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Immunology

Interaction of an immunodominant epitope with Ia molecules in T-cell activation

(peptide–Ia binding/antigen presentation/lysozyme)

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ABSTRACT **The amino acid sequence corresponding to residues 107–116 of hen egg-white lysozyme (HEL) has been identified as containing an immunodominant T-cell epitope recognized in association with the I-E^d molecule. The immunodominance of this epitope in HEL-primed *H-2^d* mice was demonstrated by analysis of the T-cell proliferative response induced by synthetic peptides covering almost the entire HEL sequence. All the T-cell hybridomas from *H-2^d* mice analyzed recognize the HEL sequence 107–116 in association with the I-E^d molecule. Correlating with the restriction of T-cell recognition, HEL-(105–120)-peptide binds to I-E^d but not to I-A^d molecules. Conservative or semiconservative substitutions at positions 113 (Asn→Lys), 114 (Arg→His), or 115 (Cys→Ala) abrogate the ability of HEL-(105–120) to activate T cells. Substitutions at residues 113 and 115 affect T-cell recognition but not the binding to I-E^d molecules, whereas, as shown by binding data and competition experiments, an Arg→His substitution at position 114 profoundly impairs the capacity of the peptide to interact with I-E^d molecules. In agreement with these results, [Lys^{113}]HEL-(105–120)-peptide but not [His^{114}]HEL-(105–120)-peptide was found to be immunogenic in *H-2^d* mice. Thus, a single semiconservative substitution drastically reduces binding capacity and abolishes immunogenicity, suggesting that a strict correlation exists between binding of a peptide to Ia molecules and its immunogenicity.**

Unlike immunoglobulins, which recognize soluble, native protein antigens, the antigen receptor of T lymphocytes recognizes denatured or partially degraded antigens in association with a molecule encoded by the major histocompatibility complex (MHC) (1, 2).

The finding that antigen and the MHC molecule are recognized by a single T-cell receptor (3–5) implies that the two components of the ligand may physically interact on the membrane of antigen-presenting cells (APCs) prior to T-cell recognition (4, 5). Direct experimental support of this hypothesis was the demonstration by Babbitt *et al.* (6) that a synthetic peptide corresponding to amino acids 46–61 of hen egg-white lysozyme (HEL) has a measurable affinity for the I-A^k molecule (a murine class II, or Ia, MHC molecule) and is also recognized preferentially by T cells together with this molecule. In contrast, the I-A^d molecule neither binds HEL-(46–61)-peptide nor acts as a restriction element for T-cell responses to this peptide. This observation was confirmed and extended by Buus *et al.* (7), who showed that the chicken ovalbumin-(323–339)-peptide, recognized in the context of I-A^d in T-cell activation, binds preferentially to I-A^d. Moreover, analysis of 12 immunodominant peptides recognized together with MHC molecules has demonstrated preferential binding to the restriction element for 11 of the peptides (8). In all these experiments, a correlation was found between the capacity of a given peptide to inhibit the binding of antigen to Ia and the capacity of the same peptide to inhibit antigen presentation by accessory cells (9), as previously suggested by the observation that related synthetic amino acid polymers (10, 11) or synthetic peptides of known sequence (12) competed for presentation to T cells. The concept emerging from these studies is that MHC molecules possess binding sites, probably a single binding site per molecule, for antigenic peptides, but the nature of the antigen–MHC interaction is still obscure.

In this study, we have used synthetic peptides of HEL to analyze an immunodominant epitope for T cells of the *H-2^d* haplotype that is contained in the HEL sequence 107–116 and is recognized in association with I-E^d class II MHC molecules. The HEL sequence 106–129 contains an antigenic determinant recognized by T cells of the *H-2^d* haplotype (13). Experiments employing single residue substitutions within the sequence 107–116 indicated that not only T-cell activation but also the peptide–Ia interaction can be drastically reduced and functionally abolished by a single amino acid substitution.

