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Identification of a major I-E^k-restricted determinant of hen egg lysozyme: Limitations of lymph node proliferation studies in defining immunodominance and crypticity

(antigen processing/major histocompatibility complex/peptide sequencing/peptide immunization)

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ABSTRACT **We have chemically analyzed the peptides presented by I-E^k molecules after processing of hen egg lysozyme (HEL) by a murine B-lymphoma line or by splenocytes. In both cases, the identified peptides were derived from a single region of HEL, containing the core residues 85–96 with heterogeneous N and C termini. This was a surprising result because this determinant had previously been described as cryptic—i.e., not presented after processing of intact HEL. Examination of the specificities of T hybridomas isolated after immunization with either HEL or 84–96 peptide (p84–96) provided an explanation for this controversy. Whereas hybridomas induced by immunization with HEL responded equally well to HEL and p84–96, those induced by peptide immunization showed a marked preference for p84–96 over intact HEL. In other words, hybridomas isolated after p84–96 immunization responded poorly to forms of the 84–96 determinant produced by natural processing, leading to the possible erroneous interpretation that 84–96 is a hidden determinant. We conclude that (*i*) p84–96 is efficiently presented on I-E^k molecules after processing of HEL, (*ii*) the explanation for the weak lymph node response to this epitope after immunization with HEL lies at the level of the T cell, not the antigen-presenting cell, and (*iii*) crypticity cannot be defined on the basis of T-cell proliferation studies alone.**

CD4-positive T cells recognize linear peptide sequences displayed on the surface of antigen-presenting cells (APCs) in combination with class II major histocompatibility complex (MHC) molecules (1, 2). Although many potential T-cell determinants may exist within a given protein antigen, only a limited number provide the focus for the immune response. Those determinants eliciting the greatest responses are termed dominant and those stimulating weaker responses are termed subdominant (3–6). Other peptides, synthesized from known protein sequences, have been described, which are themselves strongly immunogenic yet do not stimulate a significant lymph node response after immunization with the native protein. These are referred to as cryptic epitopes (6, 7), implying that they are produced with very low efficiency by normal routes of antigen processing.

At present, the mechanisms by which T-cell determinants are selected for presentation on class II MHC molecules remain poorly defined. We propose that identification of the natural end products of antigen processing is a prerequisite for gaining a greater understanding of the relevant biochemical pathways. In a previous study we showed that the predominant determinants of hen egg lysozyme (HEL) recovered from I-A^k molecules contain the immunodominant I-A^k-restricted epitope, 52–61 (8). In the current study we extend these findings to a different MHC molecule, I-E^k. However, in this case the recovered peptides were derived from a determinant previously reported as cryptic for I-E^k-restricted responses. The explanation for this apparent paradox and the implications for the definition of crypticity are discussed.

MATERIALS AND METHODS

Mice. B10.A/SgSnJ (B10.A), CBA/J (CBA), and C3H/HeJ (C3H) mice, of 12–18 weeks of age, were purchased from The Jackson Laboratory. These strains are of *H-2^a* (B10.A) or *H-2^k* (CBA, C3H) haplotype and express I-A^k and I-E^k class II MHC molecules on APCs.

Lymph Node Proliferation Studies. Groups of four or five mice were immunized with either 7 nmol of HEL or 5 nmol of peptide corresponding to HEL residues 84–96 (p84–96) in phosphate-buffered saline (PBS) emulsified 1:1 with complete Freund's adjuvant. Draining lymph nodes were harvested at day 10 and a single cell suspension was prepared. Lymph node cells (5×10^5 per 200-μl well) were cultured in triplicate, either in medium alone [Dulbecco's minimal essential medium (DMEM) containing antibiotics and 0.5% normal mouse serum] or with the indicated doses of antigen. Proliferation was assessed by uptake of [^{3}H]thymidine (0.4 μCi per well; 1 Ci = 37 GBq) present during the final 16 hr of a 4-day culture period.

