^d$ and $H\text{-}2^k$ mouse strains; Myo(106–118), Ha(111–122), and Ha(130–142) have not been tested in the $H\text{-}2^k$ haplotype. Also, we do not know how strong a binding is required in order to be relevant for antigen presentation.

The majority of the peptides (9 of 12) showed a very strong binding to their restriction element—50 percent inhibition at 5 to 100 μ*M* (Table 3)—suggesting that, for a peptide to be a good immunogen, an Ia peptide interaction with K_D less than 100 μ*M* is generally required. Alternatively, the binding to Ia molecules not used as MHC restriction elements could be explained by postulating that T cell unresponsiveness (T cell clonal deletion or T cell suppression), overrides the potential immunogenicity caused by an effective Ia-antigen interaction. In comparing the binding data shown in Tables 2 and 3 with the known MHC restriction data (Table 1) there is a single instance of strong binding to an Ia molecule that is not consistent with the known pattern of MHC restriction: λ-repr(12–26) bound very strongly to $I\text{-}E^d$ but is restricted by $I\text{-}A^d$. Thus, the data with λ-repr (12–26) (and perhaps some of the other weaker interations with nonrestriction elements) indicate that binding to Ia is not in itself a sufficient event to ensure immunogenicity of a peptide. The possible implications of these bindings for the immune response to λ-repr(12–26) has been discussed (*25*).

All the peptides that were restricted to $I\text{-}A^d$ inhibited the binding of Ova(323–339) to $I\text{-}A^d$ (Table 3). Similar statements can be made for the peptides restricted to either $I\text{-}E^d$, $I\text{-}A^k$, or $I\text{-}E^k$. Besides the four labeled peptide-Ia combinations shown in Table 3 we also

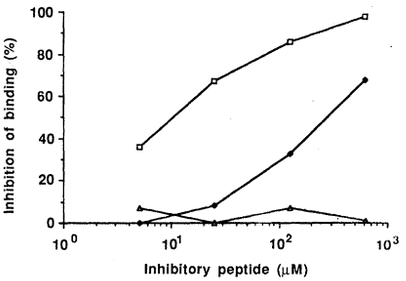

Fig. 1. Inhibition of binding of ^{125}I-labeled HEL-(46–61) to $I\text{-}A^k$. Purified $I\text{-}A^k$ (at 40 μ*M*) was incubated with labeled peptide and a dose range of inhibitory peptide for 2 days at room temperature. The degree of binding of labeled peptide to Ia was determined by gel filtration and the percent inhibition of binding was plotted against the concentration of inhibitory peptide used. The inhibitory peptides used were: Ha(130–142) (□), HEL(74–86) (◆), Myo(132–153) (△).

studied the inhibition pattern for ^{125}I-labeled λ-repr(12–26) binding to $I\text{-}E^d$ and ^{125}I-labeled Ha(130–142) binding to $I\text{-}A^k$. The inhibition pattern obtained when the 12 peptides were used to inhibit the binding of Myo(132–153) to $I\text{-}E^d$ was identical to that obtained when inhibiting the binding of λ-repr(12–26) to $I\text{-}E^d$. The same was true when the two inhibition patterns obtained with HEL(46–61) and Ha(130–142) binding to $I\text{-}A^k$ were compared. Thus, all the inhibition data obtained are consistent with the hypothesis that each Ia molecule has a single type of binding site for peptide interaction (which may have one or more copies per molecule) and that this site is used by all of the peptides capable of interacting with that molecule. However, an alternative hypothesis that there are multiple binding sites with different specificities, but that the binding of a peptide to a site with one specificity causes allosteric effects that prevent the binding of other peptides to other sites with different specificities cannot be excluded from our data.

The binding of moth and pigeon cytochrome c peptides to $I\text{-}E^d_\alpha E^b_\beta$. The work of Schwartz and his colleagues on the pigeon cytochrome c immune response has revealed a striking heteroclitic response when moth or pigeon cytochrome c was presented by B10.A(5R) accessory cells to T cell clones that had been obtained

Table 3. The capacity of peptides to inhibit binding to Ia^d and Ia^k. Ia at 40 μ*M* was incubated with 0.2 to 0.6 μ*M* ^{125}I-labeled peptide and a dose-range of inhibitory peptide for 2 days at room temperature. The degree of inhibition of binding of labeled peptide to Ia was determined by gel filtration. Known MHC restrictions are underlined.