MATERIALS AND METHODS

Mice. (B6 × DBA/2)F$_1$ (BDF1), B10.D2, and DBA/2 mice were obtained from Olac (Bicester, U.K.) and used when 2–3 months old.

Antigens. HEL was obtained from Societa' Prodotti Antibiotici (Milan). Peptides were synthesized by the Merrifield technique (14) and purified by reverse-phase HPLC.

T-Cell Proliferation. Mice were immunized subcutaneously at the base of the tail and in the hind footpads with HEL (100 μg per mouse, or the molar equivalent for peptides) emulsified in complete Freund's adjuvant (CFA) (Difco). T-cell proliferation was assayed by [^{3}H]thymidine incorporation (15).

T-Cell Hybridomas. T-cell hybridomas were established by poly(ethylene glycol)-induced fusion of lymph node cells with the T-cell lymphoma line BW5147. Lymph node cells were obtained from mice immunized with HEL/CFA and restimulated *in vitro* for 3 days with HEL (100 μg/ml) before cell fusion. After fusion, cells were cultured in selective medium and growing hybrids were screened for reactivity to HEL by assaying HEL- and APC-dependent production of interleukin 2 (IL-2). Cultures containing 5 × 10^4 hybridoma cells and 2.5 × 10^5 syngeneic irradiated (2400 rads; 1 rad =

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Abbreviations: HEL, hen egg-white lysozyme; IL-2, interleukin 2; MHC, major histocompatibility complex; APC, antigen-presenting cell; CFA, complete Freund's adjuvant.

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0.01 Gy) spleen cells were set up with or without HEL (100 μg/ml) in 0.2 ml of culture medium. In some experiments, syngeneic spleen cells were replaced with 2.5×10^4 cloned Ia^+ lymphoma cells [A20 (*H-2*^{d}) or LK35.2 (*H-2*$^{k/d}$)] obtained from the American Type Culture Collection. After 24 hr of culture, 50 μl of supernatant medium was transferred to microculture wells containing 10^4 CTLL cells. After 24 hr of incubation, the presence of IL-2 was assessed by [^{3}H]-thymidine incorporation during the last 4 hr of culture. IL-2 production was quantitated either by probit transformation of titration data (16) or by direct [^{3}H]thymidine incorporation by CTLL cells.

The Ia molecule involved in antigen recognition by individual T-cell hybridomas was determined by inhibition of IL-2 production with monoclonal anti-Ia antibodies. Ascitic fluid from hybridomas 25.5.16 (anti-I-A^b), 25.9.17 (anti-I-A public), 17.3.3 (anti-I-E^{b,k,r,s}), and 14.4.4 (anti-I-E public), kindly provided by K. Ozato (National Institutes of Health, Bethesda, MD), were added at a final dilution of 1:1000 to hybridomas and APCs in the presence of antigen.

Competition experiments were performed by incubating 2.5×10^5 normal spleen cells for 18 hr at 37°C in microculture wells with the indicated concentrations of competitor and 1 μM HEL-(105–120)-peptide. The cells were then washed three times and irradiated, and 5×10^4 hybridoma cells were added. After 24 hr, IL-2 production was quantitated.

Binding of Peptides to Ia Molecules. I-A^d and I-E^d molecules were purified from Nonidet P-40 lysates of A20 (*H-2*^{d}) cells by affinity chromatography using the monoclonal antibodies MK-D6 (anti-I-A^d) or 14.4.4 (anti-I-E^d) coupled to Sepharose 4B beads (Pharmacia). The I-E^d-binding λ repressor-(12–26)-peptide was modified by the addition of a tyrosine residue to the NH_2 terminus to allow ^{125}I labeling. The gel filtration assay used to determine the degree of association between peptides and Ia has been described (17).