Cell Lines. All cell lines were maintained in DMEM containing antibiotics and 5% heat-inactivated fetal calf serum, referred to as complete medium (CM). The B-cell lymphoma 5B/2.2 was created by cotransfection of a full-length cDNA for Eβ^k in the plasmid pcEXV3 (a gift from R. Germain, National Institutes of Health) (9), together with a plasmid containing a hygromycin-resistance gene, into the I-A^k-expressing line M12.C3.F6 (10), which has an intact Eα^d gene but lacks a functional Eβ chain. This resulted in expression of a hybrid molecule (Eα^dEβ^k), which, because of the close homology between Eα^d and Eα^k, is indistinguishable from wild-type I-E^k for antibody binding or in T-cell assays. T-cell hybridomas were generated from lymph node blasts restimulated *in vitro* for 3 days with the same antigen used for immunization (5×10^6 cells per ml, 10 μM antigen). Viable cells were enriched by Ficoll density centrifugation and fused with the thymoma line BW5147$\alpha\beta^-$ (11). Hybridoma lines with specificities of interest were subcloned twice by limiting dilution. The MHC restriction of T hybridomas was ascertained by comparing their responses to antigen presented by either M12.C3.F6 or 5B/2.2.

T-Hybridoma Assays. T-hybridoma cells (10^5) were cultured with either 5×10^4 5B/2.2 cells or 5×10^5 irradiated (2000 rads; 1 rad = 0.01 Gy) spleen cells as APCs (8). An aliquot of each supernatant was removed at 20 hr and the interleukin 2

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Abbreviations: APC, antigen-presenting cell; HEL, hen egg lysozyme; MHC, major histocompatibility complex; p84–96, peptide corresponding to hen egg lysozyme residues 84–96; IL-2, interleukin 2.

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(IL-2) content was assessed by proliferation of the IL-2-dependent line CTLL-2.

Antigens. Peptides were synthesized using F-moc chemistry (Applied Biosystems model 432A) and purified by reverse-phase HPLC. All peptides used were shown to consist of a single species of the correct molecular weight by mass spectrometry. HEL (grade V) was obtained from Sigma.

Peptide Elution and Analysis. Peptides were eluted from I-E^k exactly as detailed (8), with the modification that the monoclonal antibody used for affinity chromatography was 17-3-3s, specific for Eβ^k (12). For elution of peptides from 5B/2.2, 40 liters of cells were grown in CM. HEL was added at 1 mg/ml when the cell density reached 2×10^5 per ml and culture was continued overnight, yielding 10^{10} cells in total. To obtain peptides from splenic APCs, spleens from 200 C3H mice were harvested and a single cell suspension was prepared (1.8×10^{10} cells). The cells were then cultured in CM with 2 mg of HEL per ml for 36 hr. N-terminal sequence analysis of the HPLC fractions was performed by automated Edman degradation on an Applied Biosystems 470A gas-phase peptide sequencer.

RESULTS

Analysis of Peptides Eluted from I-E^k Molecules of APCs. I-E^k molecules were immunopurified from the B-lymphoma 5B/2.2 after culture overnight with HEL at 1 mg/ml. The peptides bound to I-E^k were released by treatment at low pH and fractionated by reverse-phase HPLC. Fractions that eluted at acetonitrile concentrations of 15–60% were subjected to analysis by Edman degradation and the sequences obtained were compared with that of HEL. Peptides eluting in six discrete regions of the gradient were found to have homology with the sequence of HEL. Furthermore, these were all derived from the same region of HEL, residues 84–96 (Table 1). The N termini of these peptides were either residue 84 or 85. However, the amount of peptide material recovered was insufficient for the C termini to be accurately determined using Edman degradation. The identities of these peptides were therefore deduced by comparing their retention times with those of synthetic peptides with N termini of 84 or 85 and C termini of 96, 97, 98, 99, or 100 under the same HPLC conditions (Table 1). By comparison with standard peptides, we estimate that the maximum amount of peptide in any single fraction was 20 pmol and that the total amount of HEL 84–96 peptide obtained was at least 120 pmol. Assuming that each of the 10^{10} APCs expressed 10^5 I-E^k molecules, a minimum of 7% of these must have been occupied by peptides containing the 84–96 determinant for this amount of material to be recovered.