Unlabeled peptide	Concentration of unlabeled peptide required for 50 percent inhibition of binding of ^{125}I-labeled*			
	Ova-(323–339) to A^d	Myo-(132–153) to E^d	HEL-(46–61) to A^k	p Cytc-(88–104) to E^k
Ova(323–339)	<u>++++</u>	−	++	+
Ha(130–142)	<u>++++</u>	−	++++	−
Myo(106–118)	<u>++++</u>	−	++	+/−
λ-repr(12–26)	<u>++</u>	++++	−	<u>++</u>
HEL(46–61)	+	+	<u>++++</u>	+
HEL(74–86)	++	−	<u>++</u>	−
HEL(81–96)	+	−	++	<u>++++</u>
Ha(111–122)	+/−	<u>++</u>	−	+
Myo(132–153)	−	<u>++++</u>	−	++
HSV(8–23)	−	<u>+++</u>	−	<u>+/−</u>
p Cytc(88–104)	++	+/−	++	<u>++++</u>
m Cytc(88–103)	−	++	−	<u>++++</u>

*++++, 50 percent inhibition obtained with 5 to 50 μ*M* of inhibitory peptide; +++, 50 percent inhibition obtained with 51 to 100 μ*M* of inhibitory peptide; ++, 50 percent inhibition obtained with 101 to 500 μ*M* of inhibitory peptide; +, 50 percent inhibition extrapolated to 501 to 1000 μ*M* of inhibitory peptide; +/−, 50 percent inhibition extrapolated to 1001 to 2500 μ*M* of inhibitory peptide; −, no inhibition detected.

Table 4. The capacity of pigeon and moth cytochrome c peptides to inhibit the binding of ^{125}I-labeled cytochrome c peptides to $E^d_\alpha E^b_\beta$ and E^k. The experiment was conducted as indicated in Table 3.

Unlabeled peptide	Concentration (μM) of unlabeled peptide required for 50 percent inhibition of binding of ^{125}I-labeled		
	m Cytc-(88–103) to $E^d_\alpha E^b_\beta$	m Cytc-(88–103) to E^k	p Cytc-(88–104) to E^k
p Cytc(88–104)	405	35*	24
m Cytc(88–103)	18	61	18

*In a given experiment only differences greater than twofold are considered significant.

from H-2^k mice immunized with pigeon cytochrome c (*23*). These T cells, specific for p Cytc(88–104) responded equally well to the pigeon and moth antigen when presented by I-E^k bearing accessory cells; however, when B10.A(5R) presenting cells, which express $E^k_\alpha E^b_\beta$, were used, the moth cytochrome c still stimulated a response whereas, the immunogen, pigeon cytochrome c did not. Since the T cell clones could clearly co-recognize the hybrid I-E molecule (when moth cytochrome c was used) and could also recognize pigeon cytochrome c (when I-E^k expressing presenting cells were used), it was reasoned that the failure of pigeon cytochrome c to be recognized in the context of the hybrid I-E molecule was due to the failure of the hybrid I-E molecule to interact with the pigeon cytochrome c (*25*). To test this hypothesis, we compared the capacity of the m Cytc(88–103) and the p Cytc(88–104) to inhibit the interaction between the ^{125}I-labeled moth cytochrome c peptide and $E^d_\alpha E^b_\beta$ (this was a suitable molecule to use since E^d_α and E^k_α are virtually identical). As a control, we also compared the capacity of the two peptides to inhibit the interaction between the moth and pigeon cytochrome c peptides and I-E^k. The data obtained indicate that the moth peptide was a much more efficient inhibitor of the binding of moth peptide to $E^d_\alpha E^b_\beta$, than was the pigeon peptide (Table 4). In contrast, both peptides were similarly effective inhibitors of the binding of the moth and pigeon peptides to I-E^k. Thus, these data substantiate the hypothesis put forward by Schwartz and his colleagues that the basis of the heteroclitic response to moth cytochrome c when B10.A(5R) presenting cells are used, is the capacity of moth Cytc(88–103) and the failure of p Cytc(88–104) to interact with the $E^k_\alpha E^b_\beta$ restriction element.