RESULTS

HEL sequence 107–116 contains an immunodominant epitope recognized in association with I-E^d molecules. DBA/2 and BALB/c mice were injected subcutaneously with HEL/CFA, and the draining lymph node cells were restimulated *in vitro* with HEL and with a panel of 10 synthetic peptides encompassing 80% of the HEL molecule. In both strains of mice, antigen-dependent T-cell proliferation was induced by HEL and by HEL synthetic peptides centered on residues 101–129 (Fig. 1; see Table 2 for sequences). The response to HEL-(105–120)-peptide accounts for about 30% of the total anti-HEL proliferative response in *H-2*^{d} mice. Since peptides HEL-(101–116) and HEL-(107–129) also induce T-cell proliferation, the putative epitope is included in HEL residues 107–116. All the hybridomas derived from *H-2*^{d} mice that we have studied (five out of five from two different fusions) recognize the above HEL region.

The restriction element used by these T-cell hybridomas was determined by inhibition of IL-2 production by monoclonal anti-Ia antibodies. For example, the hybridoma 3A1, obtained from BDF1 (*H-2*$^{b/d}$) mice, was activated to produce IL-2 by HEL and DBA/2 (*H-2*^{d}) APCs and failed to respond when monoclonal anti-I-E^d antibodies were added to cultures. Similar results were obtained for all the other hybridomas examined (data not shown). Therefore, the HEL-(107–116)-peptide contains an immunodominant determinant recognized by T cells in association with I-E^d molecules.

Activation of I-E^d-Restricted T-Cell Hybridomas by Analogues of HEL-(105–120)-Peptide. Previous studies (13) showed that the cyanogen bromide peptide 106–129 of lysozymes contains a determinant including residues 113 and 114 that is recognized by *H-2*^{d} mice. Initial experiments demonstrated that ring-necked pheasant egg lysozyme,

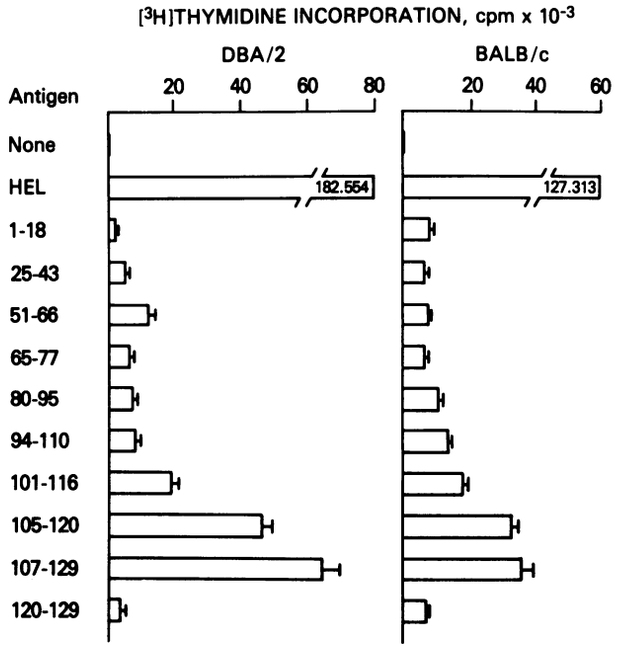

FIG. 1. T-cell proliferative responses of lymph node cells from HEL-primed *H-2*^{d} mice. DBA/2 and BALB/c mice were immunized subcutaneously with 7 μM HEL in CFA. Eight days later, lymph node cells were cultured with 7 μM HEL or with equimolar concentrations of the indicated HEL synthetic peptides. Cells were cultured for 5 days and [^{3}H]thymidine was added during the final 18 hr of culture. Columns represent mean [^{3}H]thymidine incorporation of triplicate cultures, and error bars represent the SEM.