No sequences were found that matched other I-E^k-restricted determinants of HEL, including 1–17, the previously reported dominant epitope (4). However, a number of endogenous peptide sequences were found in addition to the HEL sequences. Only two of these had 100% homology with published sequences in the GenBank data base. These were residues 31–44 of β_2-microglobulin and residues 234–248 of the cognate form of 70-kDa heat shock protein. Both of these have been identified in a previous study of peptides eluted from I-E^k-bearing spleen cells (13).

In two subsequent experiments performed using 5B/2.2, HPLC-fractionated peptides recovered from I-E^k were screened for the presence of the HEL 84–96 determinant by T-cell hybridoma response. The hybridoma used for these assays, H6.1, is I-E^k-restricted, specific for HEL 84–96, and able to respond to peptide concentrations of 3 nM (see below). IL-2 production was stimulated by peptides that eluted in six discrete regions of the HPLC gradient (Fig. 1), confirming the immunogenicity of the recovered material. The retention times of the peak fractions were 43, 48, 51, 55, 60, and 64 min, which closely correlate with those of regions 1–6 shown in Table 1. HPLC fractions were also screened for the presence of peptides from the putative dominant determinant, 1–17, using a T hybridoma of appropriate specificity. On neither occasion were any positive responses obtained. The hybridoma used is sensitive to 10 nM peptide, equivalent to 2 pmol of peptide per well. Since 20% of each fraction was used for the hybridoma assay, this indicates that no fraction contained greater than 10 pmol of 1–17 peptide.

Since it could be argued that processing of antigens by 5B/2.2 might not be representative of processing by more physiological APCs, an experiment was performed in which peptides were eluted from the I-E^k molecules of HEL-pulsed C3H spleen cells. Screening of the HPLC fractions by response of the H6.1 hybridoma again demonstrated that fractions in six discrete regions of the gradient contained peptides from the HEL 84–96 determinant (Fig. 2). This result was confirmed by sequence analysis of the positive fractions. The peak with the shortest retention time again had an N terminus of residue 85, whereas all other peptides commenced at residue 84. Again, no positive responses were obtained when the HPLC fractions were tested for recognition by a T hybridoma specific for HEL 1–17. Thus, no qualitative differences were demonstrated between the end products of HEL processing in 5B/2.2 and C3H spleen cells, although comparison of the H6.1 responses did suggest that the relative amounts of the different processing end products might vary in the two cell types. Finally, splenic APCs from three different strains presented HEL equally to the T hybridoma H6.1 (Fig. 3).

Table 1. Sequences of HEL peptides extracted from I-E^k

	Extracted peptides				Synthetic peptides
Region	Sequence*	Residues	Length	Retention time,† min	Retention time,† min
1	SSDITASVncakk	85–97	13	43–44	43.6
2	LSSDITASVNcAK	84–96	13	48–49	48.3
3	LSSDITASVncakk	84–97‡	14	51–52	51.4‡
4	LSSDITASVNcAKk	84–97‡	14	56–57	55.5‡
5	LSSDITASVncakki	84–98	15	60	60.0
6	LSSDITASVNcAKkiv(s)	84–99, 84–100§	16, 17§	64–65	65.6, 65.1§

*Peptide residues determined by automated Edman degradation are shown in uppercase. Those in lowercase were determined by comparison with the sequences of the comigrating synthetic peptides shown. The automated sequencer used does not give a detectable signal for cysteine residues.
†Time of elution on the HPLC gradient used for fractionation.
‡The synthetic HEL 84–97 peptide consistently elutes in two major peaks on this HPLC gradient despite its original purification as a single HPLC peak. This is presumably due to the adoption of different secondary conformations or dimerization in solution.
§Both 84–99 and 84–100 are possible candidates for peptides with this retention time.