Taken together, our data support a determinant selection model of MHC restriction, but they are also consistent with a model that proposes "holes" in the T cell repertoire as being critical for defining nonresponsiveness. The determinant selection hypothesis postulates that Ia molecules select peptides created during antigen processing and subsequently present them to T cells (*5, 6*). The interaction between antigen and Ia should have a certain degree of specificity and precede T cell recognition. This could explain both the phenomenon of MHC restriction and responder-nonresponder status. The strong correlation between MHC restriction and peptide binding capacity and especially the data obtained with the moth and pigeon cytochrome c peptides and $E^d_\alpha E^b_\beta$ support determinant selection as being an important factor in defining immunogenicity. An alternative line of thought (*26, 27*) explains nonresponder status as the result of limitations in the T cell repertoire. As discussed above for λ-repr(12–26), the mere binding of a peptide to Ia, although perhaps essential, is not sufficient to generate a T cell response toward that particular Ia-peptide combination. This seems reasonable since the Ia molecules themselves do not have the capacity to distinguish between self and nonself as indicated by the finding that mouse lysozyme 46–61 peptide binds efficiently to murine I-A^k (*28*). Self-nonself discrimination is entirely a T cell function, and consequently T cells must be able to override and avoid the possibility of a response created by the interaction between Ia and self or self-like antigens. Thus, the two hypotheses complement, rather than contradict, each other.

Inhibition of antigen presentation by peptides. To assess the immunologic relevance of the binding of peptide to Ia that was detected in the inhibition assay, we determined the capacity of 11 of the peptides to inhibit the presentation of antigen by fixed accessory cells. Two different antigen systems were used: (i) the presentation of Ova(323–339) by fixed A20 (H-2^d) cells to the T cell hybridoma DO-11.10; and the presentation of m Cytc(88–104) by fixed CH-12 (H-2^k) cells to the T cell hybridoma 4.1.1.7. A reduction in interleukin-2 (IL-2) secretion of 75 percent was considered significant. The concentration of inhibitory peptides required to obtain this degree of inhibition is shown in Table 5. In both antigen systems the patterns of inhibition of antigen presentation were similar to the patterns obtained with binding inhibition of Ova(323–339) and p Cytc(88–104) to purified I-A^d and I-E^k, respectively (Table 3). This suggests that the binding we observed in solution was indeed immunologically relevant and that, in contrast to the suggestions of others (*29, 30*), the Ia molecules on the surface of accessory cells are the only readily saturable molecules involved in antigen presentation. There were a few quantitative discrepancies observed [for example, the greater inhibitory capacity of cytochrome c presentation by λ-repr(12–26) compared to HEL(81–96), even though HEL(81–96) was a better inhibitor of the binding to I-E^k]. Such minor discrepancies might be anticipated on the basis of the differences in the way that the two experiments were performed. The inhibition of binding required 2 days of incubation of Ia and

Table 5. The capacity of peptides to inhibit antigen presentation. Antigen presenting cells (A20 for H-2^d or CH-12 for H-2^k) were fixed in 0.5 percent paraformaldehyde for 20 minutes at room temperature, and washed. The fixed cells were resuspended in PBS containing 0.01 percent bovine serum albumin, and the protease inhibitors PMSF (final concentration, 1 mM), EDTA (3 mg/ml), 1.10 phenanthroline (0.26 mg), pepstatin A (10 μg/ml), N^α-*p*-tosyl-L-lysine chloromethyl ketone (50 μg/ml), and benzyloxycarbonyl-phenylalanylalanine diazomethyl ketone (100 μM) (*10*). A dose of antigen that was capable of stimulating a suboptimal response by the T cell hybridomas was added to the fixed cells together with a dose range of the different inhibitory peptides and the cells were incubated for 2 hours at 37°C. These antigen-pulsed and paraformaldehyde-fixed cells were then transferred into microtiter filtration plates (Millititer SV; Millipore, Bedford, MA) (2×10^5 per well) and washed three times with PBS to remove the protease inhibitors and unbound antigen DO-11.10 or 4.1.1.7 T cell hybridomas (2×10^5 cells per well) were added and the plates were incubated for 24 hours at 37°C. The supernatants were harvested, and the secretion of IL-2 by the T cell hybridomas was assayed with the IL-2–sensitive T cell line HT-2 as previously described (*38*).

Inhibitory peptide	Restriction	Concentration (μM) of inhibitory peptide required for 75 percent inhibition	
		DO-11.10 + Ova(323–339)	4.1.1.7 + Cytc
Ova(323–339)	A^d		>1000 (+)*
Ha(130–142)	A^d	8 (++++)	>1000 (−)
λ-repr(12–26)	A^d,E^k	370 (++)	15 (++)
HEL(46–61)	A^k	>1000 (+)	>1000 (+)
HEL(74–86)	A^k	30 (++)	>1000 (−)
HEL(81–96)	E^k,A^b	600 (+)	70 (++++)
Ha(111–122)	E^d	>1000 (+/−)	650 (+)
Myo(132–153)	E^d	>1000 (−)	600 (++)
HSV(8–23)	E^d,E^k,$E^k_\alpha E^b_\beta$	>1000 (−)	>1000 (+/−)
p Cytc(88–104)	E^k	320 (++)	
m Cytc(88–103)	E^k,$E^k_\alpha E^b_\beta$	>1000 (−)	

*The symbols in parentheses indicate the relative efficiency of these peptides to inhibit the binding of labeled Ova(323–339) to I-A^d and Cytc peptide to I-E^k (Table 3).