which has an Asn→Lys substitution at position 113 and an Arg→His substitution at position 114 compared to the corresponding HEL sequence, failed to activate 3A1 cells. Synthetic peptide analogues were used to further map amino acid residues within HEL-(105–120)-peptide that are involved in activation of I-E^d-restricted T-cell hybridomas. Three hybridomas, 3A1 (from BDF1 mice) and 1H11 and 1E5 (from B10.D2 mice), which recognize the HEL-(105–120)-peptide restricted by I-E^d, displayed a similar fine antigenic specificity (Fig. 2). Peptides HEL-(105–120), [Arg^{118}]HEL-(105–120), HEL-(101–116), HEL-(107–129), and HEL-(107–116) all induce high IL-2 production, whereas the peptides [Lys^{113}]HEL-(105–120), [His^{114}]HEL-(105–120), [Ala^{115}]-HEL-(105–120), HEL-(110–129), and HEL-(112–129) failed to activate these I-E^d-restricted T-cell hybridomas. These results define amino acids 107–116 as an epitope containing the sequence required for T-cell activation. Within this region, conservative or semiconservative substitutions at positions 113, 114, and 115 abrogate T-cell activation, whereas a substitution at position 118 has no effect on T-cell activation.

Peptide Binding to I-E^d Molecules. Various synthetic peptides corresponding to sequences between residues 101 and 129 of HEL, all containing the sequence 107–116, were tested for their capacity to inhibit binding of the λ repressor-(12–26)-peptide to I-E^d (Table 1). Peptides HEL-(105–120), -(107–129), -(110–129), -(112–129), -(107–116), and -(101–116) all were found to bind to I-E^d. HEL-(107–116), the shortest peptide tested, binds to I-E^d about 10-fold better than the other peptides, as if a shorter polypeptide chain could assume more easily the proper conformation for binding to the I-E^d molecule. If HEL sequence 101–129 contains a single binding site for I-E^d, this would appear to be included within the sequence 112–116. Alternatively, the sequence 101–129 could contain more than one I-E^d binding site, one of which is the sequence 107–116. Control experiments showed that HEL-(105–120)-peptide binds to I-E^d with an affinity (dissociation