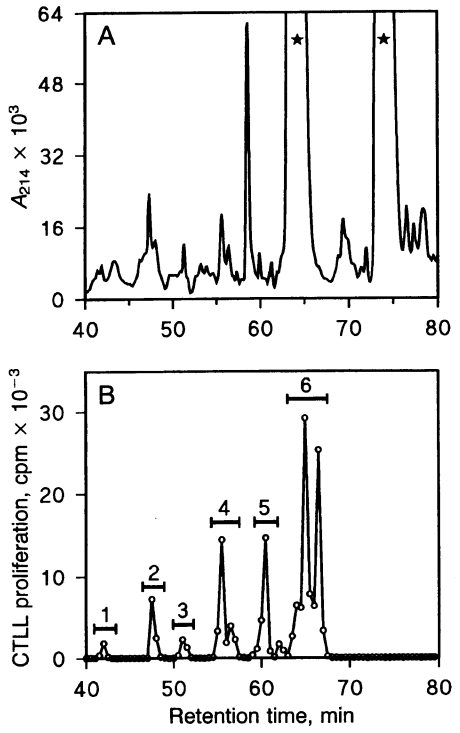

FIG. 1. Stimulation of the T-cell hybridoma H6.1 by HPLC-fractionated HEL peptides eluted from I-E^k molecules of 5B/2.2. Peptides eluted from the I-E^k molecules of the B-lymphoma 5B/2.2 after overnight culture with HEL were fractionated by reverse-phase HPLC. An aliquot of each fraction was tested for the ability to stimulate IL-2 release by the T-cell hybridoma H6.1, specific for HEL 84–96. 5B/2.2 cells were used as APCs. (*A*) Chromatogram measured at 214 nm for fractionation of the peptide material by reverse-phase HPLC. The peaks marked by stars are caused by the detergents used in the elution procedure. (*B*) Recognition of peptides from the HEL 84–96 determinant contained within the HPLC fractions by H6.1. IL-2 production by the hybridoma was measured by CTLL proliferation. Horizontal bars are shown to indicate the six regions of the gradient referred to in the text that contained peptides from the 84–96 determinant. These results are representative of two experiments.

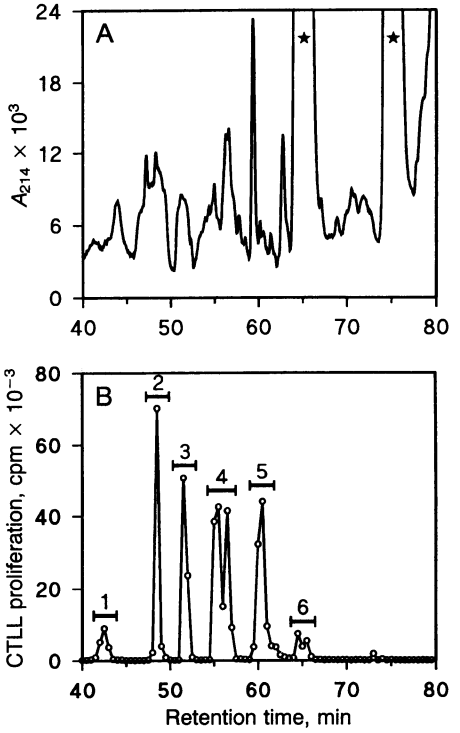

FIG. 2. Stimulation of the T-cell hybridoma H6.1 by HPLC-fractionated HEL peptides eluted from I-E^k molecules of C3H spleen cells. As for Fig. 1, except that in this experiment I-E^k molecules were immunopurified from C3H spleen cells. (*A*) Chromatogram measured at 214 nm for fractionation of the peptide material by reverse-phase HPLC. The peaks marked by stars are caused by the detergents used in the elution procedure. (*B*) Recognition of peptides from the HEL 84–96 determinant contained within the HPLC fractions by H6.1. IL-2 production by the hybridoma was measured by CTLL proliferation. Horizontal bars are shown to indicate the six regions of the gradient referred to in the text that contained peptides from the 84–96 determinant.