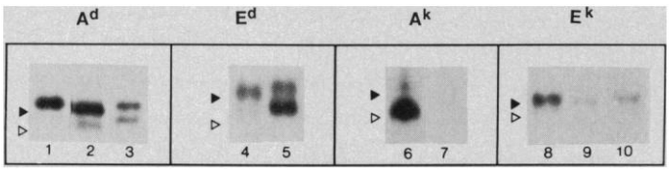

Fig. 2. Cross-linking of radiolabeled peptides to Ia. Isolated ^{125}I-labeled peptide-Ia complexes were cross-linked with 0.015 percent glutaraldehyde for 2 hours at room temperature. The reaction was stopped by the addition of L-glycine to a final concentration of 0.03*M*, and the proteins were then concentrated by vacuum dialysis, precipitated in 90 percent acetonitrile, and examined by SDS-PAGE according to the method of Laemmli (*39*). The gels were stained for proteins with Coomassie blue, dried, and subjected to autoradiography. The 30,000 to 40,000 molecular weight regions of the autoradiograms are shown and the positions of uncomplexed α (▶) and β (▷) chains of Ia as determined by Coomassie staining are indicated. Ova(323–339)/A^d (lane 1), Ha(130–142)/A^d (lane 2), λ-repr(12–26)/A^d (lane 3), Ha(111–122)/E^d (lane 4), Myo(132–153)/E^d (lane 5), HEL(46–61)/A^k (lane 6), p Cytc(88–104)/A^k (lane 7, example of negative control), λ-repr(12–26)/E^k (lane 8), p Cytc(88–104)/E^k (lane 9), and moth Cytc-(88–103)/E^k (lane 10).

peptide at room temperature in order to reach equilibrium (*10*), whereas the inhibition of antigen presentation was carried out for 2 hours at 37°C, making the latter assay sensitive to any differences in the association rates of the stimulatory and inhibitory peptides. As a further indication of the immunologic relevance of the Ia-peptide complexes that form in solution, we have recently demonstrated that isolated complexes of Ova(323–339) and I-A^d when incorporated into planar membranes are highly immunogenic for the DO-11.10 T cell hybrid (*10*). Ova(323–339) offered in the form of a peptide-Ia complex was four orders of magnitude more active in stimulating this hybrid than uncomplexed peptide and Ia.

Covalent cross-linking of peptides to Ia. Using glutaraldehyde, we have been able to cross-link peptides to Ia and analyze the complexes by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. Figure 2 shows the results obtained with Ia-peptide complexes formed between peptides that bound sufficiently to their restriction elements (see Table 2) to allow the analysis to be performed. Although this cross-linking procedure unavoidably leads to some high molecular weight aggregates of Ia and to some cross-linking of the α and β chains, it has been developed to minimize these unwanted cross-linkings and to optimize cross-linking of the labeled peptide to only the α or β chain. The ~30,000 to 40,000 molecular weight region of the gel, containing free α and β chains, is shown in Fig. 2. As previously described (*10*), the Ova(323–339) was cross-linked predominantly to the α chain of I-A^d and only slightly to the β chain. As anticipated, the peptide–α chain complex migrated slightly slower in the SDS-PAGE than free α chain. The Ha(130–142) peptide was cross-linked mainly to the α chain of I-A^d, but with somewhat more cross-linking to the β chain than observed with Ova(323–339) (lane 2). The λ-repr(12–26) peptide cross-linked equally well to both α and β chains of I-A^d (lane 3), and predominantly to the α chain of I-E^k (lane 8). The Ha(111–122) peptide was cross-linked to the α chain of I-E^d, whereas Myo(132–153) was cross-linked predominantly to the β chain of I-E^d (lanes 4 and 5). The HEL(46–61) peptide was cross-linked almost exclusively to the β chain of I-A^k (lane 6), and the two Cytc peptides were cross-linked predominantly to the α chain of I-E^k (lanes 9 and 10).