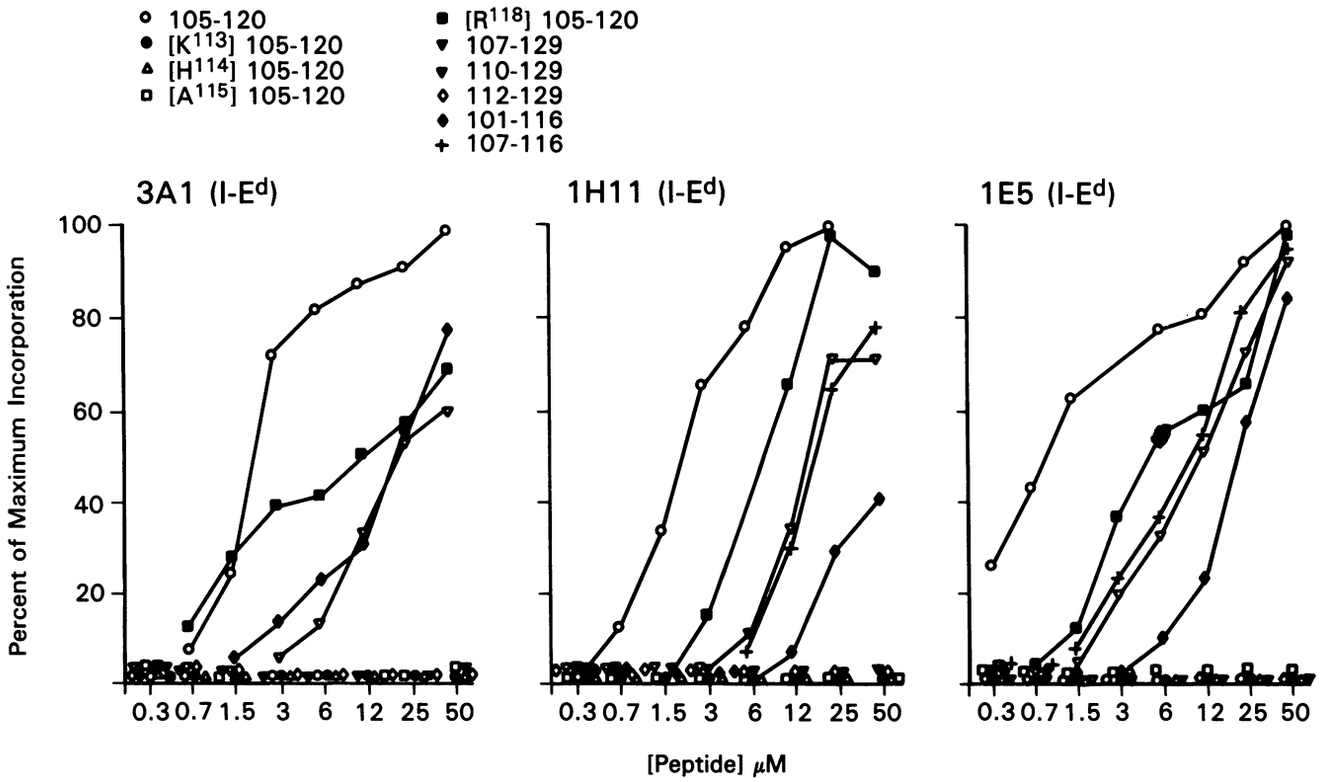

FIG. 2. Specificity of I-E^d-restricted T-cell hybridomas for synthetic peptide variants of the region 105–120 of HEL. Hybridoma cells (3A1, 1H11, and 1E5) were cultured with A20 cells in the presence of the indicated concentrations of HEL synthetic peptides. After 24 hr of culture, 50 μl of culture supernatant was incubated for 24 hr with 10^4 CTLL cells. Maximal [^{3}H]thymidine incorporation and the response in the absence of antigen were, respectively, 297,717 and 2674 cpm (3A1), 61,592 and 831 cpm (1H11), and 278,819 and 370 cpm (1E5).

constant) of about 10^{-5} M, whereas it does not have a measurable affinity to I-A^d (data not shown). Within this region, substitutions of lysine and alanine at positions 113 and 115 do not affect peptide binding to I-E^d, but an Arg→His substitution at position 114 decreased binding activity by a factor >50.

Table 1. Inhibition of binding to I-E^d and inhibition of antigen (Ag) presentation by HEL synthetic peptides

Competing peptide	IC_{50}, μM	
	Binding to I-E^d	Ag presentation
94–110	<0.01	>5000
101–116	0.74	ND
105–120	0.48	ND
107–116	4.17	ND
107–129	0.51	ND
110–129	0.26	80
112–129	0.17	170
[K^{113}]105–120	1.54	12
[H^{114}]105–120	0.02	1200
[A^{115}]105–120	1.37	10

Relative binding is expressed for each peptide as the concentration (μM) of nonradioactive λ repressor-(12–26)-peptide divided by the amount of unlabeled HEL peptide necessary to inhibit by 50% the binding of ^{125}I-labeled λ repressor-(12–26)-peptide to purified I-E^d molecules. The 50% dose for λ repressor-(12–26) prior to standardization was 9.3 μM. Data represent the averages of 2–6 independent experiments. Inhibition of antigen presentation was determined by incubating APCs for 18 hr with 1 μM HEL-(105–120) and various concentrations (1–200 μM) of nonstimulatory peptide. APCs were then irradiated and washed, and 3A1 hybridoma cells (5×10^4) were added. After 24 hr, 50-μl aliquots of culture supernatant were transferred to cultures of 10^4 CTLL cells for 24 hr of incubation. [^{3}H]Thymidine incorporation in the presence or absence of 1 μM HEL-(105–120) was 107,597 and 2976 cpm, respectively. ND, not determined.