Lymph Node Responses After Immunization with HEL and 84–96 Peptide. The finding that the 84–96 determinant was a major source of processed peptides bound to I-E^k was surprising because it has been described as being a cryptic determinant of HEL (6, 7, 14). To examine this incongruity, we first performed lymph node proliferation assays in B10.A and CBA mice immunized with HEL. In both strains, low-magnitude responses were recalled by peptides corresponding to residues 1–17 (p1–17) or 84–96 (p84–96) (Fig. 4). In comparison, much greater responses were obtained by using either intact HEL or a peptide containing the dominant I-A^k-restricted epitope, 46–61 (p46–61). HEL recall responses in *H-2*^{k}*/H-2*^{a} mice are, in fact, usually dominated by T cells specific for I-A^k-restricted epitopes (4), presumably because the precursor frequencies of such cells are higher than those of I-E^k-restricted cells. A more accurate assessment of the p1–17 and p84–96 responses could be made in mice expressing I-E^k and not I-A^k, but at present no such mouse strain is available.

To further evaluate the response to these I-E^k epitopes, a panel of T hybridomas was created after immunization of CBA mice with HEL. Of 53 HEL-specific lines isolated, 41 were I-A^k-restricted and 12 were I-E^k-restricted. The latter were screened for recognition of peptides corresponding to 1–17, 84–96, and two other previously described I-E^k determinants, 25–43 and 107–116 (4). Four lines were specific for 1–17 and three were specific for 84–96, but none responded to the 25–43 and 107–116 peptides. The specificities of the five remaining lines have not yet been determined. Thus, despite the weak recall response to p1–17 and p84–96, these results confirm that T cells specific for these determinants can be activated *in vivo* after HEL immunization.

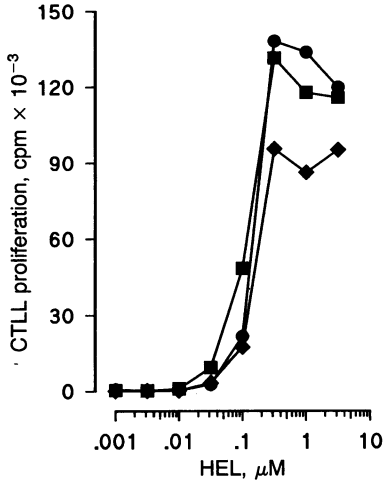

FIG. 3. Comparison of the ability of I-E^k-bearing spleen cells of different mouse strains to present the 84–96 determinant from intact HEL. Presentation of HEL to the 84–96-specific T-hybridoma H6.1 by splenocytes from C3H (●), B10.A (■), and CBA (◆) mice. IL-2 production by the hybridoma was measured in triplicate cultures by proliferation of the CTLL-2 line.

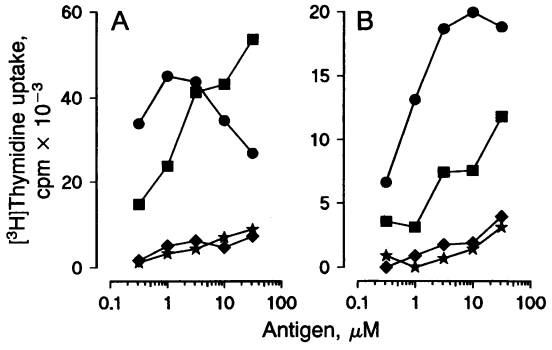

FIG. 4. Lymph node proliferative responses of CBA and B10.A mice immunized with HEL. Ten days after immunization with HEL, lymph node blasts were cultured with HEL (●), p46–61 (■), p84–96 (◆), or p1–17 (★). Proliferative responses are given as the mean of triplicate cultures after subtraction of background proliferation in the absence of added antigen. (*A*) CBA mice (background proliferation = 14,172 cpm). (*B*) B10.A mice (background proliferation = 5237 cpm).

Lymph node responses were also measured in B10.A and CBA mice after immunization with p84–96. As expected, the recall response to the same peptide was marked in both strains (Fig. 5). However, the response to HEL in the CBA mice was reduced compared to the response to peptide, and in the B10.A mice the HEL response was very weak. This was surprising because splenic APCs from these strains were certainly capable of presenting this epitope after processing of HEL (Fig. 3). To elucidate this situation, we examined the antigen specificities of a panel of T hybridomas generated from B10.A lymph node blasts after p84–96 immunization. These hybridomas showed much greater responses to p84–96 than to intact HEL (Fig. 6*A–E*). The ratio of the respective antigen doses required to elicit a 50% maximal CTLL response varied between 10- and 1000-fold. In some cases a weak response to HEL was obtained only at the highest dose used (100 μM). In contrast, hybridomas isolated after immunization with HEL responded equally well to HEL and to p84–96. Data for one such hybridoma, H6.1, are shown in Fig. 6*F*. Thus, the response after p84–96 immunization in B10.A mice was apparently dominated by T cells specific for conformations of that peptide that were produced with low efficiency by processing of intact HEL.