These cross-linking data suggest that both the α and β chain of Ia are in close proximity of the peptide binding site. This is particularly clear when the cross-linking of peptides to I-E^d were studied. Ha(111–122) was cross-linked preferentially to the α chain; whereas Myo(132–153) was preferentially cross-linked to the β chain of the I-E^d. Since the inhibition of binding data demonstrated that Ha(111–122) and Myo(132–153) interacted with the same site on I-E^d, the cross-linking of Ha(111–122) to E^d_α and of Myo(132–153) to E^d_β suggest that both chains of Ia are involved in making up the peptide interaction site. In keeping with this conclusion is the cross-linking of both chains of I-A^d to an equivalent extent with λ-repr(12–26). In other Ia-peptide combinations, both chains were also cross-linked although one chain was cross-linked preferentially over the other. Furthermore, using glutaraldehyde we were able to demonstrate cross-linking of the HEL(46–61) to A^k_β, whereas Unanue *et al.* (*31*) using a photoactivatable probe on the same peptide have demonstrated cross-linking to the A^k_α. Since the glutaraldehyde cross-linking depends on the proximity of a free amino group on the Ia to an amino group on the peptide, our failure to detect cross-linking of peptide to one of the chains of Ia may be due to the absence of a lysine residue in an appropriate position on that chain rather than the lack of involvement of that chain in the construction of the binding site.

Table 6. Amino acid sequence similarities between peptides that bind to the same Ia molecules.

I-A^k	1	2	3	4	5	6	7	8	
HEL(54-61)	G	I	L	Q	I	N	S	R	
Ha(134-141)	G	V	T	A	A	C	S	H	
I-A^d	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Ova(325-333)	Q	A	V	H	A	A	H	A	E
Myo(110-118)	A	I	I	H	V	L	H	S	R

What can be concluded about the specificity of this antigen binding site on Ia? Studies on the immunogenicity of closely related protein antigens and immunogenic peptides have indicated that Ia molecules can detect modest changes in the structure of antigens (*5, 25, 28, 32, 33*). In contrast, the present data indicate that each Ia can interact with more than 50 percent of the peptides tested. The demonstration that a given Ia molecule can bind more than one-half of this panel of peptides may not reflect the true peptide binding capacity of Ia since all the peptides used have been selected for their known immunogenicity. If we accept that the interaction of Ia with immunogenic peptides is a prerequisite for antigen presentation, it becomes clear that our panel of peptides is skewed toward Ia binding. Ideally, one should examine the immunogenicity and Ia-binding capacity of an unbiased panel of peptides. It might be expected that a smaller percentage of peptides of such a panel would bind to Ia. Regardless, it may be concluded that a single Ia molecule has the capacity to bind many different, apparently unrelated, peptides. This broad specificity clearly distinguishes antigen-Ia interactions from antigen-antibody interactions and is perhaps more analogous to the type of specificity that is observed between proteolytic enzymes and their substrates.

Using inhibition of antigen presentation (*15*), Guillet *et al.* have obtained data that suggest the presence of considerable primary sequence homology between peptides derived from unrelated proteins that compete for presentation by the same Ia molecule. The amino acid substitutions observed were conservative, with two or three exceptions, which proved to be residues involved in T cell recognition and thus may not have been involved in peptide-Ia interactions. Combining the information on the binding of peptides to Ia obtained in this article with the data available on which portion of a peptide is involved in Ia interaction, we have aligned the peptides to determine the extent of the structural homology that exists between peptides that bind strongly to the same Ia molecule. An example of such an analysis for the two peptides that bind best to I-A^k and I-A^d is shown in Table 6. We have used HEL(54–61) as the proband I-A^k binding peptide and Ova(325–333) as the I-A^d

binding peptide since there are data to suggest that these peptide regions retain most of the I-A^k and I-A^d binding capacity of the respective parental peptides (*28, 34*). The best alignment of the Ha(130–142) to HEL(54–61) and of Myo(106–118) to Ova(325–333) is shown. When Ha(130–142) and HEL(54–61) were compared, identity or conservative substitutions were found at positions 1, 2, 5, 7, and 8, with semiconservative or nonconservative substitutions at positions 3, 4, and 6. One of these positions, residue 3, has been previously implicated as being important for T cell recognition, so that this position may not be involved in Ia-antigen interaction (*28, 35*). Similarly, comparison of Myo(106–118) with Ova(325–333) revealed identity or conservative substitutions at positions 2 to 7 with semiconservative or nonconservative substitutions at positions 1, 8, and 9. Determining whether these alignments are relevant and defining the nature of the specificity of peptide-Ia interactions should be possible by construction of a series of homologous peptides and testing the effect of single amino acid substitutions on the capacity of a peptide to bind to a particular Ia molecule.

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