Competition of Nonstimulatory Analogues with HEL-(105–120)-Peptide for Presentation by APCs. To identify residues of HEL-(105–120) that are involved in interaction with the T-cell receptor and with I-E^d molecules, nonstimulatory analogues were tested for competition with HEL-(105–120)-peptide. The results (Table 1) showed that 50% inhibition of the IL-2 production induced by HEL-(105–120), in three different hybridomas, required an ≈12-fold excess of [Lys^{113}]HEL-(105–120), an ≈10-fold excess of [Ala^{115}]HEL-(105–120), an ≈80-fold excess of HEL-(110–129), or an ≈170-fold excess of HEL-(112–129), whereas [His^{114}]HEL-(105–120) produced 50% inhibition only at about 1200-fold excess. Similar results were obtained with hybridomas 1H11 and 1E5. These data correlate very closely with those obtained in the I-E^d-binding assay, suggesting that I-E^d may be the only saturable molecule involved in antigen presentation that is expressed on the surface of APCs.

The patterns of T-hybridoma activation, inhibition of antigen presentation, and inhibition of binding to I-E^d are summarized in Table 2.

Responsiveness of *H-2^d* Mice Correlates with Residues 113 and 114. Activation and competition data obtained with T-cell hybridomas that recognize HEL-(105–120)-peptide in the context of I-E^d molecules, as well as peptide binding to I-E^d molecules, indicate that residue 113 is involved in antigen–T-cell receptor interaction, whereas residue 114 primarily influences the interaction between antigen and Ia molecules. This observation predicts that immunization of *H-2^d* mice with [Lys^{113}]HEL-(105–120) would induce a response specific for the immunogen and that immunization with [His^{114}]HEL-(105–120) would not prime for a T-cell response (18). We found that immunization of *H-2^d* mice with HEL-(105–120)-peptide induced a T-cell population that exhibited the same specificity as the HEL-specific, I-E^d-restricted T-cell hybridomas; this population responded to peptides

Table 2. Summary of activation of I-E^d-restricted T-cell hybridomas, inhibition of antigen (Ag) presentation, and inhibition of binding to I-E^d by HEL synthetic peptides

Peptide	Sequence* 94 C A K K I V S D G N G M N A W V A W R N R C K G T D V Q A W I R G C R L 129	Activation of T-cell hybrids	Inhibition: Ag presentation	Inhibition: Binding to I-E^d
94–110	- - - - - - - - - - - - - - - - -	–	–	–
101–116	- - - - - - - - - - - - - - - -	+	ND	+
105–120	- - - - - - - - - - - - - - - -	+	ND	+
107–116	- - - - - - - - - -	+	ND	+
107–129	- A - -	+	ND	+
110–129	- A - -	–	+	+
112–129	- A - -	–	+	+
[K^{113}]105–120	- - - - - - - - K - - - - - - -	–	+	+
[H^{114}]105–120	- - - - - - - - - H - - - - - -	–	±	±
[A^{115}]105–120	- - - - - - - - - - A - - - - -	–	+	+

ND, not determined.
*Standard one-letter amino acid symbols.

HEL-(105–120), [Arg^{118}]HEL-(105–120), HEL-(107–129), and HEL-(101–116) but failed to respond to peptides [Lys^{113}]-HEL-(105–120), [His^{114}]HEL-(105–120), HEL-(110–129), and HEL-(112–129) or to control peptides HEL-(94–110) and -(80–95) (Fig. 3). As predicted, priming of *H-2^d* mice with [Lys^{113}]HEL-(105–120) induced a strong T-cell proliferative response to the immunogen with no crossreactivity to HEL or any of the HEL-(105–120) variants tested. In contrast, priming of *H-2^d* mice with [His^{114}]HEL-(105–120) did not induce any T-cell response to either [His^{114}]HEL-(105–120), HEL, or any of the synthetic peptides tested. A 10-fold higher dose (100 μg per mouse) of [His^{114}]HEL-(105–120) also failed to prime *H-2^d* mice to [His^{114}]HEL-(105–120), HEL, or HEL peptides. However, T-cell proliferation could be induced in these cell populations by restimulation with purified protein derivative of *Mycobacterium tuberculosis* (data not shown).