DISCUSSION

We have identified the predominant peptides of HEL associated with I-E^k molecules after antigen processing and have

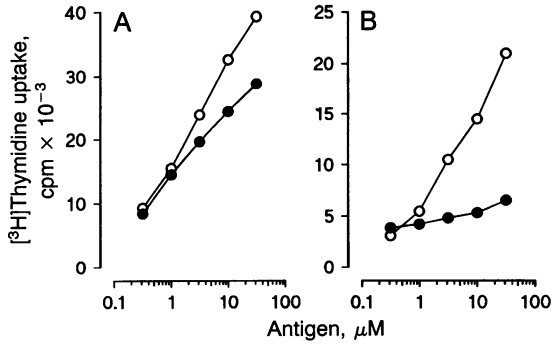

FIG. 5. Lymph node proliferative responses of CBA and B10.A mice immunized with HEL 84–96 peptide. Ten days after immunization with p84–96, lymph node blasts were cultured with HEL (●) or p84–96 (○). Proliferative responses are given as the mean of triplicate cultures after subtraction of background proliferation in the absence of added antigen. (*A*) CBA mice (background proliferation = 12,919 cpm). (*B*) B10.A mice (background proliferation = 6389 cpm).

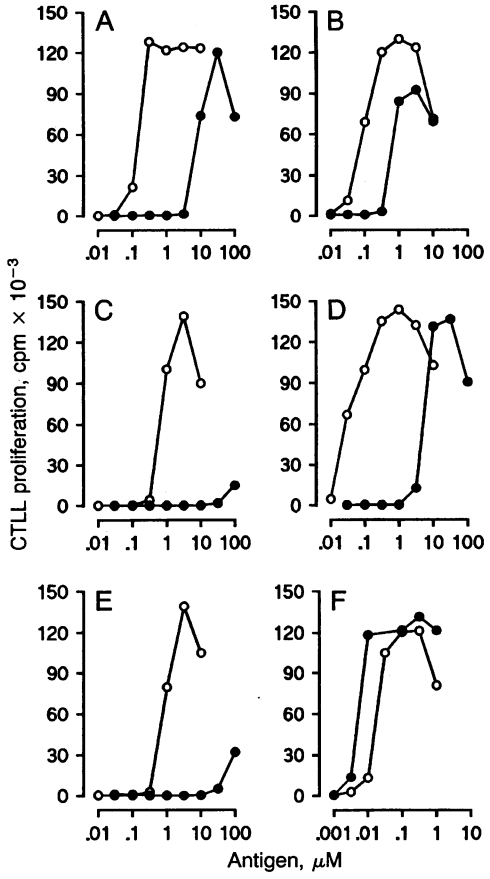

FIG. 6. Antigen responses of T hybridomas specific for HEL 84–96. Clonal T-cell hybridomas were cultured with titrated doses of HEL (●) or p84–96 (○) in the presence of 5B/2.2 as APCs. IL-2 production by the hybridomas was measured in triplicate by proliferation of the CTLL-2 line. (*A–E*) Five hybridomas obtained after immunization of B10.A mice with p84–96. (*F*) H6.1 obtained after immunization of CBA mice with HEL.

shown that these are derived from a single determinant, residues 84–96. This was the case for I-E^k-transfected B-lymphoma cells and for I-E^k-bearing splenic APCs. Although in the latter case the biochemical analysis was performed on a single strain of APC, functional assays indicated that splenic APCs from CBA, C3H, and B10.A mice all presented the 84–96 epitope of HEL equally well to a specific T hybridoma. This argues against any significant strain-specific differences in the ability to process and present this determinant from intact HEL.