DISCUSSION

The results show that the sequence 107–116 of HEL contains an immunodominant epitope recognized by T cells together with I-E^d molecules. The T-cell epitope included in the

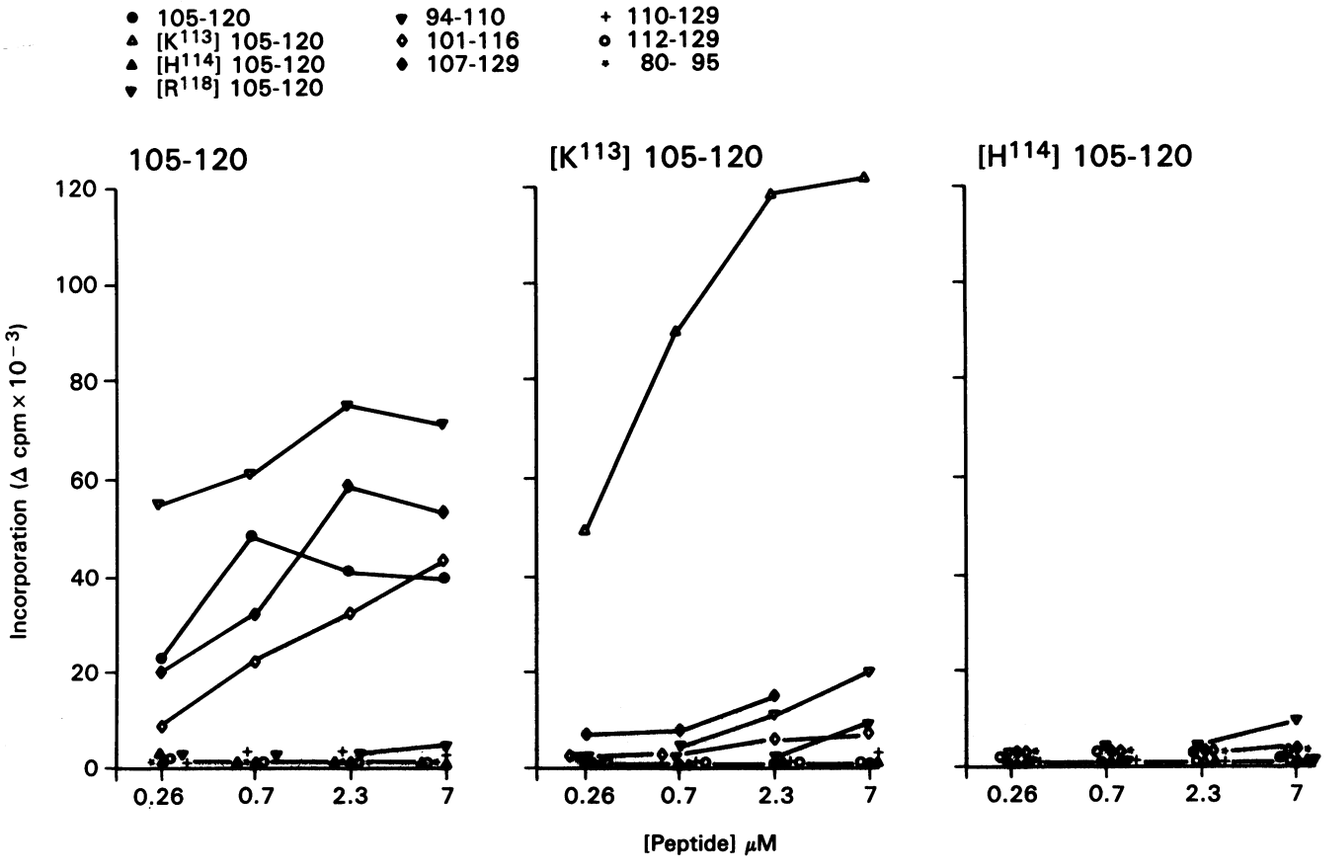

FIG. 3. T-cell proliferative responses of lymph node cells from DBA/2 mice primed with peptide HEL-(105–120), [Lys^{113}]HEL-(105–120), or [His^{114}]HEL-(105–120). DBA/2 mice were immunized subcutaneously with 7 μM peptide in CFA. Eight days later, lymph node cells were cultured with the indicated peptides. Cells were cultured for 5 days and [^{3}H]thymidine was added during the final 18 hr of culture. Results are expressed as mean [^{3}H]thymidine incorporation of triplicate cultures.

sequence 107–116 appears to be composed of an NH_2-terminal region primarily important for recognition by T cells, as truncation of the three NH_2-terminal residues abolishes T-cell activation whereas NH_2-terminal truncations up to residue 112 result in only a slight decrease in binding of the relevant peptides to the I-E^d molecule. The binding of HEL peptides to I-E^d molecules suggests that, if a single I-E^d-binding region exists within the sequence 101–129, this region is included in residues 112–116. Similar to what has previously been found (8), a very good correlation between binding and competition data was observed, indicating that, in this case, I-E^d is the only saturable molecule present on the surface of accessory cells involved in antigen presentation.

Single conservative or semiconservative substitutions at positions 113, 114, and 115 abolish T-cell recognition, whereas only the Arg→His substitution at position 114 profoundly impairs the ability of HEL-(105–120) to bind to I-E^d molecules. Thus, residues involved in the interaction with the T-cell receptor and with the I-E^d molecule appear to be contiguous and to alternate in this region of the HEL sequence. When the HEL-(105–120) analogues substituted at positions 113 and 114 were used to immunize *H-2^d* mice, only the [Lys^{113}]HEL-(105–120)-peptide, which binds I-E^d, was able to prime for a T-cell response, whereas the analogue [His^{114}]HEL-(105–120), which does not bind I-E^d, was not immunogenic. Thus the ability of a peptide to bind Ia molecules appears to be a prerequisite in determining its immunogenicity.

An Arg→His substitution at position 114 profoundly decreases the binding of HEL-(105–120) to the I-E^d molecules and prevents T-cell activation. One possible interpretation of this finding is that the difference between the size and/or shape of these two residues is recognized by the MHC molecule. Alternatively, this substitution could have a profound effect on the possible conformations of the peptide and prohibit the conformation favorable for binding to MHC.

It is clear from the sequence of HEL-(105–120)-peptide (19), and from the intermingled occurrence of residues involved in T-cell activation and in interaction with Ia molecules, that the determinant included in the sequence 107–116 does not conform with the amphipathic helix model of T-cell epitopes (19, 20). It is perhaps more likely that this peptide, as has been postulated for ovalbumin-(323–339)-peptide, assumes an extended conformation after interaction with MHC molecules (21). Two interesting properties of the HEL-(107–116)-peptide are its very high hydrophilicity (22) and the lack of the sequence "motif" common to some T-cell epitopes (23), but it remains unclear whether or not these characteristics are relevant to the peptide antigenicity. Considering the great variability in the properties of peptides that can serve as T-cell epitopes, the backbone structure of peptides, rather than the side-chain groups of amino acids, may be involved in the binding to MHC molecules. If this were the case, certain arrangements of side-chain groups may not allow the conformation(s) of a peptide required for its binding to MHC molecules. Such a mechanism would account for the binding of a multitude of peptides to any given MHC molecules and, at the same time, for the appearance of some degree of specificity.

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