From our studies in the I-A^k system, we had anticipated that the peptides eluted from I-E^k would originate from the corresponding immunodominant epitope, residues 1–17 (4). The actual source of the peptides in the current study was all the more surprising because 84–96 has been repeatedly cited as a cryptic epitope of HEL (6, 7, 14). The term "crypticity" has not been precisely defined but is used in reference to epitopes, which, in the form of synthetic peptides, satisfy the following three criteria: (*i*) that they are unable to stimulate a significant lymph node proliferation response after immunization with native antigen; (*ii*) that they themselves are immunogenic; and (*iii*) that after immunization with peptide, no significant lymph node response can be stimulated by the native protein. Although the 84–96 determinant appears to satisfy these criteria in B10.A mice, our results clearly demonstrate at a biochemical and immunological level that this epitope is not cryptic.

The solution to this paradox is that the T-cell repertoire primed by immunization with p84–96 is largely directed against forms of that determinant not readily available after processing of native HEL. It has previously been reported that more than one conformation of the same peptide/MHC complex can be recognized by T cells (15) and it is possible that discrete peptides have considerably more freedom in their interaction with class II MHC molecules than determinants selected from longer, partially processed antigens. Alternatively, the immunizing peptide could have been modified by APCs in some way before it was presented to responding T cells (16). It is implicit in the criteria for crypticity described above that the repertoire primed by peptide immunization is the same as that which would be stimulated if the determinant was presented by processing of the intact protein. This is clearly not the case for the 84–96 determinant of HEL. In fact, the repertoire available to respond to the naturally processed form of this determinant appears to be limited, accounting for the weak recall responses after immunization with HEL. Our data thus demonstrate that crypticity cannot be defined on the basis of T-cell proliferation studies alone. Furthermore, these results suggest that peptide vaccination might have some limitations as a strategy for the prevention of human disease, since the immune response generated may not be directed against the relevant native antigen.

This study complements our previous investigation of the naturally processed forms of HEL presented by I-A^k molecules (8). In that case the peptides were also derived from a single determinant. This is a striking observation, since numerous other antigenic determinants restricted by I-A^k and I-E^k are known to exist (4). For example, 5B/2.2 is able to efficiently process intact HEL for presentation to T hybridomas specific for 1–17 and 107–116 (data not shown). It is thus likely that corresponding peptides were represented in the pool extracted from I-E^k, but at levels too low to be detected by the methods used. One possible explanation for this is that these peptides have a lower binding affinity for I-E^k than p84–96 and may consequently occupy a significantly lower percentage of I-E^k molecules after processing of HEL. In addition, a lower binding affinity might result in greater losses during the elution procedure. Another possibility is that the heterogeneity of the naturally processed peptides is such that they are too widely distributed on the HPLC gradient for a detectable amount to be present in any one fraction.

Our results are consistent with a model of antigen processing in which unfolded polypeptide antigens bind to class II MHC molecules via their T-cell determinants. Those regions of the antigen external to the MHC groove are removed by proteolytic activity, while the bound determinant is protected from catabolism (17, 18). The protruding ends of the peptide are subsequently trimmed to varying extents by exopeptidases until the epitope, protected in the MHC groove, remains. Selection of T-cell determinants is therefore likely to depend on the relative affinities of different epitopes for the MHC molecule in question and greatly favor presentation of those with the highest affinities, such as 46–61 and 84–96 in the case of HEL (19).

In summary, we have identified 84–96 as the principal I-E^k-restricted determinant available for T-cell recognition after processing of HEL. This result could not have been predicted from lymph node proliferation studies. Identification of the naturally processed forms of antigenic determinants is important for a number of reasons. For example, it is evident that peptide residues outside the core determinant may profoundly influence the interaction between peptide and MHC molecule. As shown in a recent study from this laboratory (20), extensions to the HEL 52–61 core peptide favor stabilization and persistence in APCs of the I-A^k/peptide complex. Our eventual goal is to define the biochemical pathways involved in determinant selection, an aim that is unlikely to be achieved without knowledge of the precise sequences of the end products of antigen processing.

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